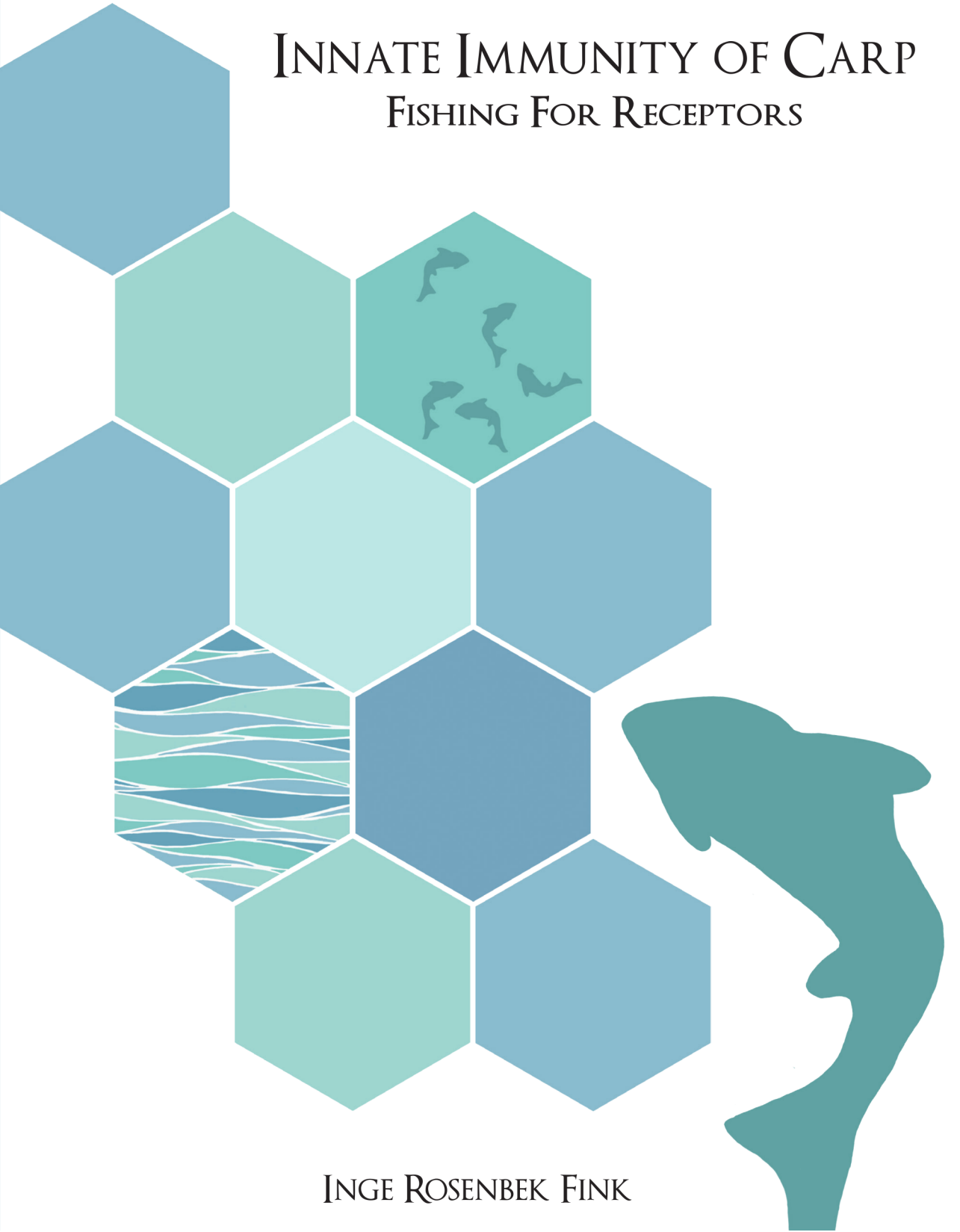
Innate Immunity of Carp

Fishing For Receptors



Inge Rosenbek Fink

Propositions

1. Although several Toll-like receptors in fish appear not to sense ligands, it makes sense for them to do so.
(this thesis)

2. The 'macrophage first' hypothesis deserves to be promoted to a dogma.
(this thesis)

3. Biologists and bioinformaticians shared a common ancestor but diverged to become different species; however, evolutionary forces will lead them to converge again.

4. Since science and populism are unequal opponents it is important to invest in applied communication science (Verweij et al. Lancet Infect Dis 2016).

5. The anti-vaccination movement should realize it is parasitizing on the sensible people of society.

6. Having children during your PhD deserves to be awarded with ECTS credits for the training portfolio.

Propositions belonging to the thesis, entitled

Innate immunity of carp. Fishing for receptors.

Inge Rosenbek Fink

Wageningen, 13 March 2017

Innate immunity of carp
Fishing for receptors

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Innate immunity of carp
Fishing for receptors

Inge Rosenbek Fink

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Chapter 1

General Introduction

Inge Rosenbek Fink

Introduction

Over the past decades, research has shown the potential of pathogen-associated molecular patterns (PAMPs) to modulate the (innate) immune system of fish. PAMPs are specific, structurally conserved components that are produced by certain broad groups of potentially pathogenic microorganisms and are absent in multicellular hosts. PAMPs being used as immunostimulants include poly I:C as a synthetic analog of double-stranded RNA present in some viruses, CpG oligodeoxynucleotides with motifs that are common in bacterial DNA but suppressed in vertebrate DNA (Carrington and Secombes, 2006), and β-glucans consisting of repeating units of glucose linked by β-glycosidic bonds (Dalmo and Bogwald, 2008). These immune modulators can be administered by injection, diet or immersion and have in common stimulating but presumed short-lived effects on the immune system, including an increase in protection against a subsequent pathogenic challenge (Sakai, 1999), (Tafalla et al., 2013), (Wang et al., 2016), (Vallejos-Vidal et al., 2016). PAMPs comprise a wide variety of structurally diverse molecules with nucleotide- and carbohydrate-based substances being well-known examples. PAMPs can be simple or complex, monomeric or repetitive, soluble or insoluble; and consequently their effects are enormously varied. Common mechanisms behind immunostimulatory effects of PAMPs are enhanced phagocytic, cytotoxic and antimicrobial activities of leukocytes as well as increased complement activity and production of pro-inflammatory cytokines. The exact effects of a single immunostimulant are highly dependent on fish species and are also affected by administration route, dosage and duration/repetition of treatment.

Despite the growing interest in the use of immunostimulants across the aquaculture industry our understanding of the underlying mechanisms of ligand recognition and activation of the fish immune response remains fragmented. Understanding the innate immune system of fish is expected to lead to a more efficient and sustainable control of diseases in aquaculture via targeted immunostimulation preferably by feed. One area of intense study has been the administration of β-glucans via fish feed. In cyprinids, β-glucans have been shown to stimulate a suite of innate immune parameters, including phagocytic capacity, oxidative burst, lysozyme and complement activity (Gopalakannan and Arul, 2010), (Lin et al., 2011), (Pionnier et al., 2013), (Pionnier et al., 2014), (Pietretti et al., 2013). Several studies in cyprinids have addressed the effect of β-glucan administration on (immune) gene expression (Falco et al., 2012), (Falco et al., 2014), (Miest et al., 2012), (van der Marel et al., 2012), and it may be clear that continuous administration of β-glucans generally results in an increased expression of at least several pro-inflammatory genes (Falco et al., 2012), (Pionnier et al., 2013). Thus, administration of β-glucans appears to stimulate the immune system of teleosts including cyprinids. Many immune stimulants, including β-glucans, have a presumed working mechanism based on modulation of the innate parts of the immune

system. However, knowledge of the receptors involved in sensing β-glucans as well as knowledge of intracellular signalling downstream of receptor activation is largely missing.

The overall aim of this thesis is to perform a molecular and functional characterization of how pathogen associated molecular patterns (PAMPs), such as β-glucans, affect the innate immune response of carp and which receptors on carp leukocytes are likely candidates to play a role in sensing such PAMPs. These studies will contribute to the valorisation and use of immunostimulants for sustainable aquaculture, aiming for a strategic improvement of fish health.

This aim will be achieved by meeting the following key objectives:

1. The characterization of thrombocytes and macrophages as innate immune cell types of carp (chapters 2, 3 and 4).

2. The characterization of candidate pattern recognition receptors on carp leukocytes that could play a role in sensing PAMPs and initiate innate immune responses (chapters 4, 5 and 6).

NEMO: an Intra-European Training Network (ITN) funded by the Marie Curie Programme (FP7)

In recent years, extensive use of antimicrobials as prophylaxis and treatment of infections has led to the emergence of pathogens with acquired resistance to commonly used anti-infective drugs. Thus, the development of alternative strategies has become crucial, and the discovery of new prophylactic molecules is of great value to veterinary medicine and maintenance of healthy livestock as well as fish. In line with this, the intra-European training network (ITN) on 'Protective immune modulation in warm water fish by feeding glucans' (2008-2012) was founded. Its purpose was to train scientists in research and generic skills in the areas of carp genetics, nutrition, health and immunology. The training network's short name 'NEMO' (network on immune modulation) was inspired by the Disney character Nemo, an energetic and happy young clownfish who is eager to go to school, very curious about everything he sees and quite friendly with anyone he meets. Central to the network was a group of 15 young European scientists who were trained in scientific and generic skills relevant for both the public and private sectors. Their scientific aim focused on the development of a sustainable and cost-effective use of β-glucan (MacroGard®) as immune-stimulant in aquaculture.

The network formed a nucleus of young scientists, of which two PhD students (Danilo Pietretti, Inge Rosenbek Fink) and one post-doc fellow (Anders Østergaard) were appointed at the Cell Biology and Immunology group of Wageningen University. Within NEMO, they worked as a team on a coordinated approach to the study of ligand-receptor interactions in common carp. Together they undertook the molecular and functional characterization of carp Toll-like receptors Tlr4 and Tlr20 (Danilo Pietretti), and scavenger receptor Scarf1 and C-type lectin receptors, among which members of the multi-gene family of immune-related lectin-like receptors (Illrs) (Anders Østergaard). The molecular and functional characterization of carp Tlr1 and Tlr2 as well as the scavenger receptor Cd36 are described in this thesis (Inge Rosenbek Fink).

The use of fish diets supplemented with immune-stimulating compounds had already shown promising results (Dalmo and Bogwald, 2008). One of the most innovative immune-stimulating compounds used in agriculture is a β-glucan component of baker's yeast cell wall. β-glucans are produced as side-products from bioethanol production and their use as immunostimulant in agriculture is a clear example of valorisation of 'waste' materials and can be produced in sufficient quantities to be economically viable also for use in fish feeding. Optimisation of the production and use of β-glucans as biotechnical tools could have a direct effect on the innate immune system and thereby lead to an improvement of fish health. The immune-stimulating effects of β-glucans depend on the position and configuration of the β-(1,3-1,6) side branches, which interact with β-glucan receptors on leukocytes. A thorough analysis of the immune-stimulating effects of β-glucans on the innate immune system of fish, especially warm water species such as common carp, was missing at the start of NEMO. The Network took an integrated approach to the study of β-glucan receptors and immune-stimulating effects of β-glucans on leukocytes of common carp as a fish species crucially important for aquaculture worldwide.

To elucidate the immune responses induced by immune stimulants a suite of immune parameters can be measured. Serum, immune organs and leukocytes can be collected to measure both humoral and cellular reactions that make up innate immunity. Cellular studies targeting macrophage activation can assess oxygen or nitrogen radical production and induced (cytokine) gene expression. Humoral studies can include the measurement of serum immunoglobulin levels, including natural antibodies, as well as complement levels. Candidate receptors on carp leukocytes that can sense β-glucans and initiate innate immune responses can be studied in vitro, for example by overexpression in cell lines transfected with plasmids carrying carp genes. All these approaches were included in the scientific program of NEMO to help ascertain how β-glucans modulate innate immune responses in carp. Thereby, the Network took an integrated approach to establish protocols for the use of β-glucans in the strategic improvement of fish health.

The Network comprised principal investigators from leading European research groups in common carp breeding, immunology, disease, genetics and nutrition (Keele University, United Kingdom; Wageningen University, The Netherlands; Danmarks Tekniske Universitet, Denmark; Tierärztliche Hochschule Hannover, Germany; Institute of Ichthyobiology and Aquaculture of the Polish Academy of Sciences, Poland; University of Plymouth, UK), two European companies who specialise in diet formulation for fish feed (Biomar AS and Tetra GmbH), as well as the leading company in β-glucan production (Biorigin) (see Figure 1). NEMO therefore provided training 'from producer to user'.

Figure 1. NEMO members enjoying a progress meeting in Split, Croatia prior to attending the 15th International Conference of the European Association of Fish Pathologists (EAFP) on Diseases of Fish and Shellfish; 12-16th September 2011.

Phagocytic leukocytes in common carp

Phagocytosis is a fundamental process that enables cells to remove cell debris and pathogens from their direct environment and mediate immunity to pathogens. Thereby, phagocytosis is both an essential part of tissue homeostasis and remodelling, and an important mechanism by which leukocytes eliminate pathogens. Similar to mammals, professional phagocytes of fish include monocytes and macrophages, (neutrophilic) granulocytes and, presumably, dendritic cells (Esteban et al., 2015), (Hodgkinson et al., 2015). In addition, B lymphocytes can be important non-professional phagocytes, both in

amphibians, reptiles and mammals (Gao et al., 2012), (Nakashima et al., 2012), (Parra et al., 2012), (Zimmerman et al., 2010) and in fish (Li et al., 2006), (Esteban et al., 2015). It remains to be investigated if phagocytic B cells of carp are preferentially IgT+ or IgM+ or both.

The phagocytic capacity of thrombocytes is unique to non-mammalian vertebrates, since mammals have anucleated platelets and not the nucleated haemostatic blood cells present in amphibians and fish. Thrombocytes represent nearly half of the phagocyte population in the common carp total peripheral blood leukocyte pool. Common carp thrombocytes can ingest not only small latex beads but also bacteria. Particle internalization leads to phagolysosome fusion and killing of internalized bacteria. While the ingesting capacity of each thrombocyte may be lower than that of monocytes/macrophages and (neutrophilic) granulocytes, thrombocytes are much more numerous in the circulating blood of fish and therefore presumably play a significant role in microbe elimination (Nagasawa et al., 2014). Phagocytosis efficiency can be further enhanced by serum opsonization and requires activation factors secreted by other leukocytes (Nagasawa et al., 2015). In this thesis, we examine the role of thrombocytes and of macrophages in the immune response of fish.

In mice, a core macrophage differentiation program is established already when pre-macrophages colonize the early embryo, followed by a progressive refinement to tissue-specific macrophages via the acquisition of tissue-specific transcriptional regulators. Differentiation of resident macrophages is thus a developmental process and an integral part of organogenesis, which in most tissues is independent of postnatal changes in the environment (Mass et al., 2016). Tissue-resident macrophages include (but are not limited to) microglia, Kupffer cells, splenic marginal zone macrophages, alveolar macrophages, and Langerhans cells, each having distinct but also overlapping functions. All these sub-types are important for both homeostasis and immune responses to pathogens. In teleost fish, macrophages arise from the hematopoietic head kidney. Tissue macrophages in fish are not always sub-classified in the same way as in mammals, but the situation appears largely comparable with the presence of tissue macrophages in the liver, central nervous system and spleen, among others. Fish macrophages play critical roles in both homeostasis and protection, with various mechanisms determining and regulating highly plastic functional phenotypes, including those comprising pro-inflammatory, anti-microbial responses on one side (M1 macrophages, see also next paragraph) and those comprising anti-inflammatory, healing functions on the other side of the spectrum (M2).

Polarization of macrophage activation

Besides the strict phagocytic ability of macrophages, they also have many other functions such as producing oxygen and nitrogen radicals, acting as professional antigen-presenting cells, producing factors involved in tissue remodelling and repair, as well as producing a multitude of chemokines and cytokines involved in inflammation, among others. Depending on their overall phenotype, macrophages have been divided into subtypes, polarized states, or activation states, with the nomenclature gradually changing over time to reflect new and more sophisticated descriptions of these heterogeneous cells (Gordon, 2003), (Mantovani et al., 2004), (Mosser and Edwards, 2008), (Xue et al., 2014). Traditionally, macrophages were often viewed as responders to their surrounding cytokine environment, mainly brought about by activated T cells. T helper 1 (Th1) cells produce inflammatory cytokines such as IFNγ, which induce classically activated macrophages, whereas T helper 2 (Th2) cells produce anti-inflammatory cytokines (e.g. IL4 and IL13), which induce alternatively activated macrophages. To align with the T helper nomenclature, classically activated macrophages are referred to as M1, and alternatively activated macrophages are referred to as M2 (Martinez et al., 2009).Previous findings have established that fish macrophages, similar to mammalian macrophages, can be broadly divided into classically activated (M1-like) and alternatively activated (M2-like) macrophages, based on in vitro experiments with carp head kidney-derived macrophages (Joerink et al., 2006b) and on in vivo parasitic infections in common carp (Joerink et al., 2006a). This thesis further characterizes the classical and alternative macrophage phenotypes, both in terms of macrophage function (production of nitric oxide by M1 macrophages, and arginase activity of M2 macrophages) and in terms of gene expression profiles. The latter contribute to identifying potential molecular markers for fish M1 and M2 macrophages, some of which have already been described for polarized mammalian macrophages. Furthermore, the role of fish cytokines in macrophage polarization is discussed. It is not known, however, which receptors on carp macrophages are likely candidates to play a role in sensing various PAMPs leading to the aforementioned polarization. This thesis therefore also examines different types of fish pattern recognition receptors.

Pattern Recognition Receptors

Pattern recognition receptors (PRRs) recognize conserved patterns – either originating from exogenous sources (pathogen-associated molecular patterns (PAMPs)) or from endogenous stress signals (damage-associated molecular patterns (DAMPs)). The main functions of PRRs include pathogen-induced phagocytosis, activation of pro-inflammatory signalling pathways, opsonisation, activation of complement and coagulation cascades,

and induction of apoptosis. According to their roles, PRRs can be defined as sensing/signalling PRRs or endocytic/phagocytic PRRs. Sensing PRRs include e.g. Toll-like receptors (TLRs), RIG-I-like receptors and NOD-like receptors. In contrast to TLRs, the latter two types are strictly cytoplasmic and will not be addressed in this thesis. Phagocytic PRRs include scavenger receptors and C-type lectin receptors. Some of these receptor families are briefly introduced below, and an overview of the PRR molecules is shown in Figure 2.

C-type lectin receptors (CLRs) comprise a large superfamily of proteins that were first described as harbouring a Ca^{++}-dependent carbohydrate recognition domain (CRD). This domain is characterized by a unique structural fold, which has since been identified in many proteins, also ones that do not bind carbohydrates or even Ca^{++}. The broader term C-type lectin-like domain (CTLD) was therefore coined to describe this protein fold. More than a thousand CTLD-containing proteins have been discovered, and they have been divided into fourteen groups (I-XIV) based on their domain organization (Drickamer and Fadden, 2002), (Zelensky and Gready, 2005). Five out of the fourteen groups that make up this superfamily include proteins that play important roles in immunity (group II: asialoglycoprotein and DC receptors, III: collectins, IV: selectins, V: natural killer cell receptors, VI: multi-CTLD endocytic receptors). Although both group II and group V members comprise type II membrane receptors with a single extracellular CLTD (Zelensky and Gready, 2005), they differ greatly in ligand-binding: group II receptors are classical C-type lectin receptors that bind carbohydrates in a Ca^{++}-dependent manner, whereas group V receptors are non-classical C-type lectin receptors that mainly bind proteins independent of Ca^{++}. Dectin-1, frequently referred to as the primary membrane-bound PRR for exogenous β-glucan (Brown and Gordon, 2001), (Taylor et al., 2007), (Saijo et al., 2007), (Robinson et al., 2006), (Willment et al., 2001), is one of the best known group V members. Dectin-1-mediated phagocytosis would permit destruction of internalized particles by reactive oxygen species (ROS), nitric oxide and lytic enzymes present in the phagolysosome. Of relevance to this thesis; group V members probably are the youngest and most rapidly evolving CTLD receptors, and appear absent from bony fish genomes (Zelensky and Gready, 2004). Indeed, a search for *dectin-1* in the genome of common carp (Henkel et al., 2012) did not identify a clear ortholog. Instead a structurally related group of CTLD receptors (the Illrs) were studied by a partner in the NEMO consortium as mentioned previously and thus, this family of receptors is not addressed in this thesis.

Scavenger receptors (SRs) constitute a large family of proteins that are structurally diverse and have been implicated in a wide range of biological functions. They are cell surface receptors that typically bind multiple ligands including microbial pathogens and modified host-derived molecules and promote the removal of these non-self or altered-self

targets. These receptors are expressed predominantly by myeloid cells and often function by mechanisms that include endocytosis, phagocytosis, adhesion, and signalling that ultimately lead to the elimination of degraded or harmful substances (Mukhopadhyay and Gordon, 2004), (Plüddemann et al., 2007), (Areschoug and Gordon, 2009). Only recently, a re-classification of (mammalian) scavenger receptors into 10 classes (SR-A to SR-J) was proposed (PrabhuDas et al., 2014). A few well-known members of the scavenger receptors will be introduced here. First, a member of the SR-A class, 'macrophage receptor with collagenous structure', or MARCO (SCARA2, SR-A6). This molecule is constitutively expressed on macrophage subpopulations and can be upregulated by various microbial stimuli in a TLR-dependent manner (Doyle et al., 2004), (Bowdish et al., 2009). Indeed, recent knockdown experiments in zebrafish confirmed a role for MARCO in rapid phagocytosis of *Mycobacterium marinum* (Benard et al., 2014). A well-known member of the SR-B class is CD36 (SCARB3, SR-B2), with broad ligand specificity for endogenous targets (apoptotic cells, various forms of modified LDL, thrombospondin) and pathogenic invaders (mycobacterial lipopeptide, *Staphylococcus aureus*, and the malaria parasite *Plasmodium falciparum*) (Silverstein and Febbraio, 2009). Also, CD36 is thought to be expressed on macrophages with the possibility to act as a co-receptor for TLR2, at least in mammals (Triantafilou et al., 2006). A well-known member of the SR-F class is SCARF1 (SREC-1, SR-F1). Recently, in a study on the conservation of recognition of fungal pathogens in *Caenorhabditis elegans*, two orthologues of CD36 and SCARF1 mediated the host defence against two prototypic fungal pathogens, *Cryptococcus neoformans* and *Candida albicans* (Means et al., 2009), (Means, 2010). In fact, both receptors were required for the control of fungal burden and for expression of antimicrobial peptides and cytokines after infection with *C. neoformans*, in mice as well as in the nematode *C. elegans*. Furthermore, binding of the fungal pathogens to SCARF1 and CD36 was β-glucan dependent, which makes these scavenger receptors highly interesting molecules in the context of the NEMO project. Anders Østergaard undertook the work to examine Scarf1 in common carp, while the characterization of Cd36 is presented in this thesis.

Toll-like receptors (TLRs) are maybe the most extensively studied PRRs and represent an ancient family of molecules – they are present in species such as amphioxus (Huang et al., 2008), sea urchin (Rast et al., 2006), and Drosophila (Lemaitre et al., 1996). The TLRs are type I membrane glycoproteins and consist of an extracellular domain containing leucine-rich repeats (LRRs), a transmembrane domain, and a cytoplasmic Toll/IL-1 receptor (TIR) domain. The LRRs create a horseshoe-like structure displaying both a concave and a convex surface. These surfaces are responsible for ligand binding and ligand-induced TLR dimerization. TLRs recognize PAMPs as diverse as proteins, carbohydrates, lipids and nucleic acids (Takeda et al., 2003). Upon ligand binding to the extracellular domain, conformational changes initiate TLR homo- or heterodimerization

and signal transduction is initiated by the TIR domains by recruiting and activating adaptor proteins such as MyD88. These in turn activate signalling pathways including MAP kinases and transcription factors, e.g. NFκB and IRFs, to induce production of pro-inflammatory cytokines and type I interferons (Akira and Takeda, 2004). The TLRs have different sub-cellular localizations depending on the type of ligand they recognize, e.g. TLRs that bind bacterial or parasite-derived extracellular ligands are present on the outer surface membrane, whereas TLRs specific for viral RNA are present on endosomal membranes. The TLR family members are expressed on various immune and non-immune cells such as B cells, NK cells, dendritic cells, macrophages, fibroblasts, epithelial cells and endothelial cells. In this thesis, the expression of Tlrs by carp thrombocytes, a cell type increasingly recognized for its immune functions, will be investigated.

TLR2 may be the most important TLR for recognition of fungal cell wall components such as β-glucan (Gantner et al., 2003). In addition, TLR2 recognizes a whole range of structurally diverse PAMPs, an ability which has been attributed to a functional interaction with a number of other receptors. In mammals, TLR2 can form heterodimers with TLR1 and TLR6 to recognize triacylated lipopeptides derived from Gram-negative bacteria and diacylated lipopeptides derived from mycoplasma, respectively (Kang et al., 2009), (Farhat et al., 2008), (Zahringer et al., 2008). Furthermore, CD36 has been shown to be involved in phagocytosis and cytokine production in response to *Staphylococcus aureus* and its cell-wall components such as lipoteichoic acid (LTA), suggesting that CD36 functions as a co-receptor of TLR2/6 (Triantafilou et al., 2006).

The intricate collaborations and connections between various PRRs are only just emerging, as is the understanding of their roles in directing tailored immune responses to invading pathogens, not only at the molecular level but also at the cellular level. This thesis contributes to the highly relevant research in this area and adds to our understanding of the continuous battle between pathogens and the immune system.

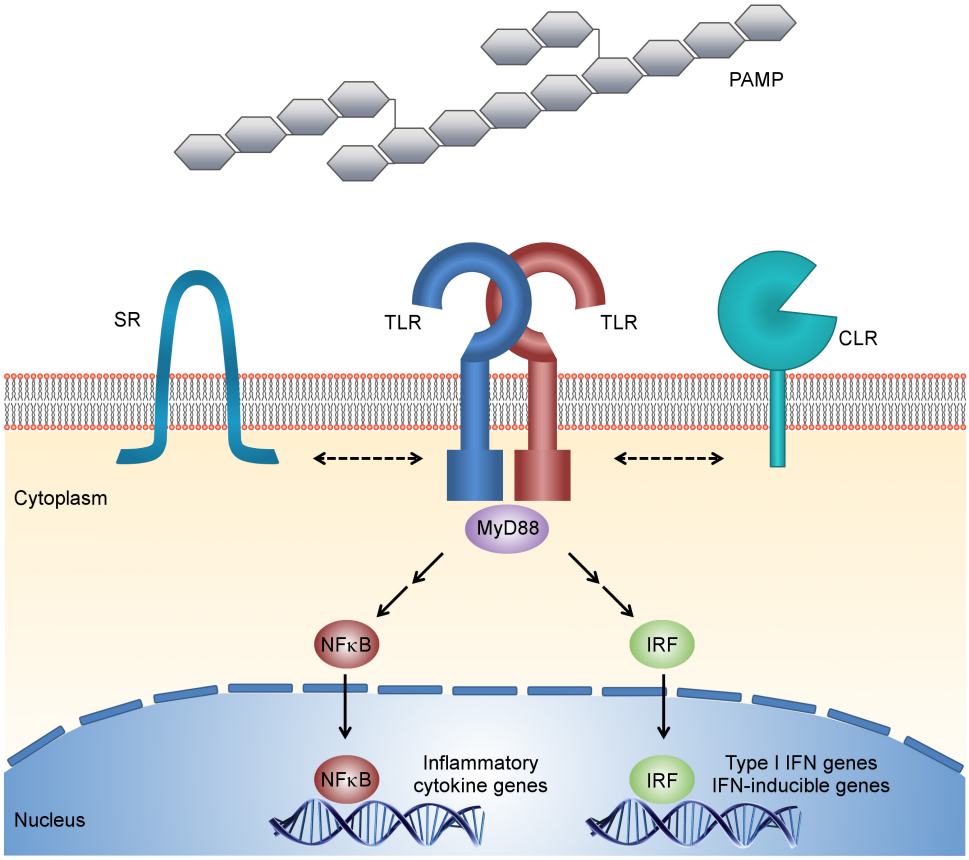

Figure 2. Pattern recognition receptors that play a role in the phagocytosis and/or signalling of PAMPS, such as β-glucans. Scavenger receptors (SRs), Toll-like receptors (TLRs), and C-type lectin receptors (CLRs) can individually or in collaboration sense the presence of PAMPs and initiate intracellular signalling. This is mediated by adaptor molecules such as MyD88, and leads to activation of transcription factors, e.g. NFκB and IRFs. Upon entering the nucleus, these mediate gene expression of inflammatory cytokines, Type I interferons (IFN) and subsequently IFN-inducible genes.

Outline of the thesis

A thorough analysis of the enhanced immunocompetence of fish, created by immune modifying PAMPs, especially in warm-water fish species such as common carp, was missing at the start of this project. Details of how immunostimulants such as β-glucans can activate pattern recognition receptors on phagocytic cell types of carp were limited because of limited knowledge on the ligand-receptor interactions. In the introductory chapter (**chapter 1**), the Initial Training Network (ITN) on 'protective immune modulation in warm water fish by feeding glucans' (short name NEMO) is explained. **The overall aim of this thesis** is to perform a molecular and functional characterization of how pathogen associated molecular patterns (PAMPs), such as β-glucans, could affect the innate immune response of carp and which receptors on carp leukocytes are likely candidates to play a role in sensing such PAMPs. These studies will contribute to the valorisation and use of immunostimulants for sustainable aquaculture, aiming for a strategic improvement of fish health.

In vivo, **carp thrombocytes**; nucleated haemostatic blood cells regarded the functional equivalent of anucleated mammalian platelets, represent nearly half of the phagocyte population in the total peripheral blood leukocyte pool. Ex vivo, carp thrombocytes are able to ingest and kill bacteria (Nagasawa, 2014). In this thesis, we investigated the role of carp thrombocytes in sensing infectious pathogens as potentially dangerous. High levels of gene expression were found for *tlr5* and *tlr8*, which would provide carp thrombocytes with the ability to sense both bacterial (Tlr5, flagellin) and viral (Tlr8, ssRNA) infections. Thus, carp thrombocytes appear able to sense and present antigens, as suggested by the expression of both *tlr* and MHC class II *dab* molecules (**chapter 2**).

Phagocytosis is the process by which a cell engulfs a solid particle to form an internal vesicle known as a phagosome, which then fuses with a lysosome to form a phagolysosome. Within the phagolysosome, enzymes and toxic peroxides digest the pathogen. The production of oxygen and nitrogen radicals by carp phagocytes has been described in detail. Within the organism's immune system, phagocytosis is a major mechanism used to remove pathogens and cell debris. **Macrophages** are probably the best-known leukocyte sub-types able to perform phagocytosis, endocytosis, secretion and microbial killing in the steady state. M1 (inflammation) and M2 (healing) polarizations contribute to a further modulation and tuning of immune responses. In this thesis, we reviewed the current knowledge on polarized macrophages in fish (**chapter 3**) and we investigated the possibility that the initial trigger for macrophage polarization into M1 or M2 could rely only on sensing microbial/parasite infection or other innate danger signals, without the influence of adaptive immunity (**chapter 4**).

Scavenger receptors such as **CD36**, part of the scavenger receptor class B family, have been studied closely in mammals where it is expressed by many different cell types and plays a role in highly diverse processes, both homeostatic and pathologic. CD36 is, among other things, important in the innate immune system, in angiogenesis, and in clearance of apoptotic cells, and it is also involved in lipid metabolism and atherosclerosis. Furthermore, CD36 has been implicated in sensing β-glucans. We therefore studied Cd36 in both common carp and zebrafish, two closely related cyprinid fish species (**chapter 5**).

TLRs are important pattern recognition receptors sensing microbial stimuli by diverse leukocyte sub-types such as phagocytic cell types, including macrophages and thrombocytes. TLRs are a family of conserved, germline-encoded pattern recognition receptors and as such activate rapid inflammatory responses upon detection of their cognate ligands. To date, little is known about the ligand specificities of individual fish TLRs, since the LRR ectodomains of TLRs are not highly conserved and sequence information alone cannot infer functional properties. We present the molecular characterization of the cDNA and genomic structure of *tlr1* from common carp along with our efforts to characterize the function of putative **Tlr1/2 heterodimers**, the subcellular localization, basal gene expression and ligand binding properties (**chapter 6**).

In the **discussion** (**chapter 7**), we review the most recent advances in the field of TLR research and discuss the evolving views on M1/M2 polarization of (fish) macrophages. We fit the data presented in this thesis with other scientific outcomes from the Initial Training Network on 'protective immune modulation in warm water fish by feeding glucans'. Thereby, the research described in this thesis comprises an evaluation of how PAMPs affect the innate immune response of carp and will hopefully contribute to the valorisation and use of PAMPs such as β-glucans as immunostimulants for sustainable aquaculture, aiming for a strategic improvement of fish health.

References

Akira, S., Takeda, K., 2004. Toll-like receptor signalling. Nature reviews. Immunology 4, 499-511.

Areschoug, T., Gordon, S., 2009. Scavenger receptors: role in innate immunity and microbial pathogenesis. Cellular microbiology 11, 1160-1169.

Benard, E.L., Roobol, S.J., Spaink, H.P., Meijer, A.H., 2014. Phagocytosis of mycobacteria by zebrafish macrophages is dependent on the scavenger receptor Marco, a key control factor of pro-inflammatory signalling. Developmental and comparative immunology 47, 223-233.

Bowdish, D.M.E., Sakamoto, K., Kim, M.J., Kroos, M., Mukhopadhyay, S., Leifer, C.A., Tryggvason, K., Gordon, S., Russell, D.G., 2009. MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and *Mycobacterium tuberculosis*. PLoS pathogens 5, e1000474.

Brown, G.D., Gordon, S., 2001. Immune recognition. A new receptor for β-glucans. Nature 413, 36-37.

Carrington, A.C., Secombes, C.J., 2006. A review of CpGs and their relevance to aquaculture. Veterinary immunology and immunopathology 112, 87-101.

Dalmo, R.A., Bogwald, J., 2008. β-glucans as conductors of immune symphonies. Fish & shellfish immunology 25, 384-396.

Doyle, S.E., O'Connell, R.M., Miranda, G.A., Vaidya, S.A., Chow, E.K., Liu, P.T., Suzuki, S., Suzuki, N., Modlin, R.L., Yeh, W.C., Lane, T.F., Cheng, G., 2004. Toll-like receptors induce a phagocytic gene program through p38. The Journal of experimental medicine 199, 81-90.

Drickamer, K., Fadden, A.J., 2002. Genomic analysis of C-type lectins. Biochem Soc Symp 69, 59-72.

Esteban, M.A., Cuesta, A., Chaves-Pozo, E., Meseguer, J., 2015. Phagocytosis in teleosts. Implications of the new cells involved. Biology 4, 907-922.

Falco, A., Frost, P., Miest, J., Pionnier, N., Irnazarow, I., Hoole, D., 2012. Reduced inflammatory response to *Aeromonas salmonicida* infection in common carp (*Cyprinus carpio* L.) fed with β-glucan supplements. Fish & shellfish immunology 32, 1051-1057.

Falco, A., Miest, J.J., Pionnier, N., Pietretti, D., Forlenza, M., Wiegertjes, G.F., Hoole, D., 2014. β-Glucan-supplemented diets increase poly(I:C)-induced gene expression of Mx, possibly via Tlr3-mediated recognition mechanism in common carp (*Cyprinus carpio*). Fish & shellfish immunology 36, 494-502.

Farhat, K., Riekenberg, S., Heine, H., Debarry, J., Lang, R., Mages, J., Buwitt-Beckmann, U., Roschmann, K., Jung, G., Wiesmuller, K.H., Ulmer, A.J., 2008. Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. Journal of leukocyte biology 83, 692-701.

Gantner, B.N., Simmons, R.M., Canavera, S.J., Akira, S., Underhill, D.M., 2003. Collaborative induction of inflammatory responses by Dectin-1 and Toll-like receptor 2. The Journal of experimental medicine 197, 1107-1117.

Gao, J., Ma, X., Gu, W., Fu, M., An, J., Xing, Y., Gao, T., Li, W., Liu, Y., 2012. Novel functions of murine B1 cells: active phagocytic and microbicidal abilities. European journal of immunology 42, 982-992.

Gopalakannan, A., Arul, V., 2010. Enhancement of the innate immune system and disease-resistant activity in *Cyprinus carpio* by oral administration of β-glucan and whole cell yeast. Aquac Res 41, 884-892.

Gordon, S., 2003. Alternative activation of macrophages. Nature reviews. Immunology 3, 23-35.

Henkel, C.V., Dirks, R.P., Jansen, H.J., Forlenza, M., Wiegertjes, G.F., Howe, K., van den Thillart, G.E., Spaink, H.P., 2012. Comparison of the exomes of common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*). Zebrafish 9, 59-67.

Hodgkinson, J.W., Grayfer, L., Belosevic, M., 2015. Biology of bony fish macrophages. Biology 4, 881-906.

Huang, S., Yuan, S., Guo, L., Yu, Y., Li, J., Wu, T., Liu, T., Yang, M., Wu, K., Liu, H., Ge, J., Yu, Y., Huang, H., Dong, M., Yu, C., Chen, S., Xu, A., 2008. Genomic analysis of the immune gene repertoire of amphioxus reveals extraordinary innate complexity and diversity. Genome research 18, 1112-1126.

Joerink, M., Forlenza, M., Ribeiro, C.M., de Vries, B.J., Savelkoul, H.F., Wiegertjes, G.F., 2006a. Differential macrophage polarisation during parasitic infections in common carp (*Cyprinus carpio* L.). Fish & shellfish immunology 21, 561-571.

Joerink, M., Ribeiro, C.M.S., Stet, R.J.M., Hermsen, T., Savelkoul, H.F.J., Wiegertjes, G.F., 2006b. Head kidney-derived macrophages of common carp (*Cyprinus carpio* L.) show plasticity and functional polarization upon differential stimulation. Journal of immunology 177, 61-69.

Kang, J.Y., Nan, X., Jin, M.S., Youn, S.J., Ryu, Y.H., Mah, S., Han, S.H., Lee, H., Paik, S.G., Lee, J.O., 2009. Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. Immunity 31, 873-884.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., Hoffmann, J.A., 1996. The dorsoventral regulatory gene cassette *spätzle*/*Toll*/*cactus* controls the potent antifungal response in Drosophila adults. Cell 86, 973-983.

Li, J., Barreda, D.R., Zhang, Y.A., Boshra, H., Gelman, A.E., Lapatra, S., Tort, L., Sunyer, J.O., 2006. B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. Nature immunology 7, 1116-1124.

Lin, S., Pan, Y., Luo, L., Luo, L., 2011. Effects of dietary β-1,3-glucan, chitosan or raffinose on the growth, innate immunity and resistance of koi (*Cyprinus carpio koi*). Fish & shellfish immunology 31, 788-794.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., Locati, M., 2004. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 25, 677-686.

Martinez, F.O., Helming, L., Gordon, S., 2009. Alternative activation of macrophages: an immunologic functional perspective. Annual review of immunology 27, 451-483.

Mass, E., Ballesteros, I., Farlik, M., Halbritter, F., Günther, P., Crozet, L., Jacome-Galarza, C.E., Händler, K., Klughammer, J., Kobayashi, Y., Gomez-Perdiguero, E., Schultze, J.L., Beyer, M., Bock, C., Geissmann, F., 2016. Specification of tissue-resident macrophages during organogenesis. Science.

Means, T.K., 2010. Fungal pathogen recognition by scavenger receptors in nematodes and mammals. Virulence 1, 37-41.

Means, T.K., Mylonakis, E., Tampakakis, E., Colvin, R.A., Seung, E., Puckett, L., Tai, M.F., Stewart, C.R., Pukkila-Worley, R., Hickman, S.E., Moore, K.J., Calderwood, S.B., Hacohen, N., Luster, A.D., El Khoury, J., 2009. Evolutionarily conserved recognition and innate immunity to fungal pathogens by the scavenger receptors SCARF1 and CD36. The Journal of experimental medicine 206, 637-653.

Miest, J.J., Falco, A., Pionnier, N.P.M., Frost, P., Irnazarow, I., Williams, G.T., Hoole, D., 2012. The influence of dietary β-glucan, PAMP exposure and *Aeromonas salmonicida* on apoptosis modulation in common carp (*Cyprinus carpio*). Fish & shellfish immunology 33, 846-856.

Mosser, D.M., Edwards, J.P., 2008. Exploring the full spectrum of macrophage activation. Nature reviews. Immunology 8, 958-969.

Mukhopadhyay, S., Gordon, S., 2004. The role of scavenger receptors in pathogen recognition and innate immunity. Immunobiology 209, 39-49.

Nagasawa, T., Nakayasu, C., Rieger, A.M., Barreda, D.R., Somamoto, T., Nakao, M., 2014. Phagocytosis by thrombocytes is a conserved innate immune mechanism in lower vertebrates. Frontiers in immunology 5, 445.

Nagasawa, T., Somamoto, T., Nakao, M., 2015. Carp thrombocyte phagocytosis requires activation factors secreted from other leukocytes. Developmental and comparative immunology 52, 107-111.

Nakashima, M., Kinoshita, M., Nakashima, H., Habu, Y., Miyazaki, H., Shono, S., Hiroi, S., Shinomiya, N., Nakanishi, K., Seki, S., 2012. Pivotal advance: characterization of mouse liver phagocytic B cells in innate immunity. Journal of leukocyte biology 91, 537-546.

Parra, D., Rieger, A.M., Li, J., Zhang, Y.A., Randall, L.M., Hunter, C.A., Barreda, D.R., Sunyer, J.O., 2012. Pivotal advance: peritoneal cavity B-1 B cells have phagocytic and microbicidal capacities and present phagocytosed antigen to CD4+ T cells. Journal of leukocyte biology 91, 525-536.

Pietretti, D., Vera-Jimenez, N.I., Hoole, D., Wiegertjes, G.F., 2013. Oxidative burst and nitric oxide responses in carp macrophages induced by zymosan, MacroGard and selective dectin-1 agonists suggest recognition by multiple pattern recognition receptors. Fish & shellfish immunology 35, 847-857.

Pionnier, N., Falco, A., Miest, J., Frost, P., Irnazarow, I., Shrive, A., Hoole, D., 2013. Dietary β-glucan stimulate complement and C-reactive protein acute phase responses in common carp (*Cyprinus carpio*) during an *Aeromonas salmonicida* infection. Fish & shellfish immunology 34, 819-831.

Pionnier, N., Falco, A., Miest, J.J., Shrive, A.K., Hoole, D., 2014. Feeding common carp *Cyprinus carpio* with β-glucan supplemented diet stimulates C-reactive protein and complement immune acute phase responses following PAMPs injection. Fish & shellfish immunology 39, 285-295.

Plüddemann, A., Neyen, C., Gordon, S., 2007. Macrophage scavenger receptors and host-derived ligands. Methods 43, 207-217.

PrabhuDas, M., Bowdish, D., Drickamer, K., Febbraio, M., Herz, J., Kobzik, L., Krieger, M., Loike, J., Means, T.K., Moestrup, S.K., Post, S., Sawamura, T., Silverstein, S., Wang, X.Y., El Khoury, J., 2014. Standardizing scavenger receptor nomenclature. Journal of immunology 192, 1997-2006.

Rast, J.P., Smith, L.C., Loza-Coll, M., Hibino, T., Litman, G.W., 2006. Genomic insights into the immune system of the sea urchin. Science 314, 952-956.

Robinson, M.J., Sancho, D., Slack, E.C., LeibundGut-Landmann, S., Reis e Sousa, C., 2006. Myeloid C-type lectins in innate immunity. Nature immunology 7, 1258-1265.

Saijo, S., Fujikado, N., Furuta, T., Chung, S.H., Kotaki, H., Seki, K., Sudo, K., Akira, S., Adachi, Y., Ohno, N., Kinjo, T., Nakamura, K., Kawakami, K., Iwakura, Y., 2007. Dectin-1 is required for host defense against *Pneumocystis carinii* but not against *Candida albicans*. Nature immunology 8, 39-46.

Sakai, M., 1999. Current research status of fish immunostimulants. Aquaculture 172, 63-92.

Silverstein, R.L., Febbraio, M., 2009. CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior. Science Signaling 2, re3.

Tafalla, C., Bogwald, J., Dalmo, R.A., 2013. Adjuvants and immunostimulants in fish vaccines: current knowledge and future perspectives. Fish & shellfish immunology 35, 1740-1750.

Takeda, K., Kaisho, T., Akira, S., 2003. Toll-like receptors. Annual review of immunology 21, 335-376.

Taylor, P.R., Tsoni, S.V., Willment, J.A., Dennehy, K.M., Rosas, M., Findon, H., Haynes, K., Steele, C., Botto, M., Gordon, S., Brown, G.D., 2007. Dectin-1 is required for β-glucan recognition and control of fungal infection. Nature immunology 8, 31-38.

Triantafilou, M., Gamper, F.G., Haston, R.M., Mouratis, M.A., Morath, S., Hartung, T., Triantafilou, K., 2006. Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting. The Journal of biological chemistry 281, 31002-31011.

Vallejos-Vidal, E., Reyes-Lopez, F., Teles, M., MacKenzie, S., 2016. The response of fish to immunostimulant diets. Fish & shellfish immunology 56, 34-69.

van der Marel, M., Adamek, M., Gonzalez, S.F., Frost, P., Rombout, J.H.W.M., Wiegertjes, G.F., Savelkoul, H.F.J., Steinhagen, D., 2012. Molecular cloning and expression of two β-defensin and two mucin genes in common carp (*Cyprinus carpio* L.) and their up-regulation after β-glucan feeding. Fish & shellfish immunology 32, 494-501.

Wang, W., Sun, J., Liu, C., Xue, Z., 2016. Application of immunostimulants in aquaculture: current knowledge and future perspectives. Aquaculture Research.

Willment, J.A., Gordon, S., Brown, G.D., 2001. Characterization of the human β-glucan receptor and its alternatively spliced isoforms. The Journal of biological chemistry 276, 43818-43823.

Xue, J., Schmidt, S.V., Sander, J., Draffehn, A., Krebs, W., Quester, I., De Nardo, D., Gohel, T.D., Emde, M., Schmidleithner, L., Ganesan, H., Nino-Castro, A., Mallmann, M.R., Labzin, L., Theis, H., Kraut, M., Beyer, M., Latz, E., Freeman, T.C., Ulas, T., Schultze, J.L., 2014. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. Immunity 40, 274-288.

Zahringer, U., Lindner, B., Inamura, S., Heine, H., Alexander, C., 2008. TLR2 - promiscuous or specific? A critical re-evaluation of a receptor expressing apparent broad specificity. Immunobiology 213, 205-224.

Zelensky, A.N., Gready, J.E., 2004. C-type lectin-like domains in *Fugu rubripes*. BMC genomics 5, 51.

Zelensky, A.N., Gready, J.E., 2005. The C-type lectin-like domain superfamily. The FEBS journal 272, 6179-6217.

Zimmerman, L.M., Vogel, L.A., Edwards, K.A., Bowden, R.M., 2010. Phagocytic B cells in a reptile. Biology letters 6, 270-273.

Chapter 2

Immune-relevant thrombocytes of common carp undergo parasite-induced nitric oxide-mediated apoptosis

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Abstract

Common carp thrombocytes account for 30–40% of peripheral blood leukocytes and are abundant in the healthy animals' spleen, the thrombopoietic organ. We show that, *ex vivo*, thrombocytes from healthy carp express a large number of immune-relevant genes, among which several cytokines and Toll-like receptors, clearly pointing at immune functions of carp thrombocytes. Few studies have described the role of fish thrombocytes during infection. Carp are natural host to two different but related protozoan parasites, *Trypanoplasma borreli* and *Trypanosoma carassii*, which reside in the blood and tissue fluids. We used the two parasites to undertake controlled studies on the role of fish thrombocytes during these infections. *In vivo*, but only during infection with *T. borreli*, thrombocytes were massively depleted from the blood and spleen leading to severe thrombocytopenia. *Ex vivo*, addition of nitric oxide induced a clear and rapid apoptosis of thrombocytes from healthy carp, supporting a role for nitric oxide-mediated control of immune-relevant thrombocytes during infection with *T. borreli*. The potential advantage for parasites to selectively deplete the host of thrombocytes via nitric oxide-induced apoptosis is discussed.

Introduction

Thrombocytes are found in all non-mammalian vertebrates such as birds, reptiles, amphibians and fish. Thrombocytes are thought to be the nucleated equivalents of platelets in mammals; they have haemostatic functions, circulate in the blood and participate in the formation of blood clots through aggregation when the vasculature is damaged. Indeed, teleost fish do not contain anucleated platelets in their vascular system, but thrombocytes (Jagadeeswaran et al., 1999; Khandekar et al., 2012; Rombout et al., 1996). Common carp (*Cyprinus carpio* L.) thrombocytes were described almost two decades ago, when a mouse monoclonal antibody (WCL6) was used to study morphology, function and origin of this cell type. Carp thrombocytes were shown to have a round to spindle-shaped morphology with a cytoplasm that contains numerous vesicles that occasionally open to the cell surface consistent with the presence of a surface-connected canalicular system (Rombout et al., 1996).

The latter is also seen in mammalian platelets where the surface-connected canalicular system serves as the pathway for transport of substances into and out of the cells (Escolar and White, 1991). Thrombocytes in carp account for 30–40% of peripheral blood leukocytes (PBL), are also found in head kidney, thymus and intestine and are highly abundant in the spleen. The spleen is considered the thrombopoietic organ in carp (Rombout et al., 1996).

It is becoming increasingly evident that mammalian platelets have immune functions (reviewed in Semple et al., 2011). Platelets can produce antibacterial proteins such as defensins (Krijgsveld et al., 2000), express cytokines and chemokines (McRedmond et al., 2004), interact with leukocytes such as dendritic cells and neutrophils (Clark et al., 2007; Kissel et al., 2006), and express Toll-like receptors (TLRs) through which they can actively bind bacteria and their products (Aslam et al., 2006). Studies in chicken suggest that also thrombocytes have immune functions including reports on the expression of immune genes and the ability to phagocytose (Chang and Hamilton, 1979; St. Paul et al., 2012). In rainbow trout, thrombocytes express genes involved in antigen presentation and immune regulation (Kollner et al., 2004), whereas phagocytosis by thrombocytes was recently claimed a conserved innate immune mechanism in lower vertebrates, including several fish species (Nagasawa et al., 2014). Despite these indications for immune function of thrombocytes, not many studies have described the role of fish thrombocytes during infection.

Carp are natural host to two different but related protozoan parasites, *Trypanoplasma borreli* and *Trypanosoma carassii* that reside in the blood and tissue fluids. Parasitic infections can cause a massive increase in spleen size during infection (Bunnajirakul

et al., 2000). Although related, *T. borreli* and *T. carassii* induce fundamentally different immune responses in the carp host (reviewed in Forlenza et al., 2011). For example, in *T. borreli*-infected carp, gene expression profiles show up-regulated expression of cytokines including *ifnγ* (Stolte et al., 2008) and *tnfα* (Forlenza et al., 2009). At the same time, *inos* gene expression is up-regulated in head kidney, spleen, PBL and liver leading to NO production and a dramatic increase in serum nitrite levels and tissue nitration (Forlenza et al., 2008b; Joerink et al., 2006; Saeij et al., 2000, 2002). In contrast, during *T. carassii*-infections, the production of pro-inflammatory cytokines is negligible (Joerink et al., 2006) and NO production is not induced (Saeij et al., 2002). Instead, *T. carassii* infections are characterised by *arginase* gene expression and activity (Joerink et al., 2006), increased numbers of splenic neutrophils, as well as a cytokine profile associated with a Th17-like immune response (Ribeiro et al., 2010b). The possibility to adjust parasite dose and route of injection facilitates tightly-regulated infections in the laboratory, allowing for controlled *in vivo* studies into the effects of experimental infection on the thrombocyte population.

We have used a well-characterised monoclonal antibody to purify populations of carp thrombocytes and determine their immune gene profile by real-time quantitative PCR *ex vivo*. We studied, in particular, the expression of pro-and anti-inflammatory mediators and expression of Toll-like receptors. We used the *in vivo* experimental infection models with *T. borreli* and *T. carassii* to study the differential effect of these parasites on the thrombocyte population in spleen by histology and the effect on thrombocytes in peripheral blood by flow cytometry. To explain the massive depletion of thrombocytes seen during *T. borreli* infections, we studied the effect of NO, as the hallmark immune modulator induced by this parasite, on apoptosis of thrombocytes *ex vivo*. Our data provide evidence for a strategy of *T. borreli* parasites to selectively deplete the host of thrombocytes via, at least, a nitric oxide-mediated apoptosis. Carp thrombocytes are immune-relevant and may play an important role in immune responses to pathogens.

Material and methods

Animals

European common carp (*Cyprinus carpio carpio* L.) were reared in the central fish facility Carus, at Wageningen University, Wageningen, The Netherlands. Fish were kept at 23 °C in recirculating UV-treated tap water and fed pelleted dry food (Sniff, Soest, Germany) daily. R3 x R8 carp are the hybrid offspring of a cross between fish of Polish origin (R3 strain) and Hungarian origin (R8 strain) (Irnazarow, 1995). Carp were between 9 and 11 months old at the start of the experiments. All studies were performed with approval of the animal experimental committee of Wageningen University.

Infection of carp with protozoan parasites

Infection of carp with *Trypanoplasma borreli* and with *Trypanosoma carassii* was performed as described previously (Forlenza et al., 2008a; Joerink et al., 2006). At least 2 weeks before the start of the experiment fish were moved to the infection quarantine facilities and acclimatised to 20 °C, the optimal temperature for parasite growth. Carp were injected i.p. with 10^4 parasites per fish in 100 µL, or with PBS as non-infected controls. At time of sampling, carp were euthanised with 0.3 g/L tricaine methane sulfonate (TMS, Crescent Research Chemicals, Phoenix, AZ, United States) buffered with 0.6 g/L $NaHCO_3$. Carp were bled from the caudal vein using a syringe containing cRPMI (RPMI 1640 with 25 mM HEPES (Lonza, Basel, Switzerland), adjusted to an osmolality of 280 mOsm/kg with water) containing heparin (50 U/mL, Leo Pharma, Ballerup, Denmark), and the spleen was aseptically removed. Parasitaemia was determined by counting the number of parasites in a small aliquot of each blood sample in a Bürker counting chamber.

Magnetic-activated sorting of thrombocytes

For analysis of immune gene expression and for *ex vivo* assays on thrombocytes, cells were purified from PBL of healthy fish by magnetic-activated cell sorting (MACS) with the help of a well-characterised thrombocyte-specific monoclonal antibody (WCL6 (Rombout et al., 1996)). Thrombocytes were stained with WCL6 (diluted 1:100) followed by phycoerythrin (PE)-conjugated goat anti- mouse (diluted 1:75, DAKO, Glostrup, Denmark) as secondary antibody. After washing twice, 10 µL of magnetic beads (anti-PE MicroBeads, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was added per 10^8 cells. After 15 min incubation at 4 °C, cells were washed twice and resuspended in cRPMI.

Magnetic separation was performed using LS Columns and a MidiMACS Separator (Miltenyi Biotec) according to the manufacturer's instructions. The $WCL6^+$ fraction was analysed by flow cytometry (Beckman Coulter Epics XL- MCL, Brea, CA, United States) to assess their purity, which exceeded 90%. Purified thrombocytes were either lysed in RLT buffer (QIAGEN, Venlo, The Netherlands) for RNA isolation or were seeded in 96-well plates for *ex vivo* apoptosis analysis (see description later).

RNA isolation and cDNA synthesis

Total RNA was extracted using the RNeasy Mini kit according to the manufacturer's protocol (QIAGEN) including on-column DNase treatment with the RNase-free DNase set (QIAGEN). Final elution was performed with 30 µL nuclease-free water. RNA concentrations were measured by spectrophotometry (Nanodrop, Thermo Scientific, Waltham, MA, United States) and stored at -80 °C until use. Prior to cDNA synthesis, 250 ng–2 µg of total RNA was subjected to a second DNase treatment by using DNase I Amplification Grade (Invitrogen, Carlsbad, CA, United States). Synthesis of cDNA was performed with Invitrogen's SuperScript III Reverse Transcriptase and random hexamers, according to the manufacturer's instructions. For all samples a non-reverse transcriptase control was included. cDNA samples were diluted 25 times in nuclease-free water before their use as templates in real-time PCR experiments.

Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was performed in a RotorGene 6000 with a 72-well rotor (Corbett Research, QIAGEN) with the ABsolute QPCR SYBR Green Mix (Thermo Scientific, Waltham, MA, United States) as detection chemistry. All primers were from Eurogentec (Liège, Belgium) and are listed in Table 1. Some primers were designed for this study based on information from the carp genome PRJNA73579 (Henkel et al., 2012). All primers were used at a 300 nM final concentration. Master-mix for each RT-qPCR run was prepared as follows: per reaction, 2 µL of forward and reverse primer stock was mixed with 7 µL of SYBR Green. To 9 µL of master mix, 5 µL of diluted cDNA was added in a 0.1 mL tube. The following amplification program was used: one denaturation step of 15 min at 95 °C; followed by 40 cycles of a three-step amplification (15 s at 95 °C for denaturation, 20 s at 60 °C for annealing, 20 s at 72 °C for elongation). Then a hold step of 1 min at 60 °C, before a melting analysis starting from 60 °C and increasing to 90 °C in 0.5 °C steps with 5 s waiting. Fluorescence acquisition was performed at the end of each cycle and during the waiting steps of the melting analysis. RT-qPCR data were

analysed by RotorGene 6000 Series Software 1.7. The melting temperature and profile of the melting curves were used to validate the specificity of the amplification. Data were analysed by the Pfaffl method of relative quantification with efficiency correction using the average amplification efficiency for each primer pair and the cycle threshold level derived from the Comparative Quantitation Analysis software. The gene expression was calculated as the average amplification efficiency to the power of the negative value of the cycle threshold level. The gene expression of the housekeeping gene 40S ribosomal protein S11 was used to normalise the data.

Table 1. List of primer sequences used in this study.

	Gene	FW (5'-3')	RV (5'-3')	GenBank accession no.	Reference
Marker	*P-selectin*	GGAAAGAATAATGAA-GACTGTGTGG	CAGGATGCCGTGTAG-CAGAG	GQ231486	Forlenza et al. (2009)
Pro-inflammatory mediators	*il1β*	AAGGAGGCCAGT-GGCTCTGT	CCTGAAGAAGAG-GAGGCTGTCA	AJ245635	Engelsma et al. (2001)
	il1r1	ACGCCACCAAGAGCCT-TTTA	GCAGCCCATATTTGGT-CAGA	AJ843873	Metz et al. (2006)
	il6a	CAGATAGCGGACGGAG-GGGC	GCGGGTCTCTTCGT-GTCTT	KC858890	Pietretti et al. (2013b)
	il6b	GGCGTATGAAGGAG-CGAAGA	ATCTGACCGATAGAG-GAGCG	KC858889	Pietretti et al. (2013b)
	tnfα[a]	GCTGTCTGCTTCACGCT-CAA	CCTTGGAAGTGACAT-TTGCTTTT	AJ311800, AJ311801	Forlenza et al. (2009)
	ifnγ2	TCTTGAGGAACCTGAG-CAGAA	TGTGCAAGTCTTTCCT-TTGTAG	AM168523	Stolte et al. (2008)
	il11	CAGCAGCACAGCTCAG-TACCA	AGCCTCTGCTCGGGT-CATCT	AJ632159	Huising et al. (2005)
	inos[a]	AACAGGTCTGAAAGG-GAATCCA	CATTATCTCTCATGTC-CAGAGTCTCTTCT	AJ242906	Saeij et al. (2000)
	mmp9	ATGGGAAAGATG-GACTGCTG	TCAAACAGGAAGGG-GAAGTG	AB057407	Ribeiro et al. (2010b)
	p19	CTCGCTCTGAAAAACTA+-CACCAGG[b]	GGCACGCTCTCTC+-CACTTACT[b]	HM231139	Ribeiro et al. (2010b)
	p35	TGCTTCTCTGTCTCTGT-GATGGA	CACAGCTGCAGTCGT-TCTTGA	AJ580354	Huising et al. (2006)
	p40a	GAGCGCATCAACCTGAC-CAT	AGGATCGTGGATATGT-GACCTCTAC	AJ621425	Huising et al. (2006)
	p40b	TCTTGCACCGCAA-GAAACTATG	TGCAGTTGATGA-GACTAGAGTTTCG	AJ628699	Huising et al. (2006)
	p40c	TGGTTGATAAGGT-TCACCCTTCTC	TATCTGTTCTACAGGT-CAGGGTAACG	AJ628700	Huising et al. (2006)
Anti-inflammatory mediators	*il10*	CGCCAGCATAAA-GAACTCGT	TGCCAAAT-ACTGCTCGAT	AB110780	Savan et al. (2003) and Piazzon et al. (2015)
	socs3_1&2	CCTTCAGACGGACTCCAA	CAAGGAAGGGGTCT-CAAC	GQ847548	Xiao et al. (2010) and Piazzon et al. (2015)
	socs3_3&4	CCGCTGGAGAAGGTG-GAA	CTGGAGGAACTCTTG-GAGTG	-	Xiao et al. (2010) and Piazzon et al. (2015)

Chemokines and chemokine receptors	*cxcl8_l2*	TCACTTCACTGGT-GTTGCTC	GGAATTGCTGGCTCT-GAATG	AB470924	van der Aa et al. (2010)
	cxca_l1	CTGGGATTCCTGAC-CATTGGT	GTTGGCTCTCTGT-TTCAATGCA	AJ421443	van der Aa et al. (2010)
	cxcr1	GCAAATTGGTTAGCCT-GGTGA	AGGCGACTCCACTG-CACAA	AB010468	Fujiki et al. (1999)
	cxcr2	TATGTGCAAACTGAT-TTCAGGCTTAC	GCACACACTATAC-CAACCAGATGG	AB010713	van der Aa et al. (2010)
MH genes	*dab1/2*	GACA+TGG+TGTACGTT-GA+TAACTATAT[b]	ATCTCTCTGCGTTATG-TACTCCAAGTG	CAA88847, CAA88848	Rakus et al. (2009)
	dab3/4	GATAT+GGT+G-TATCTTGT+GTCACTTTCC[b]	GTTCTCTGCATCT-TTCACTCCTTCCTC	X95431, X95434	Rakus et al. (2009)
Toll-like receptors	*tlr1*	AAAAGCGACCTTGA-CATTGC	GCTAACGGTGCGTAG-GATTC	Scaffold 20691.1[c]	Henkel et al. (2012)
	tlr2[a]	TCAACA+CTCTTAATG+T-GAGCCA[b]	TGTG+CTGGAAA+GGT-TCAGAAA[b]	FJ858800	Ribeiro et al. (2010a)
	tlr3.1	GTTATCCCTGGCGCATAA-TA	TCTTCAATAATTGGTAA-GGATGATG	KF387571	Falco et al. (2014)
	tlr3.2	GTTTATCCCTGGAG-CATAACT	CTTCAATAACTGG-TAAAGACGAAC	KF387572	Falco et al. (2014)
	tlr4.1	GCTTAGAAAAACTAGTG-GTGAG	GCAATCCCTATCTCT-GAGTTTC	KF582562	Pietretti (2013)
	tlr4.2	CACTGGAAACCCTATTA-CATACA	GAATGGAGGGAGAGA-CACA	-	Pietretti (2013)
	tlr5[a]	GAAGAGCATCTGTGAA-GAAGA	CACTTGTCCCATCCTAA-CC	KC347572 and Scaffold 36490.1[c]	Duan et al. (2013) and Henkel et al. (2012)
	tlr7[a]	GGTCCTAAGAATCAGAG-GGTA	GAAGTTTGTGC-CGAGATCAATG	AB553573 and Scaffolds 11579.1 & 12210.1[c]	Tanekhy et al. (2010) and Henkel et al. (2012)
	tlr8a[a]	TATCTTGCCTCAATCTCT-CAG	AGACTCTG-TAACTCTCGGAA	Scaffolds 11579.1 & 12210.1[c]	Henkel et al. (2012)
	tlr8b[a]	GAAACCTATCCGT-TCTAAACTTG	GCCTGTTGTCAATCAAT-GAAATC	Scaffolds 8630.1 & 2277.1[c]	Henkel et al. (2012)
	tlr9	CCAGCAGTTCACACATC-TA	TCCCATACCCTTCATTA-CAAA	GU809229	Kongchum et al. (2011)
	tlr20	ATTATGTGACCGTTGAG-GGCTGC	TCCAGATTGACGAC-CGATCTTAC	KF482527	Pietretti et al. (2013a)
Housekeeping gene	*40S*	CCGTGGGTGACATCGT-TACA	TCAGGACATTGAACCT-CACTGTCT	AB012087	Huttenhuis et al. (2006)

[a] Common primers were used which recognize more than one copy of the gene.

[b] + in front of a nucleotide indicates that it contains an LNA modification.

[c] Scaffold indicates the scaffold number in the draft carp genome from bioproject PRJNA73579 (Henkel et al., 2012).

Immunohistochemistry

Cryosections were made from spleen tissue from carp infected with *T. borreli* and with *T. carassii* as well as control fish. Cryosections were processed essentially as described before (Ribeiro et al., 2010b) with the exception that the primary antibody used was WCL6. First, endogenous peroxidases were inactivated then the cryosections were treated with proteinase-K, fixed in 4% paraformaldehyde, and permeabilised with 0.1% Triton in PBS. Cryosections were blocked with normal goat serum, incubated with primary antibody WCL6 (1:100), then with the secondary antibody goat anti-mouse horse-radish peroxidase-conjugated (GAM-HRP, 1:200, DAKO, Glostrup, Denmark). The colour reaction was done with 3-amino-9-ethyl-carbazole (AEC, Sigma-Aldrich, St. Louis, MO, United States) in sodium acetate buffer containing H_2O_2. Finally, cryosections were embedded in Kaiser's glycerine gelatin (Merck, Whitehouse Station, NJ, United States). All necessary controls to prove the specificity of the reaction were carried out and found to be negative.

Flow cytometry

PBL from *T. borreli*-infected, *T. carassii*-infected and healthy control fish were stained for flow cytometry with thrombocyte-specific primary antibody WCL6 (diluted 1:100) followed by incubation with secondary antibody phycoerythrin (PE)-conjugated goat anti-mouse (diluted 1:75, DAKO, Glostrup, Denmark), before being analysed by flow cytometry using a Beckman Coulter Epics XL-MCL flow cytometer.

Measurement of NO-induced apoptosis

S-nitroso-*N*-acetylpenicillamine (SNAP; Alexis Biochemicals, San Diego, CA, United States) was used as nitric oxide (NO) donor. Prior to stimulation of thrombocytes with NO radicals, the potency of the NO donor was tested by measuring the amounts of nitrite produced by increasing concentrations of SNAP using the Griess reaction. Briefly, to 75 µL of SNAP solution 100 µL 1% (w/v) sulphanilamide in 2.5% (v/v) phosphoric acid and subsequently 100 µL 0.1% (w/v) *N*-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid were added. The absorbance was measured at 540 nm (with reference 690 nm) and nitrite concentration was calculated on the basis of a sodium nitrite standard curve.

MACS-sorted thrombocytes were resuspended in cRPMI containing 0.5% pooled carp serum and seeded in 96-well plates at a density of 1 x 10^6 cells/well. Cells were

stimulated for 48 h with the NO donor SNAP or control compound AP-SS (*N*-acetyl-D,L- penicillamine disulphide, degradation product of SNAP; Alexis Biochemicals), in the presence of *T. borreli* lysates (0.5 x 10^6 parasites/ well). Flow cytometric analysis was performed to measure the percentage of apoptotic (Annexin-V^+) and necrotic ($7AAD^+$) thrombocytes. FITC-labelled Annexin-V was from Roche Diagnostics, Basel, Switzerland, and PerCP-labelled 7AAD was from BD Pharmingen, San Diego, CA, United States. Both stainings were performed according to the manufacturer's instructions.

Statistical analysis

The effects of NO donors on cell death were assessed by one-way ANOVA followed by a Tukey post-hoc test.

Results

Immune gene expression profiles of carp thrombocytes

Carp thrombocytes were purified with magnetic sorting from PBL using a specific monoclonal antibody (WCL6). Purity of more than 90% was confirmed by flow cytometry. We measured the constitutive gene expression levels of a large number of immune-relevant genes, including pro- and anti-inflammatory cytokines and mediators, chemokines, and TLRs (Table 2). First of all, the thrombocyte marker *P-selectin*, which also functions as a cell adhesion molecule on activated mammalian platelets (Semple et al., 2011), was expressed at a very high level (above housekeeping gene level). Of the pro-inflammatory cytokines and mediators, *interleukin* (*il*)-1β was most highly expressed. Also *tnfα* and *ifnγ* were expressed by thrombocytes, but to a lower extent. The matrix metalloproteinase *mmp9* was also expressed by thrombocytes. Il12 is composed of two subunits, p35 and p40, and three forms of p40 (a–c) have been reported for carp (Huising et al., 2006). Only one of the *p40* genes (*p40c*) was expressed in thrombocytes and to a very high level (more than 2 times the housekeeping gene level), whereas the expression of the other two *p40* genes (*p40a* and *p40b*) was below the detection limit. p40 can also form a heterodimer with p19, to create the cytokine Il23. However, the gene expression of *p19* was not detectable. Of the anti-inflammatory cytokines and mediators, *suppressor of cytokine signalling (socs)-3* showed a very high gene expression (more than 11 times the housekeeping gene level). Of the chemokines and chemokine receptors tested, *cxcl8_l2* was expressed highest. Major histocompatibility (MH) class II B genes *dab1/2* and *dab3/4* were also highly expressed. Constitutive gene expression was also tested for all *tlr*s described for carp. Of interest, *tlr3* and *tlr4* gene expression was below the detection limit, whereas *tlr5* and *tlr8* were most highly expressed in carp thrombocytes.

Altogether our data indicate that thrombocytes express modulatory molecules involved in pro- as well as anti-inflammatory responses, but also genes involved in antigen presentation or pathogen detection further confirming their potential role in the regulation of immune responses.

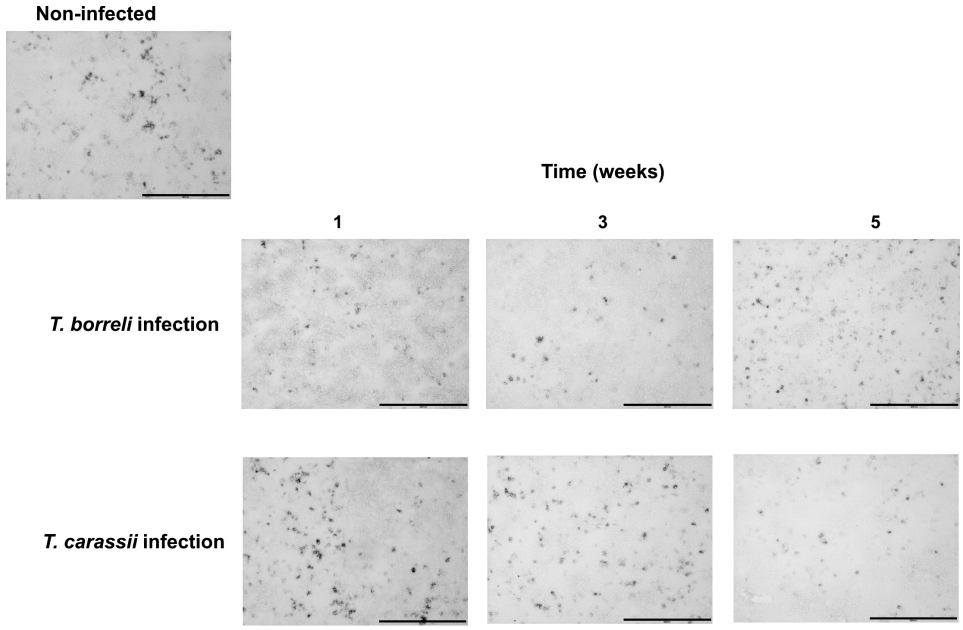
▸ **Table 2.** Carp thrombocytes express a large number of immune-relevant genes. Constitutive gene expression in thrombocytes of carp. WCL6+ thrombocytes were purified by magnetic sorting from PBL. Gene expression was measured by real-time quantitative PCR. Data were analysed by the Pfaffl method of relative quantification with efficiency correction and the expression of each gene was normalized to the housekeeping gene 40S ribosomal protein S11. Data represent the average of n=3-4 fish ± standard deviation. ND, not detectable.

	Gene	Expression
Marker	*P-selectin*	1.66 ± 1.17
Pro-inflammatory mediators	*il1β*	0.49 ± 0.46
	il1r1	0.04 ± 0.03
	il6a	0.01 ± 0.01
	il6b	0.01 ± 0.01
	tnfα[a]	0.05 ± 0.02
	ifnγ2	0.02 ± 0.01
	il11	ND
	inos[a]	0.10 ± 0.07
	mmp9	0.41 ± 0.36
	p19	ND
	p35	0.02 ± 0.01
	p40a	ND
	p40b	ND
	p40c	2.36 ± 0.57
Anti-inflammatory mediators	*il10*	0.11 ± 0.08
	socs3_1&2	11.1 ± 9.77
	socs3_3&4	1.40 ± 1.13
Chemokines and chemokine receptors	*cxcl8_l2*	0.11 ± 0.07
	cxca_l1	0.07 ± 0.09
	cxcr1	0.06 ± 0.03
	cxcr2	0.04 ± 0.02
MH genes	*dab1/2*	1.09 ± 0.29
	dab3/4	0.63 ± 0.63
Toll-like receptors	*tlr1*	0.05 ± 0.02
	tlr2[a]	0.02 ± 0.004
	tlr3.1	ND
	tlr3.2	ND
	tlr4.1	ND
	tlr4.2	ND
	tlr5[a]	0.11 ± 0.13
	tlr7[a]	0.03 ± 0.01
	tlr8a[a]	0.49 ± 0.12
	tlr8b[a]	0.01 ± 0.005
	tlr9	0.01 ± 0.003
	tlr20	0.08 ± 0.04

[a] Common primers were used which recognise more than one copy of the gene.

Thrombocyte numbers decrease in spleen during *in vivo* infection

The thrombopoietic spleen is known to massively increase in size during *T. borreli* infections. To investigate the development of the thrombocyte population in the spleen during infection, carp were infected with either *T. borreli* or *T. carassii* parasites and the spleen removed for immuno-histochemical examination. Parasitaemia was determined by weekly examination of blood samples from infected fish and was found highest at 3 weeks post-infection (w.p.i.) with *T. borreli* after which parasitaemia decreased again (data not shown), as shown previously (Steinhagen et al., 1989). Parasitaemia for *T. carassii* increased by week 3 although not as high as for *T. borreli*, but remained at moderate–high levels for 5–6 weeks post-infection (data not shown). This is also in accordance with previous studies (Overath et al., 1999). Compared to healthy control fish, thrombocyte numbers dropped most dramatically in the spleen of *T. borreli*-infected fish and were lowest at 3 w.p.i., coinciding with the peak of parasitaemia, after which their number slowly restored to pre-infection levels towards 5 w.p.i. (Fig. 1). In contrast, thrombocyte numbers showed only a slight decrease in *T. carassii*-infected fish at 5 w.p.i. These data suggest a differential role of thrombocytes during *T. borreli* infections when compared to *T. carassii* infections; on the one hand underlining the differences between these two protozoan parasites but on the other demonstrating the plasticity of carp thrombocytes to react and be differentially involved in the response to infections.



◂ **Figure 1.** Immunohistochemical detection of thrombocytopenia in the spleen of infected carp. Spleens from healthy control, *T. borreli*-infected and *T. carassii*-infected fish were collected at the indicated time points post-infection and cryosections were stained with the carp thrombocyte-specific monoclonal antibody (WCL6). The representative pictures of one of two independent experiments are shown. Scale bar 200 μm.

Carp infected with *T. borreli* parasites show thrombocytopenia

The spleen is the thrombopoietic organ in carp. Depletion of thrombocyte precursors in the spleen by parasites such as *T. borreli* or *T. carassii* is likely to directly affect thrombocyte numbers in the periphery. To investigate the development of the thrombocyte population in carp during infection, carp were injected with either *T. borreli* or *T. carassii* parasites and PBL collected over different time points post-infection. Thrombocytes were analysed in PBL rather than spleen because teasing of spleen tissue would release large amounts of collagen, causing activation and aggregation of thrombocytes.

Naive control carp were shown to have 40–45% thrombocytes in their blood (Fig. 2). Carp infected with *T. borreli* showed unaffected thrombocyte numbers 1 week after infection, when parasitaemia still is relatively low ($<10^4$ *T. borreli*/mL). At 3 w.p.i., however, carp infected with *T. borreli* ($>10^7$ *T. borreli*/mL) had developed a severe thrombocytopenia that started to recover by 5 w.p.i. (Fig. 2A). In contrast, fish infected with *T. carassii* showed only a mild reduction in thrombocyte numbers in the blood and not before 5 w.p.i. (parasitaemia 10^5/mL approximately) (Fig. 2B). These data suggest that depletion of thrombocyte precursors in the spleen during *T. borreli* infection is reflected in lower levels of circulating thrombocytes.

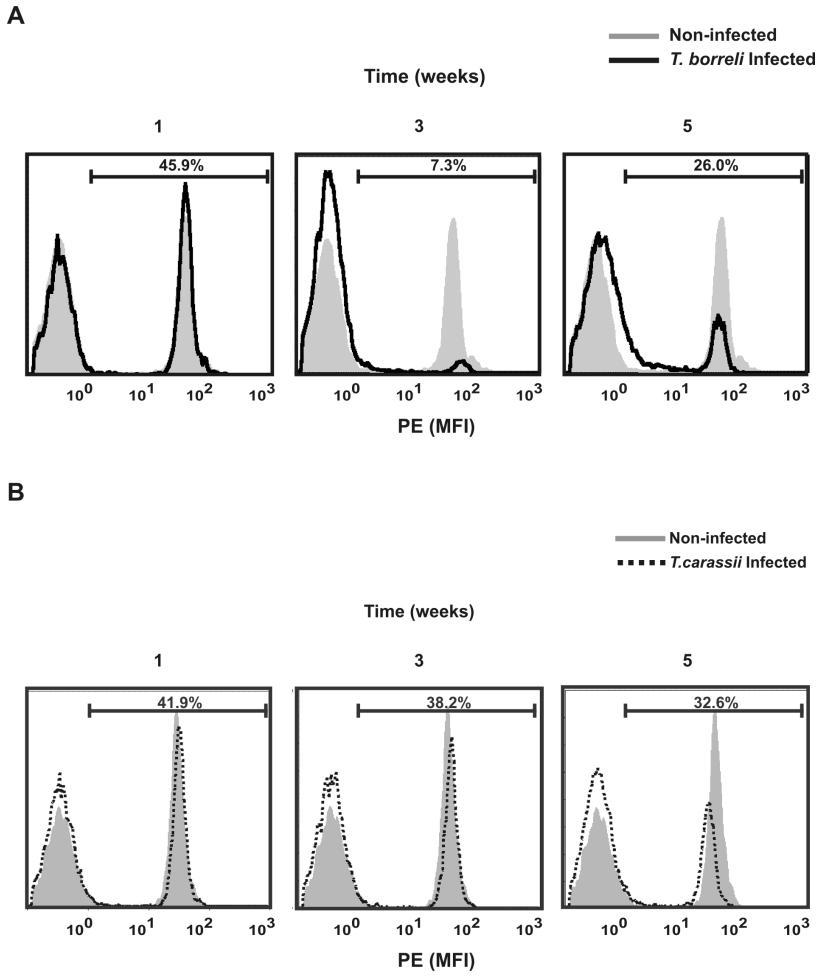


Figure 2. Flow cytometric analysis of thrombocytopenia in *T. borreli*-infected carp. PBL from non-infected, *T. borreli*-infected (A) and *T. carassii*-infected fish (B), were collected at the indicated time points post-infection and labelled with carp thrombocyte-specific monoclonal antibody (WCL6) followed by PE-labelled secondary antibody. Graphs show the percentage of $WCL6^+$ cells in PBL (mean fluorescence intensity, MFI) over time (1, 3 and 5 weeks post-infection).

Carp thrombocytes undergo nitric oxide-mediated apoptosis

The decrease in thrombocyte numbers in both spleen and blood, in particular during *T. borreli* infection, coincided with the typical peak in parasitaemia at 3 w.p.i. and associated

high nitric oxide levels, a hallmark of *T. borreli*, but not of *T. carassii* infections. To investigate a direct effect of NO on thrombocytes, thrombocytes sorted from naive fish were exposed to the NO donor SNAP or control compound (APSS) in the presence of *T. borreli* lysates and cell death was measured by flow cytometry. SNAP caused a significant increase in the number of early apoptotic thrombocytes (Annexin-V$^+$) in a dose dependent manner, whereas the number of late apoptotic (Annexin-V$^+$/7AAD$^+$) thrombocytes did not significantly change (Fig. 3A). The number of necrotic cells (7AAD$^+$) was always lower than 0.01%. The concentration of NO used on carp thrombocytes was physiologically relevant and within the range of concentrations found during *T. borreli* infection (Forlenza et al., 2008b) where serum nitrite levels can go as high as 400 µM (Fig. 3B). These data confirm that *T. borreli* not only affects thrombocyte precursors, but also mature circulating thrombocytes through an indirect mechanism based on the induction of high levels of toxic nitrogen radicals.

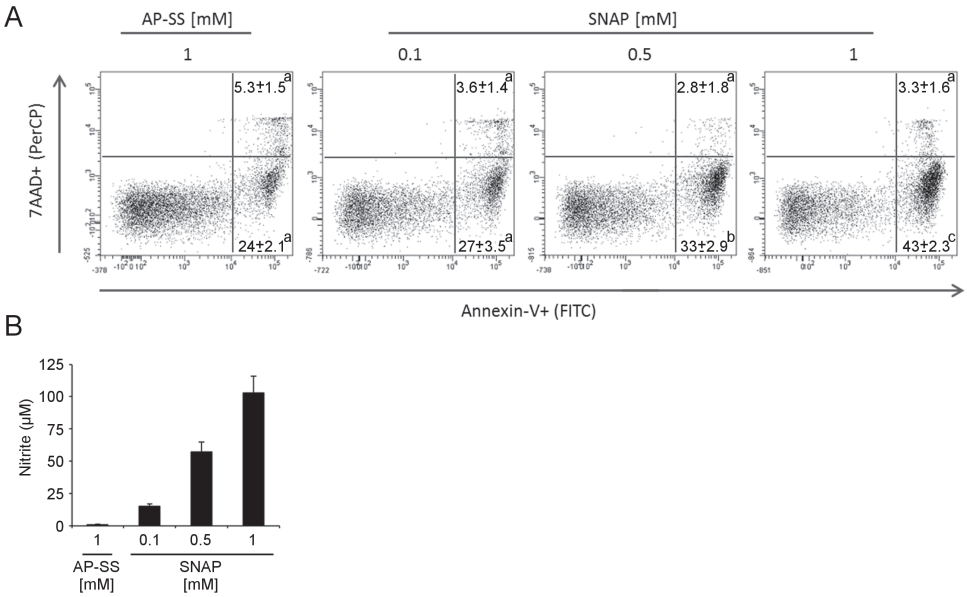


Figure 3. Nitric oxide (NO) promotes apoptosis of thrombocytes. (A) Sorted thrombocytes were treated with increasing amounts of NO donor (SNAP) or control compound (AP-SS) in the presence of parasite lysates equivalent to 0.5 x 10^6 parasites per 1 x 10^6 thrombocytes; after 48 hours, cells were stained with Annexin-V (FITC) and 7AAD (PerCP) to identify apoptotic and necrotic cells, respectively, and analysed by flow cytometry. Shown are the dot plots of one representative fish out of three, each experiment performed in triplicate. Numbers indicate averages and standard deviation of n = 3 fish; letters indicate significant differences as assessed by one-way ANOVA followed by a Tukey test. (B) Quantitation of the amount of nitrite produced by NO donor (SNAP) or control compound (AP-SS) over 24 hours measured by the Griess reaction. Bars show average + SD of duplicate measurements.

Discussion

In the present study we used a specific monoclonal antibody (WCL6) to study the involvement of carp thrombocytes in the immune response to protozoan infections. In blood, approximately 30–40% of the PBL are round- to spindle-shaped thrombocytes with canalicular and granular structures and an irregular and electron-dense nucleus, whereas in the thrombopoietic spleen several differentiation stages were identified (Rombout et al., 1996). To examine their potential role in immune responses we studied carp thrombocytes from PBL and spleen, both *ex vivo* and *in vivo* during infection with protozoan parasites. For studies on spleen we used immunochemical staining of tissue sections, as not to activate splenic thrombocytes by the release of collagen induced by the teasing of spleen tissue for cell isolation. In the spleen, known to massively increase in size during infections with the parasite *T. borreli* in particular, reduced numbers of thrombocytes are seen at 3 weeks post-infection, coinciding with the peak of parasitaemia, leading to severe thrombocytopenia. *Ex vivo*, addition of nitric oxide, the hallmark of *T. borreli* infections, induces a clear and rapid apoptosis of thrombocytes from healthy carp, supporting a role for nitric oxide-mediated control of immune-relevant thrombocytes during infection which might be to the advantage of *T. borreli*.

Gene expression studies on thrombocytes showed a clear immune profile with for instance characteristically high gene expression levels of the pro-inflammatory *il1β*. This indicates a function comparable to mammalian platelets that are recognised to have an extended role as immune cells that, among other things, can sense and respond to infectious pathogens as potentially dangerous, despite their lack of *de novo* gene expression (Jenne et al., 2013; Semple et al., 2011). In this role, platelets specialise in pro-inflammatory activities, and can secrete a large number of molecules, many of which display protective functions. Carp thrombocytes express *mmp9*, whose presence and function in mammalian platelets are being debated (Santos-Martinez et al., 2008; Seizer and May, 2013). One issue is whether *mmp9* detection in platelets is in fact caused by megakaryocyte/leukocyte contamination, which is practically unavoidable with current isolation methods (Wrzyszcz and Wozniak, 2012) and may also be the case for carp thrombocytes.

Thrombocytes express high levels of *p40c*, a subunit of Il12 and of Il23. Carp have three distinct *p40* genes (Huising et al., 2006) with *p40c* invariably highly expressed in all organs. Indeed, we found a very high gene expression of *p40c* in thrombocytes (>2 times higher than the housekeeping gene *40S* ribosomal protein *S11*) while the expression of the other two *p40* genes (*p40a* and *p40b*) was below the detection limit. Together with p35, p40 constitutes a disulphide-linked heterodimeric cytokine, Il12. In addition, together with p19, p40 can form a heterodimer to create Il23. However, both *p35* and *p19* constitutive

gene expression in thrombocytes was very low or undetectable, making it unlikely that carp thrombocytes express high constitutive levels of Il12 or Il23 protein. This is not surprising since also in humans, P35 and P19 are the factors limiting the expression of IL12 and IL23 (Gubler et al., 1991; Stern et al., 1990). Whether thrombocytes are able to up-regulate the expression of *p35* and/or *p19* would be an interesting subject of further investigation. Carp thrombocytes express high levels of not only *cxcl8*, but also anti-inflammatory *il10*. Interestingly, we found *socs3* to be very highly expressed, more than 11 times higher than the housekeeping gene. *Socs3* is also found in mammalian platelets (Burkhart et al., 2012) where it presumably plays a role in regulating megakaryocytic growth through thrombopoietin (Chaligné et al., 2009). Furthermore, carp thrombocytes express MH class II B genes (*dab*) as earlier described for rainbow trout thrombocytes (Kollner et al., 2004) but different from the mammalian situation where platelets are devoid of MHC class II molecules (Semple et al., 2011). Therefore it is clear that fish thrombocytes display a broad regulatory immune function.

Mammalian platelets have been shown to express several TLRs (Aslam et al., 2006; Cognasse et al., 2005; Shiraki et al., 2004) and similar findings have recently been observed for chicken thrombocytes (Ferdous and Scott, 2015). Constitutive gene expression was also tested for all *tlr*s presently characterised for carp. In humans, TLR1 and/or TLR2 are expressed to recognise molecular patterns from Gram-positive bacteria, TLR4 to recognise molecular patterns from Gram-negative bacteria, TLR5 to recognise flagellin and TLR9 to recognise CpG motifs in bacterial, viral or parasite DNA that generally links the activation of these TLRs to mostly sensing bacterial infections. Further, TLR3 is expressed to recognise dsRNA, and TLR7/ TLR8 are able to recognise ssRNA, linking these TLRs to the sensing of viral infections. Mammalian, as well as carp TLR2 has been linked to sensing glycosylphosphatidylinositol anchors from parasites (Campos et al., 2001; Debierre-Grockiego et al., 2007; Ribeiro et al., 2010a). The abundant presence of thrombocytes in carp blood would suggest that Tlr proteins expressed in carp thrombocytes would be present to sense infections in carp blood. High levels of gene expression were found for *tlr5* and *tlr8*, which would provide thrombocytes with the ability to sense both bacterial (*tlr5*, flagellin) and viral (*tlr8*, ssRNA) infections. The level of gene expression for *tlr3* (dsRNA) and *tlr4* (the ligand in fish is still unknown) was remarkably low. It appears that carp thrombocytes, similar to mammalian platelets, can play a role in sensing infectious pathogens as potentially dangerous. If carp thrombocytes are able to sense and present antigens, as suggested by the expression of both *tlr* and *dab* molecules, this would correlate well with the recent findings that carp thrombocytes have the capacity to phagocytose foreign particles (Nagasawa et al., 2014).

Carp are natural host for two different but related protozoan parasites *T. borreli* and *T. carassii*. Both parasites induce fundamentally different immune responses in their host (reviewed in Forlenza et al., 2011) with the most evident difference being increased levels of NO associated with *T. borreli* infections, but not *T. carassii* infections (Forlenza et al., 2008b; Joerink et al., 2006; Saeij et al., 2000, 2002). To explain the massive depletion of thrombocytes from spleen and peripheral blood typically observed during *T. borreli* infections, we studied the effect of NO on cell death of thrombocytes *ex vivo*. Addition of NO induced a clear and rapid apoptosis of thrombocytes from healthy carp, supporting a role for NO-mediated control of immune-relevant thrombocytes during infection with *T. borreli*. NO can also induce apoptosis of other cell types such as PBL-derived lymphocytes but less so head kidney-derived phagocytes, as we have shown in a previous study (Saeij et al., 2003). We also treated thrombocytes with increasing amounts of H_2O_2 which also showed an apoptotic effect (data not shown). It is possible that the combination of nitric oxide and reactive oxygen species *in vivo* can lead to the formation of peroxynitrite, which is an even more powerful oxidant and hence can have more detrimental effects on thrombocytes. The serum from *T. borreli*-infected fish could induce apoptosis of thrombocytes, unlike serum from *T. carassii*-infected or non-infected fish (unpublished data), which confirms the suspected presence of physiologically-relevant concentrations of radicals under *in vivo* circumstances, which can affect thrombocyte survival.

Our data suggest a potential strategy of *T. borreli* parasites to selectively deplete the host of thrombocytes via at least a nitric oxide-induced mechanism of apoptosis. In a previous study we could demonstrate that treatment of *T. borreli*-infected carp with the NO inhibitor aminoguanidine resulted in higher survival (Saeij et al., 2002), but thrombocyte numbers were not investigated at the time. In chickens infected with a parasite for which nitric oxide is known to be an important effector molecule (*Plasmodium gallinaceum* (Macchi et al., 2013), treatment with the NO inhibitor aminoguanidine reduced anaemia, thrombocytopenia and inflammation, and increased survival rate. It appears that both chicken and carp thrombocytes exposed to NO will undergo apoptosis. However, the situation in mammalian platelets seems slightly different; NO donors can inhibit rather than induce apoptosis of platelets but only when they are activated (Rukoyatkina et al., 2011; Sener et al., 2013). The underlying reasons for this apparent difference are not yet clear. It seems to be cell type-dependent, as exposure to NO has been reported to inhibit (Kotamraju et al., 2003) or stimulate (Wei et al., 2000) apoptosis in other cell types.

In this study we have shown that carp thrombocytes have an immune function, through gene expression of Toll-like receptors (sensing pathogens), major histocompatibility class II genes (antigen presentation), and several pro-inflammatory cytokines that can activate defence mechanisms. We used experimental infection models with *T. borreli*

and *T. carassii* parasites to show that thrombocytes are selectively depleted in the spleen and blood of *T. borreli*-infected fish, which are characterised by high NO levels. *Ex vivo* treatment of thrombocytes with an NO donor confirmed that exposure to NO resulted in thrombocyte apoptosis. This suggests a mechanism whereby *T. borreli* parasites can attenuate the host immune response by diminishing the thrombocyte population through NO-induced apoptosis.

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List of abbreviations

AP-SS: *N*-acetyl-D,L-penicillamine disulphide, IL: interleukin, MACS: magnetic-activated cell sorting, MFI: mean fluorescence intensity, MH: major histocompatibility, NO: nitric oxide, PBL: peripheral blood leukocytes, PE: phyco-erythrin, RT-qPCR: real-time quantitative PCR, SNAP: *S*-nitroso-*N*-acetylpenicillamine, SOCS: suppressor of cytokine signalling, TLR: Toll-like receptor, w.p.i.: weeks post-infection.

References

Aslam, R., Speck, E.R., Kim, M., Crow, A.R., Bang, K.W.A., Nestel, F.P., et al., 2006. Platelet Toll-like receptor expression modulates lipopolysaccharide-induced thrombocytopenia and tumor necrosis factor-α production in vivo. Blood 107, 637–641.

Bunnajirakul, S., Steinhagen, D., Hetzel, U., Körting, W., Drommer, W., 2000. A study of sequential histopathology of *Trypanoplasma borreli* (Protozoa: Kinetoplastida) in susceptible common carp *Cyprinus carpio*. Dis. Aquat. Org. 39, 221–229.

Burkhart, J.M., Vaudel, M., Gambaryan, S., Radau, S., Walter, U., Martens, L., et al., 2012. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. Blood 120, e73–e82.

Campos, M.A.S., Almeida, I.C., Takeuchi, O., Akira, S., Valente, E.P., Procopio, D.O., et al., 2001. Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. J. Immunol. 167, 416–423.

Chaligné, R., Tonetti, C., Besancenot, R., Marty, C., Kiladjian, J.J., Socié, G., et al., 2009. SOCS3 inhibits TPO-stimulated, but not spontaneous, megakaryocytic growth in primary myelofibrosis. Leukemia 23, 1186–1190.

Chang, C.F., Hamilton, P.B., 1979. The thrombocyte as the primary circulating phagocyte in chickens. J. Reticuloendothel. Soc. 25, 585–590.

Clark, S.R., Ma, A.C., Tavener, S.A., McDonald, B., Goodarzi, Z., Kelly, M.M., et al., 2007. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. Nat. Med. 13, 463–469.

Cognasse, F., Hamzeh, H., Chavarin, P., Acquart, S., Genin, C., Garraud, O., 2005. Evidence of Toll-like receptor molecules on human platelets. Immunol. Cell Biol. 83, 196–198.

Debierre-Grockiego, F., Campos, M.A., Azzouz, N., Schmidt, J., Bieker, *U.,* Resende, M.G., et al., 2007. Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived from *Toxoplasma gondii*. J. Immunol. 179, 1129–1137.

Duan, D., Sun, Z., Jia, S., Chen, Y., Feng, X., Lu, Q., 2013. Characterization and expression analysis of common carp *Cyprinus carpio* TLR5M. DNA Cell Biol. 32, 611–620.

Engelsma, M.Y., Stet, R.J.M., Schipper, H., Verburg-van Kemenade, B.M.L., 2001. Regulation of interleukin 1 beta RNA expression in the common carp, *Cyprinus carpio* L. Dev. Comp. Immunol. 25, 195–203.

Escolar, G., White, J.G., 1991. The platelet open canalicular system: a final common pathway. Blood Cells 17, 467–485.

Falco, A., Miest, J.J., Pionnier, N., Pietretti, D., Forlenza, M., Wiegertjes, G.F., et al., 2014. β-Glucan-supplemented diets increase poly(I:C)-induced gene expression of Mx, possibly via Tlr3-mediated recognition mechanism in common carp (*Cyprinus carpio*). Fish Shellfish Immunol. 36, 494–502.

Ferdous, F., Scott, T., 2015. Bacterial and viral induction of chicken thrombocyte inflammatory responses. Dev. Comp. Immunol. 49, 225–230.

Forlenza, M., de Carvalho Dias, J.D., Vesely, T., Pokorova, D., Savelkoul, H.F., Wiegertjes, G.F., 2008a. Transcription of signal-3 cytokines, IL-12 and IFNαβ, coincides with the timing of CD8αβ

up-regulation during viral infection of common carp (*Cyprinus carpio* L). Mol. Immunol. 45, 1531–1547.

Forlenza, M., Scharsack, J.P., Kachamakova, N.M., Taverne-Thiele, A.J., Rombout, J.H., Wiegertjes, G.F., 2008b. Differential contribution of neutrophilic granulocytes and macrophages to nitrosative stress in a host-parasite animal model. Mol. Immunol. 45, 3178–3189.

Forlenza, M., Magez, S., Scharsack, J.P., Westphal, A., Savelkoul, H.F., Wiegertjes, G.F., 2009. Receptor-mediated and lectin-like activities of carp (*Cyprinus carpio*) TNF-α. J. Immunol. 183, 5319–5332.

Forlenza, M., Fink, I.R., Raes, G., Wiegertjes, G.F., 2011. Heterogeneity of macrophage activation in fish. Dev. Comp. Immunol. 35, 1246–1255.

Fujiki, K., Shin, D.H., Nakao, M., Yano, T., 1999. Molecular cloning of carp (*Cyprinus carpio*) CC chemokine, CXC chemokine receptors, allograft inflammatory factor-1, and natural killer cell enhancing factor by use of suppression subtractive hybridization. Immunogenetics 49, 909–914.

Gubler, U., Chua, A.O., Schoenhaut, D.S., Dwyer, C.M., McComas, W., Motyka, R., et al., 1991. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. Proc. Natl. Acad. Sci. U.S.A. 88, 4143– 4147.

Henkel, C.V., Dirks, R.P., Jansen, H.J., Forlenza, M., Wiegertjes, G.F., Howe, K., et al., 2012. Comparison of the exomes of common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*). Zebrafish 9, 59–67.

Huising, M.O., Kruiswijk, C.P., van Schijndel, J.E., Savelkoul, H.F., Flik, G., Verburg-van Kemenade, B.M., 2005. Multiple and highly divergent IL-11 genes in teleost fish. Immunogenetics 57, 432–443.

Huising, M.O., van Schijndel, J.E., Kruiswijk, C.P., Nabuurs, S.B., Savelkoul, H.F., Flik, G., et al., 2006. The presence of multiple and differentially regulated interleukin-12p40 genes in bony fishes signifies an expansion of the vertebrate heterodimeric cytokine family. Mol. Immunol. 43, 1519–1533.

Huttenhuis, H.B., Taverne-Thiele, A.J., Grou, C.P., Bergsma, J., Saeij, J.P., Nakayasu, C., et al., 2006. Ontogeny of the common carp (*Cyprinus carpio* L.) innate immune system. Dev. Comp. Immunol. 30, 557–574.

Irnazarow, I., 1995. Genetic variability of Polish and Hungarian carp lines. Aquaculture 129, 215–219.

Jagadeeswaran, P., Sheehan, J.P., Craig, F.E., Troyer, D., 1999. Identification and characterization of zebrafish thrombocytes. Br. J. Haematol. 107, 731–738.

Jenne, C.N., Urrutia, R., Kubes, P., 2013. Platelets: bridging hemostasis, inflammation, and immunity. Int. J. Lab. Hematol. 35, 254–261.

Joerink, M., Forlenza, M., Ribeiro, C.M., de Vries, B.J., Savelkoul, H.F., Wiegertjes, G.F., 2006. Differential macrophage polarisation during parasitic infections in common carp (*Cyprinus carpio* L). Fish Shellfish Immunol. 21, 561–571.

Khandekar, G., Kim, S., Jagadeeswaran, P., 2012. Zebrafish thrombocytes: functions and origins. Adv. Hematol. 2012, 857058.

Kissel, K., Berber, S., Nockher, A., Santoso, S., Bein, G., Hackstein, H., 2006. Human platelets target dendritic cell differentiation and production of proinflammatory cytokines. Transfusion 46, 818–827.

Kollner, B., Fischer, U., Rombout, J.H., Taverne-Thiele, J.J., Hansen, J.D., 2004. Potential involvement of rainbow trout thrombocytes in immune functions: a study using a panel of monoclonal antibodies and RT-PCR. Dev. Comp. Immunol. 28, 1049–1062.

Kongchum, P., Hallerman, E.M., Hulata, G., David, L., Palti, Y., 2011. Molecular cloning, characterization and expression analysis of TLR9, MyD88 and TRAF6 genes in common carp (*Cyprinus carpio*). Fish Shellfish Immunol. 30, 361–371.

Kotamraju, S., Tampo, Y., Keszler, A., Chitambar, C.R., Joseph, J., Haas, A.L., et al., 2003. Nitric oxide inhibits H_2O_2-induced transferrin receptor-dependent apoptosis in endothelial cells: role of ubiquitin-proteasome pathway. Proc. Natl. Acad. Sci. U.S.A. 100, 10653–10658.

Krijgsveld, J., Zaat, S.A.J., Meeldijk, J., van Veelen, P.A., Fang, G., Poolman, B., et al., 2000. Thrombocidins, microbicidal proteins from human blood platelets, are C-terminal deletion products of CXC Chemokines. J. Biol. Chem. 275, 20374–20381.

Macchi, B.M.D., Miranda, F.J.B., Souza, F.S.D., Carvalho, E.C.Q.D., Albernaz, A.P., Nascimento, J.L.M.D., et al., 2013. Chickens treated with a nitric oxide inhibitor became more resistant to *Plasmodium gallinaceum* infection due to reduced anemia, thrombocytopenia and inflammation. Vet. Res. 44.

McRedmond, J.P., Park, S.D., Reilly, D.F., Coppinger, J.A., Maguire, P.B., Shields, D.C., et al., 2004. Integration of proteomics and genomics in platelets: a profile of platelet proteins and platelet-specific genes. Mol. Cell. Proteomics 3, 133–144.

Metz, J.R., Huising, M.O., Leon, K., Verb, U.K., Menade, B.M.L., Flik, G., 2006. Central and peripheral interleukin-1β and interleukin-1 receptor I expression and their role in the acute stress response of common carp, *Cyprinus carpio* L. J. Endocrinol. 191, 25–35.

Nagasawa, *T.,* Nakayasu, C., Rieger, A.M., Barreda, D.R., Somamoto, T., Nakao, M., 2014. Phagocytosis by thrombocytes is a conserved innate immune mechanism in lower vertebrates. Front. Immunol. 5, 445.

Overath, P., Haag, J., Mameza, M.G., Lischke, A., 1999. Freshwater fish trypanosomes: definition of two types, host control by antibodies and lack of antigenic variation. Parasitology 119, 591–601.

Piazzon, M.C., Savelkoul, H.F., Pietretti, D., Wiegertjes, G.F., Forlenza, M., 2015. Carp Il10 has anti-inflammatory activities on phagocytes, promotes proliferation of memory T cells, and regulates B cell differentiation and antibody secretion. J. Immunol. 194, 187–199.

Pietretti, D., 2013. Stimulation of the Innate Immune System of Carp: Role of Toll-Like Receptors. Wageningen University, Wageningen, The Netherlands, p. 216.

Pietretti, D., Scheer, M., Fink, I.R., Taverne, N., Savelkoul, H.F., Spaink, H.P., et al., 2014. Identification and functional characterization of nonmammalian Toll-like receptor 20. Immunogenetics 66, 123–141.

Pietretti, D., Vera-Jimenez, N.I., Hoole, D., Wiegertjes, G.F., 2013. Oxidative burst and nitric oxide responses in carp macrophages induced by zymosan, MacroGard(R) and selective dectin-1 agonists suggest recognition by multiple pattern recognition receptors. Fish Shellfish Immunol. 35, 847–857.

Rakus, K.L., Irnazarow, I., Forlenza, M., Stet, R.J., Savelkoul, H.F., Wiegertjes, G.F., 2009. Classical crosses of common carp (*Cyprinus carpio* L.) show co-segregation of antibody response with major histocompatibility class II B genes. Fish Shellfish Immunol. 26, 352–358.

Ribeiro, C.M., Hermsen, T., Taverne-Thiele, A.J., Savelkoul, H.F., Wiegertjes, G.F., 2010a. Evolution of recognition of ligands from Gram-positive bacteria: similarities and differences in the TLR2-mediated response between mammalian vertebrates and teleost fish. J. Immunol. 184, 2355–2368.

Ribeiro, C.M., Pontes, M.J., Bird, S., Chadzinska, M., Scheer, M., Verburg-van Kemenade, B.M., et al., 2010b. Trypanosomiasis-induced Th17-like immune responses in carp. PLoS ONE 5, e13012.

Rombout, J.H.W.M., Koumans-van Diepen, J.C.E., Emmer, P.M., Taverne-Thiele, J.J., Taverne, N., 1996. Characterization of carp thrombocytes with specific monoclonal antibodies. J. Fish Biol. 49, 521–531.

Rukoyatkina, N., Walter, U., Friebe, A., Gambaryan, S., 2011. Differentiation of cGMP-dependent and -independent nitric oxide effects on platelet apoptosis and reactive oxygen species production using platelets lacking soluble guanylyl cyclase. Thromb. Haemost. 106, 922–933.

Saeij, J.P.J., Stet, R.J.M., Groeneveld, A., Verburg-van Kemenade, L.B.M., van Muiswinkel, W.B., Wiegertjes, G.F., 2000. Molecular and functional characterization of a fish inducible-type nitric oxide synthase. Immunogenetics 51, 339–346.

Saeij, J.P.J., Van Muiswinkel, W.B., Groeneveld, A., Wiegertjes, G.F., 2002. Immune modulation by fish kinetoplastid parasites: a role for nitric oxide. Parasitology 124.

Saeij, J.P.J., van Muiswinkel, W.B., van de Meent, M., Amaral, C., Wiegertjes, G.F., 2003. Different capacities of carp leukocytes to encounter nitric oxide-mediated stress: a role for the intracellular reduced glutathione pool. Dev. Comp. Immunol. 27, 555–568.

Santos-Martinez, M.J., Medina, C., Jurasz, P., Radomski, M.W., 2008. Role of metalloproteinases in platelet function. Thromb. Res. 121, 535–542.

Savan, R., Igawa, D., Sakai, M., 2003. Cloning, characterization and expression analysis of interleukin-10 from the common carp, *Cyprinus carpio* L. Eur. J. Biochem. 270, 4647–4654.

Seizer, P., May, A.E., 2013. Platelets and matrix metalloproteinases. Thromb. Haemost. 110, 903–909.

Semple, J.W., Italiano, J.E., Jr., Freedman, J., 2011. Platelets and the immune continuum. Nat. Rev. Immunol. 11, 264–274.

Sener, A., Egemen, G., Cevik, O., Yanikkaya-Demirel, G., Apikoglu-Rabus, S., Ozsavci, D., 2013. In vitro effects of nitric oxide donors on apoptosis and oxidative/nitrative protein modifications in ADP-activated platelets. Hum. Exp. Toxicol. 32, 225–235.

Shiraki, R., Inoue, N., Kawasaki, S., Takei, A., Kadotani, M., Ohnishi, Y., et al., 2004. Expression of Toll-like receptors on human platelets. Thromb. Res. 113, 379–385.

St. Paul, M., Paolucci, S., Barjesteh, N., Wood, R.D., Schat, K.A., Sharif, S., 2012. Characterization of chicken thrombocyte responses to Toll-like receptor ligands. PLoS ONE 7, e43381.

Steinhagen, D., Kruse, P., Körting, W., 1989. The parasitemia of cloned *Trypanoplasma borreli* Laveran and Mesnil, 1901, in laboratory-infected common carp (*Cyprinus carpio* L.). J. Parasitol. 75, 685–689.

Stern, A.S., Podlaski, F.J., Hulmes, J.D., Pan, Y.-C.E., Quinn, P.M., Wolitzky, A.G., et al., 1990. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. Proc. Natl. Acad. Sci. U.S.A. 87, 6808–6812.

Stolte, E.H., Savelkoul, H.F., Wiegertjes, G., Flik, G., Lidy Verburg-van Kemenade, B.M., 2008. Differential expression of two interferon-γ genes in common carp (*Cyprinus carpio* L.). Dev. Comp. Immunol. 32, 1467–1481.

Tanekhy, M., Kono, T., Sakai, M., 2010. Cloning, characterization, and expression analysis of Toll-like receptor-7 cDNA from common carp, *Cyprinus carpio* L. Comp. Biochem. Physiol. Part D Genomics Proteomics 5, 245–255.

van der Aa, L.M., Chadzinska, M., Tijhaar, E., Boudinot, P., Verburg-van Kemenade, B.M., 2010. CXCL8 chemokines in teleost fish: two lineages with distinct expression profiles during early phases of inflammation. PLoS ONE 5, e12384.

Wei, T., Chen, C., Hou, J., Xin, W., Mori, A., 2000. Nitric oxide induces oxidative stress and apoptosis in neuronal cells. Biochim. Biophys. Acta 1498, 72–79.

Wrzyszcz, A., Wozniak, M., 2012. On the origin of matrix metalloproteinase-2 and -9 in blood platelets. Platelets 23, 467–474.

Xiao, Z.G., Liu, H., Fu, J.P., Hu, W., Wang, Y.P., Guo, Q.L., 2010. Cloning of common carp SOCS-3 gene and its expression during embryogenesis, GH-transgene and viral infection. Fish Shellfish Immunol. 28, 362–371.

Chapter 3

Heterogeneity of macrophage activation in fish

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Abstract

In this review, we focus on four different activation states of fish macrophages. *In vitro*, stimulation with microbial ligands induces the development of innate activated macrophages whereas classically activated macrophages can be induced by stimulation with LPS in combination with (recombinant) IFN-*γ*. Both types of macrophages show elevated phagocytic activity, expression of pro-inflammatory cytokine genes and radical production. Alternatively activated macrophages require the cytokines IL-4/IL-13 for induction of, among others, arginase activity. Until *in vitro* studies identify the effects of putative IL-4 and IL-13 homologues on fish macrophages, arginase enzyme activity remains the most reliable marker for the presence of alternatively activated macrophages in fish. The best evidence for the existence of regulatory macrophages, associated with the presence of IL-10, comes from *in vivo* studies, for example during parasitic infections of carp. Altogether, differentially activated macrophages in fish largely resemble the phenotypes of mammalian macrophages. However, the presence of fish-specific ligand recognition by TLRs and of duplicated genes coding for proteins with particular activities, poses additional challenges for the characterization of phenotype-specific gene signatures and cell surface markers.

Introduction

Macrophages arise from hematopoietic progenitors which differentiate directly, or *via* circulating monocytes, into subpopulations of tissue macrophages (Geissmann et al., 2010). Resident tissue macrophages of mammalian vertebrates can have various morphologic and phenotypic differences depending on the organ, and include Kupffer cells in the liver, alveolar macrophages in the lung, microglia cells in the central nervous system, osteoclasts in bone tissue and specialized macrophages in the spleen (Gordon and Taylor, 2005). All these types of macrophages are important for the maintenance of homeostasis, including the immune response to pathogens. The recent advancements in our understanding of macrophage development in teleosts, including the growth factors important for the regulation of macrophage development, have recently been summarized (Hanington et al., 2009) and are not part of this review. Rather, we will focus on the so-called macrophage activation states, reflecting the different phenotypes these cells acquire in response to distinct environmental signals. Based on the activation triggers and their resulting effector functions and cytokine profile, macrophages have been broadly divided into two types: classically activated macrophages induced in a T helper 1 (T_H1) cytokine environment, and alternatively activated macrophages, induced in a T_H2 cytokine environment (Stein et al., 1992; Goerdt and Orfanos, 1999; Mantovani et al., 2002; Gordon, 2003). Mirroring the T_H1 – T_H2 dichotomy, classically activated macrophages have also been termed M1, whereas alternatively activated macrophages have been termed M2 (Mills et al., 2000). More recently, classifications containing more subtypes of macrophage activation states have been introduced to take into account the diversity of macrophage phenotypes that are induced when these cells are exposed to different environmental signals (Mantovani et al., 2004; Mosser and Edwards, 2008).

In this review, we have adopted a definition of four different phenotypes of macrophages (Figure 1). Innate activation (i) is defined to occur when a macrophage responds to a microbial stimulus alone, whereas classical activation (ii) is defined to require a microbial stimulus plus the presence of the cytokine IFN-γ (Dalton et al., 1993). Compared to innate activated macrophages, classically activated macrophages present higher respiratory burst activity and iNOS expression as well as increased antigen presentation and co-stimulation (MHC class II and CD86, respectively) (Gordon and Taylor, 2005). It is worth noting that these activated macrophages, having such potent effector functions, must be kept under tight regulation to prevent them from causing damage to host tissues. We restrict the term alternatively activated macrophages (iii) to macrophages generated in the presence of the T_H2 cytokines IL-4 and/or IL-13. These cells, which have also been termed M2a (Mantovani et al., 2004) or wound healing macrophages (Mosser and Edwards, 2008) are characterized by increased arginase activity, production of proteins for extracellular matrix

and polyamines and indirectly counterbalance the activity of innate/classically activated macrophages by metabolizing L-arginine (Gordon and Martinez, 2010), the substrate for iNOS. Macrophages stimulated by Toll-like receptor (TLR) ligands in combination with a second signal that can be, for example, immune complexes, have been termed M2b or type-II, whereas macrophages which develop in response to IL-10, have been termed M2c or deactivating macrophages (Mantovani et al., 2004). Both, M2b and M2c macrophages produce high levels of IL-10 thereby directly contributing to the down-regulation of T_H1 immune responses. In this review, we will use the term regulatory macrophages (iv) for macrophages associated with the presence of IL-10.

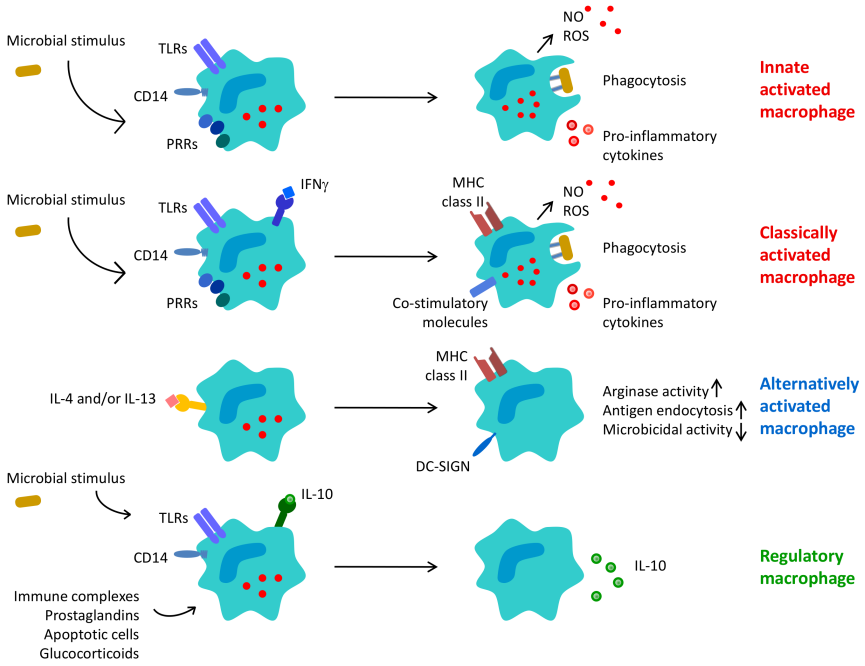

Figure 1. Activation of macrophages. Microbial stimuli are recognized by macrophages through Toll-like receptors (TLRs), CD14, or other pattern recognition receptors (PRRs). Stimulation with microbial antigens leads to the development of innate activated macrophages with increased phagocytic activity, production of pro-inflammatory cytokines, reactive oxygen species (ROS) and nitric oxide (NO). A microbial stimulus combined with IFN-γ induces classically activated macrophages that are characterized by a higher expression of MHC class II and co-stimulatory molecules in addition to the effector functions already described for innate activated macrophages. Alternatively activated macrophages develop in the presence of the cytokines IL-4 and/or IL-13 and express DC-SIGN as well as higher levels of MHC class II molecules. Alternatively activated macrophages have increased

arginase activity, antigen endocytosis and decreased microbicidal activity. Regulatory macrophages develop in response to IL-10 or upon stimulation with a microbial stimulus in combination with a second signal that can be, for example, immune complexes. Regulatory macrophages are characterized by the production of high levels of IL-10.

It is important to point out that there is not a rigid barrier between these macrophage phenotypes and that, indeed, cells exhibiting characteristic markers from more than one of these "activation states" can be observed (Bronte et al., 2003; Ghassabeh et al., 2006; Mosser and Edwards, 2008). This plasticity of macrophages has added to the confusion regarding the existence of individual macrophage sub-types. Another confounding factor is the dissimilarity between different species; given the fact that macrophages of mouse and man show important differences (Mestas and Hughes, 2004; Gordon and Martinez, 2010) it should not be surprising to observe differences between fish and mouse macrophages, or even between macrophages of different fish species. In this review we will discuss the state of the art on innate and classically activated macrophages as opposed to alternatively activated and regulatory macrophages in teleost fish.

Activation of macrophages

Innate activation of macrophages

Innate activated macrophages are induced by a microbial stimulus, which can be detected by various receptors on the macrophage surface including TLRs (Janeway and Medzhitov, 2002), scavenger receptors, C-type lectins and complement receptors (Taylor et al., 2005). These macrophages are able to phagocytose pathogens and exert major effects through the production of (pro-inflammatory) cytokines and growth factors (Mosser, 2003; Gordon and Taylor, 2005). Important molecules associated with innate activated macrophages are the microbicidal reactive oxygen species (ROS) generated during respiratory burst activity and the reactive nitrogen metabolite nitric oxide (NO), produced by the enzyme inducible nitric oxide synthase (iNOS).

For many years the most extensively studied activation state (*in vitro*) of fish macrophages has been the innate activation. The off-the-shelf availability of (lipo-/glyco-) proteins with pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS) from *Escherichia coli* (Gram-negative), lipoteichoic acid (LTA) or peptidoglycan (PGN) from *Staphylococcus aureus* (Gram-positive), flagellin from Gram-negative bacteria and polyI:C as a synthetic double-stranded RNA, unknowingly favoured the characterization of innate activated macrophages in fish. In most, if not all fish species, *in vitro* stimulation

of macrophages with microbial stimuli alone, leads to increased respiratory burst activity and associated production of oxygen radicals, increased phagocytosis and production of pro-inflammatory cytokines. These *in vitro* studies, however, have made clear that, despite a general conservation of effector functions typical of innate activated macrophages, differences between fish species do exist. For example, *in vitro* stimulation of macrophages from cyprinid fish with LPS readily induces the up-regulation of iNOS (Laing et al., 1996; Saeij et al., 2000b) and production of NO (Saeij et al., 2000b; Stafford et al., 2001b, 2002; Forlenza et al., 2008) whereas salmonid macrophages may up-regulate iNOS gene expression (Laing et al., 1999) but produce negligible amounts of NO (Stafford et al., 2001a), perhaps reflecting similar differences between mouse and human macrophages (Mestas and Hughes, 2004). No matter what, innate activated macrophages are induced by a microbial stimulus alone, with LPS being one of the most frequently used stimuli.

The molecular mechanism by which LPS stimulates an immune response has been a focus of attention in mammalian vertebrate immunology, particularly in view of the potential lethal effects of endotoxin associated with sepsis. LPS is a glycolipid present in the outer membrane of Gram-negative bacteria that is composed of (i) a hydrophobic lipid-A component, (ii) hydrophilic polysaccharides associated with the core and (iii) a glycan polymer termed O-antigen. The lipid-A portion is a conserved molecular pattern and the main inducer of biological responses to LPS (Raetz and Whitfield, 2002; Bishop, 2005). LPS is removed from the bacterial membrane and transferred to TLR4 by two accessory proteins: LPS-binding protein (LBP) and CD14, both essential for LPS recognition (Mathison et al., 1992; Tobias et al., 1995). The accessory molecule MD-2, which lacks transmembrane and intracellular regions, associates with the extracellular domain of TLR4 and is believed to be the component of the TLR4-MD-2 complex that interacts with the lipid A portion of LPS (Kim et al., 2007). Activation of macrophages *via* LPS-recognition by the TLR4 complex, generally leads to the transcription of pro-inflammatory cytokines such as TNFα, and enhancement of anti-microbial activities.

Non-mammalian vertebrates, and in particular fish, are not very susceptible to the toxic effects of LPS that lead to septic shock and in some cases even acquire tolerance to LPS upon repeated exposure. The relative insensitivity to LPS has been suggested a fish strategy aimed at the survival in the aquatic environment with high pathogenic pressure (Iliev et al., 2005; Novoa et al., 2009). Although very high (10–50 μg/ml) concentrations of LPS are needed to induce measurable amounts of NO in freshly isolated carp monocytes/macrophages, considerably lower concentrations (0.1 μg/ml, Jurecka et al., 2009) are required to stimulate NO production in cultured mature macrophages (Joerink et al., 2006b). Still, these concentrations are several-fold higher than the concentrations used to stimulate murine and human macrophages (nanogram range). Owing to several genome

initiatives and EST analyses, it is becoming clear that several if not most fish species, including pufferfish (*Takifugu rubripes*), tetraodon (*Tetraodon nigroviridis*), and possibly salmonid fish do not possess a TLR4 homologue (Leulier and Lemaitre, 2008; Rebl et al., 2010). The exception to the rule appears to be the family of Cyprinidae; both zebrafish (*Danio rerio*) and common carp (*Cyprinus carpio*) do possess a TLR4 homologue (Rebl et al., 2010). The same genome initiatives have also made clear that several if not all co-receptor molecules (LBP, CD14, MD-2) and intracellular mediators (TICAM2) essential to the recognition of LPS are absent in the fish families investigated thus far (Iliev et al., 2005). Functional studies in zebrafish confirm that the TLR4 does not recognize LPS (Sepulcre et al., 2009; Sullivan et al., 2009) conveying the important message that sequence homology does not necessarily equal functional conservation, at least with respect to the LPS binding ability of TLR4. Whether other TLR4 ligands such as extra domain A (EDA) of fibronectin (Okamura et al., 2001), can be recognized by TLR4 in cyprinid fish awaits further investigation.

As mentioned above, LPS has been extensively used for *in vitro* stimulation of fish macrophages and interestingly, stimulation with LPS does indeed lead to an innate activation of fish macrophages, despite the apparent absence in some fish species of a TLR4 receptor complex. Recently, it has been suggested that the key mediator of cytokine gene expression, at least in macrophages of rainbow trout, is not the endotoxin moiety of LPS but 'contaminating' PGN in crude preparations of LPS (MacKenzie et al., 2010). Of course, the microbial stimuli required to induce innate activation of macrophages can be diverse and are different for different groups of pathogens. In mammalian vertebrates, recognition of PAMPs such as LTA or PGN from Gram-positive bacteria is mediated by TLR2 (Aliprantis et al., 1999; Schwandner et al., 1999; Takeuchi et al., 1999; Takeda et al., 2002; Schroder et al., 2003). Less frequently, PGN from Gram-negative bacteria is studied. In rainbow trout, PGN from Gram-negative bacteria proved a potent inducer of cytokine gene expression (IL-1β, IL-6), more potent than ultrapure LPS and also more potent than PGN from Gram-positive bacteria (MacKenzie et al., 2010). Rainbow trout macrophages were hypo- or non-responsive to the prototypical TLR2 ligands LTA and synthetic lipopeptide Pam_3CSK_4(MacKenzie et al., 2010). Despite the fact that TLR2 homologues have been identified in several fish species (Rebl et al., 2010), including Antarctic fish (Acc. No. ACT64128), a TLR2 homologue has not yet been identified in salmonid fish (Palti et al., 2010), possibly explaining their unresponsiveness to prototypical TLR2 ligands. In contrast, in carp, LTA and PGN from Gram-positive bacteria (*S. aureus*), triacylated Pam_3CSK_4, but not diacylated lipopeptide MALP-2, do activate macrophages and also human HEK 293 cells transfected with carp TLR2 (Ribeiro et al., 2010a). Altogether, these results suggest a conservation of recognition of ligands from Gram-positive bacteria in cyprinid but not salmonid fish. Clearly, despite a general conservation

of effector functions typical of innate activated macrophages, large differences in TLR-based recognition of a microbial stimulus exist between fish species.

Innate activated macrophages detect microbial stimuli by various receptors on their surface including TLRs, of which different families recognize different groups of related pathogen-associated molecular patterns. Vertebrate TLRs can be subdivided into six major groups (Roach et al., 2005), including the TLR1 family (for lipopeptide), the TLR3 family (for dsRNA), the TLR4 family (for LPS), the TLR5 family (for flagellin), the TLR7–9 family (for nucleic acid and heme motifs) and the TLR11 family (ligand not well described). As discussed above for TLR2 and TLR4, it is becoming clear that not all properties of mammalian TLRs are shared by their homologues in modern bony fish and that some TLRs might not be present in all fish species. Furthermore, fish-specific TLRs, additional to the array of mammalian TLRs (Oshiumi et al., 2008), have been identified with sometimes unknown ligand specificities. Of course, fish macrophages must express a large number of innate receptors other than TLRs. For example, in mammalian vertebrates, besides TLR2, several families of pattern recognition receptors can detect PGN from Gram-positive bacteria including nucleotide-binding oligomerization domain 2 (NOD2) and peptidoglycan recognition proteins (PGRPs) (Guan and Mariuzza, 2007). The NODs are cytosolic pattern recognition receptors that detect bacterial components. Although both NOD1 and NOD2 have been described (Stein et al., 2007; Laing et al., 2008; Sha et al., 2009; Chen et al., 2010a; Chang et al., 2011) and their gene expression shown to be upregulated *in vivo* upon PGN injection of grass carp (Chen et al., 2010a), *in vitro* activation studies are required to confirm the presence of these receptors on macrophages and conclusively link them to PGN recognition. In insects, PGRPs activate the Toll or immune deficiency (Imd) signal transduction pathways or induce proteolytic cascades that generate antimicrobial products, induce phagocytosis, hydrolyze peptidoglycan and protect against infections. In zebrafish, suppression of PGRP6 significantly decreased the expression of TLR2 mRNA indirectly suggesting that TLR2 and PGRP may cooperatively recognize PGN (Chang and Nie, 2008). Thus, fish macrophages are likely to express several innate receptors that can recognize a microbial stimulus in the absence of a particular TLR. Regardless of which receptor is used for PAMP recognition and subsequent activation of the cell, *in vitro* studies clearly prove the existence of innate activated macrophages in fish.

Classical activation of macrophages

Classically activated macrophages are induced by a combination of IFN-γ and a microbial stimulus, where the latter is required for the production of TNFα (Mosser, 2003; Mantovani et al., 2004; Gordon and Taylor, 2005; Gordon, 2007; Mosser and Edwards, 2008). In

mammals, NK cells are an important early (but transient) source of IFN-γ, whereas T_H1 cells provide a more sustained production of IFN-γ once the adaptive immune response is underway (Mosser and Edwards, 2008). Once activated by IFN-γ and TNFα, classically activated macrophages produce pro-inflammatory cytokines such as IL-1, IL-6, IL-12, IL-23 and TNFα. This drives the immune response in a T_H1 direction towards cell-mediated immunity characterized by IFN-γ and activated macrophages able to destroy ingested and intracellular pathogens.

Two cytokines especially, are required for classical activation of macrophages; IFN-γ and TNFα, both of which have been described in fish (Hirono et al., 2000; Laing et al., 2001; Garcia-Castillo et al., 2002; Saeij et al., 2003; Zou et al., 2004; Igawa et al., 2006; Milev-Milovanovic et al., 2006; Robertsen, 2006; Glenney and Wiens, 2007; Haugland et al., 2007; Ordas et al., 2007; Grayfer et al., 2008; Stolte et al., 2008; Covello et al., 2009; Furnes et al., 2009; Grayfer and Belosevic, 2009). In zebrafish, catfish, common carp and goldfish, two types of IFN-γ have been identified. Both isoforms, initially named IFN-γ1 and IFN-γ2, contain typical IFN-γ signature motifs. Whereas IFN-γ1 is shorter than the mammalian vertebrate IFN-γ and does not contain a C-terminal cationic residue required for IFN-γ activity, IFN-γ2 is structurally similar to mammalian IFN-γ. Consequently, fish IFN-γ1 and IFN-γ2 are now referred to as IFN-γ related (IFN-γrel) and IFN-γ, respectively (Savan et al., 2009). Also for TNFα, the other cytokine required for classical macrophage activation, most fish species investigated show the presence of two isoforms, named TNFα1 and TNFα2. Although structurally related to each other and to their mammalian homologue, functional activities of fish TNFα appear species-specific (Zou et al., 2003b; García-Castillo et al., 2004; Grayfer et al., 2008; Roca et al., 2008; Forlenza et al., 2009a), in particular with respect to the ability of fish TNFα to directly activate phagocytes.

It is only recently that the availability of biologically active recombinant proteins for the cytokines IFN-γ and TNFα has allowed for *in vitro* studies on classically activated fish macrophages. Recombinant IFN-γ alone was shown to stimulate, in trout macrophages, gene expression of IFN-γ-inducible protein 10 (γIP-10), MHC class II β-chain and antiviral genes, and enhanced respiratory burst activity (Zou et al., 2005). In goldfish, recombinant IFN-γ alone increased the expression of several pro-inflammatory genes and increased the phagocytic and nitric oxide response of macrophages (Grayfer and Belosevic, 2009). Interestingly, stimulation of goldfish macrophages with both IFN-γ and TNFα2 resulted in a synergistic effect on nitric oxide production, respiratory burst and phagocytic activities. In carp, differently from trout or goldfish IFN-γ, IFN-γ alone was not able to enhance the expression of pro-inflammatory molecules, neither respiratory burst nor nitric oxide production of phagocytes (macrophages and neutrophilic granulocytes). However, when

recombinant carp IFN-γ was used in combination with LPS a powerful synergistic effect was observed on iNOS gene expression, nitric oxide production, respiratory burst activity and expression of pro-inflammatory cytokines (Arts et al., 2010). Additional studies on the potential (synergistic) effects of IFN-γ and TNFα are needed to further determine the effects of these cytokines on (classically activated) fish macrophages.

As mentioned above, fish possess a second IFN-γ isoform with only a low degree of sequence similarity to IFN-γ, named IFN-γrel. In carp and in grass carp, both isoforms are regulated by different stimuli (Stolte et al., 2008; Chen et al., 2010b). Carp recombinant IFN-γrel, differently from recombinant IFN-γ, failed to prime any antimicrobial activity in phagocytes, either alone or in combination with LPS (Arts et al., 2010). This would suggest IFN-γ, but not IFN-γrel, to be the functional equivalent of mammalian IFN-γ in carp. However, *in vivo* bacterial infection of zebrafish embryos indicated that IFN-γ and IFN-γrel act partly redundantly. The last conclusion is based on the absence of qualitative differences in target gene expression induced by one IFN-γ isoform or the other, and on the observation that knockdown of either IFN-γ alone did not interfere with the ability of zebrafish embryos to clear bacterial infection (Sieger et al., 2009). These findings suggest that, at least *in vivo*, zebrafish IFN-γ and IFN-γrel have largely overlapping functions. Different from this observation in zebrafish, gene expression studies in goldfish have shown that, at least *in vitro*, the two IFN-γ isoforms, alone or in combination with recombinant TNFα2, trigger the expression of distinct sets of target genes (Grayfer and Belosevic, 2009; Grayfer et al., 2010). However, with respect to the ability of recombinant goldfish IFN-γ and IFN-γrel to trigger antimicrobial activities, both isoforms presented distinct capacities to induce monocyte/macrophage antimicrobial functions. In goldfish, IFN-γrel elicited a robust but relatively short-lived priming of monocytes for respiratory burst, and down-regulated the priming potential of IFN-γ and of TNFα2. Goldfish IFN-γrel induced significantly higher phagocytosis and nitrite production in monocytes and macrophages, respectively, when compared to IFN-γ (Grayfer et al., 2010). These findings indicate that, at least in goldfish, IFN-γrel and IFN-γ mediate specific pro-inflammatory responses of myeloid cells. If true, the segregation of IFN-γ into two functional isoforms in bony fish would be different from the single gene present in all other vertebrates examined. Definitely, the above-mentioned findings in (cyprinid) fish paint a complex picture of cytokine-regulated macrophage activation.

It may be clear that, despite an apparent conservation of the role of IFN-γ in the generation of classically activated macrophages, the underlying mechanisms are not yet fully understood in fish and may not always follow the mammalian dogmas. For example, in light of the above-discussed inability of LPS to signal *via* TLR4 and putative contamination of LPS preparations with PGN, the observed synergistic effect of IFN-γ and

LPS in carp is not easily explained. Although LPS readily induces TNFα gene expression, it has been shown that TNFα is not a potent activator of macrophages (at least in carp, zebrafish and seabream) (Roca et al., 2008; Forlenza et al., 2009a), and direct stimulation of macrophages with recombinant TNFα does not result in an activation of antimicrobial activities. Furthermore, inhibition of TNFα protein expression during trypanoplasma infection in carp (Forlenza et al., 2009a) did not influence iNOS gene expression. Thus, despite the synergistic effect of IFN-γ and LPS observed *in vitro*, whether TNFα is the co-stimulatory molecule cooperating with IFN-γ is not fully understood. These studies make clear that the underlying mechanisms responsible for the biological effects of IFN-γ and TNFα on fish macrophages need to be further elucidated.

In conclusion, the cytokine-mediated mechanisms through which classical activation of macrophages is achieved may differ between fish and mammalian vertebrates. Furthermore, differences between fish (families) exist with respect to the activities of the cytokines involved and the (TLR) receptors required for recognition of microbial compounds. If for a long time, we erroneously assumed that gene duplication implied functional redundancy, recent advances in recombinant and gene technology have revealed that multiple isoforms may have been retained to achieve distinct functions. No matter what receptor route is taken to recognize microbial PAMPs, and no matter what cytokine (iso)form is required to activate the cell, *in vitro* studies clearly suggest that classically activated macrophages, characterized by an inflammatory state and enhanced antimicrobial capacity, exist in fish.

Alternative activation of macrophages

Alternatively activated macrophages are generated in the presence of the T_H2 cytokines IL-4 and/or IL-13 (Gordon and Martinez, 2010). A feature typical of alternatively activated macrophages is that they metabolize L-arginine differently from the way innate/classically activated macrophages do (Modolell et al., 1995). Where the enzyme iNOS is omnipresent in innate/classically activated macrophages and converts L-arginine into L-citrulline and NO, alternatively activated macrophages compete for the same substrate by up-regulating arginase enzymatic activity, and convert L-arginine into L-ornithine and urea (Figure 2). Thus, alternatively activated macrophages, through the activation of arginase, rendering L-arginine unavailable for conversion by iNOS into NO, may act as 'anti-inflammatory' macrophages by attenuating the production of NO (Modolell et al., 1995).

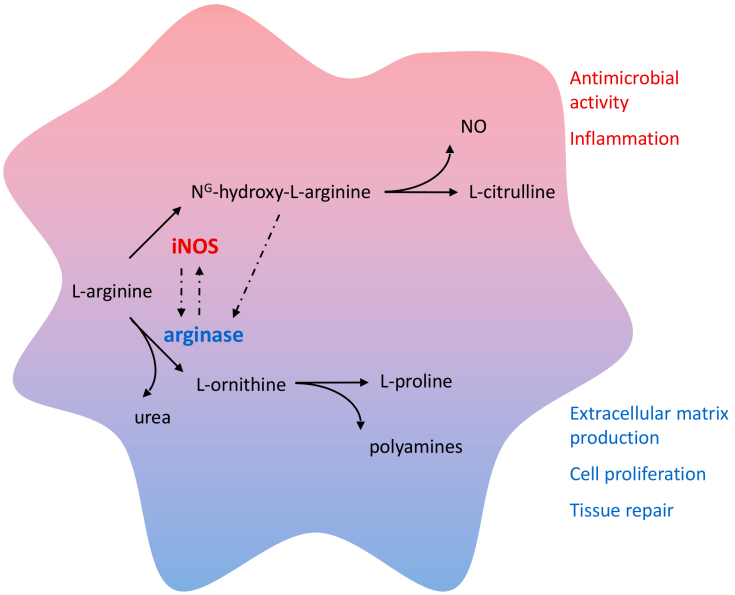

Figure 2. L-Arginine metabolism of macrophages. The enzymes iNOS and arginase use a common substrate, L-arginine, to initiate different pathways that negatively regulate each other as indicated by the dashed lines. The pathway initiated by iNOS leads to production of NO and L-citrulline and a phenotype associated with antimicrobial activity and inflammation, hallmarks of (innate and) classically activated macrophages. The pathway initiated by arginase leads to the formation of urea, and metabolites (L-proline and polyamines) linked to extracellular matrix production, cell proliferation and tissue repair and is associated with the development of alternatively activated macrophages.

In fish, two genes with homology to both IL-4 and IL-13 have been reported (Li et al., 2007; Ohtani et al., 2008). However, unlike IL-4 and IL-13 of mammalian vertebrates, the two fish genes are not located in one T_H2 locus on a single chromosome. At present, it is unknown whether the two molecules represent duplicated genes that have acquired distinct functions, similar to what has been described for the IFN-γ isoforms, or whether they display functional redundancy. The two homologous genes are tentatively referred to as IL-4-like (IL-4L) and IL-4-related (IL-4rel) and it seems that only functional studies can conclusively classify these genes as IL-4 or IL-13 orthologues. At this moment, no functional studies on the IL-4L protein have been reported. However, a recombinant IL-4rel protein has recently been produced and functional studies in zebrafish have shown that injection of IL-4rel leads to enhanced B-cell proliferation (Hu et al., 2010), which is in accordance with the functional properties of mammalian vertebrate IL-4 (Callard, 1989; Clark et al., 1989). In another study, the same recombinant IL-4rel protein

increased DC-SIGN/CD209 expression on leukocyte cell surfaces (Lin et al., 2009). The latter observation especially, is in agreement with the functional effects of IL-4/IL-13 on alternatively activated macrophages in mammalian vertebrates. These effects include increased expression of DC-SIGN along with increased endocytosis of mannosylated ligands, down-regulation of latex bead phagocytosis, increased antigen presentation to T_H2 cells and decreased microbicidal functions (Martinez et al., 2009). In this context it could be rewarding to study the presence of IL-4rel-specific receptors on fish macrophages. In mammalian vertebrates, the heterodimeric receptor complex for IL-4 and IL-13 consists of one receptor subunit in common for both cytokines (IL-4Rα), which is in fact the molecular basis for their overlapping biological functions, and one receptor subunit specific for each cytokine (Mueller et al., 2002). Although similar sequences for these T_H2 cytokine receptors are present in the zebrafish database (Acc. No. NP 001013300), no studies have been performed to link the presence of IL-4/IL-13 cytokine receptors to macrophage polarization. No doubt, functional studies on the effects of IL-4-like and IL-4-rel proteins on (purified) macrophage cell populations should be helpful to study the link between these presumed T_H2 cytokines with the generation of alternatively activated macrophages in fish.

Arginase activity as a marker for alternatively activated macrophages

Arginase is a manganese metallo-enzyme that catalyses the hydrolysis of L-arginine to L-ornithine and urea and is the main enzyme responsible for the cyclic nature of the urea cycle (Wu and Morris, 1998). In mammalian vertebrates, arginase activity can be indicative of either of two distinct isoforms encoded by two separate genes (Jenkinson et al., 1996). Arginase-1 takes part in the ornithine-urea cycle aimed at ammonia detoxification, is located in the cell cytosol and is mainly expressed in the liver, whereas arginase-2 is located in the mitochondria. Arginase is necessary for the production of proline and glutamate, important for cell proliferation and collagen production during extracellular matrix regeneration (Albina et al., 1990; Vincendeau et al., 2003). In mice it is the expression of arginase-1 in particular (Munder et al., 1999), that is associated with the presence of IL-4/IL-13 induced alternatively activated macrophages, whereas LPS+IL-10-induced regulatory macrophages are characterized by enhanced expression of arginase-2 (Lang et al., 2002).

Also in fish, arginase-1 and arginase-2 are present as distinct isoforms that cluster with their respective mammalian counterparts, suggesting a conservation of structural differences. Polyploid fish have undergone further duplications of the arginase genes, sometimes resulting in multiple copies of both arginase isoforms (Wright et al., 2004; Joerink et al., 2006c). Both fish arginases contain a mitochondrial targeting sequence and the absence of a cytosolic form of arginase in fish may be related to the fact that most

fish are ammoniotelic animals, excreting their excess nitrogenous wastes as ammonia directly into the water. In carp, arginase-1 gene expression was found mainly in the mid kidney, whereas arginase-2 gene expression was detected in all organs, with the highest expression in liver (Joerink et al., 2006c). In rainbow trout, although arginase-1 gene expression is highest in liver, fasting animals regulate arginase-2 gene expression (Wright et al., 2004). In addition, up-regulation of either arginase gene leads to increased arginase enzyme activity and may thus be associated with the presence of alternatively activated macrophages in fish. It may be clear that, until the exact role of the two arginase isoforms in fish has been resolved, it is best to determine the gene expression of both genes.

There are a number of studies in fish that have measured arginase gene expression as part of an immune response to infection. In carp, infection with the protozoan parasite *Trypanosoma carassii* induced an up-regulation of arginase-1, but not arginase-2 gene expression in head kidney (Joerink et al., 2006a); mechanical skin injury induced arginase-2, but not arginase-1 gene expression during the first few hours after skin damage (Gonzalez et al., 2007) and injection of zymosan in the peritoneum resulted in an influx of phagocytes with increased gene expression of, among others, arginase-2 (arginase-1 not measured; (Chadzinska et al., 2008)), that could be enhanced by co-injection of morphine (Chadzinska et al., 2009). In Atlantic salmon, infection with ectoparasitic caligid crustaceans induced up-regulation of arginase-1 gene expression in intact skin of infected fish (arginase-2 not measured) (Skugor et al., 2008); infection with the bacterium *Aeromonas salmonicida* showed induced gene expression for both arginase-1 and -2 (Fast et al., 2009) and injection with oil-adjuvanted vaccines against this bacterium induced granulomatous reactions associated with increased arginase-1 gene expression in head kidney (Mutoloki et al., 2010). Although it is evident that gene expression studies can only be suggestive of the presence *in vivo* of alternative macrophage activation, the use of an iNOS/arginase gene expression index (Fast et al., 2009) may be a useful way to quantify macrophage polarization during immune responses to pathogens *in vivo*.

A lack of arginase mRNA expression does not always imply a lack of arginase activity, or vice versa and total arginase enzymatic activity can be indicative of the activation of arginase-1, arginase-2 or both (Laberge et al., 2009). Thus, the most reliable marker for the presence of alternatively activated macrophages in fish is arginase enzymatic activity. Studies in mice have shown that arginase activity can be induced *via* the increase of intracellular cAMP and tyrosine kinase phosphorylation as well as by the administration of exogenous cAMP (Munder et al., 1999). Similarly, studies in carp using head kidney-derived macrophages have shown that administration of exogenous cAMP induced arginase-2, but not arginase-1 gene expression and clearly induced arginase activity but not nitric oxide production in these macrophages (Joerink et al., 2006c). In carp, as in mice,

arginase enzymatic activity could be specifically inhibited by N^G-hydroxy-L-arginine (Figure 2) which, as intermediate in the conversion of L-arginine into L-citrulline and NO, inhibits arginase by specifically interacting with the manganese-cluster of the active site of the arginase enzyme. Altogether, these *in vitro* results provide the best evidence to date that arginase enzymatic activity could be a useful marker for the presence of alternatively activated macrophages in fish.

Regulatory macrophages

Regulatory macrophages are associated with the presence of IL-10 and are therefore involved in the regulation of pro-inflammatory responses and in the dampening of inflammatory reactions. They can be generated in the presence of TLR ligands in combination with a second signal that can be immune complexes, prostaglandins, apoptotic cells, glucocorticoids or G-protein coupled receptor ligands (M2b) or IL-10 alone (M2c) (Mantovani et al., 2004). Most fish species possess homologues of the six major families of TLRs as well as of all molecules acting as signals in the generation of regulatory macrophages. However, as discussed above in the context of innate activation, several fish TLRs may have properties unique to modern bony fish. Nevertheless, all signals required for the development of regulatory macrophages should be present in fish. What remains to be proven is the presence of macrophages with a true regulatory phenotype. Homologues to mammalian vertebrate IL-10 have been identified in several teleost species (Lutfalla et al., 2003; Savan et al., 2003; Zou et al., 2003a; Inoue et al., 2005; Zhang et al., 2005; Pinto et al., 2007; Seppola et al., 2008), but the production of biologically active recombinant IL-10 protein remains elusive. More importantly, measurements of IL-10 gene expression or IL-10 proteins produced by (purified) macrophage cell populations should be performed before this presumed anti-inflammatory cytokine can be linked to the presence of regulatory macrophages. At this moment, most studies on IL-10 gene expression in fish have focused on the analysis of whole tissue samples after *in vivo* microbial infection and therefore cannot easily be linked to the presence of regulatory macrophages.

Evidence for heterogeneity of macrophage activation during parasite infections

As discussed above, the presence of innate and classically activated macrophages seems established, whereas the cytokine (IFN-γ/TNFα)-mediated regulation of classical activation of fish macrophages still reveals a complex picture. Furthermore, the putative role of cytokines involved in the generation of alternatively activated (IL-4/IL-13) or regulatory macrophages (IL-10) requires verification in fish. In addition, most studies that analysed the kinetics of arginase gene expression did so in whole organs but not in purified populations of macrophages, giving indirect evidence for the presence of alternatively activated macrophages at best. However, *in vivo* studies on the immune responses of carp to the parasites *Trypanoplasma borreli* and *Trypanosoma carassii*, that induce fundamentally different immune responses in their host, have provided us with good indications for the presence of (innate and) classically activated, alternatively activated and regulatory macrophages.

The parasites *T. borreli* and *T. carassii* are two Kinetoplastid parasites phylogenetically related to *Trypanosoma brucei* spp. that cause African sleeping sickness in humans and cattle. Similar to these mammalian parasites, *T. borreli* and *T. carassii* live extracellularly in the blood and tissue fluids of their host (carp). During experimental infection of carp with *T. borreli,* the peak of parasitemia (10^7–10^8 parasites/ml of blood) is generally observed at 3–4 weeks post-infection (p.i.), after which parasite numbers decline to the lowest detection level of 10^4/ml by week 7 and are cleared from the host. In general, approximately 50% mortality can be observed during *T. borreli* infections. In contrast, *T. carassii* infections are characterized by a more chronic persistence of parasite numbers that do not easily increase above 10^6–10^7/ml but remain at this level for up to 8–9 weeks until they are cleared. Mortalities are generally not observed during *T. carassii* infections.

During *T. borreli* infections, circumstances most favourable for development of innate/classically activated macrophages are found during the 'early' phase of infection. Within the first 3 weeks p.i., when parasitemia is highest, *T. borreli* induces a marked up-regulation of iNOS gene expression that results in a dramatic increase in serum nitrite levels and tissue nitration (Saeij et al., 2000a, 2002; Joerink et al., 2006a; Forlenza et al., 2008). Concomitantly, gene expression of IFN-γ (Stolte et al., 2008; Ribeiro et al., 2010b) and TNFα1/TNFα2 (Forlenza et al., 2009a) is up-regulated (Figure 3). The simultaneous presence of parasite antigens, pro-inflammatory cytokines and nitric oxide, all argue in favour of the presence of classically activated macrophages. This changes during the 'later' phase of infection with *T. borreli*, when optimal conditions for the development of regulatory macrophages are found. Around 4–6 weeks p.i a marked up-regulation

of IL-10 gene expression is seen (unpublished data), along with a down-regulation of gene expression of iNOS, IFN-γ, TNFα1/TNFα2 (Forlenza et al., 2008; Forlenza et al., 2009a) (Figure 3) and a reduction in tissue nitration. At this moment during infection, the production of parasite-specific antibodies and complement activation will lead to parasite clearance (Forlenza et al., 2009b), orchestrated by the presence of regulatory macrophages. Interestingly, particular carp strains that suffer from 100% mortality due to high numbers of *T. borreli* (Wiegertjes et al., 1995) show a reduced pro-inflammatory response, as measured by NO production and cytokine gene expression, associated with an early IL-10 gene expression that is up-regulated prior to peak parasitemia (unpublished data). Typically, individuals from these carp strains die before an effective antibody response can be generated (Wiegertjes et al., 1995). These experiments suggest that timing of macrophage polarization may be critically important to the host for a successful resolution of *T. borreli* infections.

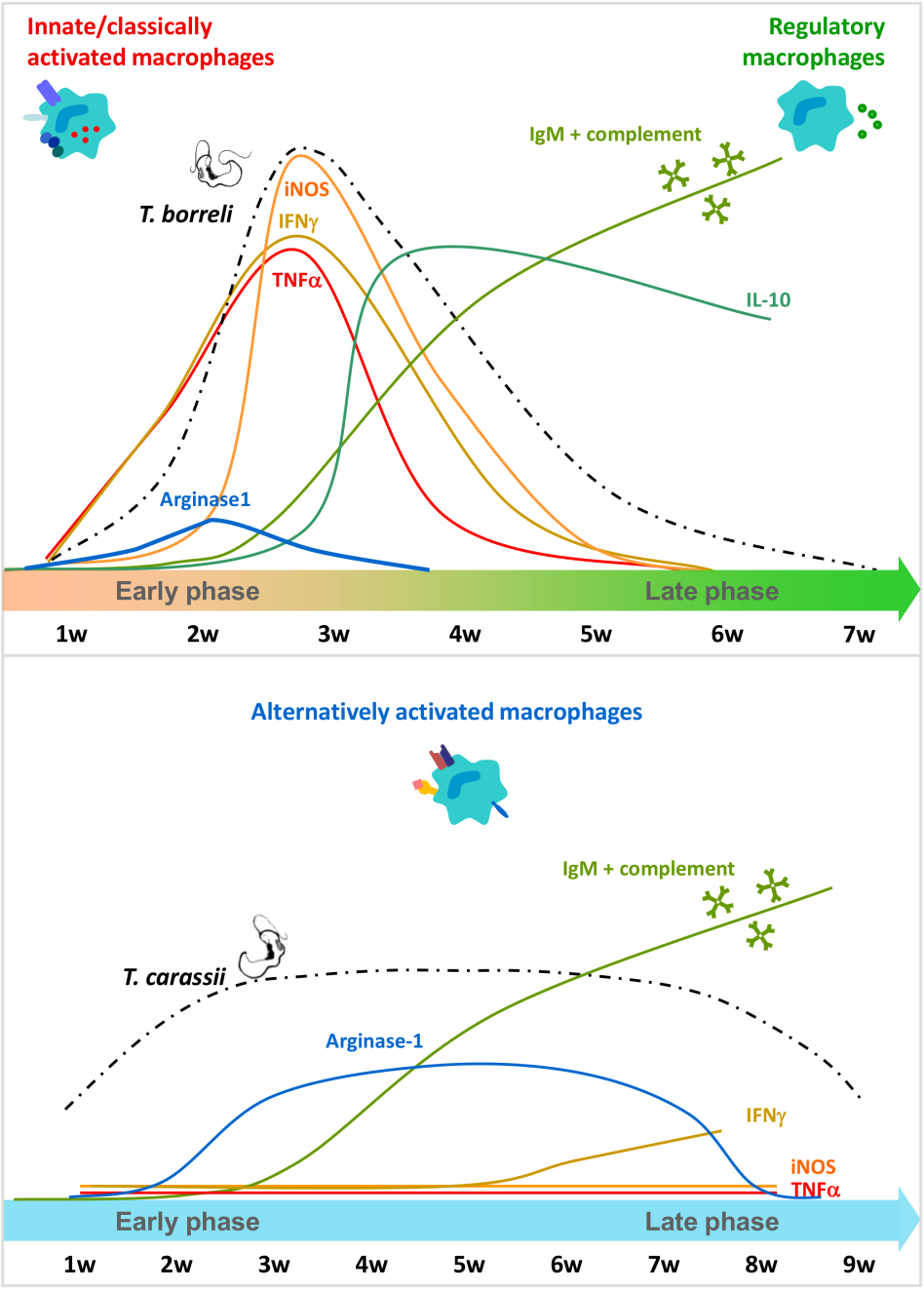
Innate/classically activated macrophages
Regulatory macrophages
IgM + complement
T. borreli
iNOS
IFNγ
TNFα
IL-10
Arginase1
Early phase
Late phase
1w
2w
3w
4w
5w
6w
7w
Alternatively activated macrophages
IgM + complement
T. carassii
Arginase-1
IFNγ
iNOS
TNFα
Early phase
Late phase
1w
2w
3w
4w
5w
6w
7w
8w
9w

Figure 3. Differential macrophage activation during protozoan infection in carp. During *Trypanoplasma borreli* infection, conditions favourable to the generation of classically activated macrophages develop in the initial phase of the immune response. At this time, up-regulation of IFN-γ and TNFα gene expression is observed. This coincides with the up-regulation of iNOS gene expression, production of elevated serum nitrite levels and tissue nitration. In the later phase of infection (>4 weeks) up-regulation of IL-10 gene expression is observed concomitantly with the decrease in pro-inflammatory genes expression. At this time, the presence of IL-10 as well as of immune complexes (*T. borreli*-specific antibodies) creates the optimal conditions for the generation of regulatory macrophages, ultimately leading to the resolution of the infection and parasite clearance. In contrast, during *Trypanosoma carassii* infections, characterized by a more chronic persistence of the parasite, conditions more favourable for the development of alternatively activated macrophages are observed. In fact, up-regulation of arginase-1 gene expression and arginase enzyme activity is observed continuously during the infection whereas iNOS and other pro-inflammatory genes are not significantly up-regulated.

Possibly, *T. borreli* parasites may try to polarize macrophages to their advantage. *Ex vivo* studies using head kidney-derived leukocytes from *T. borreli*-infected fish showed that re-stimulation with parasite antigens (or with LPS) induced significant amounts of nitric oxide, whereas re-stimulations with the same parasite antigens (or with cAMP) led to an inhibition of arginase activity (Joerink et al., 2006a). By favouring the development of classically activated macrophages with elevated iNOS activity, *T. borreli* could limit the availability of L-arginine thereby hindering arginase activity and the development of alternatively activated macrophages. The NO-inducing ability of *T. borreli* is considered a parasite strategy owing to their resistance to the toxic effects of NO and the immunosuppressive effect of this radical on the immune response of the host (Forlenza et al., 2008; Wiegertjes and Forlenza, 2010). Indeed, to *T. borreli* parasites, at least during the 'early' phase of infection, polarization towards macrophages with a classical signature must be advantageous.

Carp infected with *T. carassii* parasites show a completely different profile of macrophage activation. Clearly, *T. carassii* infections do not lead to a prominent NO production (Saeij et al., 2002, 2003). Instead, these parasites seem to induce elevated levels of arginase enzyme activity, particularly during the later phase of infection, as shown by *ex vivo* studies on head kidney-derived leukocytes from *T. carassii*-infected fish (Joerink et al., 2006a). While re-stimulation of these cells with LPS had no effect on the production of NO, re-stimulation with cAMP led to higher arginase activity. In contrast to *T. borreli*, *T. carassii* parasites seem to hinder the development of classically activated macrophages, most likely by competing for L-arginine substrate and favour the development of alternatively activated macrophages. The low resistance of *T. carassii* to the toxic effects of NO, at least *in vitro* (unpublished data), suggests that macrophage polarization away from classically

activated macrophages could be in favour of the development of *T. carassii*, again suggesting that Kinetoplastid parasites may polarize macrophages to their advantage.

The more chronic nature of *T. carassii* infections seems to confirm that the development of alternatively activated macrophages does not aid to fight infection with *T. carassii* but rather favours the persistence of this parasite in the fish host. This is a phenomenon not uncommon to mammalian vertebrates. Both, susceptibility to protozoan and helminth infections have been linked to the presence of alternatively activated macrophages (Kreider et al., 2007; Raes et al., 2007). It is thought that the production of polyamines by activation of arginase can favour parasite replication. Homologues of IL-4 have only been described recently for fish and their kinetic of gene expression, for example during *T. carassii* infections, have not been investigated yet thereby hampering the identification of alternatively activated macrophages. In mammals, the IL-4 required for the development of alternatively activated macrophages is initially produced by basophils, mast cells and neutrophilic granulocytes (Brandt et al., 2000; Reese et al., 2007). It is only later that adaptive (T_H2) immune responses drive the production of IL-4 and IL-13 for a sustained presence of alternatively activated macrophages. Possibly, the massive granulocytosis observed in the spleen of *T. carassii*-infected carp (Ribeiro et al., 2010b) could lead to the production of IL-4, favouring the development of alternatively activated macrophages. Likewise, a potential role for IL-10 towards the development of regulatory macrophages *in vivo* first requires extensive investigations of the function of this cytokine in fish.

In conclusion, although the interpretation of the above-described experiments with respect to *in vivo* heterogeneity of macrophage activation must be considered speculative, parasite infection studies in carp have provided us with good indications of the presence of (innate and) classically activated, alternatively activated and maybe even regulatory macrophages. In general, cytokine gene expression profiles but also different modes of macrophage activation in response to specific stimuli *in vitro*, *in vivo* and *ex vivo*, do indicate the existence of differentially activated macrophages in fish.

Future perspectives

The interpretation of data on the presence of polarized macrophages in teleosts is hampered by the use of different methodologies applied by different research laboratories and several, often phylogenetically distant, fish species. For example, the frequent use of mixed myeloid cell populations does not allow for easy comparison with data obtained from head kidney-derived macrophages or macrophage cell lines. Furthermore, different culture systems are employed in studying the effects of recombinant (cyprinid) cytokines,

which may lead to discrepancies in respective findings. For future experiments, it is imperative that we adopt common, standardized procedures for the isolation and *in vitro* culture of pure macrophage populations possibly of fewer fish species.

On the one hand, modern bony fish do seem to have TLR homologues representing all six major TLR families, although it is clear that not all properties of mammalian TLRs are shared and that some TLRs might not be present in all fish species. On the other hand, fish-specific TLRs have been identified for which ligand-binding studies will be even more essential to assess the importance of these receptors for macrophage activation.

The cytokines that are thought to play determining roles in macrophage activation are ill-defined in fish. Fish TNFα has been shown to have species-specific activities especially with respect to its ability to directly activate phagocytes. Additionally, it appears that the macrophage-modulating activity of mammalian IFN-γ may be carried out by two different IFN-γ isoforms in fish, each possessing distinct capacities to mediate activation of myeloid cells. Although recombinant IL-4rel protein seems to lead to enhanced B-cell proliferation, neither recombinant IL-4 nor IL-10 protein has been studied. Further functional studies are required to conclusively assign a role for these cytokines in the polarization of fish macrophages.

Distinctive gene signatures and cell surface markers specific for the different macrophage activation states are missing in teleosts. In mammals, despite the fact that the general functions of murine and human macrophages are thought to be preserved, the markers of each activation state are largely confined to murine macrophages. Therefore, instead of aiming at the identification of common signatures and surface markers specific for each macrophage phenotype in the numerous teleost species, it may be more rewarding to focus on general effector functions of fish macrophages. In this context, the characterization of macrophage phenotypes during the immune response to infections, *in vivo* or *ex vivo*, may help to define the heterogeneity of fish macrophages.

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References

Albina, J.E., Mills, C.D., Henry Jr., W.L., Caldwell, M.D., 1990. Temporal expression of different pathways of 1-arginine metabolism in healing wounds. J. Immunol. 144 (10), 3877–3880.

Aliprantis, A.O., Yang, R.B., Mark, M.R., Suggett, S., Devaux, B., Radolf, J.D., Klimpel, G.R., Godowski, P., Zychlinsky, A., 1999. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science 285 (5428), 736–739.

Arts, J.A., Tijhaar, E.J., Chadzinska, M., Savelkoul, H.F., Verburg-van Kemenade, B.M., 2010. Functional analysis of carp interferon-gamma: evolutionary conservation of classical phagocyte activation. Fish Shellfish Immunol. 29 (5), 793–802.

Bishop, R.E., 2005. Fundamentals of endotoxin structure and function. Contrib. Microbiol., 121–127.

Brandt, E., Woerly, G., Younes, A.B., Loiseau, S., Capron, M., 2000. IL-4 production by human polymorphonuclear neutrophils. J. Leukoc. Biol. 68 (1), 125–130.

Bronte, V., Serafini, P., De Santo, C., Marigo, I., Tosello, V., Mazzoni, A., Segal, D.M., Staib, C., Lowel, M., Sutter, G., Colombo, M.P., Zanovello, P., 2003. IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. J. Immunol. 170 (1), 270–278.

Callard, R.E., 1989. Cytokine regulation of B-cell growth and differentiation. Br. Med. Bull. 45 (2), 371–388.

Chadzinska, M., Leon-Kloosterziel, K.M., Plytycz, B., Lidy Verburg-van Kemenade, B.M., 2008. In vivo kinetics of cytokine expression during peritonitis in carp: evidence for innate and alternative macrophage polarization. Dev. Comp. Immunol. 32 (5), 509–518.

Chadzinska, M., Savelkoul, H.F., Verburg-van Kemenade, B.M., 2009. Morphine affects the inflammatory response in carp by impairment of leukocyte migration. Dev. Comp. Immunol. 33 (1), 88–96.

Chang, M., Wang, T., Nie, P., Zou, J., Secombes, C.J., 2011. Cloning of two rainbow trout nucleotide-binding oligomerization domain containing 2 (NOD2) splice variants and functional characterization of the NOD2 effector domains. Fish Shellfish Immunol. 30 (1), 118–127.

Chang, M.X., Nie, P., 2008. RNAi suppression of zebrafish peptidoglycan recognition protein 6 (zfPGRP6) mediated differentially expressed genes involved in Toll-like receptor signaling pathway and caused increased susceptibility to *Flavobacterium columnare*. Vet. Immunol. Immunopathol. 124 (3–4), 295–301.

Chen, W.Q., Xu, Q.Q., Chang, M.X., Nie, P., Peng, K.M., 2010a. Molecular characterization and expression analysis of nuclear oligomerization domain proteins NOD1 and NOD2 in grass carp *Ctenopharyngodon idella*. Fish Shellfish Immunol. 28 (1), 18–29.

Chen, W.Q., Xu, Q.Q., Chang, M.X., Zou, J., Secombes, C.J., Peng, K.M., Nie, P., 2010b. Molecular characterization and expression analysis of the IFN-gamma related gene (IFN-gammarel) in grass carp *Ctenopharyngodon idella*. Vet. Immunol. Immunopathol. 134 (3–4), 199–207.

Clark, E.A., Shu, G.L., Luscher, B., Draves, K.E., Banchereau, J., Ledbetter, J.A., Valentine, M.A., 1989. Activation of human B cells. Comparison of the signal transduced by IL-4 to four different competence signals. J. Immunol. 143 (12), 3873–3880.

Covello, J.M., Bird, S., Morrison, R.N., Battaglene, S.C., Secombes, C.J., Nowak, B.F., 2009. Cloning and expression analysis of three striped trumpeter (*Latris lineata*) pro-inflammatory cytokines, TNF-α, IL-1β and IL-8, in response to infection by the ectoparasitic, *Chondracanthus goldsmidi*. Fish Shellfish Immunol. 26 (5), 773–786.

Dalton, D.K., Pitts-Meek, S., Keshav, S., Figari, I.S., Bradley, A., Stewart, T.A., 1993. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. Science 259 (5102), 1739–1742.

Fast, M.D., Tse, B., Boyd, J.M., Johnson, S.C., 2009. Mutations in the *Aeromonas salmonicida* subsp. *salmonicida* type III secretion system affect Atlantic salmon leucocyte activation and downstream immune responses. Fish Shellfish Immunol. 27 (6), 721–728.

Forlenza, M., Magez, S., Scharsack, J.P., Westphal, A., Savelkoul, H.F., Wiegertjes, G.F., 2009a. Receptor-mediated and lectin-like activities of carp (*Cyprinus carpio*) TNF-alpha. J. Immunol. 183 (8), 5319–5332.

Forlenza, M., Nakao, M., Wibowo, I., Joerink, M., Arts, J.A., Savelkoul, H.F., Wiegertjes, G.F., 2009b. Nitric oxide hinders antibody clearance from the surface of *Trypanoplasma borreli* and increases susceptibility to complement-mediated lysis. Mol. Immunol. 46 (16), 3188–3197.

Forlenza, M., Scharsack, J.P., Kachamakova, N.M., Taverne-Thiele, A.J., Rombout, J.H., Wiegertjes, G.F., 2008. Differential contribution of neutrophilic granulocytes and macrophages to nitrosative stress in a host-parasite animal model. Mol. Immunol. 45 (11), 3178–3189.

Furnes, C., Seppola, M., Robertsen, B., 2009. Molecular characterisation and expression analysis of interferon gamma in Atlantic cod (*Gadus morhua*). Fish Shellfish Immunol. 26 (2), 285–292.

García-Castillo, J., Chaves-Pozo, E., Olivares, P., Pelegín, P., Meseguer, J., Mulero, V., 2004. The tumor necrosis factor-α of the bony fish seabream exhibits the in vivo proinflammatory and proliferative activities of its mammalian counterparts, yet it functions in a species-specific manner. Cell. Mol. Life Sci. (CMLS) 61 (11), 1331–1340.

Garcia-Castillo, J., Pelegrin, P., Mulero, V., Meseguer, J., 2002. Molecular cloning and expression analysis of tumor necrosis factor alpha from a marine fish reveal its constitutive expression and ubiquitous nature. Immunogenetics 54 (3), 200–207.

Geissmann, F., Manz, M.G., Jung, S., Sieweke, M.H., Merad, M., Ley, K., 2010. Development of monocytes, macrophages, and dendritic cells. Science 327 (5966), 656–661.

Ghassabeh, G.H., De Baetselier, P., Brys, L., Noel, W., Van Ginderachter, J.A., Meerschaut, S., Beschin, A., Brombacher, F., Raes, G., 2006. Identification of a common gene signature for type II cytokine-associated myeloid cells elicited in vivo in different pathologic conditions. Blood 108 (2), 575–583.

Glenney, G.W., Wiens, G.D., 2007. Early diversification of the TNF superfamily in teleosts: genomic characterization and expression analysis. J. Immunol. 178 (12), 7955–7973.

Goerdt, S., Orfanos, C.E., 1999. Other functions, other genes: alternative activation of antigen-presenting cells. Immunity 10 (2), 137–142.

Gonzalez, S.F., Huising, M.O., Stakauskas, R., Forlenza, M., Lidy Verburg-van Kemenade, B.M., Buchmann, K., Nielsen, M.E., Wiegertjes, G.F., 2007. Real-time gene expression analysis in carp (*Cyprinus carpio* L.) skin: inflammatory responses to injury mimicking infection with ectoparasites. Dev. Comp. Immunol. 31 (3), 244–254.

Gordon, S., 2003. Alternative activation of macrophages. Nat. Rev. Immunol. 3 (1), 23–35.

Gordon, S., 2007. The macrophage: past, present and future. Eur. J. Immunol. 37 (Suppl. 1), S9–17.

Gordon, S., Martinez, F.O., 2010. Alternative activation of macrophages: mechanism and functions. Immunity 32 (5), 593–604.

Gordon, S., Taylor, P.R., 2005. Monocyte and macrophage heterogeneity. Nat.Rev. Immunol. 5 (12), 953–964.

Grayfer, L., Belosevic, M., 2009. Molecular characterization, expression and functional analysis of goldfish (*Carassius aurutus* L.) interferon gamma. Dev. Comp. Immunol. 33 (2), 235–246.

Grayfer, L., Garcia, E.G., Belosevic, M., 2010. Comparison of macrophage antimicrobial responses induced by type II interferons of the goldfish (*Carassius auratus* L.). J. Biol. Chem. 285 (31), 23537–23547.

Grayfer, L., Walsh, J.G., Belosevic, M., 2008. Characterization and functional analysis of goldfish (*Carassius auratus* L.) tumor necrosis factor-alpha. Dev. Comp. Immunol. 32 (5), 532–543.

Guan, R., Mariuzza, R.A., 2007. Peptidoglycan recognition proteins of the innate immune system. Trends Microbiol. 15 (3), 127–134.

Hanington, P.C., Tam, J., Katzenback, B.A., Hitchen, S.J., Barreda, D.R., Belosevic, M., 2009. Development of macrophages of cyprinid fish. Dev. Comp. Immunol. 33 (4), 411–429.

Haugland, Ø., Mercy, I.S., Romøren, K., Torgersen, J., Evensen, Ø., 2007. Differential expression profiles and gene structure of two tumor necrosis factor-alpha variants in Atlantic salmon (*Salmo salar* L.). Mol. Immunol. 44 (7), 1652–1663.

Hirono, I., Nam, B.H., Kurobe, T., Aoki, T., 2000. Molecular cloning, characterization, and expression of TNF cDNA and gene from Japanese flounder *Paralychthys olivaceus*. J. Immunol. 165 (8), 4423–4427.

Hu, Y.-L., Xiang, L.-X., Shao, J.-Z., 2010. Identification and characterization of a novel immunoglobulin Z isotype in zebrafish: Implications for a distinct B cell receptor in lower vertebrates. Mol. Immunol. 47 (4), 738–746.

Igawa, D., Sakai, M., Savan, R., 2006. An unexpected discovery of two interferon gamma-like genes along with interleukin (IL)-22 and -26 from teleost: IL-22 and -26 genes have been described for the first time outside mammals. Mol. Immunol. 43 (7), 999–1009.

Iliev, D.B., Roach, J.C., Mackenzie, S., Planas, J.V., Goetz, F.W., 2005. Endotoxin recognition: in fish or not in fish? FEBS Lett. 579 (29), 6519–6528.

Inoue, Y., Kamota, S., Ito, K., Yoshiura, Y., Ototake, M., Moritomo, T., Nakanishi, T., 2005. Molecular cloning and expression analysis of rainbow trout (*Oncorhynchus mykiss*) interleukin-10 cDNAs. Fish Shellfish Immunol. 18 (4), 335–344.

Janeway Jr., C.A., Medzhitov, R., 2002. Innate immune recognition. Annu. Rev. Immunol., 20197–20216.

Jenkinson, C.P., Grody, W.W., Cederbaum, S.D., 1996. Comparative properties of arginases. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 114 (1), 107–132.

Joerink, M., Forlenza, M., Ribeiro, C.M.S., de Vries, B.J., Savelkoul, H.F.J., Wiegertjes, G.F., 2006a. Differential macrophage polarisation during parasitic infections in common carp (*Cyprinus carpio* L.). Fish Shellfish Immunol. 21 (5), 561–571.

Joerink, M., Ribeiro, C.M.S., Stet, R.J.M., Hermsen, T., Savelkoul, H.F.J., Wiegertjes, G.F., 2006b. Head kidney-derived macrophages of common carp (*Cyprinus carpio* L.) show plasticity and functional polarization upon differential stimulation. J. Immunol. 177 (1), 61–69.

Joerink, M., Savelkoul, H.F.J., Wiegertjes, G.F., 2006c. Evolutionary conservation of alternative activation of macrophages: Structural and functional characterization of arginase 1 and 2 in carp (*Cyprinus carpio* L.). Mol. Immunol. 43 (8), 1116–1128.

Jurecka, P., Irnazarow, I., Stafford, J.L., Ruszczyk, A., Taverne, N., Belosevic, M., Savelkoul, H.F.J., Wiegertjes, G.F., 2009. The induction of nitric oxide response of carp macrophages by transferrin is influenced by the allelic diversity of the molecule. Fish Shellfish Immunol. 26 (4), 632–638.

Kim, H.M., Park, B.S., Kim, J.I., Kim, S.E., Lee, J., Oh, S.C., Enkhbayar, P., Matsushima, N., Lee, H., Yoo, O.J., Lee, J.O., 2007. Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. Cell 130 (5), 906–917.

Kreider, T., Anthony, R.M., Urban Jr., J.F., Gause, W.C., 2007. Alternatively activated macrophages in helminth infections. Curr. Opin. Immunol. 19 (4), 448–453.

Laberge, T., Walsh, P.J., McDonald, M.D., 2009. Effects of crowding on ornithine-urea cycle enzyme mRNA expression and activity in gulf toadfish (*Opsanus beta*). J. Exp. Biol. 212 (Pt 15), 2394–2402.

Laing, K.J., Grabowski, P.S., Belosevic, M., Secombes, C.J., 1996. A partial sequence for nitric oxide synthase from a goldfish (*Carassius auratus*) macrophage cell line. Immunol. Cell Biol. 74 (4), 374–379.

Laing, K.J., Hardie, L.J., Aartsen, W., Grabowski, P.S., Secombes, C.J., 1999. Expression of an inducible nitric oxide synthase gene in rainbow trout *Oncorhynchus mykiss*. Dev. Comp. Immunol. 23 (1), 71–85.

Laing, K.J., Purcell, M.K., Winton, J.R., Hansen, J.D., 2008. A genomic view of the NOD- like receptor family in teleost fish: identification of a novel NLR subfamily in zebrafish. BMC Evol. Biol., 842.

Laing, K.J., Wang, T., Zou, J., Holland, J., Hong, S., Bols, N., Hirono, I., Aoki, T., Secombes, C.J., 2001. Cloning and expression analysis of rainbow trout *Oncorhynchus mykiss* tumour necrosis factor-alpha. Eur. J. Biochem. 268 (5), 1315–1322.

Lang, R., Patel, D., Morris, J.J., Rutschman, R.L., Murray, P.J., 2002. Shaping gene expression in activated and resting primary macrophages by IL-10. J. Immunol. 169 (5), 2253–2263.

Leulier, F., Lemaitre, B., 2008. Toll-like receptors – taking an evolutionary approach. Nat. Rev. Genet. 9 (3), 165–178.

Li, J.H., Shao, J.Z., Xiang, L.X., Wen, Y., 2007. Cloning, characterization and expression analysis of pufferfish interleukin-4 cDNA: the first evidence of Th2-type cytokine in fish. Mol. Immunol. 44 (8), 2078–2086.

Lin, A.-F., Xiang, L.-X., Wang, Q.-L., Dong, W.-R., Gong, Y.-F., Shao, J.-Z., 2009. The DC-SIGN of zebrafish: insights into the existence of a CD209 homologue in a lower vertebrate and its involvement in adaptive immunity. J. Immunol. 183 (11), 7398–7410.

Lutfalla, G., Crollius, H., Stange-thomann, N., Jaillon, O., Mogensen, K., Monneron, D., 2003. Comparative genomic analysis reveals independent expansion of a lineage-specific gene family in vertebrates: The class II cytokine receptors and their ligands in mammals and fish. BMC Genomics 4 (1), 29.

MacKenzie, S.A., Roher, N., Boltana, S., Goetz, F.W., 2010. Peptidoglycan, not endotoxin, is the key mediator of cytokine gene expression induced in rainbow trout macrophages by crude LPS. Mol. Immunol. 47 (7–8), 1450–1457.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., Locati, M., 2004. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 25 (12), 677–686.

Mantovani, A., Sozzani, S., Locati, M., Allavena, P., Sica, A., 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. 23 (11), 549–555.

Martinez, F.O., Helming, L., Gordon, S., 2009. Alternative activation of macrophages: an immunologic functional perspective. Annu. Rev. Immunol., 27451–27483.

Mathison, J.C., Tobias, P.S., Wolfson, E., Ulevitch, R.J., 1992. Plasma lipopolysaccharide (LPS)-binding protein. A key component in macrophage recognition of gram-negative LPS. J. Immunol. 149 (1), 200–206.

Mestas, J., Hughes, C.C.W., 2004. Of mice and not men: differences between mouse and human immunology. J. Immunol. 172 (5), 2731–2738.

Milev-Milovanovic, I., Long, S., Wilson, M., Bengten, E., Miller, N.W., Chinchar, V.G., 2006. Identification and expression analysis of interferon gamma genes in channel catfish. Immunogenetics 58 (1), 70–80.

Mills, C.D., Kincaid, K., Alt, J.M., Heilman, M.J., Hill, A.M., 2000. M-1/M-2 macrophages and the Th1/Th2 paradigm. J. Immunol. 164 (12), 6166–6173.

Modolell, M., Corraliza, I.M., Link, F., Soler, G., Eichmann, K., 1995. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. Eur. J. Immunol. 25 (4), 1101–1104.

Mosser, D.M., 2003. The many faces of macrophage activation. J. Leukoc. Biol. 73(2), 209–212.

Mosser, D.M., Edwards, J.P., 2008. Exploring the full spectrum of macrophage activation. Nat. Rev. Immunol. 8 (12), 958–969.

Mueller, T.D., Zhang, J.L., Sebald, W., Duschl, A., 2002. Structure, binding, and antagonists in the IL-4/IL-13 receptor system. Biochim. Biophys. Acta 1592 (3), 237–250.

Munder, M., Eichmann, K., Moran, J.M., Centeno, F., Soler, G., Modolell, M., 1999. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. J. Immunol. 163 (7), 3771–3777.

Mutoloki, S., Cooper, G.A., Marjara, I.S., Koop, B.F., Evensen, O., 2010. High gene expression of inflammatory markers and IL-17A correlates with severity of injection site reactions of Atlantic salmon vaccinated with oil-adjuvanted vaccines. BMC Genomics, 11336.

Novoa, B., Bowman, T.V., Zon, L., Figueras, A., 2009. LPS response and tolerance in the zebrafish (*Danio rerio*). Fish Shellfish Immunol. 26 (2), 326–331.

Ohtani, M., Hayashi, N., Hashimoto, K., Nakanishi, T., Dijkstra, J.M., 2008. Comprehensive clarification of two paralogous interleukin 4/13 loci in teleost fish. Immunogenetics 60 (7), 383–397.

Okamura, Y., Watari, M., Jerud, E.S., Young, D.W., Ishizaka, S.T., Rose, J., Chow, J.C., Strauss 3rd, J.F., 2001. The extra domain A of fibronectin activates Toll-like receptor 4. J. Biol. Chem. 276 (13), 10229–10233.

Ordas, M.C., Costa, M.M., Roca, F.J., Lopez-Castejon, G., Mulero, V., Meseguer, J., Figueras, A., Novoa, B., 2007. Turbot TNFalpha gene: molecular characterization and biological activity of the recombinant protein. Mol Immunol. 44 (4), 389–400.

Oshiumi, H., Matsuo, A., Matsumoto, M., Seya, T., 2008. Pan-vertebrate toll-like receptors during evolution. Curr. Genomics 9 (7), 488–493.

Palti,Y.,Rodriguez,M.F.,Gahr,S.A.,Purcell,M.K.,Rexroad3rd,C.E.,Wiens,G.D.,2010. Identification, characterization and genetic mapping of TLR1 loci in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol. 28 (5–6), 918–926.

Pinto, R.D., Nascimento, D.S., Reis, M.I.R., do Vale, A., dos Santos, N.M.S., 2007. Molecular characterization, 3D modelling and expression analysis of sea bass (*Dicentrarchus labrax* L.) interleukin-10. Mol. Immunol. 44 (8), 2056–2065.

Raes, G., Beschin, A., Ghassabeh, G.H., De Baetselier, P., 2007. Alternatively activated macrophages in protozoan infections. Curr. Opin. Immunol. 19 (4), 454–459.

Raetz, C.R., Whitfield, C., 2002. Lipopolysaccharide endotoxins. Annu. Rev. Biochem., 71635–71700.

Rebl, A., Goldammer, T., Seyfert, H.M., 2010. Toll-like receptor signaling in bony fish. Vet. Immunol. Immunopathol. 134 (3–4), 139–150.

Reese, T.A., Liang, H.E., Tager, A.M., Luster, A.D., Van Rooijen, N., Voehringer, D., Locksley, R.M., 2007. Chitin induces accumulation in tissue of innate immune cells associated with allergy. Nature 447 (7140), 92–96.

Ribeiro, C.M., Hermsen, T., Taverne-Thiele, A.J., Savelkoul, H.F., Wiegertjes, G.F., 2010a. Evolution of recognition of ligands from Gram-positive bacteria: similarities and differences in the TLR2-mediated response between mammalian vertebrates and teleost fish. J. Immunol. 184 (5), 2355–2368.

Ribeiro, C.M.S., Pontes, M.J.S.L., Bird, S., Chadzinska, M., Scheer, M., Verburg-van Kemenade, B.M.L., Savelkoul, H.F.J., Wiegertjes, G.F., 2010b. Trypanosomiasis-induced Th17-like immune responses in carp. PLoS One 5 (9), e13012.

Roach, J.C., Glusman, G., Rowen, L., Kaur, A., Purcell, M.K., Smith, K.D., Hood, L.E., Aderem, A., 2005. The evolution of vertebrate Toll-like receptors. Proc. Natl. Acad. Sci. U. S. A. 102 (27), 9577–9582.

Robertsen, B., 2006. The interferon system of teleost fish. Fish Shellfish Immunol. 20 (2), 172–191.

Roca, F.J., Mulero, I., Lopez-Munoz, A., Sepulcre, M.P., Renshaw, S.A., Meseguer, J., Mulero, V., 2008. Evolution of the inflammatory response in vertebrates: fish TNF-alpha is a powerful activator of endothelial cells but hardly activates phagocytes. J. Immunol. 181 (7), 5071–5081.

Saeij, J.P., Stet, R.J., de Vries, B.J., van Muiswinkel, W.B., Wiegertjes, G.F., 2003. Molecular and functional characterization of carp TNF: a link between TNF polymorphism and trypanotolerance? Dev. Comp. Immunol. 27 (1), 29–41.

Saeij, J.P., Stet, R.J., Groeneveld, A., Verburg-van Kemenade, L.B., van Muiswinkel, W.B., Wiegertjes, G.F., 2000a. Molecular and functional characterization of a fish inducible-type nitric oxide synthase. Immunogenetics 51 (4–5), 339–346.

Saeij, J.P., Van Muiswinkel, W.B., Groeneveld, A., Wiegertjes, G.F., 2002. Immune modulation by fish kinetoplastid parasites: a role for nitric oxide. Parasitology 124 (Pt 1), 77–86.

Saeij, J.P.J., Stet, R.J.M., Groeneveld, A., Verburg-van Kemenade, B.M.L., van Muiswinkel, W.B., Wiegertjes, G.F., 2000b. Molecular and functional characterization of a fish inducible-type nitric oxide synthase. Immunogenetics 51 (4–5), 339–346.

Savan, R., Igawa, D., Sakai, M., 2003. Cloning, characterization and expression analysis of interleukin-10 from the common carp, *Cyprinus carpio* L. Eur. J. Biochem. 270 (23), 4647–4654.

Savan, R., Ravichandran, S., Collins, J.R., Sakai, M., Young, H.A., 2009. Structural conservation of interferon gamma among vertebrates. Cytokine Growth Factor Rev. 20 (2), 115–124.

Schroder, N.W., Morath, S., Alexander, C., Hamann, L., Hartung, T., Zahringer, U., Gobel, U.B., Weber, J.R., Schumann, R.R., 2003. Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. J. Biol. Chem. 278 (18), 15587–15594.

Schwandner, R., Dziarski, R., Wesche, H., Rothe, M., Kirschning, C.J., 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J. Biol. Chem. 274 (25), 17406–17409.

Seppola, M., Larsen, A.N., Steiro, K., Robertsen, B., Jensen, I., 2008. Characterisation and expression analysis of the interleukin genes, IL-1[beta]. IL-8 and IL-10, in Atlantic cod (*Gadus morhua* L.). Mol. Immunol. 45 (4), 887–897.

Sepulcre, M.P., Alcaraz-Perez, F., Lopez-Munoz, A., Roca, F.J., Meseguer, J., Cayuela, M.L., Mulero, V., 2009. Evolution of lipopolysaccharide (LPS) recognition and signaling: fish TLR4 does not recognize LPS and negatively regulates NF-kappaB activation. J. Immunol. 182 (4), 1836–1845.

Sha, Z., Abernathy, J.W., Wang, S., Li, P., Kucuktas, H., Liu, H., Peatman, E., Liu, Z., 2009. NOD-like subfamily of the nucleotide-binding domain and leucine-rich repeat containing family receptors and their expression in channel catfish. Dev. Comp. Immunol. 33 (9), 991–999.

Sieger, D., Stein, C., Neifer, D., van der Sar, A.M., Leptin, M., 2009. The role of gamma interferon in innate immunity in the zebrafish embryo. Dis. Model Mech. 2 (11–12), 571–581.

Skugor, S., Glover, K.A., Nilsen, F., Krasnov, A., 2008. Local and systemic gene expression responses of Atlantic salmon (*Salmo salar* L.) to infection with the salmon louse (*Lepeophtheirus salmonis*). BMC Genomics, 9498.

Stafford, J.L., Galvez, F., Goss, G.G., Belosevic, M., 2002. Induction of nitric oxide and respiratory burst response in activated goldfish macrophages requires potassium channel activity. Dev. Comp. Immunol. 26 (5), 445–459.

Stafford, J.L., McLauchlan, P.E., Secombes, C.J., Ellis, A.E., Belosevic, M., 2001a. Generation of primary monocyte-like cultures from rainbow trout head kidney leukocytes. Dev. Comp. Immunol. 25 (5–6), 447–459.

Stafford, J.L., Neumann, N.F., Belosevic, M., 2001b. Products of proteolytic cleavage of transferrin induce nitric oxide response of goldfish macrophages. Dev. Comp. Immunol. 25 (2), 101–115.

Stein, C., Caccamo, M., Laird, G., Leptin, M., 2007. Conservation and divergence of gene families encoding components of innate immune response systems in zebrafish. Genome Biol. 8 (11), R251.

Stein, M., Keshav, S., Harris, N., Gordon, S., 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. J. Exp. Med. 176 (1), 287–292.

Stolte, E.H., Savelkoul, H.F.J., Wiegertjes, G., Flik, G., Verburg-van Kemenade, B.M.L., 2008. Differential expression of two interferon-gamma genes in common carp (*Cyprinus carpio* L.). Dev. Comp. Immunol. 32 (12), 1467–1481.

Sullivan, C., Charette, J., Catchen, J., Lage, C.R., Giasson, G., Postlethwait, J.H., Millard, P.J., Kim, C.H., 2009. The gene history of zebrafish tlr4a and tlr4b is predictive of their divergent functions. J. Immunol. 183 (9), 5896–5908.

Takeda, K., Takeuchi, O., Akira, S., 2002. Recognition of lipopeptides by Toll-like receptors. J. Endotoxin Res. 8 (6), 459–463.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., Akira, S., 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 11 (4), 443–451.

Taylor, P.R., Martinez-Pomares, L., Stacey, M., Lin, H.H., Brown, G.D., Gordon, S., 2005. Macrophage receptors and immune recognition. Annu. Rev. Immunol., 23901–23944.

Tobias, P.S., Soldau, K., Gegner, J.A., Mintz, D., Ulevitch, R.J., 1995. Lipopolysaccharide binding protein-mediated complexation of lipopolysaccharide with soluble CD14. J. Biol. Chem. 270 (18), 10482–10488.

Vincendeau, P., Gobert, A.P., Daulouede, S., Moynet, D., Mossalayi, M.D., 2003. Arginases in parasitic diseases. Trends Parasitol. 19 (1), 9–12.

Wiegertjes, G.F., Forlenza, M., 2010. Nitrosative stress during infection-induced inflammation. Curr. Pharm. Des. 16 (38), 4193–4201.

Wiegertjes, G.F., Groeneveld, A., van Muiswinkel, W.B., 1995. Genetic variation in susceptibility to *Trypanoplasma borreli* infection in common carp (*Cyprinus carpio* L.). Vet. Immunol. Immunopathol. 47 (1–2), 153–161.

Wright, P.A., Campbell, A., Morgan, R.L., Rosenberger, A.G., Murray, B.W., 2004. Dogmas and controversies in the handling of nitrogenous wastes: expression of arginase Type I and II genes in rainbow trout: influence of fasting on liver enzyme activity and mRNA levels in juveniles. J. Exp. Biol. 207 (Pt 12), 2033–2042.

Wu, G., Morris, S.M., 1998. Arginine metabolism: nitric oxide and beyond. Biochem. J. 336 (1), 1–17.

Zhang, D.C., Shao, Y.Q., Huang, Y.Q., Jiang, S.G., 2005. Cloning, characterization and expression analysis of interleukin-10 from the zebrafish (*Danio rerio*). J. Biochem. Mol. Biol. 38 (5), 571–576.

Zou, J., Carrington, A., Collet, B., Dijkstra, J.M., Yoshiura, Y., Bols, N., Secombes, C., 2005. Identification and bioactivities of IFN-gamma in rainbow trout *Oncorhynchus mykiss*: the first Th1-type cytokine characterized functionally in fish. J. Immunol. 175 (4), 2484–2494.

Zou, J., Clark, M.S., Secombes, C.J., 2003a. Characterisation, expression and promoter analysis of an interleukin 10 homologue in the puffer fish, *Fugu rubripes*. Immunogenetics 55 (5), 325–335.

Zou, J., Secombes, C.J., Long, S., Miller, N., Clem, L.W., Chinchar, V.G., 2003b. Molecular identification and expression analysis of tumor necrosis factor in channel catfish (*Ictalurus punctatus*). Dev. Comp. Immunol. 27 (10), 845–858.

Zou, J., Yoshiura, Y., Dijkstra, J.M., Sakai, M., Ototake, M., Secombes, C., 2004. Identification of an interferon gamma homologue in Fugu, Takifugu rubripes. Fish Shellfish Immunol. 17 (4), 403–409.

Chapter 4

Cytokine-independent polarization points at an intrinsic property of carp macrophages to polarize into M1- and M2-type activation states

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Abstract

Since Metchnikoff's description of phagocytosis, macrophages are recognized as cell types critical to the regulation of innate immunity. Macrophages of higher vertebrates can express a range of activation states, with the extremes also termed M1 and M2. The M1-M2 dichotomy could be an intrinsic property of macrophages which arose early in evolution, prior to the development of adaptive immunity. Very little, however, is known about the evolutionary conservation of these activation states. We hypothesized that macrophages from teleost fish would show activation profiles and gene signatures analogous to those typical of macrophages from higher vertebrates. Confirming this hypothesis, we show that macrophages of carp can polarize into activation states typical of classical (M1) and alternative (M2) extremes. Here, we describe that cytokine-independent activation with microbial LPS leads to activated macrophages which display a typical M1-type state as shown by high levels of nitric oxide, whereas exogenous cAMP drives macrophages to an alternative M2-type state as shown by high levels of arginase activity. We identify a number of genes that may serve as markers for activated teleost M1 and M2 macrophages, some being unique to teleosts and some which are conserved from teleosts to human and/or mouse. Furthermore, we report that cytokine-dependent activation with Ifnγ amplifies the activation state induced by LPS alone. Thus, from an evolutionary viewpoint, our findings suggest that polarization towards M1 and M2 activation states is guided by evolutionarily conserved principles.

Introduction

Over the last 10-20 years, a conceptual framework has been developed for the description of macrophage activation, with macrophages polarized into classically (M1) or alternatively (M2) activated cells representing two extreme activation states of a wider spectrum (Biswas and Mantovani, 2010). The spectrum includes innate activated macrophages (generated in the presence of microbial ligands), which have a high resemblance to classically activated macrophages (induced by stimulation with microbial ligands in combination with pro-inflammatory cytokines like IFNγ or TNFα). Unless specified otherwise, here we include both innate and classically activated macrophages in the 'M1' definition. Recently, the current M1 versus M2 polarization model was refined to describe at least nine distinct macrophage activation programs in human monocyte-derived macrophages (Xue et al., 2014) and the list appears to be growing, necessitating a revision of nomenclature and reporting guidelines (Murray et al., 2014). Substantial differences in gene expression profiles, however, may exist between human and mouse macrophages (Schroder et al., 2012), (Shay et al., 2013) and it remains unclear to which degree activation pathways in macrophages are conserved across species (Murray and Wynn, 2011a). To date, the vast majority of studies on macrophage activation have been performed on cells from human and mouse, restricting all investigations of macrophage polarization to studies in mammals. Here, we examined the hypothesis that macrophages from teleost fish, in particular of the model species common carp (*Cyprinus carpio*), show activation profiles and gene signatures analogous to those typical of macrophages from mammals, by performing an in-depth study of M1 and M2 activities and associated transcription profiles.

The presence in mice and humans of the T cell-derived cytokines IFNγ and IL4 or IL13, respectively, can drive in vitro cultures of macrophages from bone marrow (mice) and peripheral blood monocytes (human) into M1 and M2 populations (Mantovani et al., 2004). It is possible, however, that the M1-M2 dichotomy arose early in evolution prior to the development of mammalian vertebrates, and would be an evolutionarily conserved, intrinsic property of macrophages associated with transitions from M1 to M2, or vice versa. Indeed, the ability of macrophage-like cells to phagocytose foreign objects and repair cellular damage already existed in the first primitive animals, whereas the requirement for cytokine-driven adaptive immunity developed later in evolution (Mills and Ley, 2014). Teleost fish are excellently positioned to examine macrophage polarization in the absence or presence of T-cell derived cytokines since they represent ancestral bony vertebrates with an adaptive immune system influenced by homologous cytokines with activities similar to, but also different from, those of higher vertebrates (Wang and Secombes, 2013). Fish Ifnγ exists as a teleost-specific monomeric Ifnγ-'related' (Ifnγ-rel) cytokine with distinct anti-viral activities (Shibasaki et al., 2014), and as a dimeric Ifnγ with structure and

activities similar to mammalian IFNγ (Zou and Secombes, 2011). Fish Il4/13 is ancestral to mammalian IL4 and IL13 and exists as Il4/13A, which may provide a basal level of type-2 immunity, and as Il4/13B which, when induced, provides an enhanced type-2 immunity, at least in rainbow trout (Wang et al., 2016). Previously, we developed a well-characterized in vitro culture system for carp macrophages, based on in vitro cultures from head kidney (Joerink et al., 2006a), the functional and structural homologue of mammalian bone marrow (Zapata, 1981). We characterized innate activation of carp macrophages induced by microbial ligands such as LPS (Joerink et al., 2006a), classical activation induced by LPS in combination with IFNγ (Arts et al., 2010) and alternative activation induced by cyclic AMP (cAMP) (Joerink et al., 2006b). In mammals, extracellular cAMP can mimic the effect of IL13 as the primary signalling pathway responsible for activation of arginase (Chang et al., 2000). Thus, stimulation with LPS or cAMP allowed us to examine the possibility that the M1-M2 dichotomy would be an evolutionarily conserved, intrinsic property of macrophages and already present in macrophages of teleost fish.

We performed our studies in common carp (*Cyprinus carpio* L.), a very close relative of zebrafish (*Danio rerio* L.) (Henkel et al., 2012), which has become a popular organism for the study of vertebrate gene function (Howe et al., 2013). The large body size of carp facilitated both functional studies and collection of RNA from polarized macrophages, whereas the well-annotated zebrafish genome was used to identify carp genes transcribed in datasets from polarized macrophages. Never before did a study address function and associated transcription profile of polarized macrophages of a lower vertebrate. Here, using as microbial ligand LPS to polarize macrophages into the M1 state, we measured the production of nitric oxide (NO) to quantify M1 polarization. On the other side of the spectrum, we used extracellular cAMP to polarize macrophages into the M2 state and measured arginase activity to quantify M2 polarization. Polarization profiles, described as transcriptomes obtained upon RNA sequencing, were confirmed by real-time quantitative PCR (RT-qPCR) of the most regulated genes. It appeared that fish cytokines orthologous to IFNγ or IL4 or IL13, are not required per se for the establishment of polarized states of fish macrophages, since LPS and cAMP alone could polarize carp macrophages into M1 or M2. Cytokine-dependent activation with Ifnγ amplified the innate activation state induced by LPS alone. It remains to be investigated to what extent fish Il4/13 can influence macrophage polarization. In accordance with our hypothesis, here we show that M1 and M2 profiles of macrophages derived from head kidney, the bone marrow equivalent in teleost fish, are strikingly similar to those found in mice and humans. The M1-M2 dichotomy could therefore be an intrinsic property of macrophages which arose early in evolution.

Materials and Methods

Animals

European common carp (*Cyprinus carpio carpio* L.) were reared in the central fish facility Carus, at Wageningen University and Research, Wageningen, Netherlands. Fish were kept at 23°C in recirculating UV-treated tap water and fed pelleted dry food (Sniff, Soest, Germany) daily. R3xR8 carp are the hybrid offspring of a cross between fish of Polish origin (R3 strain) and Hungarian origin (R8 strain) (Irnazarow, 1995). Carp were between 9 and 11 months old at the start of the experiments. All studies were performed with approval from the local animal welfare committee (DEC) of Wageningen University.

In vitro culture of head kidney-derived macrophages

Carp head kidney-derived macrophages were obtained essentially as described before (Joerink et al., 2006a). Briefly, fish were euthanized, bled, and head kidneys were removed. These were gently passed through a nylon mesh to obtain a single-cell suspension using incomplete NMGFL-15 medium. The suspension was layered on 51% (1.071 g/mL) Percoll and centrifuged to obtain an interface layer of leukocytes. These cells were collected, washed, and seeded in flasks with complete NMGFL-15 medium. After 6 days of culture at 27°C the head kidney-derived macrophages were harvested and ready for stimulation.

Each day of culture, the progress of the cells was assessed by microscopy, and representative pictures were obtained with a CCD camera (DP50, Olympus, Zoeterwoude, Netherlands) connected to an Axiovert 10 Inverted Microscope (Carl Zeiss, Sliedrecht, Netherlands).

In order to monitor possible variability between individual fish, an aliquot of head kidney-derived macrophages from each fish was tested for a consistent cellular response (in terms of nitrite production) to 24 hours stimulation with 50 μg/mL LPS, since this is a fast protocol to assess culture quality (see below for details on stimuli and nitrite production).

Stimuli used for in vitro polarization of head kidney-derived macrophages

In order to polarize head kidney-derived macrophages into innate activated cells, LPS (*Escherichia coli*, L2880, Sigma-Aldrich) was used at 30 µg/mL. To polarize cells towards classically activated macrophages, cells were stimulated with 30 µg/mL LPS and 100 ng/mL recombinant carp Ifnγ2 (Arts et al., 2010). To polarize cells towards alternatively activated macrophages, cells were stimulated with 0.5 mg/mL of the cAMP analogue, dibutyryl cAMP (N^6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate sodium, D0627, Sigma-Aldrich).

All concentrations mentioned above were chosen after optimization experiments (Joerink et al., 2006a), (Arts et al., 2010).

Quantification of nitrite production

Head kidney-derived macrophages were seeded at a density of $0.5x10^6$ cells in 90 µL 50% complete NMGFL-15 medium in 96-well flat-bottom culture plates. Each well received 10 µL stimulus to reach the desired concentration as indicated previously, or 50% complete NMGFL-15 medium as control. After 24 hours at 27°C, the nitrite production was measured by the Griess reaction as described previously (Saeij et al., 2000). The absorbance was measured at 535 nm in a FilterMax™ F5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) and the nitrite concentration was calculated on the basis of a sodium nitrite standard curve.

Arginase assay

Head kidney-derived macrophages were seeded at a density of $0.2x10^6$ cells and stimulated as described above. After 24 hours, arginase enzyme activity was measured with the QuantiChrom™ Arginase Assay Kit (DARG-200, BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions, with some minor changes. Briefly, only $0.2x10^6$ cells were used per sample. The cells were lysed on ice using 100 µL lysis buffer containing 20 mM Tris-HCl (pH 7.5-8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, and phenylmethylsulfonyl fluoride added immediately prior to use to a final concentration of 1 mM. After addition of substrate reagent, the plate was incubated at 37°C for 90 min, after which the arginase reaction was stopped. The amount of urea produced was then determined by addition of urea reagent and measuring the optical density after

1 hour incubation at room temperature. The optical density was measured at 435 nm in a FilterMax™ F5 microplate reader (Molecular Devices) and the arginase activity was calculated according to the manufacturer's description by comparison to a urea standard sample provided with the assay.

RNA isolation

For RNA isolation, 1-3 x10^6 cells/450 µL 50% complete NMGFL-15 medium were seeded in 24-well flat-bottom culture plates. Each well received 50 µL stimulus to reach the desired concentration as indicated previously, or 50% complete NMGFL-15 medium as control. Alternatively, cells were seeded at a density of 0.5x10^6 cells in 90 µL 50% complete NMGFL-15 medium in triplicate wells of 96-well flat-bottom culture plates, and each well received 10 µL stimulus or 50% complete NMGFL-15 medium as control. After 6 hours of stimulation (time-point chosen after optimization experiments), cells were lysed and collected in 350 µL RLT buffer (QIAgen, Venlo, Netherlands) and stored at -80°C until RNA extraction.

Total RNA was extracted using the RNeasy Mini kit according to the manufacturer's protocol (QIAgen) including on-column DNase treatment with the RNase-free DNase set (QIAgen). RNA sizing, quantitation and quality were assessed using the Agilent Bioanalyzer. RNA was stored at -80°C until use.

Illumina sequencing and data analysis

Libraries were made with the TruSeq Stranded total RNA library prep kit according to manufacturer's description (Illumina Inc., San Diego, CA, USA). Paired-end libraries were sequenced with a read length of 2×50 nucleotides using an Illumina HiSeq 2500 according to the manufacturer's description, using Illumina software (HCS) for basecalling. Tophat version 2.0.5 (Kim et al., 2013) was used to align the reads to the reference genome (Bioproject PRJNA73579) (Henkel et al., 2012). For each read pair, secondary alignments (which meet alignment criteria but are less likely to be correct) were filtered out using SAMtools version 0.1.18 (Li et al., 2009). For each predicted gene, read counts were obtained from the alignment file using HTSeq-count version 0.5.3.p9 (Anders et al., 2015) using the 'intersection-strict' setting to ignore reads not aligning to annotated exons. Data quality was assessed using the statistical package R (Team, 2015). Raw RNA-seq counts were normalized to correct for sequencing depth and (optionally) transcript length by

dividing counts by sequencing depth (the total number of reads) or by empirical estimates of sequencing depth (available in the edgeR package, v. 3.12.0 (Robinson et al., 2010)).

cDNA synthesis

Prior to cDNA synthesis, 250 ng-1 µg of total RNA was subjected to an additional DNase treatment by using DNase I Amplification Grade (Invitrogen) according to the manufacturer's protocol. Synthesis of cDNA was performed with Invitrogen's SuperScript III Reverse Transcriptase and random primers, according to the manufacturer's instructions. For each sample a non-reverse transcriptase control was performed. cDNA samples were diluted 25 times in nuclease-free water before use as templates in real-time PCR experiments.

Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was performed in a Rotor-Gene 6000 with a 72-well rotor (QIAgen, The Netherlands) with the ABsolute QPCR SYBR Green Mix (Thermo Scientific) as detection chemistry as described previously (Forlenza et al., 2012). Primer sequences are given in Table 1. RT-qPCR data were analysed by Rotor-Gene 6000 Series Software 1.7. The melting temperature and profile of the melting curves were used to validate the specificity of the amplification. The gene expression was analysed using the average amplification efficiency for each primer pair and the take-off value as calculated by the comparative quantitation analysis of the software. Gene expression was calculated as the average amplification efficiency to the power of the negative value of the take-off value. The expression of the housekeeping gene *40s ribosomal protein s11* was used to normalize the data.

Construction of phylogenetic trees

Protein sequences corresponding to genes of interest were obtained for human, mouse, chicken, *Xenopus*, spotted gar, cod, fugu, and zebrafish using Ensembl (http://www.ensembl.org/). In a few cases, for genes which were not annotated in Ensembl we obtained the sequences from NCBI (http://www.ncbi.nlm.nih.gov/protein/). The multiple sequence alignments for the phylogenetic trees were made with ClustalX 2.1 (Larkin et al., 2007) and the trees were constructed using the Neighbour Joining method with the number of bootstrap trials set to 10000. The phylogenetic trees were visualized with MEGA6.

Statistical analysis

To determine differentially expressed genes RNA-seq counts were analysed using the DESeq package from Bioconductor (v3.3) (Anders and Huber, 2010) in R statistical software (3.0.2) (Team, 2015). Counts from stimulated and unstimulated samples were normalized taking into account the sequencing depth of each sample and differential expression was assessed using the negative binomial distribution. Results were considered statistically significant when Padj<0.05. Venn diagrams were constructed using the R package VennDiagram (1.6.17) (Chen, 2016).

Results

Functional characterization of cytokine-independent polarization of carp macrophages

Leukocytes were isolated from carp head kidney and cultured for 6 days without addition of stimuli or cytokines. During this time most lymphocytes die and monocytes differentiate into macrophages ((Joerink et al., 2006a); see also Figure 1A). To characterize the cytokine-independent polarization of carp macrophages, cells were polarized with LPS to induce innate activation, or with cAMP to induce alternative activation. Differences in macrophage activity associated with these different activation states were verified by measuring production of nitric oxide (Figure 1B) and arginase activity (Figure 1C). The observed effects were stimulus-specific, since adding LPS did not significantly increase arginase activity, nor did cAMP lead to nitrite production (data not shown). Overall, carp head kidney-derived macrophages could be polarized, in a cytokine-independent manner, into different activation states using LPS and cAMP, as shown by the functional characteristics of the cells in terms of nitric oxide production and arginase activity, respectively.

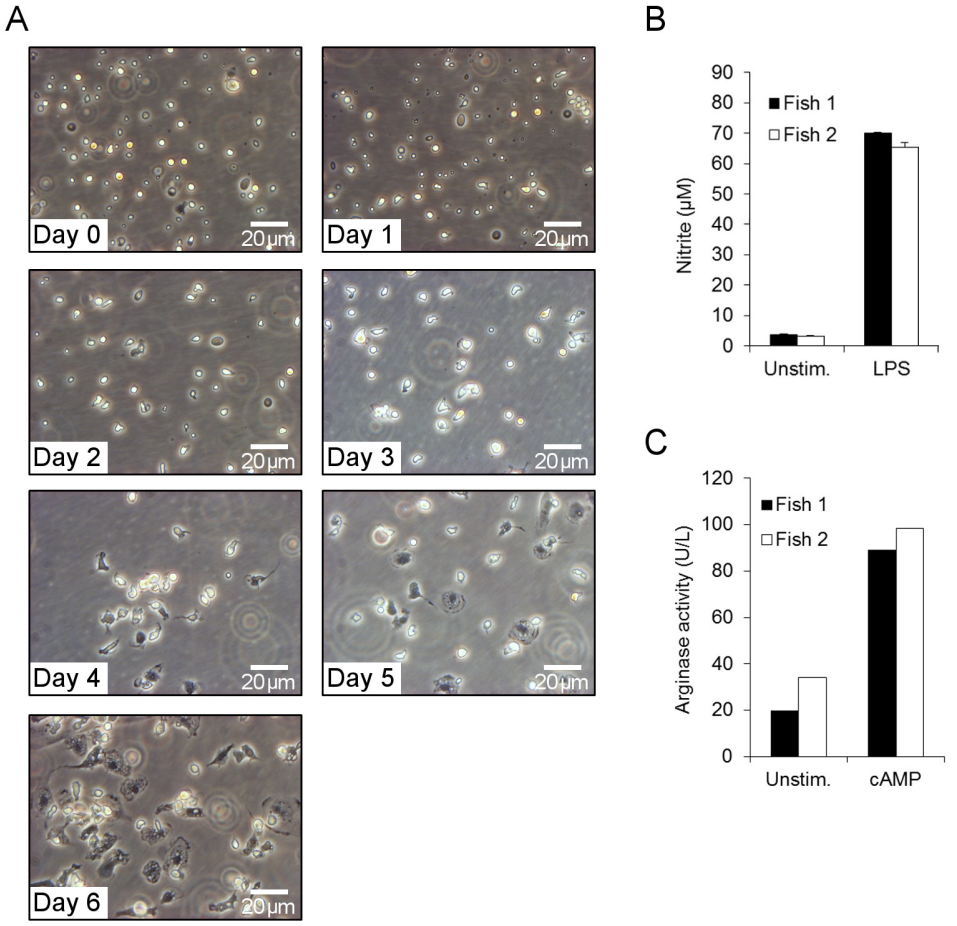

Figure 1. Differential activation of head kidney-derived carp macrophages suggests polarization into innate or alternative activation states. (A) Photographs of freshly isolated leukocytes from head kidney (Day 0) and their progression during in vitro culture towards an enriched population of differentiated carp macrophages at Day 6. (B) Production of nitric oxide by carp macrophages stimulated with LPS (innate activation) compared to unstimulated cells. Values on the y-axis represent nitrite concentrations. Bars are mean ± SD of triplicate measurements on macrophages from n=2 individual fish per treatment. (C) Arginase activity of carp macrophages stimulated with cAMP (alternative activation) or left unstimulated. Values on the y-axis represent arginase activity measured as conversion of L-arginine to urea in units per litre of sample. Bars are measurements on macrophages from n=2 individual fish per treatment. The polarization of carp macrophages was confirmed with cells from n>8 fish; Figures 1B-C show the data for those macrophages used for subsequent RNA analysis.

Transcriptional characterization of cytokine-independent polarized carp macrophages

After having assessed the ability of LPS and cAMP to polarize macrophages into distinct states, each having a clear differential activation with respect to nitric oxide production and arginase activity, we next investigated the molecular signatures specific for each of these two subsets. In particular we were interested in identifying specific gene expression signatures, and for this purpose transcriptional profiling using RNA-Seq was chosen. In Figure 2A we show the number of differentially expressed genes and their fold change after stimulation with LPS or cAMP in comparison with their respective unstimulated controls (obtained from the same macrophage cultures). Overall, for both LPS- and cAMP-stimulated cells, more than 1000 genes were differentially expressed with fold-change >2.

The degree of similarity between gene expression profiles of innate activated macrophages (stimulated with LPS) and alternatively activated macrophages (stimulated with cAMP) is visualized by Venn diagrams in Figure 2B. Approximately 2-4-fold more genes were up- rather than down-regulated, which was true for both stimuli. Most importantly, LPS and cAMP stimulation induced unique profiles with only a small number of shared transcripts between the two stimuli. Thus, sequencing-based RNA analysis identifies functional innate activated carp macrophages as a population clearly distinct from functional alternatively activated macrophages.

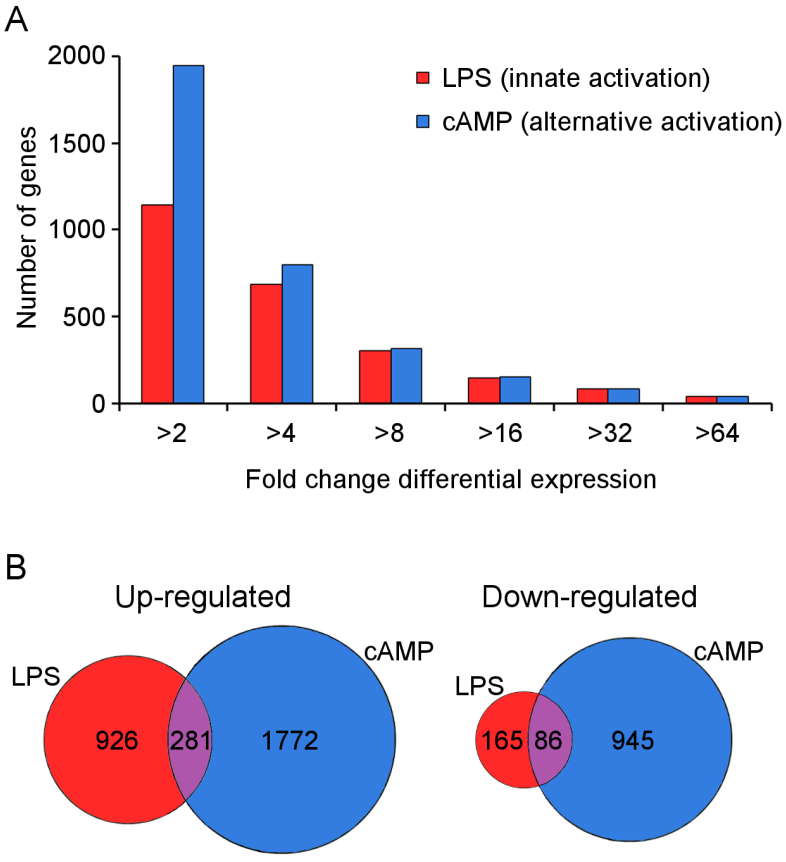

Figure 2. Transcriptional analysis of cytokine-independent polarized carp macrophages. RNA was isolated from carp macrophages stimulated with 30 µg/mL LPS (innate activation) or 0.5 mg/mL cAMP (alternative activation) and their corresponding unstimulated controls. RNA was used to prepare cDNA libraries for Illumina RNA-Seq. (A) The bar graph shows the number of differentially regulated transcripts in polarized macrophages significantly (Padj<0.05) different from their corresponding unstimulated controls. (B) Venn diagrams show the overall unique or shared gene signatures between LPS- and cAMP-stimulated cells.

Table 2 shows the top 30 (most up-regulated) genes in innate activated macrophages stimulated with LPS. Table 3 shows the top 30 genes in alternatively activated macrophages stimulated with cAMP. As indicated in these tables, a large number of the genes listed have previously been shown to be involved in either the innate or alternative activation of human or mouse macrophages. Some genes however appear to be uniquely up-regulated in polarized macrophages of fish.

A subset of the most up-regulated genes in innate activated macrophages was chosen for subsequent verification of expression by real-time qPCR: the pro-inflammatory cytokines interleukin-1 and -12, (*il1β* and *il12a* (*p35*)), the soluble pattern recognition receptor called long pentraxin 3a (*ptx3a*), the acute phase protein serum amyloid A (*saa*) which recruits immune cells to inflammatory sites and induces enzymes that degrade extracellular matrix, and inducible nitric oxide synthase (*nos2b*) which is paramount for production of nitric oxide by activated macrophages. Similarly, a number of genes most up-regulated in alternatively activated macrophages were also chosen for confirmation by qPCR: cysteine-rich angiogenic inducer 61, a secreted, extracellular matrix-associated signalling protein (*cyr61*); inhibin beta subunit, which dimerizes to form activin, an important regulator of wound repair (*inhba*), tissue inhibitor of metalloproteinases (*timp2*) which regulates turnover of extracellular matrix, transglutaminase (*tgm2*), which binds to proteins of the extracellular matrix, and finally arginase (*arg2*). While *arg2* was not among the top 30 genes, it was still in the top 50 with a 26-fold up-regulation; it is known as a valuable marker for alternatively activated macrophages, and was therefore included in the qPCR experiments. Regulation of expression of these selected genes, based on the RNA sequencing data, was verified by RT-qPCR on macrophages from n=4 individuals (Figure 3). Without exception, RT-qPCR confirmed RNA sequencing, albeit with fold-changes 5-10-fold lower than corrected counts. Phylogenetic analyses confirm the orthology of the chosen genes, thus indicating the potential use of these molecules as gene signatures for innate- or alternatively-activated macrophages.

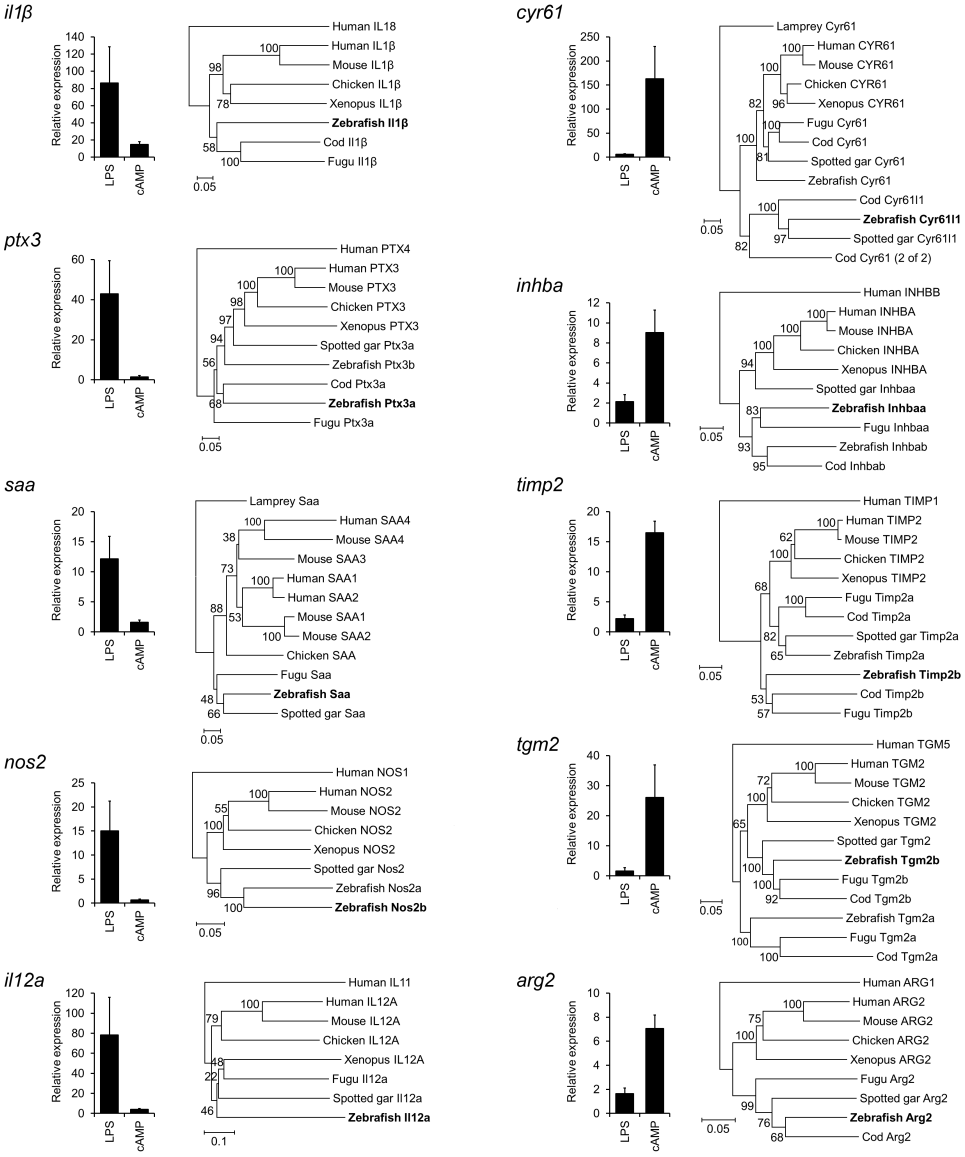

Figure 3. Gene signatures for innate and alternatively activated macrophages. Five genes which could be potential signatures for innate activated macrophages (left) or for alternatively activated macrophages (right) were studied in further detail. The bar graphs show the relative expression levels obtained by RT-qPCR analysis (n=4), where expression data were normalized to the housekeeping gene *40s ribosomal protein s11* and expressed relative to the unstimulated control. Furthermore, the phylogenetic trees visualize the evolutionary relationships between the relevant protein sequences

in various species: human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), clawed frog (*Xenopus tropicalis*), spotted gar (*Lepisosteus oculatus*), cod (*Gadus morhua*), fugu (*Takifugu rubripes*), and zebrafish (*Danio rerio*). Accession numbers for all protein sequences used to construct the phylogenetic trees can be found in Table 4.

Characterization of cytokine-dependent macrophage activation

Stimulating carp macrophages with LPS only, readily polarizes cells into M1-like innate activated macrophages, as shown above. We then sought to establish whether the pro-inflammatory cytokine Ifnγ would enhance the effect of LPS as is the case in human and mouse, thereby polarizing cells into classically activated macrophages (Biswas and Mantovani, 2010). Thus, in order to study potential additive effects of Ifnγ, this cytokine was added to macrophages in combination with LPS, and gene expression was analysed by RNA-Seq. The degree of similarity between gene expression profiles of innate activated carp macrophages (stimulated with LPS only) and classically activated macrophages (stimulated with LPS+Ifnγ) is visualized in Figure 4A, with the Venn diagram showing a substantial overlap in both up- and down-regulated transcripts. In fact, 80-90% of the genes up- or down-regulated by LPS were also up- or down-regulated by LPS+Ifnγ. This substantial overlap in gene signature is also reflected in the top 30 of genes most highly up-regulated by LPS+Ifnγ (Table 5): almost all highly up-regulated genes in this table overlap with the top 30 genes found in macrophages stimulated with LPS alone (Table 2). The additive effect of Ifnγ was clear (Figure 4A), and especially highlighted by the higher fold change induced by LPS+Ifnγ in comparison to LPS only (Table 5). Thus, sequencing-based RNA analysis identifies innate and classically activated carp macrophages as two populations with very similar profiles, with Ifnγ enhancing the effect of LPS alone. In clear contrast, the number of transcripts shared between classically activated macrophages (stimulated with LPS+Ifnγ) and alternatively activated macrophages (stimulated with cAMP) (Figure 4B) is very low. Only 10-20% of the genes up- or down-regulated by cAMP were also up- or down-regulated by LPS+Ifnγ. Thus, sequencing-based RNA analysis identifies classically and alternatively activated carp macrophages as two populations with very distinct profiles.

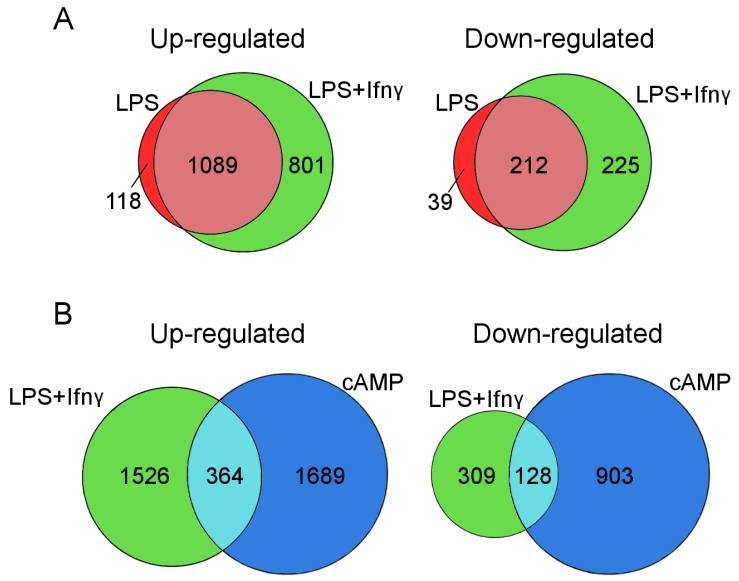

Figure 4. Fish Ifnγ augments the response of macrophages to LPS. RNA was isolated from carp macrophages stimulated with 30 μg/mL LPS in combination with recombinant carp Ifnγ (100 ng/mL). RNA was subsequently used for paired-end cDNA libraries for Illumina RNA-Seq. Venn diagrams show the overall gene expression similarities and differences between (A) LPS and LPS+Ifnγ, and (B) LPS+Ifnγ and cAMP using sequencing data for LPS and cAMP alone previously shown in Figure 2.

Discussion

Previous studies have shown that carp head kidney-derived macrophages, upon in vitro stimulation with LPS or cAMP, can develop functionally different polarization states (Joerink et al., 2006a). Our experiments have confirmed that stimulation with LPS leads to an increase in production of nitric oxide, whereas cAMP stimulation leads to increased arginase activity. LPS preparations from Gram-negative bacteria such as *Escherichia coli* are widely used as a microbial stimulus to activate macrophages. In mammals, the recognition of LPS by the TLR4 receptor complex triggers the activation of a signalling pathway leading to activation of transcription factors such as NFκB and AP-1. These in turn up-regulate the expression of pro-inflammatory cytokines, typical of M1 phenotypes. In fish, the recognition of LPS may not go through the exact same Tlr4 receptor complex; much higher concentrations of LPS are needed for stimulation (Iliev et al., 2005) and Tlr4 may not always be involved – several studies have dissected the function of zebrafish Tlr4 and concluded it does not recognize LPS, while some fish species (e.g. *Fugu rubripes*) even seem to lack entirely a *tlr4* gene in their genome (Sepulcre et al., 2009), (Sullivan et al., 2009), (Oshiumi et al., 2003), (Pietretti and Wiegertjes, 2014). Nevertheless, regardless of the exact receptor sensing LPS, we show that LPS polarizes fish macrophages into an innate/M1-like phenotype as evidenced by the increased production of nitric oxide and up-regulation of pro-inflammatory genes.

With regards to generating an alternative/M2 phenotype in macrophages, it has previously been shown that the addition of exogenous cAMP to human monocytes can lead to an anti-inflammatory state via adenosine receptors and activation of adenylyl cyclases and increase in intracellular second messenger cAMP (Sciaraffia et al., 2014). Furthermore, cAMP indirectly leads to arginase activation – one of the hallmarks of alternatively activated macrophages, also used by us to characterise M2-like fish macrophages, in a manner dependent on protein kinase A (Chang et al., 2000). Although this exact mechanism has not been confirmed for fish cells, there also is no reason to assume the mechanism would be different in fish macrophages responding to cAMP. Thus, similar to macrophages of higher vertebrates, we show that addition of cAMP generates fish macrophages with an alternative/M2-like phenotype, as evidenced by an increase of arginase activity and expression of genes involved in tissue repair and regulation.

Of particular interest, carp macrophages do not seem to require the presence of Ifnγ or Il4/13 cytokines to differentiate into cells with functional characteristics of M1 and M2 macrophages. Possibly, macrophages have developed the ability to polarize already in an evolutionarily distant past, perhaps even before T cell subsets and their associated

cytokines developed fully, and it could therefore be an intrinsic property of these cells (Wiegertjes et al., 2016).

We combined our in vitro culture system measuring the nitric oxide production or arginase activity of functionally polarized carp macrophages, with an RNA-Seq approach to identify gene signatures for activated carp M1 and M2 macrophages. Our analyses show that innate (M1-like) and alternatively activated (M2-like) macrophages have very different gene expression profiles. Among the many genes that make up the gene signatures of either M1 or M2, we have chosen a limited number for subsequent confirmation by qPCR. From the top 30 most up-regulated genes representing the profile of innate activated macrophages, here we discuss the following five as potential signature molecules for M1 macrophages; *il1β*, *ptx3a*, *saa*, *nos2b*, and *il12a*.

Il1β is a well-known pro-inflammatory cytokine, while pentraxin 3 (a fluid-phase pattern recognition receptor (Deban et al., 2011)) and serum amyloid A (Uhlar and Whitehead, 1999), (Ye and Sun, 2015) are part of the acute-phase response. Their up-regulation in LPS-stimulated macrophages is therefore not surprising. That is also the case for *nos2b*, which encodes the inducible nitric oxide synthase iNOS (its role in polarized macrophages is reviewed by Wiegertjes *et al*. (Wiegertjes et al., 2016)). Il12a (p35) forms part of the heterodimeric Il12 cytokine along with Il12b (p40). In mammals, IL12 skews naive CD4+ T cells towards a Th1 phenotype thus promoting IFNγ production, which in a positive feedback loop leads to further macrophage activation (Hamza et al., 2010). In concert with IFNγ, IL12 also antagonizes Th2 differentiation and restricts the production of Th2 cytokines IL4, IL5, and IL13 (Watford et al., 2003). In human monocytes, expression of p35 seems to be the limiting factor for heterodimer production (Snijders et al., 1996). This might also be the case in carp macrophages, where we observe a 155-447-fold up-regulation of *p35* in LPS-stimulated cells based, in particular, on an extremely low count in unstimulated macrophages. Indeed, the second chain of the Il12 heterodimer, *p40*, already is very highly expressed in unstimulated cells (approx. 10,000 counts), with a 3-fold increase only, when cells are stimulated with LPS. It should be noted that carp possess several paralogs of *p40* (Huising et al., 2006), and the very high basal expression is the case only for the *p40b* paralog. Although more work is needed to ascertain the functional implications of the different Il12 heterodimers, the five genes discussed above all comply with the M1 macrophage profile.

From the top 30 most up-regulated genes representing the profile of classically activated macrophages, the majority of potential markers is identical to those listed for innate activated macrophages, albeit that most genes display a higher fold change. This suggests that LPS alone indeed can induce an M1-like profile in teleost macrophages. It also

suggests that the pro-inflammatory cytokine Ifnγ plays a similar role in fish as in higher vertebrates, augmenting the response of macrophages to microbial stimuli such as LPS. This is in line with a previous study in carp (Arts et al., 2010), and with mammalian studies which have found that LPS+Ifnγ (classically) activated macrophages have higher respiratory burst activity as well as increased ability for antigen presentation (major histocompatibility complex (MHC) class II) and co-stimulation (CD86) than have LPS-only stimulated macrophages (reviewed by Taylor *et al*. (Taylor et al., 2005)).

From the transcriptional profile of alternatively activated macrophages, here we discuss the following five as potential gene signatures for M2 macrophages; *cyr61*, *inhba*, *timp2*, *tgm2*, and *arg2*. The latter gene, encoding Arginase-2, is significantly up-regulated in cAMP-stimulated macrophages, although it does not reach a position in the top 30 list. Obviously, the top 30 is merely a random cut-off and there are many more genes that contribute to the phenotype of alternatively activated macrophages, among which *arg2* which was in the top 50 with a highly significant fold change. The up-regulation of *arg2* gene expression is in accordance with the increased arginase enzyme activity that we measured. Arginase-2 and iNOS activity leads to opposing outcomes in macrophages through their different metabolism of L-arginine (Wiegertjes et al., 2016).

cyr61 is the most up-regulated gene in the cAMP-stimulated macrophages. It encodes the matricellular protein Cysteine-rich 61, an extracellular matrix (ECM) protein that plays a minimal role in matrix structural integrity, but regulates a multitude of cellular responses. It modulates inflammation, wound healing, and tissue repair, and it supports cell adhesion through direct binding to integrin receptors (Lau, 2011). *cyr61* is expressed by fibroblasts (Chen et al., 2001) and to our knowledge has not previously been directly linked to alternative macrophage activation, but it seems highly relevant to study further within this context.

The *inhba* gene encodes the protein Inhibin beta A, which forms a subunit of both Activin and Inhibin, two closely related glycoproteins with contrasting biological effects. Activin is a dimer composed of two Inhibin beta subunits. Inhibin is also a dimer wherein the first component is a beta subunit similar or identical to the beta subunit in Activin. However, in contrast to Activin, the second component of the Inhibin dimer is a more distantly-related alpha subunit. In our datasets only the beta subunit is expressed (and highly up-regulated by cAMP-stimulation), which means the formation of Activin is the result. This molecule is an important regulator of wound repair (Sulyok et al., 2004), and is associated with alternative activation of macrophages (Ogawa et al., 2006).

Next, we consider *timp2* (TIMP metallopeptidase inhibitor 2), which is an endogenous inhibitor of the ECM-degrading matrix metalloproteinases MMP14 (Zucker et al., 1998)

and MMP2 (Morgunova et al., 2002). TIMP2 regulates ECM turnover and ensures uncontrolled MMP-mediated tissue damage does not occur, thereby contributing to the maintenance of tissue homeostasis. As such, the relation to alternatively activated macrophages is clear.

Last but not least, the *tgm2* gene is up-regulated in cAMP-stimulated macrophages. *tgm2* encodes Transglutaminase 2, and has been identified as a conserved M2 marker in both human and mouse macrophages (Martinez et al., 2013). TGM2 is an enzyme associated with multiple biological functions, including phagocytosis of apoptotic cells, crosslinking of cellular proteins, and extracellular matrix generation (Mehta et al., 2010). Since our study has identified TGM2 as being up-regulated also in fish M2 macrophages, it is likely to exhibit functions conserved across species.

In conclusion, the five genes discussed above all comply with the M2 macrophage profile and it may be clear that our transcriptomic approach has allowed for the identification of M1 as well as M2 gene signatures for fish macrophages. These genes, along with many other genes identified in our RNA-Seq datasets, have potential to be used in future studies of polarized immune responses in fish. In addition, the annotation of the RNA-Seq datasets used in this study is continually being improved. This will lay the foundation for a broader analysis of up- and down-regulated pathways and gene functions based on e.g. GO (gene ontology) analysis, which can further group the differentially regulated genes according to cellular component, molecular function, and biological process. So, while we here have extracted information on gene signatures that are immediately useful in our studies of polarized macrophages, further insights await regarding an integrated view on pathways relevant to the study of these cells. Drawing on the strength of combining research in carp with zebrafish, the gene signatures we here identify in carp could for instance be used to track zebrafish macrophage subsets in vivo during e.g. tumour development, infection or inflammatory processes. An example of this was shown already for TNFα (Nguyen-Chi et al., 2015), but even more suitable genes can now be chosen, that better reflect the polarized macrophage subsets.

In the present study we have focused on genes up-regulated with high fold changes, as these could be considered most relevant when searching for markers relevant for M1- and M2-like phenotypes of fish macrophages. Of course, further information can be obtained from our dataset when genes would be ranked according to P-values, as some genes with a lower fold change in some cases can have a lower P-value. In addition, more emphasis can be placed on down-regulated genes, which also have a story to tell about the differences between macrophage subtypes. Also, total counts in datasets of unstimulated carp macrophages provide information on macrophage phenotypes prior to polarization.

The transcriptome of carp could be annotated using the comprehensively described zebrafish genome, with zebrafish being a close relative of carp and therefore a suitable reference (Henkel et al., 2012), (Kolder et al., 2016). However, the carp genome has undergone an additional whole-genome duplication event compared to zebrafish, which means there are often two copies of each gene in carp which correspond to a single gene in zebrafish. This was apparent throughout the transcriptome, visible in the top 30 tables, and was often confirmed by RT-qPCR where we intentionally designed common primers to detect both paralogs and correspondingly identified two peaks in the melting curve analysis. It may be of interest for future studies to design paralog-specific primers to ascertain which paralog, if any, would be most relevant to further refine the gene signature profiles of polarized macrophages in fish.

We hypothesized that macrophages from teleost fish would show activation profiles and gene signatures analogous to those typical of macrophages from higher vertebrates. It may be clear from our data that macrophages of carp can indeed polarize into activation states typical of innate/classical (M1) and alternative (M2) extremes. Further, we describe that cytokine-independent activation with microbial LPS leads to M1-like macrophages as shown by high levels of nitric oxide, whereas exogenous cAMP drives macrophages into alternative M2-like states as shown by high levels of arginase. Our RNA sequencing approach confirmed the existence of a large set of gene signatures for M1 and M2 macrophages which appear conserved from fish to mammals. Further comparisons between fish and mammalian species have yet to be made and will help to address similarities and differences in a broader perspective. Certainly, cytokine-dependent activation with Ifnγ amplified the innate activation state induced by LPS alone, as described for mammalian species, whereas we are presently studying the possibility that carp Il4/13 could amplify alternative activation states.

Huge advances have been made in the field of macrophages since the process of phagocytosis was first described by Metchnikoff in 1882. It seems appropriate for the initial description to have been made in an invertebrate species, since these animals (lacking adaptive immunity) are so very dependent on innate immunity and indeed macrophages for survival. As such, macrophage (-like) cells are found across the animal kingdom and have to deal with very diverse circumstances. Having the ability to adjust to different stimuli and environments allows macrophages to respond in an appropriate manner, producing nitric oxide, reactive oxygen species, and pro- or anti-inflammatory cytokines and regulating expression of genes for tissue maintenance and repair. This versatility is of great advantage to the host, and is well described for mice and humans. We show here that archetypal polarization of macrophages also occurs in a teleost species and describe conserved gene signatures associated with such polarization. In conclusion,

from an evolutionary viewpoint, our findings suggest that the extremes of the macrophage activation states, M1 and M2, are guided by evolutionarily conserved principles.

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Table 1. Primers for RT-qPCR.

	Gene*	FW (5'-3')	RV (5'-3')	GenBank accession no.	Reference
M1-like macrophages	*il1β*	AAGGAGGCCAGTGGCTCTGT	CCTGAAGAAGAGGAGGCTGTCA	AJ245635	(Engelsma et al., 2001)
	*ptx3a**	GCAGCAAAGGAAGCATAC	ACCACATTACCACGGAT	cypCar_00014116	This study
	*saa**	CCCAGGACAAGCCATTG	GCAGCATCATAGTTCCC	cypCar_00037333, cypCar_00036204	This study
	*nos2b**	AACAGGTCTGAAAGGGAATCCA	CATTATCTCTCATGTCCAGAGTCTCTTCT	AJ242906	(Saeij et al., 2000)
	il12a (p35)	TGCTTCTCTGTCTCTGTGATGGA	CACAGCTGCAGTCGTTCTTGA	AJ580354	(Huising et al., 2006)
M2-like macrophages	*cyr61**	AGTAGTCGGCTGCGTC	AATGCGGTTGTCATCATC	cypCar_00001309	This study
	*inhba**	CAGCCACGGACTGTC	GCATTGAGGAGAGCAGC	cypCar_00008046, cypCar_00010506	This study
	*timp2**	TGGCAAAAAGGAATACCTG	CTCAGAGACTCCCAAGATTC	cypCar_00030755, cypCar_00034223	This study
	*tgm2**	GCCTGGTATTTTGGACAGT	GCACTCAGCACTCTTGT	cypCar_00034483, cypCar_00030329, cypCar_00041907	This study
	*arg2**	GGAGACCTGGCCTTCAAGCATCT	CTGATTGGCACGTCCAACT	AJ618955	(Joerink et al., 2006b)
Housekeeping gene	*40S rps11*	CCGTGGGTGACATCGTTACA	TCAGGACATTGAACCTCACTGTCT	AB012087	(Huttenhuis et al., 2006)

*Genes for which common primers were designed to recognize more than one paralog.

Table 2. The top 30 genes most up-regulated by LPS ranked by fold change.

Gene	Description	Contig*	Fold change	Padj	Main function	Reference
sost	Sclerostin	cypCar_00018891	605	4.85E-32	Prostaglandin E2 decreases *sost* transcription via cAMP. *sost* inhibits bone formation by osteoblasts	(Genetos et al., 2011)
il12a, il12-p35	interleukin 12 subunit a, p35	cypCar_00024698, cypCar_00024699	447, 155	1.63E-49, 1.02E-40	differentiation of naive T cells into Th1 cells, stimulates the production of IFNγ and TNFα from T cells and NK cells	(Holscher, 2004)
ptx3a	pentraxin 3a	cypCar_00014116	372	2.59E-64	acute-phase response protein, pathogen recognition, leukocyte recruitment	(Deban et al., 2011)
il1b	interleukin 1β	cypCar_00043439, cypCar_00043440	295, 283	3.37E-64, 8.49E-102	pro-inflammatory cytokine	(Mantovani et al., 2004), (Jaguin et al., 2013)
sich211-242b18.1, pde4dip	phosphodiesterase 4D interacting protein	cypCar_00007456	270	7.78E-17	serves to anchor PDE4D at Golgi/centrosome region of the cell, where PDE4D degrades cAMP	(Verde et al., 2001)
ccl20, mip3a	chemokine (C-C motif) ligand 20	cypCar_00004353	261	9.25E-33	chemotactic factor that attracts lymphocytes	(Schutyser et al., 2000)
adcyap1a	adenylate cyclase activating polypeptide 1 (pituitary)	cypCar_00018838, cypCar_00029617	202, 47	4.91E-08, 2.96E-09	pro- and anti-inflammatory factor, increase phagocytic ability of macrophages	(Sherwood et al., 2000), (El Zein et al., 2007)
avpr2ab	arginine vasopressin receptor 2	cypCar_00004651	135	8.23E-03	possible role in the regulation of inflammation	(Fleisher-Berkovich et al., 2004)
adgrb3	adhesion G protein-coupled receptor b3	cypCar_00032513	129	8.98E-08	brain-specific angiogenesis inhibitor; high-affinity receptor for C1q-like proteins	(Bolliger et al., 2011)
arhgef25b	Rho guanine nucleotide exchange factor 25	cypCar_00002651	106	6.65E-03	involved in formation of actin stress fibers, regulates axon and dendrite formation	(van Unen et al., 2015)
sorcs2	sortilin related VPS10 domain containing receptor 2	cypCar_00007700	102	4.22E-06	facilitate rapid endocytosis; involved in intracellular sorting	(Hermey et al., 2004)
cyp27c1	cytochrome P450 family 27 subfamily C member 1	cypCar_00041703	97	2.79E-24	converts vitamin A_1 into A_2	(Enright et al., 2015)
steap4	STEAP4 metalloreductase	cypCar_00042005	91	2.08E-72	metalloreductase; upregulated in rainbow trout macrophages stimulated with LPS	(Iliev et al., 2006)
sidkey-257i7.5	uncharacterized protein	cypCar_00005123	86	2.60E-02	unknown, novel protein similar to Spi-C transcription factor (Spi-1/PU.1 related)	www.ensembl.org

agrn	agrin	cypCar_00029572	82	7.11E-06	monocyte and macrophage maturation and survival; formation of the phagocytic synapse	(Mazzon et al., 2012)
lacc1	laccase domain-containing protein 1	cypCar_00008998, cypCar_00009189	81, 57	6.06E-06, 5.46E-04	association with auto-inflammatory diseases: Crohn's, leprosy, juvenile idiopathic arthritis	(Wakil et al., 2015)
tmc2b	transmembrane channel like 2b	cypCar_00006710	78	5.78E-06	transmembrane protein that is necessary for mechanotransduction in cochlear hair cells of the inner ear	(Pan et al., 2013)
saa	serum amyloid a	cypCar_00037333, cypCar_00036204	77, 28	1.24E-06, 4.65E-14	acute-phase protein; pro-inflammatory and in part homeostatic properties	(Uhlar and Whitehead, 1999), (Ye and Sun, 2015)
aknad1	AKNA domain containing 1	cypCar_00023198	72	2.58E-04	unknown	
mcoln3a	mucolipin 3	cypCar_00027296	71	2.97E-27	Ca(2+) channel protein, regulation of autophagy	(Choi and Kim, 2014)
neurl1aa	neuralized E3 ubiquitin protein ligase 1Aa	cypCar_00005365	71	4.19E-04	hippocampal-dependent synaptic plasticity; E3 ubiquitin-protein ligase, regulates the Notch pathway	(Teider et al., 2010)
gpr114, adgrg5	probable G-protein coupled receptor 114	cypCar_00003051	67	1.87E-02	orphan receptor	
gpr52	G protein-coupled receptor 52	cypCar_00031308	51	8.45E-03	orphan receptor	
nos2b, inos	nitric oxide synthase 2	cypCar_00024539, cypCar_00004424	48, 44	4.21E-60, 1.14E-57	expressed by M1 macrophages; metabolizes arginine to nitric oxide	(Rath et al., 2014)
lama2	laminin subunit alpha 2	cypCar_00002504	47	1.64E-02	extracellular protein, major component of the basement membrane, interacts with other extracellular matrix components	(Schéele et al., 2007)
tnni2b.2	troponin I2, fast skeletal type	cypCar_00015014	47	1.86E-05	inhibitory subunit of the troponin complex, calcium regulation of muscle contraction and relaxation	(Sheng and Jin, 2016)
Uncharacterized		cypCar_00010254	47	1.48E-02	unknown	
sich211-147m6.1		cypCar_00047183	45	1.96E-02	unknown	
efna1a	ephrin A1	cypCar_00017998	45	3.86E-05	cell adhesion	(Poliakov et al., 2004)
ptgs2a	prostaglandin-endoperoxide synthase 2, COX-2	cypCar_00026925	40	1.09E-04	linked to M1 activation	(Murray and Wynn, 2011b)

*Only contigs which are significantly up-regulated are included.

Table 3. The top 30 genes most up-regulated by cAMP ranked by fold change.

Gene	Description	Contig*	Fold change	Padj	Main function	Reference
cyr61	cysteine-rich angiogenic inducer 61 protein-like protein 1	cypCar_00001309	674	3.11E-225	matricellular protein, modulates wound healing and tissue repair	(Lau, 2011)
npy	Neuropeptide Y	cypCar_00010618, cypCar_00004502	384, 74	1.26E-31, 9.49E-06	anti-inflammatory, involved in wound healing	(Dimitrijevic et al., 2008), (Pradhan Nabzdyk et al., 2013)
timp2b	tissue inhibitor of metalloproteinase 2b	cypCar_00030755, cypCar_00034223	260, 17	0, 1.64E-122	inhibitor of ECM-degrading MMPs, maintains tissue homeostasis	(Zucker et al., 1998), (Morgunova et al., 2002)
inhbaa	activin beta A subunit	cypCar_00008046, cypCar_00010506	226, 10	1.85E-23, 7.46E-03	forms homodimer Activin, regulates wound repair, promotes alternative activation of macrophages	(Sulyok et al., 2004), (Ogawa et al., 2006)
arhgap28	Rho GTPase activating protein 28	cypCar_00048151, cypCar_00046740, cypCar_00045015, cypCar_00048150	214, 197, 55, 39	8.95E-35, 3.94E-16, 2.54E-12, 9.40E-07	up-regulated during Rho-dependent assembly of ECM	(Yeung et al., 2014)
mcamb	melanoma cell adhesion molecule, CD146	cypCar_00017065	187	9.57E-03	cell adhesion molecule, associated with the actin cytoskeleton	(Kratzer et al., 2013)
tgm2b	transglutaminase 2	cypCar_00034483, cypCar_00030329, cypCar_00041907	152, 136, 52	2.83E-91, 6.92E-86, 1.81E-46	crosslinking of proteins, conserved M2 marker	(Martinez et al., 2013)
pde3a	phosphodiesterase 3A, cGMP-inhibited	cypCar_00012460	130	1.32E-10	hydrolyzes cAMP to regulate the amplitude and duration of intracellular cAMP signals	(Ahmad et al., 2012)
il11a	interleukin 11	cypCar_00003776, cypCar_00004183	116, 10	3.77E-66, 2.30E-08	regulates macrophage effector function through inhibition of TNFα, IL1β , IL12, and nitric oxide production	(Trepicchio et al., 1996)
anxa6	annexin A6	cypCar_00014605	107	1.50E-08	associates with plasma membrane lipid rafts and links these to the cytoskeleton	(Cornely et al., 2011)
klhl38b	Kelch-like family member 38	cypCar_00006019	95	1.42E-07	unknown	
sich211-14c7.2		cypCar_00014543	95	1.42E-07	unknown	
xirp1	xin actin binding repeat containing 1	cypCar_00034573	91	5.25E-21	binds to and stabilizes the actin-based cytoskeleton	(Pacholsky et al., 2004)
LOC100003647	three-finger protein 5	cypCar_00018983, cypCar_00018982	71, 45	2.01E-05, 1.31E-18	unknown	
Uncharacterized		cypCar_00041288	67	5.45E-39	unknown	

sgip1b	SH3-domain GRB2-like (endophilin) interacting protein 1	cypCar_00020190, cypCar_00024880	65, 20	6.61E-05, 5.35E-06	recruits proteins essential to the formation of functional clathrin-coated pits and mediates endocytosis	(Dergai et al., 2010)
osbpl7	oxysterol binding protein-like 7	cypCar_00031716, cypCar_00018211	64, 2	6.74E-05, 1.60E-02	intracellular lipid receptor	(Suchanek et al., 2007)
has1	hyaluronan synthase 1	cypCar_00033285	64	3.77E-03	produces hyaluronan, a constituent of the extracellular matrix, active during wound healing and tissue repair	(Triggs-Raine and Natowicz, 2015)
sema3e	semaphorin 3E	cypCar_00022777	57	5.05E-03	chemoattractant for macrophages, may however be linked to M1 macrophages in mice	(Wanschel et al., 2013)
Agt	angiotensinogen	cypCar_00002786	55	8.32E-06	increases blood pressure and is associated with M2 macrophages	(Sumida et al., 2015), (Moore et al., 2015)
arl4d	ADP ribosylation factor like GTPase 4D	cypCar_00025470	53	1.09E-11	regulates actin remodelling and cell migration	(Li et al., 2007)
vipr1b	vasoactive intestinal peptide receptor 1	cypCar_00022000	49	1.19E-03	leads to cAMP production, anti-inflammatory activity, associated with M2 macrophages	(Leceta et al., 2000)
LOC100535149	ortholog of CRISPLD2	cypCar_00039278	49	1.33E-10	CRISPLD2 regulates anti-inflammatory effects of glucocorticoids, associated with wound healing	(Himes et al., 2014), (Zhang et al., 2015)
ramp2	receptor activity modifying protein 2	cypCar_00022158	49	1.69E-39	transports calcitonin-receptor-like receptor (CRLR) to the plasma membrane	(Hay et al., 2016)
apcdd1l	adenomatosis polyposis coli down-regulated 1 like	cypCar_00004844	43	1.00E-68	inhibits Wnt signalling	(Shimomura et al., 2010)
dfna5a	deafness associated tumor suppressor	cypCar_00035581	43	1.40E-185	induces apoptosis, tumour supressor	(Op de Beeck et al., 2011)
gprc6a	G protein-coupled receptor class C group 6 member A	cypCar_00007229	42	4.30E-09	this receptor is activated by certain amino acids, e.g. L-arginine	(Clemmensen et al., 2014)
scg2a	secretogranin II	cypCar_00025881	41	6.70E-03	the derivative secretoneurin attracts fibroblasts to assist in matrix remodelling	(Kähler et al., 1996)
fam129ba	family with sequence similarity 129 member B	cypCar_00006618, cypCar_00011439	34, 3	4.42E-02, 4.06E-06	linked to wound healing	(Oishi et al., 2012)
tusc5a	tumor suppressor candidate 5	cypCar_00002791	33	2.25E-02	regulated by PPARγ, expressed by adipocytes and peripheral neurons	(Oort et al., 2007)

*Only contigs which are significantly up-regulated are included.

Table 4. Accession numbers for phylogenetic trees.

Species	Protein name	Accession number
Human	IL1β	ENSP00000263341
Mouse	IL1β	ENSMUSP00000028881
Chicken	IL1β	NP_989855.1
Xenopus	IL1β	ENSXETP00000002737
Fugu	Il1β	ENSTRUP00000040919
Cod	Il1β	gb_ABV59377.1
Zebrafish	Il1β	ENSDARP00000002293
Human	IL18	ENSP00000280357
Human	PTX3	ENSP00000295927
Mouse	PTX3	ENSMUSP00000029421
Chicken	PTX3	ENSGALP00000042091
Xenopus	PTX3	ENSXETP00000026752
Fugu	Ptx3a	ENSTRUP00000032565
Spotted gar	Ptx3a	ENSLOCP00000003969
Cod	Ptx3a	ENSGMOP00000009769
Zebrafish	Ptx3a	ENSDARP00000089444
Zebrafish	Ptx3b	ENSDARP00000100459
Human	PTX4	ENSP00000293922
Human	SAA1	ENSP00000384906
Human	SAA2	ENSP00000256733
Human	SAA4	ENSP00000278222
Mouse	SAA1	ENSMUSP00000119150
Mouse	SAA2	ENSMUSP00000075365
Mouse	SAA3	ENSMUSP00000006956
Mouse	SAA4	ENSMUSP00000006952
Chicken	SAA	ENSGALP00000010113
Fugu	Saa	ENSTRUP00000044293
Spotted gar	Saa	ENSLOCP00000008205
Zebrafish	Saa	ENSDARP00000067628
Lamprey	Saa	ENSPMAP00000010482

Species	Protein name	Accession number
Human	NOS2	ENSP00000327251
Mouse	NOS2	ENSMUSP00000018610
Chicken	NOS2	ENSGALP00000034026
Xenopus	NOS2	ENSXETP00000027335
Spotted gar	Nos2	ENSLOCP00000006803
Zebrafish	Nos2a	ENSDARP00000106851
Zebrafish	Nos2b	ENSDARP00000048846
Human	NOS1	ENSP00000320758
Human	IL12A	ENSP00000303231
Mouse	IL12A	ENSMUSP00000029345
Chicken	IL12A	ENSGALP00000015585
Xenopus	IL12A	XP_004914898.1
Fugu	Il12a	ENSTRUP00000011200
Spotted gar	Il12a	ENSLOCP00000004421
Zebrafish	Il12a	ENSDARP00000056760
Human	IL11	ENSP00000264563
Human	CYR61	ENSP00000398736
Mouse	CYR61	ENSMUSP00000029846
Chicken	CYR61	ENSGALP00000014090
Xenopus	CYR61	ENSXETP00000006718
Fugu	Cyr61	ENSTRUP00000047275
Spotted gar	Cyr61	ENSLOCP00000006500
Spotted gar	Cyr61l1	ENSLOCP00000002941
Cod	Cyr61	ENSGMOP00000004048
Cod	Cyr61 (2 of 2)	ENSGMOP00000012019
Cod	Cyr61l1	ENSGMOP00000006741
Zebrafish	Cyr61	ENSDARP00000036965
Zebrafish	Cyr61l1	ENSDARP00000051335
Lamprey	Cyr61	ENSPMAP00000003248

Species	Protein name	Accession number
Human	INHBA	ENSP00000242208
Mouse	INHBA	ENSMUSP00000132085
Chicken	INHBA	ENSGALP00000036647
Xenopus	INHBA	ENSXETP00000003924
Fugu	Inhbaa	ENSTRUP00000044783
Spotted gar	Inhbaa	ENSLOCP00000014729
Cod	Inhbab	ENSGMOP00000020884
Zebrafish	Inhbaa	ENSDARP00000006400
Zebrafish	Inhbab	ENSDARP00000047467
Human	INHBB	ENSP00000295228
Human	TIMP2	ENSP00000262768
Mouse	TIMP2	ENSMUSP00000017610
Chicken	TIMP2	ENSGALP00000041954
Xenopus	TIMP2	ENSXETP00000043611
Fugu	Timp2a	ENSTRUP00000046417
Fugu	Timp2b	ENSTRUP00000028366
Spotted gar	Timp2a	ENSLOCP00000015631
Cod	Timp2a	ENSGMOP00000001570
Cod	Timp2b	ENSGMOP00000017202
Zebrafish	Timp2a	ENSDARP00000081499
Zebrafish	Timp2b	ENSDARP00000092838
Human	TIMP1	ENSP00000218388
Human	TGM2	ENSP00000355330
Mouse	TGM2	ENSMUSP00000099411
Chicken	TGM2	ENSGALP00000038435
Xenopus	TGM2	NP_001123852.1
Fugu	Tgm2a	ENSTRUP00000040288
Fugu	Tgm2b	ENSTRUP00000033114
Spotted gar	Tgm2	ENSLOCP00000001329
Cod	Tgm2a	ENSGMOP00000015990
Cod	Tgm2b	ENSGMOP00000010875
Zebrafish	Tgm2a	ENSDARP00000093487
Zebrafish	Tgm2b	ENSDARP00000099554
Human	TGM5	ENSP00000220420

Species	Protein name	Accession number
Human	ARG2	ENSP00000261783
Mouse	ARG2	ENSMUSP00000021550
Chicken	ARG2	NP_001186633.1
Xenopus	ARG2	ENSXETP00000017510
Fugu	Arg2	ENSTRUP00000009375
Spotted gar	Arg2	ENSLOCP00000017484
Cod	Arg2	ENSGMOP00000015002
Zebrafish	Arg2	ENSDARP00000057376
Human	ARG1	ENSP00000357066

Table 5. The genes most highly upregulated by combined stimuli LPS+IFNγ ranked by fold change.

Gene*	Description	Contig**	Fold change***	Padj	Function	Reference
il12a, il12-p35	interleukin 12 subunit a, p35	cypCar_00024698, cypCar_00024699	2238, 809 (447, 155)	8.75E-13, 2.81E-17	differentiation of naive T cells into Th1 cells, stimulates the production of IFNγ and TNFα from T cells and NK cells	(Holscher, 2004)
sost	sclerostin	cypCar_00018891	1268 (605)	2.49E-46	Prostaglandin E_2 decreases *sost* transcription via cAMP. *sost* inhibits bone formation by osteoblasts	(Genetos et al., 2011)
il1b	interleukin 1β	cypCar_00043439, cypCar_00043440	525, 482 (295, 283)	4.60E-105, 1.18E-119	pro-inflammatory cytokine	(Mantovani et al., 2004), (Jaguin et al., 2013)
adcyap1a	adenylate cyclase activating polypeptide 1 (pituitary)	cypCar_00018838, cypCar_00029617	390, 112 (202, 47)	3.83E-19, 2.17E-12	pro- and anti-inflammatory factor, increase phagocytic ability of macrophages	(Sherwood et al., 2000), (El Zein et al., 2007)
ptx3a	pentraxin 3a	cypCar_00014116	344 (372)	7.30E-64	acute-phase response protein, pathogen recognition, leukocyte recruitment	(Deban et al., 2011)
sich2l1-242b18.1, pde4dip	phosphodiesterase 4D interacting protein	cypCar_00007456	302 (270)	9.29E-19	serves to anchor PDE4D at Golgi/centrosome region of the cell, where PDE4D degrades cAMP	(Verde et al., 2001)
lacc1	laccase domain-containing protein 1	cypCar_00008998, cypCar_00009189	167, 104 (81, 57)	1.49E-36, 1.99E-12	association with autoinflammatory diseases: Crohn's, leprosy, juvenile idiopathic arthritis	(Wakil et al., 2015)
mcoln3a	mucolipin 3	cypCar_00027296	164 (71)	1.92E-48	Ca(2+) channel protein, regulation of autophagy	(Choi and Kim, 2014)
gpr114, adgrg5	probable G-protein coupled receptor 114	cypCar_00003051	160 (67)	4.18E-05	orphan receptor	
ccl20, mip3a	chemokine (C-C motif) ligand 20	cypCar_00004353	155 (261)	7.06E-35	chemotactic factor that attracts lymphocytes	(Schutyser et al., 2000)
avpr2ab	arginine vasopressin receptor 2	cypCar_00004651	151 (135)	1.51E-02	possible role in the regulation of inflammation	(Fleisher-Berkovich et al., 2004)
sema3e	semaphorin 3e	cypCar_00022777	151	5.32E-03	expressed by M1 macrophages, regulates macrophage retention in atherosclerotic plaques	(Wanschel et al., 2013)
adgrb3	adhesion G protein-coupled receptor b3	cypCar_00032513, cypCar_00037857	147, 63 (129)	5.70E-04, 6.95E-03	brain-specific angiogenesis inhibitor; high-affinity receptor for C1q-like proteins	(Bolliger et al., 2011)
arhgef25b	Rho guanine nucleotide exchange factor 25	cypCar_00002651	142 (106)	6.69E-03	involved in formation of actin stress fibers, regulates axon and dendrite formation	(van Unen et al., 2015)
tmc2b	transmembrane channel like 2b	cypCar_00006710	141 (78)	2.08E-12	transmembrane protein that is necessary for mechanotransduction in cochlear hair cells of the inner ear	(Pan et al., 2013)

cyp27c1	cytochrome P450 family 27 subfamily C member 1	cypCar_00041703	134 (97)	2.19E-31	converts vitamin A_1 into A_2	(Enright et al., 2015)
sema3b	semaphorin 3b	cypCar_00010298, cypCar_00002842	130, 20	2.17E-08, 2.64E-05	tumour suppressor through induction of apoptosis; inhibits axonal extension	(Loginov et al., 2015), (Püschel et al., 1995)
saa	serum amyloid a	cypCar_00037333, cypCar_00036204	119, 37 (77, 28)	2.32E-15, 1.52E-17	acute-phase protein; proinflammatory and in part homeostatic properties	(Uhlar and Whitehead, 1999), (Ye and Sun, 2015)
sidkey-257i7.5	uncharacterized protein	cypCar_00005123	119 (86)	3.64E-02	unknown, novel protein similar to Spi-C transcription factor (Spi-1/PU.1 related)	www.ensembl.org
neurl1aa	neuralized E3 ubiquitin protein ligase 1Aa	cypCar_00005365, cypCar_00005366	118, 52 (71)	2.52E-10, 9.50E-05	hippocampal-dependent synaptic plasticity; E3 ubiquitin-protein ligase, regulates the Notch pathway	(Teider et al., 2010)
slc6a9	solute carrier family 6 member 9; sodium- and chloride-dependent glycine transporter 1	cypCar_00001569	115	6.86E-33	regulation of glycine levels in NMDA receptor-mediated neurotransmission	(Aragon and Lopez-Corcuera, 2005)
sorbs3	sorbin and SH3 domain containing 3; vinexin	cypCar_00022668, cypCar_00031014	102, 21	3.00E-58, 6.95E-03	cell spreading, migration and anchorage-independent growth	(Mizutani et al., 2007)
agrn	agrin	cypCar_00029572	99 (82)	9.55E-09	monocyte and macrophage maturation and survival; formation of the phagocytic synapse	(Mazzon et al., 2012)
arhgap28	Rho GTPase activating protein 28	cypCar_00046740, cypCar_00045015, cypCar_00048151	89, 30, 20	1.21E-05, 3.07E-03, 5.23E-07	involved in reorganization of the actin cytoskeleton and stress fibers, remodelling of extracellular matrix	(Yeung et al., 2014)
steap4	STEAP4 metalloreductase	cypCar_00042005	87 (91)	1.72E-74	metalloreductase; upregulated in rainbow trout macrophages stimulated with LPS	(Iliev et al., 2006)
nos2b, inos	nitric oxide synthase 2	cypCar_00024539, cypCar_00004424	79, 71 (48, 44)	1.40E-74, 1.33E-71	expressed by M1 macrophages; metabolizes arginine to nitric oxide	(Rath et al., 2014)
stap2a	signal transducing adaptor family member 2a	cypCar_00019239, cypCar_00026583	75, 22	2.95E-09, 2.97E-20	enhances the production of inflammatory cytokines in macrophages	(Sekine, 2014)
aknad1	AKNA domain containing 1	cypCar_00023198	67 (72)	4.79E-04	unknown	
gdpd2	glycerophosphodiester phosphodiesterase domain containing 2, GDE3	cypCar_00007949	66	5.75E-22	hydrolyses glycerophosphoinositol to produce inositol 1-phosphate and glycerol	(Corda et al., 2009)
kctd12.1	potassium channel tetramerization domain containing 12	cypCar_00007902	65	2.87E-03	regulates the activity of voltage-gated potassium channels	(Resendes et al., 2004)

*Underlined genes were not in the top 30 list of LPS-only stimulated cells.
**Only contigs which are significantly up-regulated are included.
***For comparison, the fold change in LPS-only stimulated cells is given in parenthesis.

References

Ahmad, F., Degerman, E., Manganiello, V.C., 2012. Cyclic nucleotide phosphodiesterase 3 signaling complexes. Horm Metab Res 44, 776-785.

Anders, S., Huber, W., 2010. Differential expression analysis for sequence count data. Genome biology 11, R106.

Anders, S., Pyl, P.T., Huber, W., 2015. HTSeq - a Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166-169.

Aragon, C., Lopez-Corcuera, B., 2005. Glycine transporters: crucial roles of pharmacological interest revealed by gene deletion. Trends in pharmacological sciences 26, 283-286.

Arts, J.A.J., Tijhaar, E.J., Chadzinska, M., Savelkoul, H.F.J., Verburg-van Kemenade, B.M.L., 2010. Functional analysis of carp interferon-g: Evolutionary conservation of classical phagocyte activation. Fish & shellfish immunology 29, 793-802.

Biswas, S.K., Mantovani, A., 2010. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nature immunology 11, 889-896.

Bolliger, M.F., Martinelli, D.C., Südhof, T.C., 2011. The cell-adhesion G protein-coupled receptor BAI3 is a high-affinity receptor for C1q-like proteins. Proceedings of the National Academy of Sciences of the United States of America 108, 2534-2539.

Chang, C.-I., Zoghi, B., Liao, J.C., Kuo, L., 2000. The involvement of tyrosine kinases, cyclic AMP/protein kinase A, and p38 mitogen-activated protein kinase in IL-13-mediated arginase I induction in macrophages: Its implications in IL-13-inhibited nitric oxide production. Journal of immunology 165, 2134-2141.

Chen, C.C., Mo, F.E., Lau, L.F., 2001. The angiogenic factor Cyr61 activates a genetic program for wound healing in human skin fibroblasts. The Journal of biological chemistry 276, 47329-47337.

Chen, H., 2016. VennDiagram: Generate high-resolution Venn and Euler plots, R package version 1.6.17.

Choi, S., Kim, H.J., 2014. The Ca^{2+} channel TRPML3 specifically interacts with the mammalian ATG8 homologue GATE16 to regulate autophagy. Biochemical and biophysical research communications 443, 56-61.

Clemmensen, C., Smajilovic, S., Wellendorph, P., Bräuner-Osborne, H., 2014. The GPCR, class C, group 6, subtype A (GPRC6A) receptor: from cloning to physiological function. British Journal of Pharmacology 171, 1129-1141.

Corda, D., Kudo, T., Zizza, P., Iurisci, C., Kawai, E., Kato, N., Yanaka, N., Mariggio, S., 2009. The developmentally regulated osteoblast phosphodiesterase GDE3 is glycerophosphoinositol-specific and modulates cell growth. The Journal of biological chemistry 284, 24848-24856.

Cornely, R., Rentero, C., Enrich, C., Grewal, T., Gaus, K., 2011. Annexin A6 is an organizer of membrane microdomains to regulate receptor localization and signalling. IUBMB life 63, 1009-1017.

Deban, L., Jaillon, S., Garlanda, C., Bottazzi, B., Mantovani, A., 2011. Pentraxins in innate immunity: lessons from PTX3. Cell and tissue research 343, 237-249.

Dergai, O., Novokhatska, O., Dergai, M., Skrypkina, I., Tsyba, L., Moreau, J., Rynditch, A., 2010. Intersectin 1 forms complexes with SGIP1 and Reps1 in clathrin-coated pits. Biochemical and biophysical research communications 402, 408-413.

Dimitrijevic, M., Stanojevic, S., Mitic, K., Kustrimovic, N., Vujic, V., Miletic, T., Kovacevic-Jovanovic, V., 2008. The anti-inflammatory effect of neuropeptide Y (NPY) in rats is dependent on dipeptidyl peptidase 4 (DP4) activity and age. Peptides 29, 2179-2187.

El Zein, N., Badran, B.M., Sariban, E., 2007. The neuropeptide pituitary adenylate cyclase activating protein stimulates human monocytes by transactivation of the Trk/NGF pathway. Cellular signalling 19, 152-162.

Engelsma, M.Y., Stet, R.J.M., Schipper, H., Verburg-van Kemenade, B.M.L., 2001. Regulation of interleukin 1 beta RNA expression in the common carp, *Cyprinus carpio* L. Developmental and comparative immunology 25, 195-203.

Enright, J.M., Toomey, M.B., Sato, S.Y., Temple, S.E., Allen, J.R., Fujiwara, R., Kramlinger, V.M., Nagy, L.D., Johnson, K.M., Xiao, Y., How, M.J., Johnson, S.L., Roberts, N.W., Kefalov, V.J., Guengerich, F.P., Corbo, J.C., 2015. Cyp27c1 red-shifts the spectral sensitivity of photoreceptors by converting vitamin A_1 into A_2. Current biology : CB 25, 3048-3057.

Fleisher-Berkovich, S., Kagan, E., Grossman, N., Danon, A., 2004. Multiple effects of arginine vasopressin on prostaglandin E_2 synthesis in fibroblasts. European Journal of Pharmacology 485, 53-59.

Forlenza, M., Kaiser, T., Savelkoul, H.F.J., Wiegertjes, G.F., 2012. The use of real-time quantitative PCR for the analysis of cytokine mRNA levels. Methods in molecular biology 820, 7-23.

Genetos, D.C., Yellowley, C.E., Loots, G.G., 2011. Prostaglandin E_2 signals through *PTGER2* to regulate sclerostin expression. PloS one 6, e17772.

Hamza, T., Barnett, J.B., Li, B., 2010. Interleukin 12 a key immunoregulatory cytokine in infection applications. International journal of molecular sciences 11, 789-806.

Hay, D.L., Walker, C.S., Gingell, J.J., Ladds, G., Reynolds, C.A., Poyner, D.R., 2016. Receptor activity-modifying proteins; multifunctional G protein-coupled receptor accessory proteins. Biochemical Society transactions 44, 568-573.

Henkel, C.V., Dirks, R.P., Jansen, H.J., Forlenza, M., Wiegertjes, G.F., Howe, K., van den Thillart, G.E., Spaink, H.P., 2012. Comparison of the exomes of common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*). Zebrafish 9, 59-67.

Hermey, G., Plath, N., Hübner, C.A., Kuhl, D., Schaller, H.C., Hermans-Borgmeyer, I., 2004. The three sorCS genes are differentially expressed and regulated by synaptic activity. J Neurochem 88, 1470-1476.

Himes, B.E., Jiang, X., Wagner, P., Hu, R., Wang, Q., Klanderman, B., Whitaker, R.M., Duan, Q., Lasky-Su, J., Nikolos, C., Jester, W., Johnson, M., Panettieri, R.A., Jr., Tantisira, K.G., Weiss, S.T., Lu, Q., 2014. RNA-Seq transcriptome profiling identifies CRISPLD2 as a glucocorticoid responsive gene that modulates cytokine function in airway smooth muscle cells. PloS one 9, e99625.

Holscher, C., 2004. The power of combinatorial immunology: IL-12 and IL-12-related dimeric cytokines in infectious diseases. Medical microbiology and immunology 193, 1-17.

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J.C., Koch, R., Rauch, G.J., White, S., Chow, W., Kilian, B., Quintais, L.T.,

Guerra-Assuncao, J.A., Zhou, Y., Gu, Y., Yen, J., Vogel, J.H., Eyre, T., Redmond, S., Banerjee, R., Chi, J., Fu, B., Langley, E., Maguire, S.F., Laird, G.K., Lloyd, D., Kenyon, E., Donaldson, S., Sehra, H., Almeida-King, J., Loveland, J., Trevanion, S., Jones, M., Quail, M., Willey, D., Hunt, A., Burton, J., Sims, S., McLay, K., Plumb, B., Davis, J., Clee, C., Oliver, K., Clark, R., Riddle, C., Elliot, D., Threadgold, G., Harden, G., Ware, D., Begum, S., Mortimore, B., Kerry, G., Heath, P., Phillimore, B., Tracey, A., Corby, N., Dunn, M., Johnson, C., Wood, J., Clark, S., Pelan, S., Griffiths, G., Smith, M., Glithero, R., Howden, P., Barker, N., Lloyd, C., Stevens, C., Harley, J., Holt, K., Panagiotidis, G., Lovell, J., Beasley, H., Henderson, C., Gordon, D., Auger, K., Wright, D., Collins, J., Raisen, C., Dyer, L., Leung, K., Robertson, L., Ambridge, K., Leongamornlert, D., McGuire, S., Gilderthorp, R., Griffiths, C., Manthravadi, D., Nichol, S., Barker, G., Whitehead, S., Kay, M., Brown, J., Murnane, C., Gray, E., Humphries, M., Sycamore, N., Barker, D., Saunders, D., Wallis, J., Babbage, A., Hammond, S., Mashreghi-Mohammadi, M., Barr, L., Martin, S., Wray, P., Ellington, A., Matthews, N., Ellwood, M., Woodmansey, R., Clark, G., Cooper, J., Tromans, A., Grafham, D., Skuce, C., Pandian, R., Andrews, R., Harrison, E., Kimberley, A., Garnett, J., Fosker, N., Hall, R., Garner, P., Kelly, D., Bird, C., Palmer, S., Gehring, I., Berger, A., Dooley, C.M., Ersan-Urun, Z., Eser, C., Geiger, H., Geisler, M., Karotki, L., Kirn, A., Konantz, J., Konantz, M., Oberlander, M., Rudolph-Geiger, S., Teucke, M., Lanz, C., Raddatz, G., Osoegawa, K., Zhu, B., Rapp, A., Widaa, S., Langford, C., Yang, F., Schuster, S.C., Carter, N.P., Harrow, J., Ning, Z., Herrero, J., Searle, S.M., Enright, A., Geisler, R., Plasterk, R.H., Lee, C., Westerfield, M., de Jong, P.J., Zon, L.I., Postlethwait, J.H., Nusslein-Volhard, C., Hubbard, T.J., Roest Crollius, H., Rogers, J., Stemple, D.L., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498-503.

Huising, M.O., van Schijndel, J.E., Kruiswijk, C.P., Nabuurs, S.B., Savelkoul, H.F., Flik, G., Verburg-van Kemenade, B.M., 2006. The presence of multiple and differentially regulated interleukin-12p40 genes in bony fishes signifies an expansion of the vertebrate heterodimeric cytokine family. Molecular immunology 43, 1519-1533.

Huttenhuis, H.B., Taverne-Thiele, A.J., Grou, C.P., Bergsma, J., Saeij, J.P., Nakayasu, C., Rombout, J.H., 2006. Ontogeny of the common carp (*Cyprinus carpio* L.) innate immune system. Developmental and comparative immunology 30, 557-574.

Iliev, D.B., Goetz, G.W., MacKenzie, S., Planas, J.V., Goetz, F.W., 2006. Pathogen-associated gene expression profiles in rainbow trout macrophages. Comparative biochemistry and physiology. Part D, Genomics & proteomics 1, 416-422.

Iliev, D.B., Roach, J.C., Mackenzie, S., Planas, J.V., Goetz, F.W., 2005. Endotoxin recognition: in fish or not in fish? FEBS letters 579, 6519-6528.

Irnazarow, I., 1995. Genetic variability of Polish and Hungarian carp lines. Aquaculture 129, 215-219.

Jaguin, M., Houlbert, N., Fardel, O., Lecureur, V., 2013. Polarization profiles of human M-CSF-generated macrophages and comparison of M1-markers in classically activated macrophages from GM-CSF and M-CSF origin. Cellular immunology 281, 51-61.

Joerink, M., Ribeiro, C.M.S., Stet, R.J.M., Hermsen, T., Savelkoul, H.F.J., Wiegertjes, G.F., 2006a. Head kidney-derived macrophages of common carp (*Cyprinus carpio* L.) show plasticity and functional polarization upon differential stimulation. Journal of immunology 177, 61-69.

Joerink, M., Savelkoul, H.F., Wiegertjes, G.F., 2006b. Evolutionary conservation of alternative activation of macrophages: Structural and functional characterization of arginase 1 and 2 in carp (*Cyprinus carpio* L.). Molecular immunology 43, 1116-1128.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., Salzberg, S.L., 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome biology 14, R36.

Kolder, I.C.R.M., van der Plas-Duivesteijn, S.J., Tan, G., Wiegertjes, G.F., Forlenza, M., Guler, A.T., Travin, D.Y., Nakao, M., Moritomo, T., Irnazarow, I., den Dunnen, J.T., Anvar, S.Y., Jansen, H.J., Dirks, R.P., Palmblad, M., Lenhard, B., Henkel, C.V., Spaink, H.P., 2016. A full-body transcriptome and proteome resource for the European common carp. BMC genomics 17, 701.

Kratzer, A., Chu, H.W., Salys, J., Moumen, Z., Leberl, M., Bowler, R., Cool, C., Zamora, M., Taraseviciene-Stewart, L., 2013. Endothelial cell adhesion molecule CD146: implications for its role in the pathogenesis of COPD. The Journal of pathology 230, 388-398.

Kähler, C.M., Bellmann, R., Reinisch, N., Schratzberger, P., Gruber, B., Wiedermann, C.J., 1996. Stimulation of human skin fibroblast migration by the neuropeptide secretoneurin. Eur J Pharmacol 304, 135-139.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947-2948.

Lau, L.F., 2011. CCN1/CYR61: the very model of a modern matricellular protein. Cellular and molecular life sciences : CMLS 68, 3149-3163.

Leceta, J., Gomariz, R.P., Martinez, C., Abad, C., Ganea, D., Delgado, M., 2000. Receptors and transcriptional factors involved in the anti-inflammatory activity of VIP and PACAP. Annals of the New York Academy of Sciences 921, 92-102.

Li, C.C., Chiang, T.-C., Wu, T.-S., Pacheco-Rodriguez, G., Moss, J., Lee, F.-J.S., 2007. ARL4D recruits cytohesin-2/ARNO to modulate actin remodeling. Molecular biology of the cell 18, 4420-4437.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., Subgroup, G.P.D.P., 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25, 2078-2079.

Loginov, V.I., Dmitriev, A.A., Senchenko, V.N., Pronina, I.V., Khodyrev, D.S., Kudryavtseva, A.V., Krasnov, G.S., Gerashchenko, G.V., Chashchina, L.I., Kazubskaya, T.P., Kondratieva, T.T., Lerman, M.I., Angeloni, D., Braga, E.A., Kashuba, V.I., 2015. Tumor suppressor function of the *SEMA3B* gene in human lung and renal cancers. PloS one 10, e0123369.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., Locati, M., 2004. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 25, 677-686.

Martinez, F.O., Helming, L., Milde, R., Varin, A., Melgert, B.N., Draijer, C., Thomas, B., Fabbri, M., Crawshaw, A., Ho, L.P., Ten Hacken, N.H., Cobos Jimenez, V., Kootstra, N.A., Hamann, J., Greaves, D.R., Locati, M., Mantovani, A., Gordon, S., 2013. Genetic programs expressed in resting and IL-4 alternatively activated mouse and human macrophages: similarities and differences. Blood 121, e57-69.

Mazzon, C., Anselmo, A., Soldani, C., Cibella, J., Ploia, C., Moalli, F., Burden, S.J., Dustin, M.L., Sarukhan, A., Viola, A., 2012. Agrin is required for survival and function of monocytic cells. Blood 119, 5502-5511.

Mehta, K., Kumar, A., Kim, H.I., 2010. Transglutaminase 2: a multi-tasking protein in the complex circuitry of inflammation and cancer. Biochemical pharmacology 80, 1921-1929.

Mills, C.D., Ley, K., 2014. M1 and M2 macrophages: the chicken and the egg of immunity. Journal of innate immunity 6, 716-726.

Mizutani, K., Ito, H., Iwamoto, I., Morishita, R., Deguchi, T., Nozawa, Y., Asano, T., Nagata, K.I., 2007. Essential roles of ERK-mediated phosphorylation of vinexin in cell spreading, migration and anchorage-independent growth. Oncogene 26, 7122-7131.

Moore, J.P., Vinh, A., Tuck, K.L., Sakkal, S., Krishnan, S.M., Chan, C.T., Lieu, M., Samuel, C.S., Diep, H., Kemp-Harper, B.K., Tare, M., Ricardo, S.D., Guzik, T.J., Sobey, C.G., Drummond, G.R., 2015. M2 macrophage accumulation in the aortic wall during angiotensin II infusion in mice is associated with fibrosis, elastin loss, and elevated blood pressure. Am J Physiol Heart Circ Physiol 309, H906-917.

Morgunova, E., Tuuttila, A., Bergmann, U., Tryggvason, K., 2002. Structural insight into the complex formation of latent matrix metalloproteinase 2 with tissue inhibitor of metalloproteinase 2. Proceedings of the National Academy of Sciences of the United States of America 99, 7414-7419.

Murray, P.J., Allen, J.E., Biswas, S.K., Fisher, E.A., Gilroy, D.W., Goerdt, S., Gordon, S., Hamilton, J.A., Ivashkiv, L.B., Lawrence, T., Locati, M., Mantovani, A., Martinez, F.O., Mege, J.L., Mosser, D.M., Natoli, G., Saeij, J.P., Schultze, J.L., Shirey, K.A., Sica, A., Suttles, J., Udalova, I., van Ginderachter, J.A., Vogel, S.N., Wynn, T.A., 2014. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity 41, 14-20.

Murray, P.J., Wynn, T.A., 2011a. Obstacles and opportunities for understanding macrophage polarization. Journal of leukocyte biology 89, 557-563.

Murray, P.J., Wynn, T.A., 2011b. Protective and pathogenic functions of macrophage subsets. Nature reviews. Immunology 11, 723-737.

Nguyen-Chi, M., Laplace-Builhe, B., Travnickova, J., Luz-Crawford, P., Tejedor, G., Phan, Q.T., Duroux-Richard, I., Levraud, J.-P., Kissa, K., Lutfalla, G., Jorgensen, C., Djouad, F., 2015. Identification of polarized macrophage subsets in zebrafish. eLife 4, e07288.

Ogawa, K., Funaba, M., Chen, Y., Tsujimoto, M., 2006. Activin A functions as a Th2 cytokine in the promotion of the alternative activation of macrophages. Journal of immunology 177, 6787-6794.

Oishi, H., Itoh, S., Matsumoto, K., Ishitobi, H., Suzuki, R., Ema, M., Kojima, T., Uchida, K., Kato, M., Miyata, T., Takahashi, S., 2012. Delayed cutaneous wound healing in *Fam129b*/*Minerva*-deficient mice. Journal of biochemistry 152, 549-555.

Oort, P.J., Warden, C.H., Baumann, T.K., Knotts, T.A., Adams, S.H., 2007. Characterization of Tusc5, an adipocyte gene co-expressed in peripheral neurons. Molecular and cellular endocrinology 276, 24-35.

Op de Beeck, K., Van Camp, G., Thys, S., Cools, N., Callebaut, I., Vrijens, K., Van Nassauw, L., Van Tendeloo, V.F.I., Timmermans, J.P., Van Laer, L., 2011. The DFNA5 gene, responsible for hearing loss and involved in cancer, encodes a novel apoptosis-inducing protein. European journal of human genetics : EJHG 19, 965-973.

Oshiumi, H., Tsujita, T., Shida, K., Matsumoto, M., Ikeo, K., Seya, T., 2003. Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, *Fugu rubripes*, genome. Immunogenetics 54, 791-800.

Pacholsky, D., Vakeel, P., Himmel, M., Löwe, T., Stradal, T., Rottner, K., Fürst, D.O., van der Ven, P.F.M., 2004. Xin repeats define a novel actin-binding motif. J Cell Sci 117, 5257-5268.

Pan, B., Géléoc, G.S., Asai, Y., Horwitz, G.C., Kurima, K., Ishikawa, K., Kawashima, Y., Griffith, A.J., Holt, J.R., 2013. TMC1 and TMC2 are components of the mechanotransduction channel in hair cells of the mammalian inner ear. Neuron 79, 504-515.

Pietretti, D., Wiegertjes, G.F., 2014. Ligand specificities of Toll-like receptors in fish: indications from infection studies. Developmental and comparative immunology 43, 205-222.

Poliakov, A., Cotrina, M., Wilkinson, D.G., 2004. Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. Developmental cell 7, 465-480.

Pradhan Nabzdyk, L., Kuchibhotla, S., Guthrie, P., Chun, M., Auster, M.E., Nabzdyk, C., Deso, S., Andersen, N., Gnardellis, C., LoGerfo, F.W., Veves, A., 2013. Expression of neuropeptides and cytokines in a rabbit model of diabetic neuroischemic wound healing. Journal of vascular surgery 58, 766-775.

Püschel, A.W., Adams, R.H., Betz, H., 1995. Murine Semaphorin D/Collapsin is a member of a diverse gene family and creates domains inhibitory for axonal extension. Neuron 14, 941-948.

Rath, M., Müller, I., Kropf, P., Closs, E.I., Munder, M., 2014. Metabolism via arginase or nitric oxide synthase: two competing arginine pathways in macrophages. Frontiers in immunology 5, 532.

Resendes, B.L., Kuo, S.F., Robertson, N.G., Giersch, A.B.S., Honrubia, D., Ohara, O., Adams, J.C., Morton, C.C., 2004. Isolation from cochlea of a novel human intronless gene with predominant fetal expression. Journal of the Association for Research in Otolaryngology : JARO 5, 185-202.

Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139-140.

Saeij, J.P.J., Stet, R.J.M., Groeneveld, A., Verburg-van Kemenade, L.B.M., van Muiswinkel, W.B., Wiegertjes, G.F., 2000. Molecular and functional characterization of a fish inducible-type nitric oxide synthase. Immunogenetics 51, 339-346.

Schéele, S., Nyström, A., Durbeej, M., Talts, J.F., Ekblom, M., Ekblom, P., 2007. Laminin isoforms in development and disease. J Mol Med 85, 825-836.

Schroder, K., Irvine, K.M., Taylor, M.S., Bokil, N.J., Le Cao, K.A., Masterman, K.A., Labzin, L.I., Semple, C.A., Kapetanovic, R., Fairbairn, L., Akalin, A., Faulkner, G.J., Baillie, J.K., Gongora, M., Daub, C.O., Kawaji, H., McLachlan, G.J., Goldman, N., Grimmond, S.M., Carninci, P., Suzuki, H., Hayashizaki, Y., Lenhard, B., Hume, D.A., Sweet, M.J., 2012. Conservation and divergence in Toll-like receptor 4-regulated gene expression in primary human versus mouse macrophages. Proceedings of the National Academy of Sciences of the United States of America 109, E944-953.

Schutyser, E., Struyf, S., Menten, P., Lenaerts, J.P., Conings, R., Put, W., Wuyts, A., Proost, P., Van Damme, J., 2000. Regulated production and molecular diversity of human liver and activation-regulated chemokine/macrophage inflammatory protein-3a from normal and transformed cells. Journal of immunology 165, 4470-4477.

Sciaraffia, E., Riccomi, A., Lindstedt, R., Gesa, V., Cirelli, E., Patrizio, M., De Magistris, M.T., Vendetti, S., 2014. Human monocytes respond to extracellular cAMP through A2A and A2B adenosine receptors. Journal of leukocyte biology 96, 113-122.

Sekine, Y., 2014. Adaptor protein STAP-2 modulates cellular signaling in immune systems. Biol Pharm Bull 37, 185-194.

Sepulcre, M.P., Alcaraz-Perez, F., Lopez-Munoz, A., Roca, F.J., Meseguer, J., Cayuela, M.L., Mulero, V., 2009. Evolution of lipopolysaccharide (LPS) recognition and signaling: fish TLR4 does

not recognize LPS and negatively regulates NF-kB activation. Journal of immunology 182, 1836-1845.

Shay, T., Jojic, V., Zuk, O., Rothamel, K., Puyraimond-Zemmour, D., Feng, T., Wakamatsu, E., Benoist, C., Koller, D., Regev, A., Consortium, I., 2013. Conservation and divergence in the transcriptional programs of the human and mouse immune systems. Proceedings of the National Academy of Sciences of the United States of America 110, 2946-2951.

Sheng, J.J., Jin, J.P., 2016. *TNNI1*, *TNNI2* and *TNNI3*: Evolution, regulation, and protein structure-function relationships. Gene 576, 385-394.

Sherwood, N.M., Krueckl, S.L., McRory, J.E., 2000. The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. Endocr Rev 21, 619-670.

Shibasaki, Y., Yabu, T., Araki, K., Mano, N., Shiba, H., Moritomo, T., Nakanishi, T., 2014. Peculiar monomeric interferon gammas, IFNgrel 1 and IFNgrel 2, in ginbuna crucian carp. The FEBS journal 281, 1046-1056.

Shimomura, Y., Agalliu, D., Vonica, A., Luria, V., Wajid, M., Baumer, A., Belli, S., Petukhova, L., Schinzel, A., Brivanlou, A.H., Barres, B.A., Christiano, A.M., 2010. APCDD1 is a novel Wnt inhibitor mutated in hereditary hypotrichosis simplex. Nature 464, 1043-1047.

Snijders, A., Hilkens, C.M., van der Pouw Kraan, T.C., Engel, M., Aarden, L.A., Kapsenberg, M.L., 1996. Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit. Journal of immunology 156, 1207-1212.

Suchanek, M., Hynynen, R., Wohlfahrt, G., Lehto, M., Johansson, M., Saarinen, H., Radzikowska, A., Thiele, C., Olkkonen, V.M., 2007. The mammalian oxysterol-binding protein-related proteins (ORPs) bind 25-hydroxycholesterol in an evolutionarily conserved pocket. The Biochemical journal 405, 473-480.

Sullivan, C., Charette, J., Catchen, J., Lage, C.R., Giasson, G., Postlethwait, J.H., Millard, P.J., Kim, C.H., 2009. The gene history of zebrafish *tlr4a* and *tlr4b* is predictive of their divergent functions. Journal of immunology 183, 5896-5908.

Sulyok, S., Wankell, M., Alzheimer, C., Werner, S., 2004. Activin: an important regulator of wound repair, fibrosis, and neuroprotection. Molecular and cellular endocrinology 225, 127-132.

Sumida, T., Naito, A.T., Nomura, S., Nakagawa, A., Higo, T., Hashimoto, A., Okada, K., Sakai, T., Ito, M., Yamaguchi, T., Oka, T., Akazawa, H., Lee, J.K., Minamino, T., Offermanns, S., Noda, T., Botto, M., Kobayashi, Y., Morita, H., Manabe, I., Nagai, T., Shiojima, I., Komuro, I., 2015. Complement C1q-induced activation of b-catenin signalling causes hypertensive arterial remodelling. Nature communications 6, 6241.

Taylor, P.R., Martinez-Pomares, L., Stacey, M., Lin, H.H., Brown, G.D., Gordon, S., 2005. Macrophage receptors and immune recognition. Annual review of immunology 23, 901-944.

Team, R.C., 2015. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Teider, N., Scott, D.K., Neiss, A., Weeraratne, S.D., Amani, V.M., Wang, Y., Marquez, V.E., Cho, Y.J., Pomeroy, S.L., 2010. Neuralized1 causes apoptosis and downregulates Notch target genes in medulloblastoma. Neuro-oncology 12, 1244-1256.

Trepicchio, W.L., Bozza, M., Pedneault, G., Dorner, A.J., 1996. Recombinant human IL-11 attenuates the inflammatory response through down-regulation of proinflammatory cytokine release and nitric oxide production. Journal of immunology 157, 3627-3634.

Triggs-Raine, B., Natowicz, M.R., 2015. Biology of hyaluronan: Insights from genetic disorders of hyaluronan metabolism. World journal of biological chemistry 6, 110-120.

Uhlar, C.M., Whitehead, A.S., 1999. Serum amyloid A, the major vertebrate acute-phase reactant. Eur J Biochem 265, 501-523.

van Unen, J., Reinhard, N.R., Yin, T., Wu, Y.I., Postma, M., Gadella, T.W.J., Goedhart, J., 2015. Plasma membrane restricted RhoGEF activity is sufficient for RhoA-mediated actin polymerization. Scientific reports 5, 14693.

Verde, I., Pahlke, G., Salanova, M., Zhang, G., Wang, S., Coletti, D., Onuffer, J., Jin, S.L., Conti, M., 2001. Myomegalin is a novel protein of the Golgi/centrosome that interacts with a cyclic nucleotide phosphodiesterase. The Journal of biological chemistry 276, 11189-11198.

Wakil, S.M., Monies, D.M., Abouelhoda, M., Al-Tassan, N., Al-Dusery, H., Naim, E.A., Al-Younes, B., Shinwari, J., Al-Mohanna, F.A., Meyer, B.F., Al-Mayouf, S., 2015. Association of a mutation in *LACC1* with a monogenic form of systemic juvenile idiopathic arthritis. Arthritis & rheumatology 67, 288-295.

Wang, T., Johansson, P., Abós, B., Holt, A., Tafalla, C., Jiang, Y., Wang, A., Xu, Q., Qi, Z., Huang, W., Costa, M.M., Diaz-Rosales, P., Holland, J.W., Secombes, C.J., 2016. First in-depth analysis of the novel Th2-type cytokines in salmonid fish reveals distinct patterns of expression and modulation but overlapping bioactivities. Oncotarget 7, 10917-10946.

Wang, T., Secombes, C.J., 2013. The cytokine networks of adaptive immunity in fish. Fish & shellfish immunology 35, 1703-1718.

Wanschel, A., Seibert, T., Hewing, B., Ramkhelawon, B., Ray, T.D., van Gils, J.M., Rayner, K.J., Feig, J.E., O'Brien, E.R., Fisher, E.A., Moore, K.J., 2013. Neuroimmune guidance cue Semaphorin 3E is expressed in atherosclerotic plaques and regulates macrophage retention. Arteriosclerosis, thrombosis, and vascular biology 33, 886-893.

Watford, W.T., Moriguchi, M., Morinobu, A., O'Shea, J.J., 2003. The biology of IL-12: coordinating innate and adaptive immune responses. Cytokine Growth Factor Rev 14, 361-368.

Wiegertjes, G.F., Wentzel, A.S., Spaink, H.P., Elks, P.M., Fink, I.R., 2016. Polarization of immune responses in fish: The 'macrophages first' point of view. Molecular immunology 69, 146-156.

Xue, J., Schmidt, S.V., Sander, J., Draffehn, A., Krebs, W., Quester, I., De Nardo, D., Gohel, T.D., Emde, M., Schmidleithner, L., Ganesan, H., Nino-Castro, A., Mallmann, M.R., Labzin, L., Theis, H., Kraut, M., Beyer, M., Latz, E., Freeman, T.C., Ulas, T., Schultze, J.L., 2014. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. Immunity 40, 274-288.

Ye, R.D., Sun, L., 2015. Emerging functions of serum amyloid A in inflammation. Journal of leukocyte biology 98, 923-929.

Yeung, C.-Y.C., Taylor, S.H., Garva, R., Holmes, D.F., Zeef, L.A., Soininen, R., Boot-Handford, R.P., Kadler, K.E., 2014. Arhgap28 is a RhoGAP that inactivates RhoA and downregulates stress fibers. PloS one 9, e107036.

Zapata, A., 1981. Lymphoid organs of teleost fist. II. Ultrastructure of renal lymphoid tissue of *Rutilus rutilus* and *Gobio gobio*. Developmental and comparative immunology 5, 685-690.

Zhang, H., Sweezey, N.B., Kaplan, F., 2015. LGL1 modulates proliferation, apoptosis, and migration of human fetal lung fibroblasts. Am J Physiol Lung Cell Mol Physiol 308, L391-402.

Zou, J., Secombes, C.J., 2011. Teleost fish interferons and their role in immunity. Developmental and comparative immunology 35, 1376-1387.

Zucker, S., Drews, M., Conner, C., Foda, H.D., DeClerck, Y.A., Langley, K.E., Bahou, W.F., Docherty, A.J.P., Cao, J., 1998. Tissue inhibitor of metalloproteinase-2 (TIMP-2) binds to the catalytic domain of the cell surface receptor, membrane type 1-matrix metalloproteinase 1 (MT1-MMP). The Journal of biological chemistry 273, 1216-1222.

Chapter 5

Molecular and functional characterization of the scavenger receptor CD36 in zebrafish and common carp

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Abstract

CD36 is a scavenger receptor which has been studied closely in mammals where it is expressed by many different cell types and plays a role in highly diverse processes, both homeostatic and pathologic. It is among other things important in the innate immune system, in angiogenesis, and in clearance of apoptotic cells, and it is also involved in lipid metabolism and atherosclerosis. Recently, in the cephalochordate amphioxus a primitive CD36 family member was described, which was present before the divergence of CD36 from other scavenger receptor B family members, SCARB1 and SCARB2. Not much is known on the Cd36 molecule in teleost fish. We therefore studied Cd36 in both zebrafish and common carp, two closely related cyprinid fish species. Whereas a single *cd36* gene is present in zebrafish, carp has two *cd36* genes, and all show conserved synteny compared to mammalian CD36. The gene expression of carp *cd36* is high in brain, ovary and testis but absent in immune organs. Although in mammals CD36 expression in erythrocytes, monocytes and macrophages is high, gene expression studies in leukocyte subtypes of adult carp and zebrafish larvae, including thrombocytes and macrophages provided no indication for any substantial expression of *cd36* in immune cell types. Surprisingly, analysis of the *cd36* promoter region does show the presence of several binding sites for transcription factors known to regulate immune responses. Overexpression of carp *cd36* locates the receptor on the cell surface of mammalian cell lines consistent with the predicted topology of cyprinid Cd36 with a large extracellular domain, two transmembrane domains, and short cytoplasmic tails at both ends. Gene expression of *cd36* is down-regulated during infection of zebrafish with *Mycobacterium marinum*, whereas knockdown of *cd36* in zebrafish larvae led to higher bacterial burden upon such infection. We discuss the putative role for Cd36 in immune responses of fish in the context of other members of the scavenger receptor class B family.

Introduction

Scavenger receptors are soluble or membrane-associated molecules which have in common the ability to bind chemically modified lipoproteins or other poly-anionic ligands (Plüddemann et al., 2007). Each molecule additionally binds to other ligands, and scavenger receptors are thus functionally diverse. They have been grouped together into eight classes (A-H) where members within a class display structural homology. However, between the eight classes the scavenger receptors are structurally very different. CD36 is a member of the scavenger receptor class B family, which also contains scavenger receptor class B member 1 and 2 (SCARB1 and SCARB2) (Calvo and Vega, 1993; Calvo et al., 1995). SCARB1 is important in lipid metabolism as a receptor mediating cholesterol transfer to and from high density lipoprotein HDL (Brundert et al., 2005; Chadwick and Sahoo, 2013). SCARB2 (also referred to as lysosomal integral membrane protein-2, LIMP-2) is required for the normal biogenesis and maintenance of lysosomes and endosomes and as such plays a role in various human diseases (Gonzalez et al., 2014). CD36 is an integral membrane glycoprotein with two transmembrane domains, a large extracellular loop containing multiple glycosylation sites, and two short intracellular tails. In mammals, CD36 is expressed by many different cell types such as platelets, monocytes and macrophages, endothelial cells and hepatocytes, and CD36 seems to play a role in highly diverse processes, both homeostatic and pathologic (Febbraio et al., 2001). Mammalian CD36 was originally identified as a macrophage receptor for oxidized LDL (Endemann et al., 1993), but its ligand-binding ability is very extensive and encompasses endogenous as well as exogenous ligands (reviewed in Silverstein and Febbraio, 2009). Among other molecules, CD36 recognizes microbial diacylglycerides such as macrophage-activating lipopeptide 2 from *Mycoplasma pneumoniae* (MALP-2) and lipoteichoic acid from *Staphylococcus aureus* and acts as a co-receptor for these molecules together with Toll-like receptor (TLR) 2/6 heterodimers (Hoebe et al., 2005; Stuart et al., 2005). As a receptor recognizing exogenous pathogen-associated molecular patterns (PAMPs), CD36 is acknowledged as a pattern recognition receptor (PRR) important in innate immunity.

CD36 orthologs have been described in many vertebrate species such as human, mouse (Endemann et al., 1993), rat (Abumrad et al., 1993), cow (Greenwalt et al., 1990) and chicken (Shu et al., 2011). Ancestral homologs are also found in invertebrate species such as *C. elegans* (Means et al., 2009), sea urchin and fruit fly, and a recent study reported several homologs in amphioxus, a basal chordate (Zhang et al., 2013). Presumably due to amphioxus-specific gene duplication events there are five *cd36* paralogs in this species. These genes are thought to represent the primitive form of scavenger receptor class B before the divergence of CD36, SCARB1 and SCARB2. One of the amphioxus genes was cloned and studied in more detail: its expression was up-regulated in the gills upon

feeding and down-regulated during fasting. In addition, studies in Atlantic salmon and rainbow trout have focused on fatty acid transport proteins including Cd36 and described gene expression changes regulated by insulin, fasting and dietary composition (Sanchez-Gurmaches et al., 2011, 2012; Torstensen et al., 2011). This suggests a conservation of the role of SCARB family members in at least nutritional control and/or lipid metabolism. Contrary to the mammalian species, knowledge of the immune function of Cd36 in teleost fish is lacking.

In mammals, there is evidence of CD36 interaction with pre-formed Toll-like receptor TLR2-TLR6 heterodimers, where CD36 recognizes specific lipids or lipoproteins and brings the ligand to the TLRs. Ligand/receptor clusters localize within lipid rafts of the plasma membrane, after which intracellular signalling and phagocytosis is initiated (Triantafilou et al., 2006). The intracellular signalling goes via MyD88 and a series of phosphorylation and ubiquitination events of cytosolic proteins, leading to activation of the transcription factor NFκB and expression of immune related genes (Akira and Takeda, 2004). Of interest, out of eight accessory molecules classified in mammals as important mediators of ligand delivery and/or recognition and required for TLR function (Lee et al., 2012) only four molecules, including CD36, seem to have clear orthologs in fish genomes (Pietretti et al., 2013). Given our interest in the function of TLRs (Pietretti and Wiegertjes, 2014) among which Tlr2 (Ribeiro et al., 2010), we studied Cd36 in zebrafish and common carp, two closely related cyprinid fish species, taking advantage of the complementary tools available for these two species to achieve a more thorough understanding of the function of Cd36 in teleost fish.

We characterize for the first time in detail Cd36 of zebrafish and two Cd36 molecules of common carp finding a molecular structure consistent with mammalian CD36. Phylogenetic analysis as well as a synteny approach allowed us to confidently designate the cyprinid molecules as homologs of the mammalian CD36. Gene expression was studied in tissues and leukocyte subtypes, indicating a complete absence of *cd36* basal gene expression in immune organs and leukocytes of adult carp. However, knock-down of *cd36* in zebrafish embryos by a morpholino approach and subsequent *Mycobacterium marinum* infection indicated a role for Cd36 in controlling bacterial burden. We discuss the possibility that the division of functions between the Cd36 family members Cd36, Scarb1 and Scarb2, all of which are present in teleosts, may not be exactly the same as in their mammalian counterparts.

Materials and methods

Animals

European common carp (*Cyprinus carpio carpio* L.) were reared in the central fish facility Carus, at Wageningen University, Wageningen, Netherlands. Fish were kept at 23 °C in recirculating UV-treated tap water and fed pelleted dry food (Sniff, Soest, Germany) daily. R3xR8 carp are the hybrid offspring of a cross between fish of Polish origin (R3 strain) and Hungarian origin (R8 strain) (Irnazarow, 1995). Carp were between 9 and 11 months old at the start of the experiments. All studies were performed with approval from the local animal welfare committee (DEC) of Wageningen University.

Zebrafish were handled in compliance with the local animal welfare regulations, maintained according to standard protocols (zfin.org), and culture was approved by the local animal welfare committee (DEC) of Leiden University. Embryos from the zebrafish AB/TL line were used for the infection experiments. Embryos were grown at 28.5 °C in egg water (60 µg/mL Instant Ocean sea salts). For the duration of bacterial injections and stereo fluorescence imaging embryos were kept under anaesthesia in egg water containing 200 µg/mL tricaine methane sulfonate (Sigma-Aldrich, St. Louis, MO, USA). Embryos used for stereo fluorescence imaging were kept in egg water containing 0.003% 1-phenyl-2-thiourea (Sigma-Aldrich) to prevent melanisation which can produce background fluorescence during imaging. For fluorescence activated cell sorting (FACS) isolation of macrophages and neutrophils from zebrafish larvae, the transgenic lines *Tg(mpeg1:mCherry-F)*^{UMSF001} (Bernut et al., 2014) and *Tg(mpx:egfp)*^{i114} (Renshaw et al., 2006) were used, respectively.

Organ isolation

Carp aged 9-11 months were euthanized with 0.3 g/L tricaine methane sulfonate (TMS, Crescent Research Chemicals, Phoenix, AZ, USA) buffered with 0.6 g/L $NaHCO_3$. Carp were bled from the caudal vein using a needle and syringe containing cRPMI medium (RPMI 1640 with 25 mM HEPES, (Lonza, Basel, Switzerland) adjusted to an osmolality of 280 mOsm/kg with sterile water) containing 50 U/mL heparin (Leo Pharma, Ballerup, Denmark), 50 U/mL penicillin G (Sigma-Aldrich), and 50 µg/mL streptomycin sulphate (Sigma-Aldrich). For isolation of peripheral blood leukocytes (PBL), the heparinized blood was centrifuged at 100 × *g* for 5 min at 4 °C and then another 5 min at 300 × *g*. The buffy coat was collected, carefully layered on Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, UK) and centrifuged at 800 × *g* for 25 min at 4 °C without brake. The leukocyte

layer was collected and washed twice with cRPMI. The obtained PBL were stored at −80 °C until used for RNA isolation. After bleeding the fish, the organs of interest were aseptically removed and immediately frozen in liquid nitrogen and stored at −80 °C until used for RNA isolation.

Zebrafish embryos for RNA isolation were snap-frozen in liquid nitrogen and stored at −80 °C.

Isolation of leukocyte subtypes

Carp leukocyte subtypes were isolated by density gradient separation and/or magnetic cell sorting using specific antibodies as described before for thrombocytes (Rombout et al., 1996), thymocytes (Stolte et al., 2008), granulocytes (Forlenza et al., 2008), B cells (Koumans-van Diepen et al., 1995; Secombes et al., 1983), and macrophages (Romano et al., 1998). In short, PBL or single-cell suspensions derived from carp organs were incubated with primary mouse monoclonal antibody: WCL-6 for thrombocytes (from blood), TCL-BE8 for neutrophils (from mid kidney), WCI-12 for B cells (from blood), and WCL-15 for monocytes/macrophages (from spleen). After incubation and washing, cells were stained with phycoerythrin (PE)-conjugated goat anti-mouse secondary antibody. After washing and counting of cells, magnetic beads (anti-PE MicroBeads, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added and allowed to bind, before washing and magnetic separation on LS Midi Columns using a MidiMACS Separator (Miltenyi Biotec).

For isolation of leukocytes from *Tg(mpeg1:mCherry-F)*^{UMSF001} and *Tg(mpx:egfp)*^{i114} zebrafish, larvae at 5-6 days post fertilization (dpf) were dissociated according to Covassin et al. (2006). In short, anaesthetized embryos were dechorionated using pronase treatment, rinsed in calcium-free Ringer solution, followed by digestion with 0.25% trypsin and 1 mM EDTA. The obtained cell suspension was centrifuged, rinsed with PBS and resuspended in Leibovitz medium L15 without phenol red, 1% foetal calf serum, 0.8 mM $CaCl_2$, penicillin 50 U/mL and streptomycin 0.05 mg/mL. The single cell suspension was subjected to FACS at room temperature using a FACSAria (Becton Dickinson) with the BD FACSDiva software version 5.0.3 and a Coherent Sapphire solid state laser 488 nm with 13 mW power. The fluorescence-positive and negative cell fractions were collected in medium as above but with 10% foetal calf serum.

RNA isolation

Total RNA from carp organs and leukocytes was extracted using the RNeasy Mini kit according to the manufacturer's protocol (Qiagen, Venlo, Netherlands) including on-column DNase treatment with the RNase-free DNase set (Qiagen). Final elution was performed with 30 µL nuclease-free water. The integrity of the RNA was determined by agarose gel electrophoresis and the RNA quality and concentrations were assessed spectrophotometrically by measuring the absorbance at 260 nm and 280 nm (Nanodrop, Thermo Scientific, Waltham, MA, USA). RNA from *M. marinum*-infected zebrafish was isolated at 4 dpi as described in Stockhammer et al. (2009). RNA from zebrafish larval leukocytes was isolated directly after FACS sorting using the RNAqueous-Micro Kit (Ambion, Carlsbad, CA, USA). Zebrafish RNA quality was checked by lab-on-a-chip analysis with an Agilent Bioanalyzer 2100 using the RNA 6000 Pico kit (Agilent, Santa Clara, CA, USA). RNA was stored at −80 °C until use.

cDNA synthesis

Prior to cDNA synthesis, 500 ng–2 µg of carp total RNA was subjected to an additional DNase treatment by using DNase I Amplification Grade (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNA was performed with Invitrogen's SuperScript III Reverse Transcriptase, according to the manufacturer's instructions. As control for genomic contamination, for each sample a reaction without SuperScript III Reverse Transcriptase was performed. cDNA samples were diluted 25 times in nuclease-free water before use as templates in real-time quantitative PCR experiments. For zebrafish samples, cDNA synthesis was performed as described previously (Stockhammer et al., 2009), using iScript reverse transcriptase (Bio-Rad Laboratories, Hercules, CA, USA). Zebrafish cDNA was diluted 10 times before further use.

Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) on samples from carp was performed in a Rotor-Gene 6000 with a 72-well rotor (Corbett Research, Qiagen) with the ABsolute QPCR SYBR Green Mix (Thermo Scientific) as detection chemistry. All primers were from Eurogentec (Liège, Belgium) and were used as forward and reverse primer mix at a 2.1 µM final concentration. Master-mix for each RT-qPCR run was prepared as follows: per reaction, 2 µL of primer stock was mixed with 7 µL of SYBR Green. To 9 µL of master-mix, 5 µL of diluted cDNA was added in a 0.1 mL tube. The following amplification

program was used: one denaturation step of 15 min at 95 °C; followed by 40 cycles of a three-step amplification (15 s at 95 °C for denaturation, 20 s at 60 °C for annealing, 20 s at 72 °C for elongation). Then a short hold for 1 min at 60 °C, before a melting analysis starting from 60 °C and increasing to 90 °C in 0.5 °C steps with 5 s waiting. Fluorescence acquisition was performed at the end of each cycle and during the waiting steps of the melting analysis.

RT-qPCR data were analysed by Rotor-Gene 6000 Series Software 1.7. The melting temperature and profile of the melting curves were used to validate the specificity of the amplification. The gene expression was analysed using the average amplification efficiency for each primer pair and the take-off value of each sample (as derived from the Comparative Quantitation Analysis of the Rotor-Gene 6000 Series Software 1.7). The relative gene expression was calculated as the average amplification efficiency to the power of the take-off value. The gene expression of the housekeeping gene *40S ribosomal protein S11* was used to normalize the data.

RT-qPCR analysis of zebrafish samples was performed as described in Stockhammer et al. (2009). Reactions contained 10 µL of 2× iQ SYBR Green Supermix (Bio-Rad Laboratories), 5 pmol of each primer, and 5 µL diluted cDNA in a total of 20 µL. All reactions were performed with biological triplicates and technical duplicates. Data from zebrafish leukocytes were normalised to the expression of *eukaryotic translation initiation factor 4A*, *isoform 1B* (*eif4*), whereas data from *M. marinum*-infected zebrafish embryos were normalized to *peptidylprolyl isomerase A-like* (*ppial*). For analysis purposes an amplification efficiency of 2 was assumed.

The sequences of primers used in this study are given in Table 1.

Table 1. List of primers and morpholinos used in this study. Restriction sites are double underlined, Kozak sequence is shown in bold, and sequence encoding an HA-tag is single underlined.

Oligo name	Sequence 5' → 3'	Purpose
CycaCD36_FW1	GCTGCTGCTCGCCATC	Cloning of carp *cd36a*
CycaCD36_RV3	GTTTTACTGAAATTGTGCTGACATAC	Cloning of carp *cd36a*
CycaCD36_RV2	CCACAGTACGACCACATAATG	Semi-nested PCR for cloning of carp *cd36a*
CD36_pcDNA3_FW	ACGAGGATCC**AACATGGCA**TACCCATACGATGTTCCAGATTACGCTATGACCTGCTGTGATCTGAAATG	Cloning of carp *cd36a*

CD36_pcDNA3_RV	CATGCTCGAGACAATGAAGATTTTTTTTGT-TATTGC	Cloning of carp *cd36a*
CD36_qFW1	CGGGACTTTAGCATTTGAC	RT-qPCR of carp *cd36a*
CD36_qRV1	AGTGAAGTTATCATTGAAGGTG	RT-qPCR of carp *cd36a*
CD36b_qFW2	GCTGATTGGTCGGATGGAGT	RT-qPCR of carp *cd36b*
CD36b_qRV2	GAAGTTTGGTATTTTTCGGTTTCA	RT-qPCR of carp *cd36b*
CD36_qFW2	CACCTTCAATGATAACTTCACTT	RT-qPCR of carp *cd36a*
CD36_qRV2	AGCGTTGGATGAGCAAG	RT-qPCR of carp *cd36a*
CD36_qFW3	GACAGATGGTTCCTCCTTC	RT-qPCR of carp *cd36a* and *cd36b*
CD36_qRV3	TTCCCTTCAGGTCAACAG	RT-qPCR of carp *cd36a* and *cd36b*
CD36_qFW4	GGGCTCATCACAGGGAC	RT-qPCR of carp *cd36a*
CD36_qRV4	TTGGCGTGTCTACAGACG	RT-qPCR of carp *cd36a*
CD36_qFW5	GACCTGCTGTGATCTGAAATG	RT-qPCR of carp *cd36a* and *cd36b*
CD36_qRV5	GTCCCGTTTTCCAACACTG	RT-qPCR of carp *cd36a* and *cd36b*
CD36_qFW6	GTGAACCATCGCTGAGT	RT-qPCR of carp *cd36a* and *cd36b*
CD36_qRV6	GTCAACAGTTCCCTCATACTC	RT-qPCR of carp *cd36a* and *cd36b*
40S_FW	CCGTGGGTGACATCGTTACA	RT-qPCR of carp *40S ribosomal protein S11*
40S_RV	TCAGGACATTGAACCTCACTGTCT	RT-qPCR of carp *40S ribosomal protein S1*1
zf_cd36_qFW2	CACAGGCAAAGATGACATT	RT-qPCR of zebrafish *cd36*
zf_cd36_qRV2	CGGGTGGAAAGAGGAAC	RT-qPCR of zebrafish *cd36*
eif4a1b_FW	TTCAGAAACTCAGTACTAGCATACA	RT-qPCR of zebrafish *eif4a1b*
eif4a1b_RV	GTGACATCCAACACCTCTGC	RT-qPCR of zebrafish *eif4a1b*
ppial_FW	ACACTGAAACACGGAGGCAAAG	RT-qPCR of zebrafish *ppial*
ppial_REV	CATCCACAACCTTCCCGAACAC	RT-qPCR of zebrafish *ppial*
MO1-CD36_e2i2	CTATGAGGCCACAAATATTACCTGT	Zebrafish morpholino knockdown
MO2-CD36-e3i3	TTTGAAATACATACTGCGACAGCAA	Zebrafish morpholino knockdown
MO-FW	TGCTTGGCGGAATCCTCATC	RT-PCR to verify zebrafish knockdown
MO-RV	TCGGATAAGCCAATCTGCCAG	RT-PCR to verify zebrafish knockdown

Cloning full-length *cd36a* from common carp

Carp head kidney leukocytes were obtained essentially as described previously (Verburg-van Kemenade et al., 1994). RNA was isolated as described above and then used as template in reverse-transcription PCR using the LongRange 2Step RT-PCR kit (Qiagen). Reverse transcription was done with an oligo-dT primer and the subsequent PCR was done with gene-specific primers (CycaCD36_FW1 and CycaCD36_RV3, see Table 1) whose sequence was based on preliminary information from the carp genome. Semi-nested PCR was then performed with the Expand High Fidelity PCR System (Roche Diagnostics, Basel, Switzerland) with primers CycaCD36_FW1 and CycaCD36_RV2 (see Table 1). The PCR product was subsequently cloned into pGEM-T Easy (Promega, Madison, WI, USA) and transformed into *E. coli* JM109 competent cells (Promega). Eight clones were sequenced using the ABI prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) and analysed using a 3730 DNA Analyzer. Sequence data were analysed with Sequencher version 4.10 (Gene Codes, Ann Arbor, MI, USA) and aligned with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). A clone with the consensus sequence was used for further studies.

Bioinformatics

The nucleotide sequence of zebrafish *cd36* is available at Ensembl (ENSDARG00000032639). We obtained the coding sequence of carp *cd36a* after cloning, and the carp *cd36b* coding sequence is predicted based on genome sequence data (Bioproject PRJNA73579) (Henkel et al., 2012). In the present manuscript, to distinguish zebrafish from carp *cd36*, the genes are denoted as *Cyprinus carpio* (*cc*) or *Danio rerio* (*dr*) *cd36* with labelling *a* or *b* for the two carp *cd36*. Exon-intron structure was studied by multiple alignments and open reading frame predictions (FGENESH at http://linux1.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind, and GENSCAN at http://genes.mit.edu/GENSCAN.html), and exon-intron structure was visualized using WormWeb (http://www.wormweb.org/exonintron). Nucleotide sequences were translated into protein sequence using the EMBL-EBI tool (http://www.ebi.ac.uk/Tools/st/) and these were aligned with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Molecular weights were calculated with ProtParam (http://web. expasy.org/protparam/). Transmembrane regions were predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and *N*-glycosylation sites were predicted with NetNGlyc v. 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). *O*-glycosylation sites were predicted using NetOGlyc v. 4.0 (http://www.cbs.dtu.dk/services/NetOGlyc/). Synteny analysis

was performed on the basis of sequence information from Ensembl release 74 (http://www.ensembl.org/). The multiple sequence alignment for the phylogenetic tree was made with ClustalX 2.1 (Larkin et al., 2007) and the tree was constructed using the Neighbour Joining method with the number of bootstrap trials set to 10,000, also using ClustalX 2.1. The phylogenetic tree was subsequently visualized with MEGA6 and displayed with the moderately related zebrafish scavenger receptor Scarf1 as the root. Amino acid identity and similarity between Cd36 proteins was calculated with the software MatGAT 2.0. The transcriptional start site for zebrafish *cd36* was determined by Cap Analysis Gene Expression (CAGE) analysis. MatInspector (http://www.genomatix.de/) was used to predict the presence of various vertebrate transcription factor binding sites in the promoter regions of human, mouse, chicken and zebrafish *cd36*.

cd36a-mCherry expression plasmid

The gene encoding the fluorescent protein mCherry was cloned into pcDNA3 using the BamHI and XbaI sites. An XhoI site and a short linker sequence of 15 nucleotides encoding the amino acids GGSGG was placed upstream of the mCherry sequence. Subsequently, the full-length carp *cd36a* sequence was modified from the above-mentioned pGEM-T Easy construct to include sequence to encode an HA-tag at the N-terminus, and to remove the stop codon, using primers CD36_pcDNA3_FW and CD36_pcDNA3_RV (see Table 1). The modified *cccd36a* was then cloned between the BamHI site and the newly created XhoI site in pcDNA3, thus creating a fusion of *cccd36a*-mCherry in pcDNA3. Isolation of highly pure plasmid DNA suitable for transfection was performed with the S.N.A.P. MidiPrep Kit (Invitrogen) according to the manufacturer's instructions.

Subcellular localization of carp Cd36a

Human embryonic kidney 293 (HEK) cells were cultured at 37 °C at 5% CO_2 in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (Gibco), 2 mM L-glutamine (Merck, Darmstadt, Germany), 50 U/mL penicillin G (Sigma-Aldrich), and 50 µg/mL streptomycin sulphate (Sigma-Aldrich). HEK cells were seeded directly on untreated glass cover slips, 4.5 10^4 cells each. The following day, cells were transfected with 2 µg *cccd36a*-mCherry-pcDNA3 or mCherry-pcDNA3 using jetPRIME (Polyplus Transfection, Illkirch, France) according to the manufacturer's instructions at a 1:2 ratio of plasmid:transfection reagent.

The Epithelioma papulosum cyprini (EPC) cell line, which is an adherent cell type derived from fathead minnow, was cultured at 27 °C at 5% CO_2 in RPMI 1640 (Lonza) supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin G, and 50 µg/mL streptomycin sulphate. EPC cells were seeded on untreated glass cover slips in six-well plates, 1 10^6 cells/well, and allowed to adhere. EPC cells were transfected the following day with 2 µg *cccd36a*-mCherry-pcDNA3 or mCherry-pcDNA3 using FuGENE HD Transfection Reagent (Promega) according to the manufacturer's instructions at a 1:3.5 ratio of plasmid:transfection reagent.

Three days after transfecting HEK and EPC cells with carp *cd36a*-mCherry-pcDNA3 or mCherry-pcDNA3, the cover slips were carefully washed with HBSS, fixed for 20 min at room temperature with 4% paraformaldehyde, washed with HBSS and stained with wheat germ agglutinin-Alexa Fluor 488 conjugate (Molecular Probes, Invitrogen) for 15 min at room temperature, before final washing with HBSS and mounting with Vectashield (Vector Laboratories, Burlingame, CA, USA).

Cells were visualized with a Zeiss LSM-510 (Zeiss, Oberkochen, Germany) confocal laser scanning microscope with a Plan-Apochromat 63×/1.4 oil immersion objective. Green fluorescent signal (Alexa Fluor 488) was excited with a 488 nm argon laser and detected using a band-pass filter (505–550 nm). Red fluorescence (mCherry protein) was excited with a 543 nm helium-neon laser and detected using a long-pass filter (585 nm). Image processing was performed with ImageJ (http://imagej.nih.gov/ij/).

Morpholino knockdown

Morpholino oligonucleotides (Gene Tools, Philomath, OR, USA) were diluted to the desired concentration in 1× Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES; pH 7.6) containing 1% phenol red (Sigma-Aldrich) and 1 nL was injected into the yolk at the 1-2 cell stage using a Femtojet injector (Eppendorf, Hamburg, Germany). For knockdown of *cd36*, two morpholinos were used, one targeting the exon 2-intron 2 splice junction (*cd36* Mo1: 5'CTATGAGGCCACAAATATTACCTGT3', 0.2 mM), and the other targeting the exon 3-intron 3 splice junction (*cd36* Mo2: 5'TTTGAAATACATACTGCGACAGCAA3', 0.6 mM). As a control, the standard control morpholino from Gene Tools was used at the same concentrations as the corresponding *cd36* morpholino. Knockdown of *cd36* with both morpholinos was verified at 1 dpf by RT-PCR with the SuperScript® One-Step RT-PCR System (Invitrogen). Whole embryo RNA isolation was performed as described in Stockhammer et al. (2009) and 50 ng of DNAse treated (Invitrogen) RNA template was used for the RT-PCR reaction. RT-PCR primers

for *cd36* knockdown verification were MO-FW and MO-RV, whose sequence is given in Table 1. The following adjustments were made to the PCR settings: 56 °C for 30 s for the annealing step of the PCR amplification with 35 cycles. To verify that *cd36* knockdown did not affect leukocyte development an L-plastin immunostaining was performed as described (Mathias et al., 2007).

M. marinum infection

Mycobacterium marinum infections were performed using the wildtype Mma20 strain expressing mCherry in a pSMT3 vector (van der Sar et al., 2004). Embryos were staged at 24 hours post fertilization (hpf) by morphological criteria and manually dechorionated. Bacteria were prepared and injected into the blood circulation at 28 hpf and plated during injections to confirm the number of injected colony forming units (cfu) (Benard et al., 2012). At 4 days post injection (dpi) the uninfected and infected embryos were imaged with a stereo fluorescence microscope, and RNA was isolated for subsequent gene expression analysis. Knockdown with morpholino 1 was repeated four times with 174 morphants and 135 control embryos and the infection phenotype was confirmed with knockdown using morpholino 2 with 35 morphants and 60 control embryos. Fluorescence images were taken with a Leica MZ16FA stereo fluorescence microscope equipped with a DFC420 C digital colour camera. Overlay images of bright field and fluorescence stereomicroscopy were made in Fiji. *M. marinum* pixel count values were calculated using dedicated pixel quantification software (Stoop et al., 2011; Nezhinsky et al., 2012). All data for *M. marinum* pixel count (mean±SEM) were analysed using unpaired, two-tailed *t*-tests for comparisons between two groups (ns, no significant difference; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Results

Identification of cyprinid *cd36* genes

We identified a single *cd36* gene in zebrafish (ENSDARG00000032639) and two *cd36* genes in common carp (Bioproject PRJNA73579). BLAST analyses in the zebrafish and carp genomes did not identify additional *cd36* genes. Furthermore, the presence of two genes in carp versus a single gene in zebrafish is very common, considering the additional whole genome duplication event that has taken place in carp compared to zebrafish (Henkel et al., 2012).

All three cyprinid *cd36* genes are composed of 12 exons and 11 introns, similar to the coding parts of the human *cd36* gene (Figure 1). The full-length sequences (*drcd36, cccd36a* and *cccd36b*) have open reading frames ranging from 1398 to 1410 bp encoding for proteins of 465-469 aa with predicted molecular weights of 51.6–52.1 kDa. All three cyprinid molecules are predicted to be integral membrane glycoproteins with two transmembrane domains, a large extracellular loop and two short intracellular tails, one at the N- and one at the C-terminus (Figure 2). The extracellular part is predicted to contain multiple *N*-glycosylation sites as is the case for mammalian Cd36 (see Figure 2). Furthermore, there are several conserved cysteines of which six are located in the extracellular part and two in the N-terminal intracellular tail. By similarity to the human Cd36 protein, the six extracellular cysteines form disulphide bonds as follows: 243–311, 272–333, and 313–322 (human numbering). The two cysteines in the N-terminal tail are known to be palmitoylated in the human Cd36 protein (Tao et al., 1996), acting as tethers to the plasma membrane and localizing the protein to lipid rafts. Also these amino acids are conserved in the cyprinid molecules. Although the above-mentioned conservation of structural characteristics could point at a conservation of function, the C-terminal tail appears less conserved and the alignment in this region is weak.

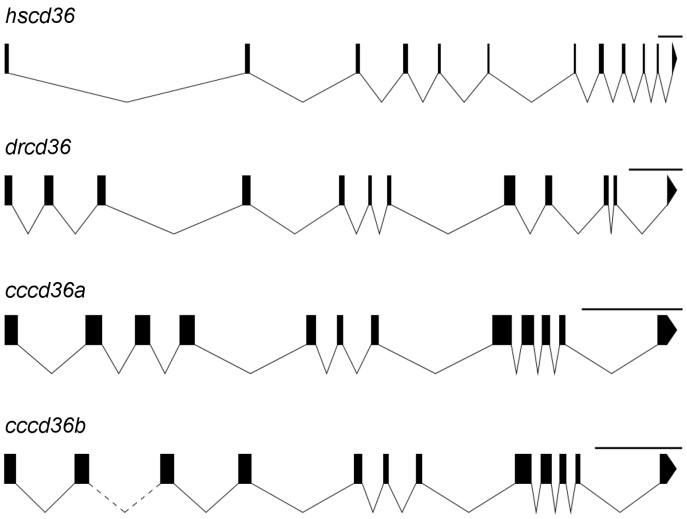


Figure 1. Exon-intron structure of the *cd36* genes of human, zebrafish and carp. The exon-intron structure is shown for human (*hscd36 Homo sapiens* ENST00000309881), zebrafish (*drcd36 Danio rerio* ENSDART00000050753), and carp (*cccd36a Cyprinus carpio* Scaffold 349; *cccd36b Cyprinus carpio* Scaffolds 102854 and 15047). Exons are shown as boxes, introns as lines. Only coding exons are shown. Intron 2 of *cccd36b* is composed of sequence from Scaffolds 102854 and 15047, which could not be precisely joined in the current assembly of the carp genome; this is indicated by the dotted line. Scale bar represents 1000 bp.

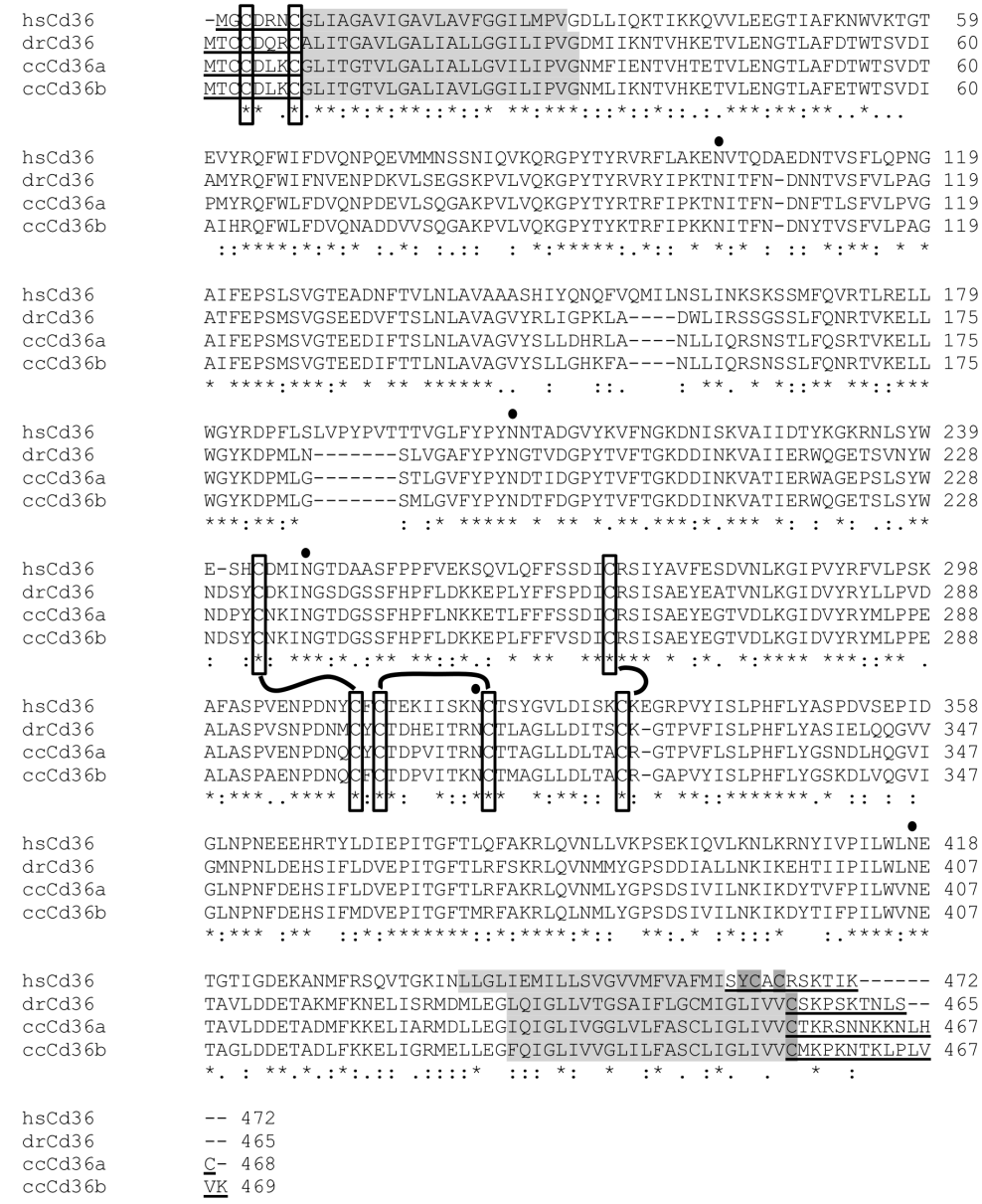


Figure 2. Multiple alignment of Cd36 shows conservation of two transmembrane domains, multiple glycosylation sites, and presence of two short intracellular tails. Multiple alignment of amino acid sequences of human (hsCd36 *Homo sapiens* ENSP00000399421), zebrafish (drCd36 *Danio rerio* ENSDARP00000050752) and two carp Cd36 proteins (ccCd36a *Cyprinus carpio* KM030422) and

ccCd36b Bioproject PRJNA73579). Underlined amino acids are intracellular, light grey shading indicates transmembrane regions, while dark grey shading indicates important amino acids in the C terminal regions. Conserved cysteines in the extracellular part of the protein are boxed and disulphide bonds are schematically shown. Conserved *N*-glycosylation sites are indicated with black dots above the sequence.

There are three amino acids in the C-terminus of the human protein which are important for the function, namely Y463, C464, and C466 (human numbering, dark grey shading in Figure 2). The two cysteines are palmitoylated similar to the cysteines in the N-terminal tail, and the tyrosine is potentially a site of phosphorylation. Both Y463 and C464 are important for phagocytosis of bacteria and for subsequent signalling via TLR2/6, leading to NFκB activation and cytokine production (Stuart et al., 2005). The cyprinid Cd36 molecules lack a tyrosine in the C-terminal tail, and they only have a single cysteine (C456, cyprinid numbering, dark grey shading in Figure 2). In conclusion, cyprinid Cd36 molecules are integral membrane glycoproteins with two transmembrane domains, a large extracellular loop and two short intracellular tails, similar to mammalian Cd36.

Phylogenetic analysis

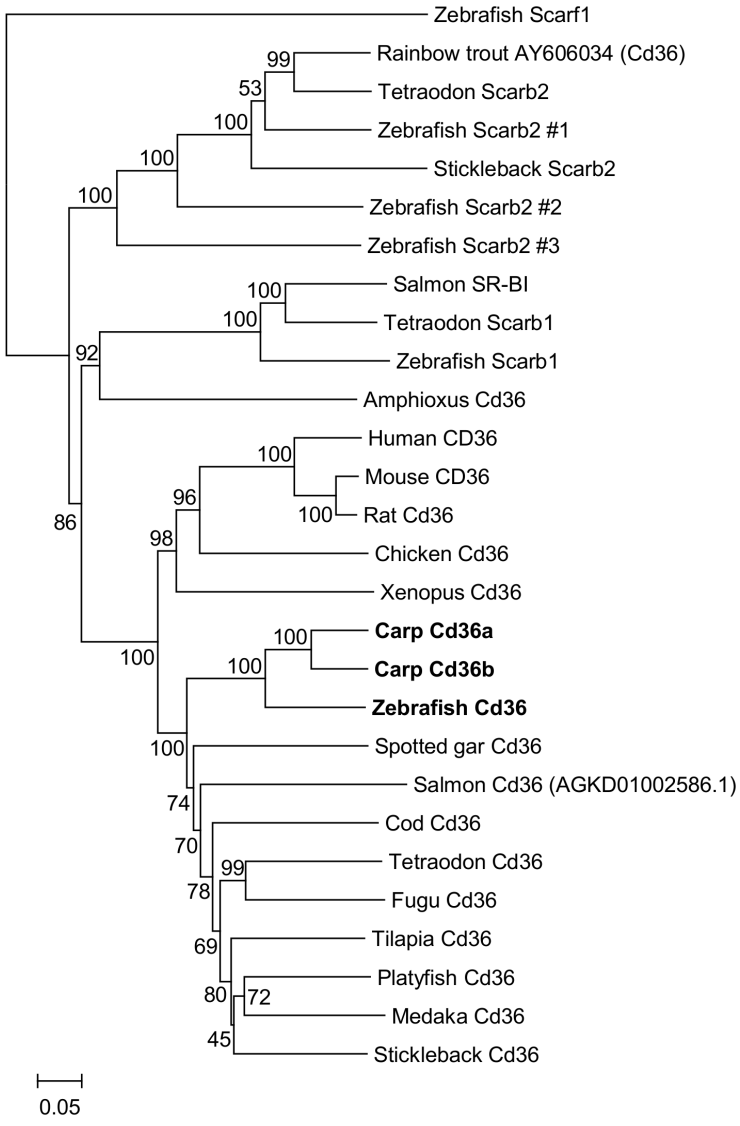
An amino acid sequence alignment indicates a high degree of similarity, not only among the cyprinid Cd36 proteins (88.7–93%) but also between cyprinid and other fish species, chicken, xenopus and mammalian Cd36 proteins (all >70%; Table 2). Also percentages of identity are very high with human to fish amino acid identities ranging from 50.8 to 52.3%. This indicates that the molecule has been well conserved during evolution. The amphioxus Cd36 protein stands out as being most different from all the other proteins with identities around 35% only.

▸ **Table 2**. Percentage identity and similarity between Cd36 of various species. Identities (upper right) and similarities (lower left) were based on the following amino acid sequences: human (*Homo sapiens* ENSP00000399421), mouse (*Mus musculus* ENSMUSP00000080974), chicken (*Gallus gallus* ENSGALP00000038958), xenopus (*Xenopus tropicalis* ENSXETP00000017231), fugu (*Takifugu rubripes* ENSTRUP00000041077), stickleback (*Gasterosteus aculeatus* ENSGACP00000026370), spotted gar (*Lepisosteus oculatus* ENSLOCP00000019733), zebrafish (*Danio rerio* ENSDARP00000050752), carp a (*Cyprinus carpio* KM030422), carp b (*Cyprinus carpio* Bioproject PRJNA73579), and amphioxus (*Branchiostoma floridae* JGI126670).

	Human	Mouse	Chicken	Xenopus	Fugu	Stickleback	Spotted gar	Zebrafish	Carp a	Carp b	Amphioxus
Human		84.1	61.4	54.9	52.3	51.1	51.6	51.5	51.2	50.8	35.6
Mouse	94.1		62.9	55.5	53.9	51.7	52.3	52.5	52.6	52.7	34.8
Chicken	81.8	81.4		56.2	49.8	50.9	51.7	52.9	51.5	50.1	36.9
Xenopus	75.4	74.6	76		50.8	52.2	51.4	51.2	49.3	47.1	36.8
Fugu	72.8	72.6	72.8	71.6		65.4	57.5	56.5	54.5	56.1	34.2
Stickleback	73.5	72.5	71.5	72.3	80.2		59.3	58.8	58.3	57.7	35.3
Spotted gar	74	73.4	73.2	70.9	74.2	76.9		56.3	56.1	57.5	37.2
Zebrafish	73.1	71.6	74.3	70.2	74.9	77.4	73.4		77.4	76.1	35.6
Carp a	72.9	72.2	72	70.9	73.5	75.5	74	88.7		87	37.3
Carp b	73.1	72.5	73.2	70.9	75.2	76.5	75.5	88.7	93		36.1
Amphioxus	59.2	57.7	60	59.6	58.5	58.8	59.4	59	60.8	60.8	

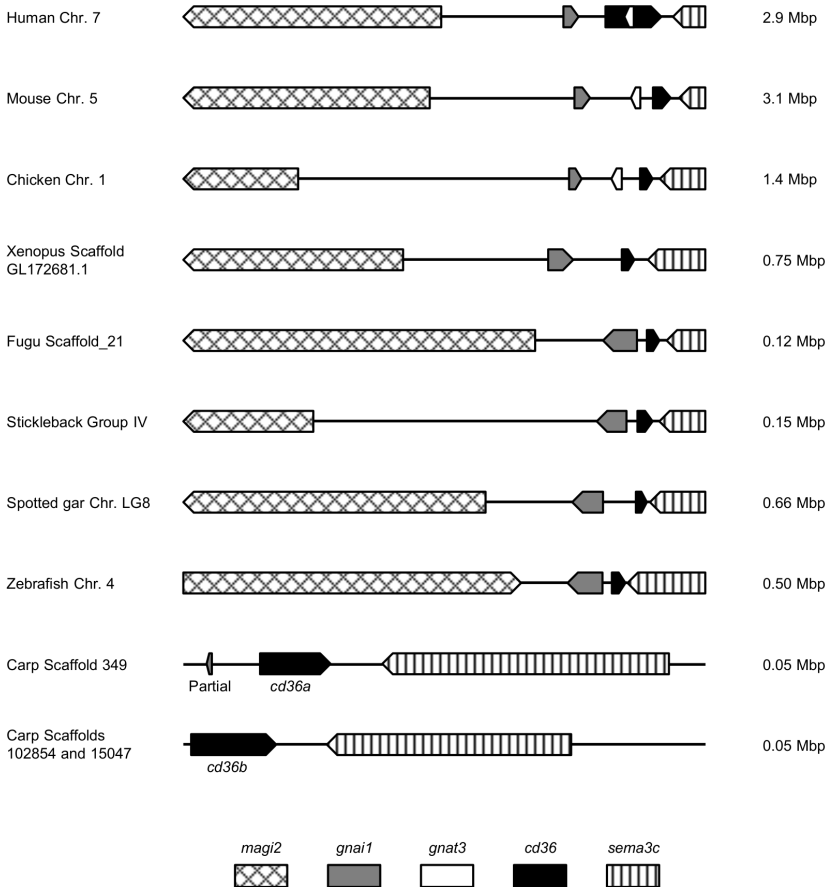
Phylogenetic analysis was performed on amino acid sequences of multiple Cd36 and Scarb molecules (Figure 3). A moderately related scavenger receptor, zebrafish Scarf1, was used as an outgroup to root the phylogenetic tree. The overall topology of the phylogenetic tree shows that the Cd36, Scarb1 and Scarb2 molecules form separate clusters. In this study we have identified a putative Cd36 of Atlantic salmon, a sequence we obtained by performing a BLAST search in salmon whole genome shotgun sequences using the zebrafish Cd36 sequence. This putative Cd36 molecule conforms with the phylogenetic tree contrary to the rainbow trout Cd36, whose position in the tree suggests it should be renamed into Scarb2. The ancestral amphioxus Cd36 seems to be closer related to the Scarb1 family. Amongst the remaining Cd36 molecules, the tetrapods (mammalian, bird and amphibian molecules) form one cluster and all the fish molecules branch out in a separate cluster. Zebrafish Cd36 and the two carp Cd36 molecules cluster together as expected from the high identities found between these. Bootstrap values are high in the entire tree with only a single exception, namely the split between stickleback and platyfish/medaka Cd36 molecules. Overall, the phylogenetic tree highlights the close relationship of all the teleost Cd36 proteins, visibly separate from the teleost Scarb1 and Scarb2 molecules and from the tetrapod proteins.

▸ **Figure 3.** Phylogenetic tree of Cd36 and Scarb sequences. Cd36, Scarb1 and Scarb2 molecules from various species were included in the phylogenetic analysis. A moderately related scavenger receptor (zebrafish Scarf1) was used as an outgroup. Carp Cd36a and Cd36b, as well as zebrafish Cd36 are indicated in bold. Amino acid sequences used: Zebrafish Scarf1 (*Danio rerio* ENSDARP00000113737), rainbow trout Cd36/Scarb2 (*Oncorhynchus mykiss* AY606034), tetraodon Scarb2 (*Tetraodon nigroviridis* ENSTNIP00000018658), zebrafish Scarb2 #1 (*Danio rerio* ENSDARP00000110051), stickleback Scarb2 (*Gasterosteus aculeatus* ENSGACP00000018249), zebrafish Scarb2 #2 (*Danio rerio* ENSDARP00000076810), zebrafish Scarb2 #3 (*Danio rerio* ENSDARP00000110098), salmon SR-BI (*Salmo salar* DQ914655), tetraodon Scarb1 (*Tetraodon nigroviridis* ENSTNIP00000016587), zebrafish Scarb1 (*Danio rerio* ENSDARP00000106590), amphioxus Cd36 (*Branchiostoma* floridae JGI126670), human CD36 (*Homo sapiens* ENSP00000399421), mouse CD36 (*Mus musculus* ENSMUSP00000080974), rat Cd36 (*Rattus norvegicus* ENSRNOP00000058398), chicken Cd36 (*Gallus gallus* ENSGALP00000038958), xenopus Cd36 (*Xenopus tropicalis* ENSXETP00000017231), carp Cd36a (*Cyprinus carpio* KM030422), carp Cd36b (*Cyprinus carpio* Bioproject PRJNA73579), zebrafish Cd36 (*Danio rerio* ENSDARP00000050752), spotted gar Cd36 (*Lepisosteus oculatus* ENSLOCP00000019733), salmon Cd36 (*Salmo salar*, predicted from AGKD01002586.1), cod Cd36 (*Gadus morhua* ENSGMOP00000012522), tetraodon Cd36 (*Tetraodon nigroviridis* ENSTNIP00000014345), fugu Cd36 (*Takifugu rubripes* ENSTRUP00000041077), tilapia Cd36 (*Oreochromis niloticus* ENSONIP00000000108), platyfish Cd36 (*Xiphophorus maculatus* ENSXMAP00000004793), medaka Cd36 (*Oryzias latipes* ENSORLP00000020106), stickleback Cd36 (*Gasterosteus aculeatus* ENSGACP00000026370).


Zebrafish Scarf1
Rainbow trout AY606034 (Cd36)
Tetraodon Scarb2
Zebrafish Scarb2 #1
Stickleback Scarb2
Zebrafish Scarb2 #2
Zebrafish Scarb2 #3
Salmon SR-BI
Tetraodon Scarb1
Zebrafish Scarb1
Amphioxus Cd36
Human CD36
Mouse CD36
Rat Cd36
Chicken Cd36
Xenopus Cd36
Carp Cd36a
Carp Cd36b
Zebrafish Cd36
Spotted gar Cd36
Salmon Cd36 (AGKD01002586.1)
Cod Cd36
Tetraodon Cd36
Fugu Cd36
Tilapia Cd36
Platyfish Cd36
Medaka Cd36
Stickleback Cd36
99
53
100
100
100
100
100
92
100
96
100
86
98
100
100
100
74
70
99
78
69
80
72
45
0.05

Synteny analysis

Conservation of synteny was investigated by comparing the genomic regions immediately up- and down-stream of human, mouse, chicken and xenopus *cd36* with the genomic regions up- and down-stream of *cd36* in a number of fish species with an annotated genome, including zebrafish and common carp (Figure 4). Human, mouse and chicken genomes contain a block of five genes: *magi2*, *gnai1*, *gnat3*, *cd36*, and *sema3c*. This block is fairly conserved in the other species with the exception of xenopus and fish genomes lacking the *gnat3* gene. For carp, although the scaffolds are limited in length, the analysis does confirm conservation of synteny with *sema3c* downstream of *cd36a* and *cd36b*, and a partial *gnai1* sequence upstream of *cd36a*. Overall, analysis of the genes adjacent to *cd36* in cyprinid fish indicates conserved synteny between teleost *cd36* and non-teleost *cd36*.



◂ **Figure 4.** Comparison of genomic regions in the vicinity of *cd36*. Synteny analysis comparing the genomic organisation of *cd36* in various species. Five genes are included in this figure, each represented by a unique colour/shaded box. The arrow indicates the direction of transcription. Intergenic regions are shown as a black line. Carp *cd36b* is split over two scaffolds. The size of each depicted genomic area is indicated to the right, in megabasepairs (Mbp).

Promoter analysis

In addition to synteny analysis we also studied in detail the transcription factor binding sites in the promoter region of zebrafish *cd36* and compared this to human, mouse and chicken (Supplementary Figure 1). The transcriptional start site has not been determined for either of the carp *cd36* genes, so promoter analysis was not performed for these. The putative transcriptional start site for zebrafish *cd36* has been experimentally verified by CAGE analysis (personal communication, H.P. Spaink), while human, mouse and chicken transcriptional start sites and promoters are already annotated in MatInspector. The promoter analyses suggest regulation of *cd36* transcription by the common transcription factors Pu.1 (part of the ETS family of transcription factors) (Zakrzewska et al., 2010), p53 (Lane and Levine, 2010), NF-κB (Oeckinghaus and Ghosh, 2009), and Stat1 (Reich, 2013). Although the exact locations of the transcription factor binding sites are not necessarily conserved and their functionality has not been experimentally verified, the presence of binding sites for all four transcription factors likely influences *cd36* gene expression levels in various tissues and cell types, including those with an immune function, and thus could be of importance for Cd36 function.

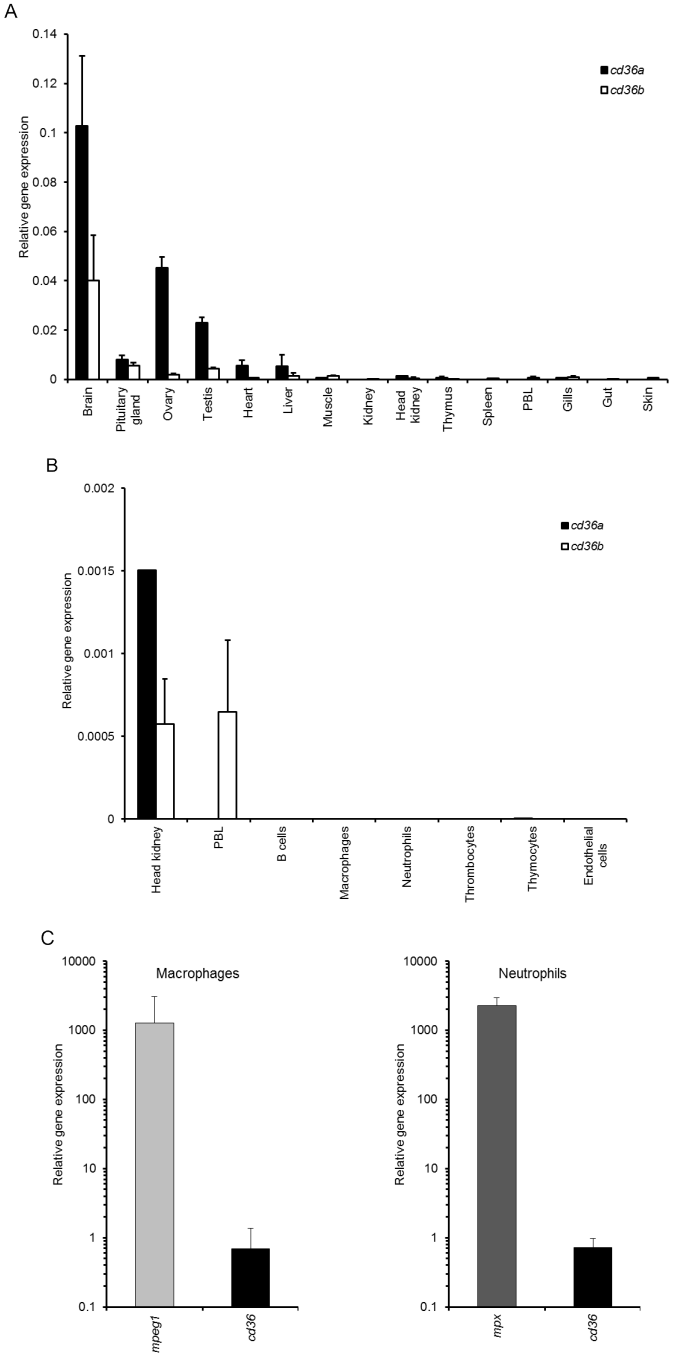
Gene expression

Constitutive gene expression of *cccd36a* and *cccd36b* was measured in organs of four healthy carp and normalized to the expression of the housekeeping gene. The results from 15 organs are shown in Figure 5A. The gene expression of *cccd36a* is generally higher than that of *cccd36b*. Highest expression for both genes is found in brain tissue (0.10 and 0.04 times the housekeeping gene, respectively), with the pituitary gland contributing slightly to the total brain gene expression. High-to-moderate gene expression was also detected in the reproductive organs, ovary (0.05 and 0.002) and testis (0.02 and 0.004), followed by low-to-moderate expression (≤0.005) levels in heart and liver. The remaining organs, including typical immune organs such as head kidney and spleen, have a very low to undetectable *cd36* gene expression level. The relatively high gene expression in brain, ovary and testis, and low to non-detectable expression in other tissues including the

immune organs tested was confirmed by transcriptome analysis of the same organs from carp (data not shown). In addition, several primer pairs were used for RT-qPCR yielding similar results. The sequences of these primers are listed in Table 1, and their location is shown in Supplementary Figure 2. Furthermore, *cd36* gene expression was undetectable by RT-qPCR using as template various immune cell types from carp purified by magnetically activated cell sorting (MACS), *i.e.* thrombocytes, thymocytes, neutrophils, B cells, and macrophages (Figure 5B).

Complementary to the analysis of multiple organs and cell types of adult carp, we took advantage of the zebrafish model to investigate *cd36* expression in leukocytes. To this end we used transgenic zebrafish lines with different fluorescently marked leukocyte populations, dissociated larvae at 6 dpf and isolated the fluorescent and non-fluorescent cell fractions by fluorescence activated cell sorting (FACS). RT-qPCR analysis of FACS isolated macrophages (*mpeg1*-promoter driven fluorescence) and neutrophils (*mpx*-promoter driven fluorescence) from zebrafish 6 dpf larvae revealed a very low *cd36* gene expression compared to the levels of macrophage- or neutrophil-specific markers (*mpeg1* and *mpx*, respectively), as shown in Figure 5C.

▸ **Figure 5**. Constitutive gene expression of *cd36*. (A) Carp organs and (B) Carp cell types. The figure shows gene expression of *cccd36a* (black bars) and *cccd36b* (white bars) in various tissues and cell types, including neuronal, reproductive and immune organs, normalized to the expression of the *40S ribosomal protein S11* housekeeping gene. For comparison, head kidney and PBL are shown next to the carp cell types in (B). Note the different scale on the *y*-axis in (A) and (B). The bars indicate average gene expression of $n = 4$ adult fish and standard deviations are indicated. PBL = peripheral blood leukocytes. (C) Expression of *drcd36* in macrophages (*mpeg1*-positive cells) and neutrophils (*mpx*-positive cells) from zebrafish larvae relative to *mpeg1*- and *mpx*-negative cells, respectively. (*mpeg1* and *mpx* gene expression data from Benard et al., 2014a). Data represent $n = 3$ biological replicates and are normalized to the *eif4* housekeeping gene.


A
Relative gene expression
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
cd36a
cd36b
Brain
Pituitary gland
Ovary
Testis
Heart
Liver
Muscle
Kidney
Head kidney
Thymus
Spleen
PBL
Gills
Gut
Skin
B
Relative gene expression
0.002
0.0015
0.001
0.0005
0
cd36a
cd36b
Head kidney
PBL
B cells
Macrophages
Neutrophils
Thrombocytes
Thymocytes
Endothelial cells
C
Macrophages
Neutrophils
Relative gene expression
10000
1000
100
10
1
0.1
mpeg1
cd36
mpx
cd36

Subcellular localization

In order to investigate the subcellular localization of cyprinid Cd36 we first cloned and sequenced the full-length *cccd36a* coding region. We then transfected the HEK cell line with a construct expressing carp Cd36a fused to the fluorescent protein mCherry. The cell membrane was visualized by staining with wheat germ agglutinin-Alexa 488 conjugate which exhibits green fluorescence. Confocal microscopy showed the presence of Cd36a-mCherry at the rim of the cells, co-localizing with the green fluorescent surface stain (Figure 6A). In contrast, the fluorescent protein mCherry alone does not localize to the cell membrane but is evenly distributed throughout the cell (Figure 6B), meaning the Cd36 molecule is responsible for the redirection of the fluorescent protein to the cell membrane. Similar results were obtained with EPC cells transfected with the same Cd36 construct (data not shown). The surface localization of carp Cd36a is in agreement with the localization of the mammalian Cd36 molecule as well as the predicted structure of the carp molecule with two transmembrane regions, an extracellular part, and two cytoplasmic tails.

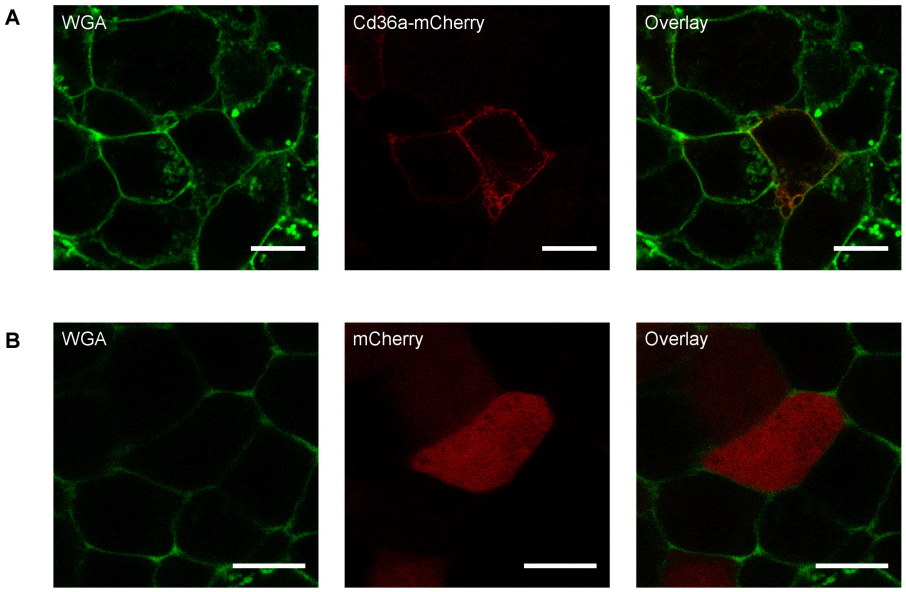


Figure 6. Subcellular localization of Cd36. (A) HEK cells stained with wheat germ agglutinin-Alexa Fluor 488 (green, left) and expressing carp Cd36a-mCherry (red, centre). An overlay is shown to the right. (B) HEK cells stained with wheat germ agglutinin-Alexa Fluor 488 (green, left) and expressing mCherry (red, centre). An overlay is shown to the right.

Role of Cd36 in *M. marinum* infection

To further study the function of teleost Cd36, we took advantage of the rapid gene knockdown technology available for zebrafish (Sumanas and Larson, 2002). We used antisense morpholino oligonucleotides to knockdown *cd36* in zebrafish embryos, which were subsequently subjected to infection with mCherry-labelled *M. marinum*. The bacterial fluorescence per embryo was quantified at 4 dpi by image analysis. At this stage of *M. marinum* infection, the embryos have developed granuloma-like structures consisting of infected and uninfected leukocytes and the bacterial fluorescence within these granulomas can be detected with stereo fluorescence microscopy (Benard et al., 2012). Two different splice blocking morpholinos were used targeting different exon-intron splice junctions of the *cd36* gene. The knockdown effect was verified by RT-PCR (Supplementary Figure 3) and embryos were stained with antibody against the pan-leukocytic marker L-plastin, showing that *cd36* knockdown did not affect leukocyte development in duct of Cuvier or in the tail region (Supplementary Figure 4). Both *cd36* morphant groups showed a significantly increased bacterial fluorescence compared to embryos injected with a control morpholino (quantification shown in Figure 7A, and representative pictures shown in Figure 7B). We further analysed gene expression of *cd36* in *M. marinum*-infected zebrafish embryos at 4 dpi (Figure 7C). Compared to uninfected fish, *cd36* expression was down-regulated approximately 3-fold in infected individuals.

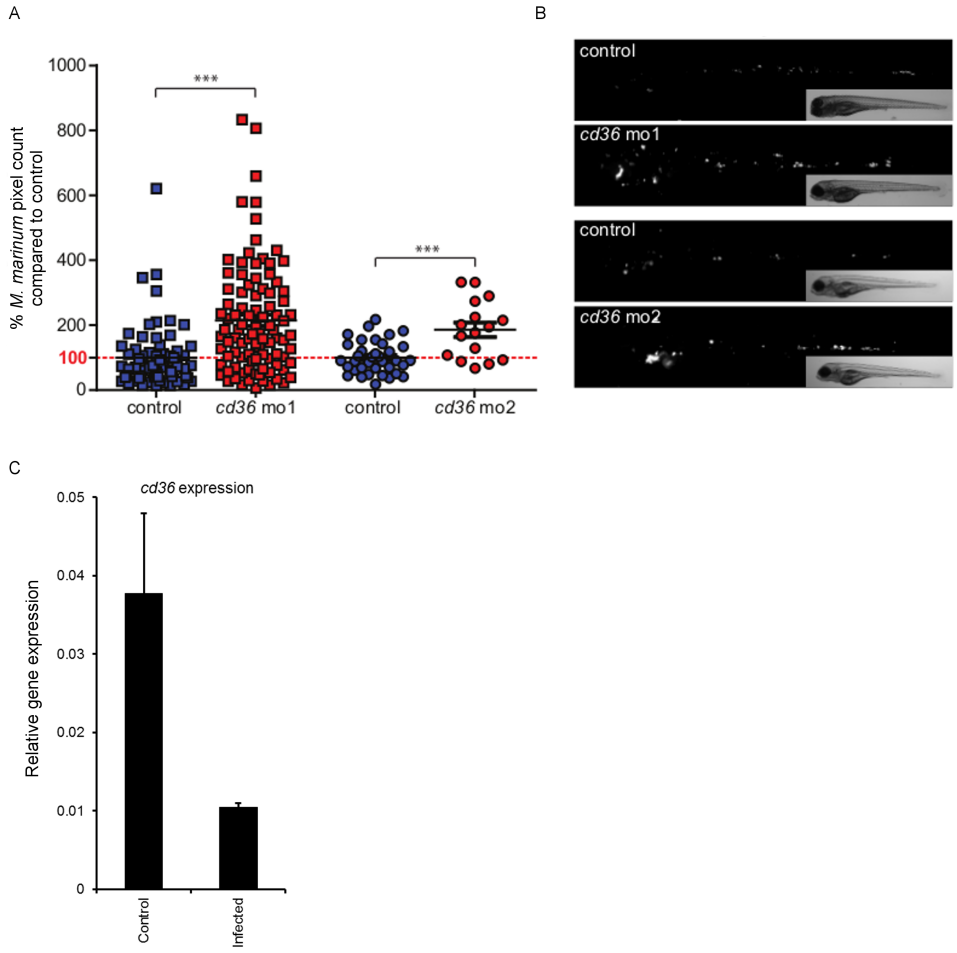


Figure 7. Interplay between *cd36* and *Mycobacterium marinum* during infection of zebrafish embryos. *cd36* knockdown impairs the control of *M. marinum* infection. AB/TL embryos were injected with two different splice blocking morpholinos against *cd36*, subsequently injected with mCherry-expressing *M. marinum* at 28 hpf and imaged at 4 dpi. (A) Bacterial burden was quantified by determining the number of fluorescent bacterial pixels per embryo with dedicated software and is represented as a percentage of the average control pixel count. (B) Representative stereo fluorescence images of an infected embryo per group are depicted with their bright field channel shown as inlay. (C) Gene expression of *cd36* is decreased during *M. marinum* infection of zebrafish embryos. Zebrafish embryos were infected as above and at 4 dpi gene expression was analysed for *cd36* and normalized to the *ppial* housekeeping gene. Data represent the average and standard deviation obtained from $n = 3$ biological replicates.

Discussion

Given our interest in the function of TLRs and the evidence, at least in mammals, of CD36 interaction with preformed TLR2-TLR6 heterodimers, we aimed to characterize the function of Cd36 in zebrafish and common carp, two closely related cyprinid fish species. Cyprinid Cd36 molecules are integral membrane glycoproteins with two transmembrane domains, a large extracellular loop and two short intracellular tails and are similar to mammalian Cd36 at the sequence level. Similar to mammalian Cd36 proteins the disulphide bonds are conserved in the cyprinid proteins, and so is the presence of multiple *N*-glycosylation sites. At present, immune functions for the scavenger receptor class B family member Cd36 have not been described for teleost fish.

Although the cyprinid Cd36 sequences seem relatively similar to mammalian Cd36, there also appear to be a number of intriguing differences. In contrast to the human protein that has no *O*-glycosylation sites, there is one *O*-glycosylation site predicted in drCd36, three sites in ccCd36a, and one site in ccCd36b. The significance of these potential *O*-glycosylation sites in the fish Cd36 proteins is not known. Most notably, there is a clear difference between the mammalian and fish Cd36 sequences in the C-terminal tail which, at least in the human protein, contains two key amino acids, namely Y463 and C464. The cysteine is palmitoylated and plays a role in the localization of Cd36 to lipid raft domains in the plasma membrane (Tao et al., 1996), whereas the tyrosine is a potential site of phosphorylation and could provide a docking site for SH2 domain-containing proteins such as the Src family of tyrosine kinases and the actin regulator p130Cas (Stuart et al., 2005). Src kinases that reside in lipid rafts have previously been shown to initiate downstream signalling cascades upon interaction with Cd36, and the actin regulator p130Cas is presumably involved in Cd36-dependent cytoskeletal rearrangements that are required for phagocytosis (Stuart et al., 2007). Upon examination of the C-terminal tail of cyprinid Cd36 molecules it is clear that no tyrosine is present but a single cysteine is found. Possibly, the presence of the cysteine could suggest that cyprinid Cd36 may localize to lipid rafts in the plasma membrane, but the absence of the tyrosine could have a determining effect on signalling and phagocytosis.

In our study we could demonstrate the presence of Cd36 on the plasma membrane by confocal microscopy. We could not clearly demonstrate, however, a role for Cd36 in phagocytosis; morpholino knockdown of zebrafish *cd36* did not affect phagocytosis of *M. marinum* (Benard et al., 2014b) and transfection of cell lines with carp *cd36* did not confer the ability to phagocytose *S. aureus* or zymosan particles (unpublished data, I. R. Fink). This suggests that differences in key amino acids in the C-terminal tail of cyprinid Cd36 may affect function of fish Cd36 as compared to function of mammalian Cd36.

We further used cell lines transfected with carp *cd36* to study its putative role as a pattern recognition receptor, by stimulating the cells with various ligands and measuring downstream transcription factor activation. Since we could not see any effect of *cd36* in these studies, this could indicate that *cd36* might primarily function as a co-receptor and as such would always require the presence of another receptor to fulfil its role.

High gene expression levels of cyprinid *cd36* were found in brain, ovary and testis. In contrast, gene expression was undetectable in immune organs and undetectable in all leukocyte subtypes tested, including monocytes/macrophages of adult carp. Also macrophages and neutrophils from zebrafish larvae had very low levels of *cd36* expression. In mammals, *cd36* is expressed in many different tissues and cell types, *e.g.* skeletal and smooth muscle, intestinal epithelia, adipocytes, endothelial cells, platelets, dendritic cells and monocytes/macrophages (Febbraio et al., 2001). We refute the option that the surprising absence of *cd36* gene expression in immune organs and cell types from (cyprinid) fish could be due to the design of our primers; design of several additional primer pairs located in different parts of *cd36* did not provide any indication of *cd36* expression in immune organs and immune cell types. We consider it unlikely that all the primer combinations we tested for carp *cd36* would have failed to amplify particular truncated splice variants uniquely expressed in immune organs and immune cell types. Although our primers would not detect the possible existence of tissue-specific splice variants arising from alternative promoter usage (Andersen et al., 2006), CAGE analysis indicates the presence of only a single transcription start site for zebrafish *cd36*, which suggests tissue-specific splice variants of *cd36* may not be commonly seen in fish. Furthermore, transcriptome analyses of a multitude of carp organs revealed an expression pattern for *cd36* which confirms our findings (unpublished data). In fact, we have found no indication for alternative splicing of cyprinid *cd36*. Possibly, differences in transcriptional regulation of *cd36* and/or absence of splice variants in cyprinid *cd36* molecules might help explain differences in function between mammalian and fish Cd36. Of interest, particular mutations in the human *cd36* gene can give rise to aberrant expression patterns and phenotypes. One example is a rather commonly occurring mutation (*C268T*), which leads to lack of *cd36* expression in monocytes and platelets, a condition called type I deficiency (Rac et al., 2007). Although this particular nucleotide is not mutated in the cyprinid genes and thus does not explain the observed absence of *cd36* gene expression in these cell types in fish, clearly mammalian CD36 is prone to mutations that affect expression in monocytes and platelets, a phenotype comparable to what we observe in the teleosts we studied. In conclusion, the complete absence of *cd36* gene expression in immune organs and leukocyte cell types of cyprinid fish suggests that maybe not all prototypical immune functions ascribed to the function of CD36 as a PRR important in innate immunity would be present in (cyprinid) fish. Clearly, the exact function of fish Cd36 remains a subject of investigation.

Presence of high levels of cyprinid *cd36* gene expression particularly in brain, testis and ovary, tissues with high lipid and/or cholesterol uptake for *e.g.* conversion to neurosteroid and sex hormones, could be indicative of a function for Cd36 mediating cholesterol transfer, a normal characteristic of the Scarb1 protein, in particular. Previous studies in salmon and rainbow trout (Trattner et al., 2008; Torstensen et al., 2011; Sanchez-Gurmaches et al., 2012, and others) on Cd36 have pointed to lipid metabolism as a function conserved between mammalian and teleost Cd36. Although it may well be true that fish members of the class B family of scavenger receptors, including Cd36 and Scarb molecules have overlapping functions, our phylogenetic analysis shows that the rainbow trout Cd36 sequence is more likely a Scarb2 homolog, which would suggest these data have to be interpreted with care. Although we identified a putative Cd36 sequence in salmon that also exhibits conserved synteny and does cluster with other fish Cd36 sequences in the phylogenetic tree, we could not easily identify a *cd36* sequence in rainbow trout. In fact all candidates in rainbow trout are more closely related to Scarb2 molecules than to Cd36. Several *scarb1* and *scarb2* paralogs are present in both the zebrafish and carp genome and interestingly, in contrast to *cd36*, transcriptome data reveal moderate to high expression of both *scarb1* and *scarb2* genes in macrophages (unpublished data, I. R. Fink). This finding reinforces the possibility that the division of functions between members of the class B family of scavenger receptors could be different between mammals and fish.

The ancestral *cd36* gene in amphioxus could be up-regulated by *in vivo* challenge with *E. coli*, and by *in vitro* LTA stimulation of a flounder cell line transfected with an amphioxus *cd36*-pcDNA3 construct (Zhang et al., 2013). However, our *in vivo* study showed that *cd36* gene expression in *M. marinum*-infected zebrafish embryos was down-regulated, which is in line with published data on infected adult zebrafish (van der Sar et al., 2009).

Although a null mutant would be required to confirm our results, knockdown of *cd36* in zebrafish embryos did not affect general development of leukocytes or the migration of neutrophils or macrophages towards locally induced inflammation, nor phagocytosis of *M. marinum* but did result in higher bacterial load following infection with *M. marinum*, suggesting a possible but presently undefined role for fish Cd36 in the control of bacterial infection. Given the suggestion that the division of functions between members of the class B family of scavenger receptors could be different between mammals and fish, future studies could address immune functions of the different class B family members found in teleost fish and explore further their functional relationships.

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Abbreviations

CD36, cluster of differentiation 36; dpf, days post fertilization; dpi, days post injection; hpf, hours post fertilization; LDL, low density lipoprotein; LTA, lipoteichoic acid; PBL, peripheral blood leukocytes; RT-PCR, reverse transcription polymerase chain reaction; RT-qPCR, real-time quantitative polymerase chain reaction; SCARB, scavenger receptor class B; TLR, Toll-like receptor.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/ j.molimm.2014.09.010.

References

Abumrad, N.A., El-Maghrabi, M.R., Amri, E.Z., Lopez, E., Grimaldi, P.A., 1993. Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. Homology with human CD36. J. Biol. Chem. 268, 17665–17668.

Akira, S., Takeda, K., 2004. Toll-like receptor signalling. Nat. Rev. Immunol. 4, 499–511.

Andersen, M., Lenhard, B., Whatling, C., Eriksson, P., Odeberg, J., 2006. Alternative promoter usage of the membrane glycoprotein CD36. BMC Mol. Biol. 7, 8.

Benard, E.L., Racz, P.I., Rougeot, J., Nezhinsky, A.E., Verbeek, F.J., Spaink, H.P., Meijer, A.H., 2014a. Macrophage-expressed perforins Mpeg1 and Mpeg1.2 have an anti-bacterial function in zebrafish. J. Innate. Immun., http://dx.doi.org/10.1159/000366103.

Benard, E.L., Roobol, S.J., Spaink, H.P., Meijer, A.H., 2014b. Phagocytosis of mycobacteria by zebrafish macrophages is dependent on the scavenger receptor Marco, a key control factor of pro-inflammatory signalling. Dev. Comp. Immunol. 47, 223–233.

Benard, E.L., van der Sar, A.M., Ellett, F., Lieschke, G.J., Spaink, H.P., Meijer, A.H., 2012. Infection of zebrafish embryos with intracellular bacterial pathogens. J. Visualized Exp.: JoVE 61, e3781.

Bernut, A., Herrmann, J.L., Kissa, K., Dubremetz, J.F., Gaillard, J.L., Lutfalla, G., Kremer, L., 2014. Mycobacterium abscessus cording prevents phagocytosis and promotes abscess formation. Proc. Natl. Acad. Sci. U.S.A. 111, E943–E952.

Brundert, M., Ewert, A., Heeren, J., Behrendt, B., Ramakrishnan, R., Greten, H., Merkel, M., Rinninger, F., 2005. Scavenger receptor class B type I mediates the selective uptake of high-density lipoprotein-associated cholesteryl ester by the liver in mice. Arterioscler. Thromb. Vasc. Biol. 25, 143–148.

Calvo, D., Dopazo, J., Vega, M.A., 1995. The CD36, CLA-1 (CD36L1), and LIMPII (CD36L2) gene family: cellular distribution, chromosomal location, and genetic evolution. Genomics 25, 100–106.

Calvo, D., Vega, M.A., 1993. Identification, primary structure, and distribution of CLA-1, a novel member of the CD36/LIMPII gene family. J. Biol. Chem. 268, 18929–18935.

Chadwick, A.C., Sahoo, D., 2013. Functional genomics of the human high-density lipoprotein receptor scavenger receptor BI: an old dog with new tricks. Curr. Opin. Endocrinol. Diabetes Obes. 20, 124–131.

Covassin, L., Amigo, J.D., Suzuki, K., Teplyuk, V., Straubhaar, J., Lawson, N.D., 2006. Global analysis of hematopoietic and vascular endothelial gene expression by tissue specific microarray profiling in zebrafish. Dev. Biol. 299, 551–562.

Endemann, G., Stanton, L.W., Madden, K.S., Bryant, C.M., White, R.T., Protter, A.A., 1993. CD36 is a receptor for oxidized low density lipoprotein. J. Biol. Chem. 268, 11811–11816.

Febbraio, M., Hajjar, D.P., Silverstein, R.L., 2001. CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. J. Clin. Invest. 108, 785–791.

Forlenza, M., Scharsack, J.P., Kachamakova, N.M., Taverne-Thiele, A.J., Rombout, J.H., Wiegertjes, G.F., 2008. Differential contribution of neutrophilic granulocytes and macrophages to nitrosative stress in a host-parasite animal model. Mol. Immunol. 45, 3178–3189.

Gonzalez, A., Valeiras, M., Sidransky, E., Tayebi, N., 2014. Lysosomal integral membrane protein-2: a new player in lysosome-related pathology. Mol. Genet. Metab. 111, 84–91.

Greenwalt, D.E., Watt, K.W., Hasler, T., Howard, R.J., Patel, S., 1990. Structural, functional, and antigenic differences between bovine heart endothelial CD36 and human platelet CD36. J. Biol. Chem. 265, 16296–16299.

Henkel, C.V., Dirks, R.P., Jansen, H.J., Forlenza, M., Wiegertjes, G.F., Howe, K., van den Thillart, G.E., Spaink, H.P., 2012. Comparison of the exomes of common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*). Zebrafish 9, 59–67.

Hoebe, K., Georgel, P., Rutschmann, S., Du, X., Mudd, S., Crozat, K., Sovath, S., Shamel, L., Hartung, T., Zähringer, U., Beutler, B., 2005. CD36 is a sensor of diacylglycerides. Nature 433, 523–527.

Irnazarow, I., 1995. Genetic variability of Polish and Hungarian carp lines. Aquaculture 129, 215–219.

Koumans-van Diepen, J.C.E., Egberts, E., Peixoto, B.R., Taverne, N., Rombout, J.H.W.M., 1995. B cell and immunoglobulin heterogeneity in carp (*Cyprinus carpio* L.); an immuno(cyto)chemical study. Dev. Comp. Immunol. 19, 97–108.

Lane, D., Levine, A., 2010. p53 Research: the past thirty years and the next thirty years. Cold Spring Harbor Perspect. Biol. 2, a000893.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.

Lee, C.C., Avalos, A.M., Ploegh, H.L., 2012. Accessory molecules for Toll-like receptors and their function. Nat. Rev. Immunol. 12, 168–179.

Mathias, J.R., Dodd, M.E., Walters, K.B., Rhodes, J., Kanki, J.P., Look, A.T., Huttenlocher, A., 2007. Live imaging of chronic inflammation caused by mutation of zebrafish Hai1. J. Cell Sci. 120, 3372–3383.

Means, T.K., Mylonakis, E., Tampakakis, E., Colvin, R.A., Seung, E., Puckett, L., Tai, M.F., Stewart, C.R., Pukkila-Worley, R., Hickman, S.E., Moore, K.J., Calderwood, S.B., Hacohen, N., Luster, A.D., El Khoury, J., 2009. Evolutionarily conserved recognition and innate immunity to fungal pathogens by the scavenger receptors SCARF1 and CD36. J. Exp. Med. 206, 637–653.

Nezhinsky, A.E., Stoop, E., van der Sar, A., Verbeek, F.J., 2012. Numerical Analysis of Image Based High Throughput Zebrafish Infection Screens-Matching Meaning with Data., pp. 257–262.

Oeckinghaus, A., Ghosh, S., 2009. The NF-κB family of transcription factors and its regulation. Cold Spring Harbor Perspect. Biol. 1, a000034.

Pietretti, D., Spaink, H.P., Falco, A., Forlenza, M., Wiegertjes, G.F., 2013. Accessory molecules for Toll-like receptors in teleost fish. Identification of TLR4 interactor with leucine-rich repeats (TRIL). Mol. Immunol. 56, 745–756.

Pietretti, D., Wiegertjes, G.F., 2014. Ligand specificities of Toll-like receptors in fish: indications from infection studies. Dev. Comp. Immunol. 43, 205–222.

Plüddemann, A., Neyen, C., Gordon, S., 2007. Macrophage scavenger receptors and host-derived ligands. Methods 43, 207–217.

Rac, M.E., Safranow, K., Poncyljusz, W., 2007. Molecular basis of human CD36 gene mutations. Mol. Med. 13, 288–296.

Reich, N.C., 2013. STATs get their move on. Jak-Stat 2, e27080.

Renshaw, S.A., Loynes, C.A., Trushell, D.M.I., Elworthy, S., Ingham, P.W., Whyte, M.K.B., 2006. A transgenic zebrafish model of neutrophilic inflammation. Blood 108, 3976–3978.

Ribeiro, C.M., Hermsen, T., Taverne-Thiele, A.J., Savelkoul, H.F., Wiegertjes, G.F., 2010. Evolution of recognition of ligands from Gram-positive bacteria: similarities and differences in the TLR2-mediated response between mammalian vertebrates and teleost fish. J. Immunol. 184, 2355–2368.

Romano, N., Picchietti, S., Taverne-Thiele, J.J., Taverne, N., Abelli, L., Mastrolia, L., Verburg-van Kemenade, B.M.L., Rombout, J.H.W.M., 1998. Distribution of macrophages during fish development: an immunohistochemical study in carp (*Cyprinus carpio, L.*). Anat. Embryol. 198, 31–41.

Rombout, J.H.W.M., Koumans-van Diepen, J.C.E., Emmer, P.M., Taverne-Thiele, J.J., Taverne, N., 1996. Characterization of carp thrombocytes with specific monoclonal antibodies. J. Fish Biol. 49, 521–531.

Sanchez-Gurmaches, J., Cruz-Garcia, L., Gutierrez, J., Navarro, I., 2012. mRNA expression of fatty acid transporters in rainbow trout: in vivo and in vitro regulation by insulin, fasting and inflammation and infection mediators. Comp. Biochem. Physiol. A: Mol. Integr. Physiol. 163, 177–188.

Sanchez-Gurmaches, J., Østbye, T.-K., Navarro, I., Torgersen, J., Hevrøy, E.M., Ruyter, B., Torstensen, B.E., 2011. In vivo and in vitro insulin and fasting control of the transmembrane fatty acid transport proteins in Atlantic salmon (*Salmo salar*). Am. J. Physiol. Regul. Integr. Comp. Physiol. 301, R947–R957.

Secombes, C.J., van Groningen, J.J.M., Egberts, E., 1983. Separation of lymphocyte subpopulations in carp *Cyprinus carpio* L. by monoclonal antibodies: immuno-histochemical studies. Immunol. 48, 165–175.

Shu, G., Liao, W.Y., Feng, J.Y., Yu, K.F., Zhai, Y.F., Wang, S.B., Khondowe, P., Wang, X.Q., Jiang, Q.Y., 2011. Active immunization of fatty acid translocase specifically decreased visceral fat deposition in male broilers. Poult. Sci. 90, 2557–2564.

Silverstein, R.L., Febbraio, M., 2009. CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior. Sci. Signal. 2, re3.

Stockhammer, O.W., Zakrzewska, A., Hegedus, Z., Spaink, H.P., Meijer, A.H., 2009. Transcriptome profiling and functional analyses of the zebrafish embryonic innate immune response to *Salmonella* infection. J. Immunol. 182, 5641–5653.

Stolte, E.H., Savelkoul, H.F., Wiegertjes, G., Flik, G., Lidy Verburg-van Kemenade, B.M., 2008. Differential expression of two interferon-γ genes in common carp (*Cyprinus carpio* L.). Dev. Comp. Immunol. 32, 1467–1481.

Stoop, E.J.M., Schipper, T., Rosendahl Huber, S.K., Nezhinsky, A.E., Verbeek, F.J., Gurcha, S.S., Besra, G.S., Vandenbroucke-Grauls, C.M.J.E., Bitter, W., van der Sar, A.M., 2011. Zebrafish embryo screen for mycobacterial genes involved in the initiation of granuloma formation reveals a newly identified ESX-1 component. Dis. Models Mech. 4, 526–536.

Stuart, L.M., Bell, S.A., Stewart, C.R., Silver, J.M., Richard, J., Goss, J.L., Tseng, A.A., Zhang, A., El Khoury, J.B., Moore, K.J., 2007. CD36 signals to the actin cytoskeleton and regulates microglial migration via a p130Cas complex. J. Biol. Chem. 282, 27392–27401.

Stuart, L.M., Deng, J., Silver, J.M., Takahashi, K., Tseng, A.A., Hennessy, E.J., Ezekowitz, R.A., Moore, K.J., 2005. Response to *Staphylococcus aureus* requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain. J. Cell Biol. 170, 477–485.

Sumanas, S., Larson, J.D., 2002. Morpholino phosphorodiamidate oligonucleotides in zebrafish: a recipe for functional genomics? Brief Funct. Genomic Proteomic 1, 239–256.

Tao, N., Wagner, S.J., Lublin, D.M., 1996. CD36 is palmitoylated on both N- and C-terminal cytoplasmic tails. J. Biol. Chem. 271, 22315–22320.

Torstensen, B.E., Espe, M., Stubhaug, I., Lie, O., 2011. Dietary plant proteins and vegetable oil blends increase adiposity and plasma lipids in Atlantic salmon (*Salmo salar* L.). Br. J. Nutr. 106, 633–647.

Trattner, S., Ruyter, B., Ostbye, T.K., Gjoen, T., Zlabek, V., Kamal-Eldin, A., Pickova, J., 2008. Sesamin increases alpha-linolenic acid conversion to docosahexaenoic acid in atlantic salmon (*Salmo salar* L.) hepatocytes: role of altered gene expression. Lipids 43, 999–1008.

Triantafilou, M., Gamper, F.G., Haston, R.M., Mouratis, M.A., Morath, S., Hartung, T., Triantafilou, K., 2006. Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting. J. Biol. Chem. 281, 31002–31011.

van der Sar, A.M., Abdallah, A.M., Sparrius, M., Reinders, E., Vandenbroucke-Grauls, C.M., Bitter, W., 2004. *Mycobacterium marinum* strains can be divided into two distinct types based on genetic diversity and virulence. Infect. Immun. 72, 6306–6312.

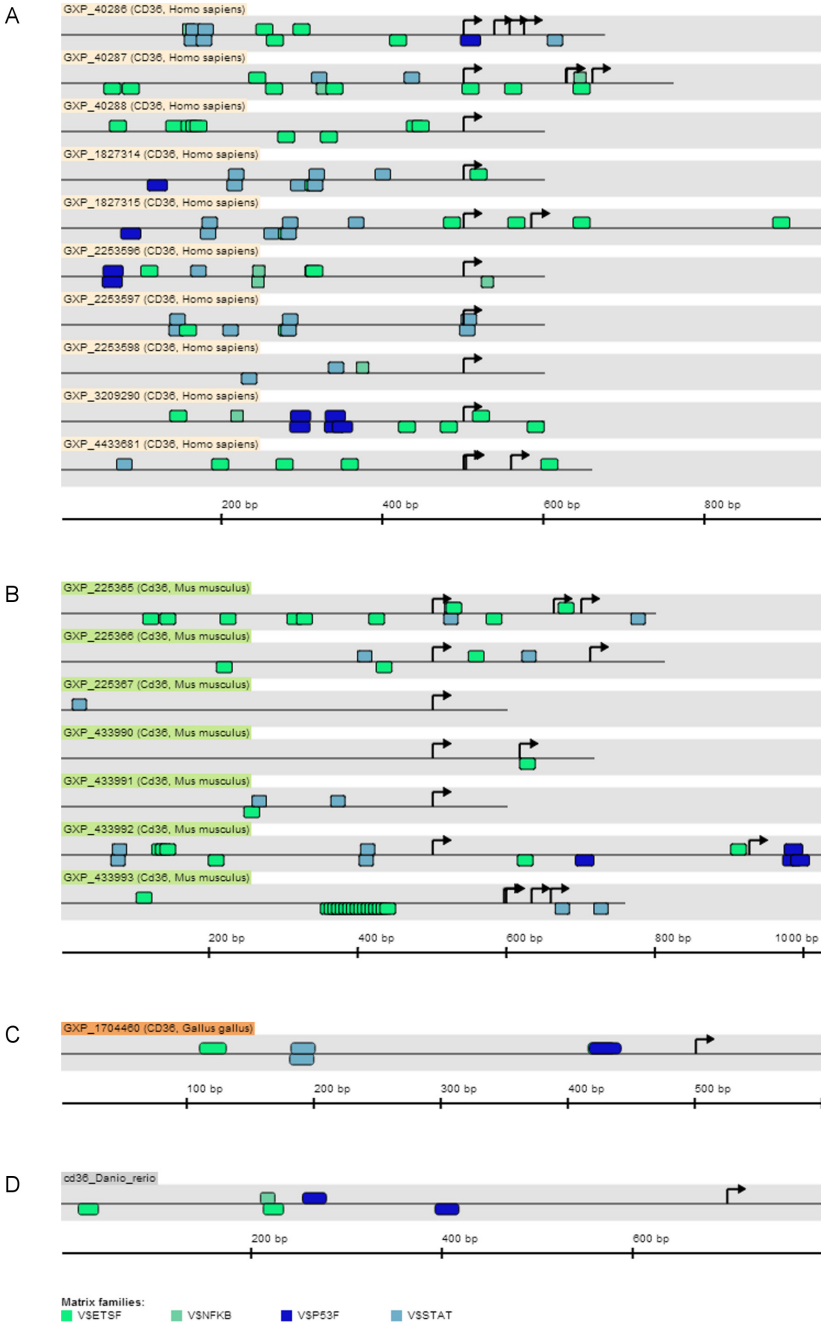
van der Sar, A.M., Spaink, H.P., Zakrzewska, A., Bitter, W., Meijer, A.H., 2009. Specificity of the zebrafish host transcriptome response to acute and chronic mycobacterial infection and the role of innate and adaptive immune components. Mol. Immunol. 46, 2317–2332.

Verburg-van Kemenade, B.M.L., Groeneveld, A., van Rens, B.T.T.M., Rombout, J.H.W.M., 1994. Characterization of macrophages and neutrophilic granulocytes from the pronephros of carp (*Cyprinus carpio*). J. Exp. Biol. 187, 143–158.

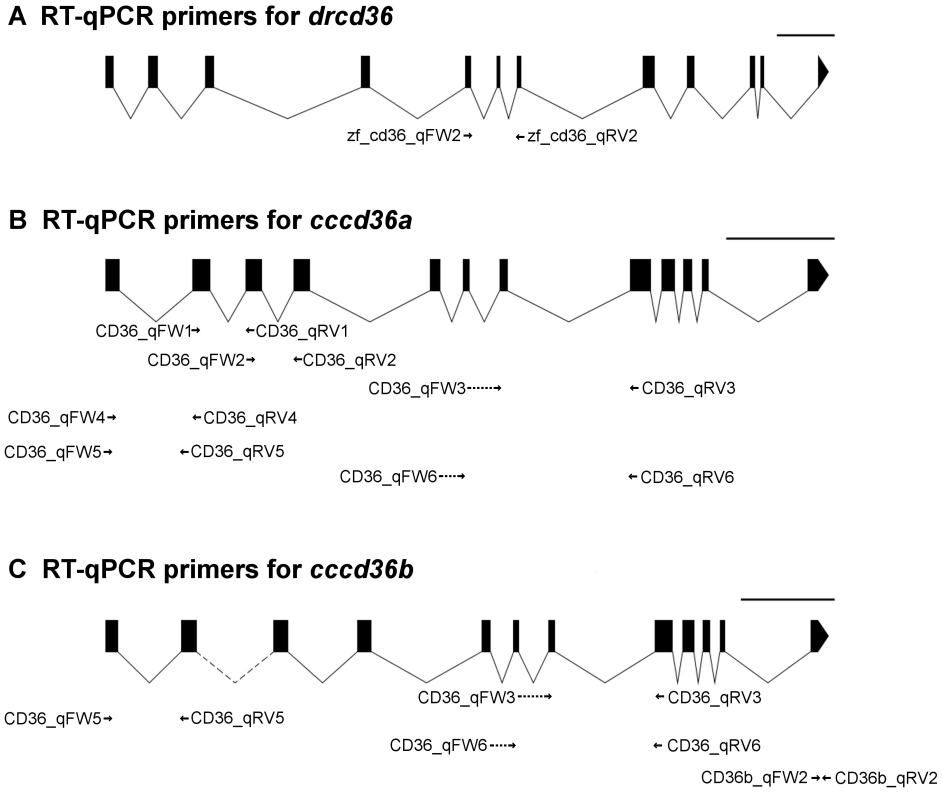
Zakrzewska, A., Cui, C., Stockhammer, O.W., Benard, E.L., Spaink, H.P., Meijer, A.H., 2010. Macrophage-specific gene functions in Spi1-directed innate immunity. Blood 116, e1–e11.

Zhang, M., Xu, Y., Li, L., Wei, S., Zhang, S., Liu, Z., 2013. Identification, evolution and expression of a CD36 homolog in the basal chordate amphioxus *Branchiostoma japonicum*. Fish Shellfish Immunol. 34, 546–555.

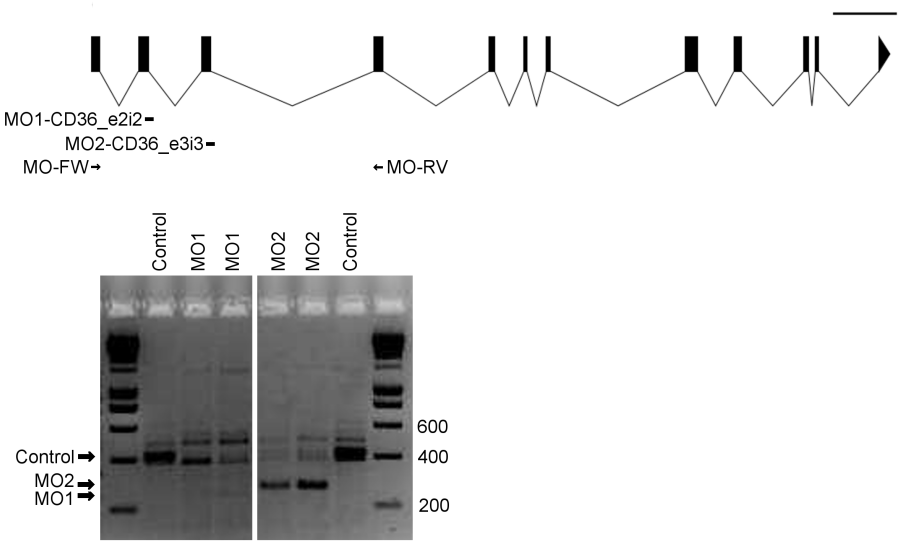
Supplementary figures



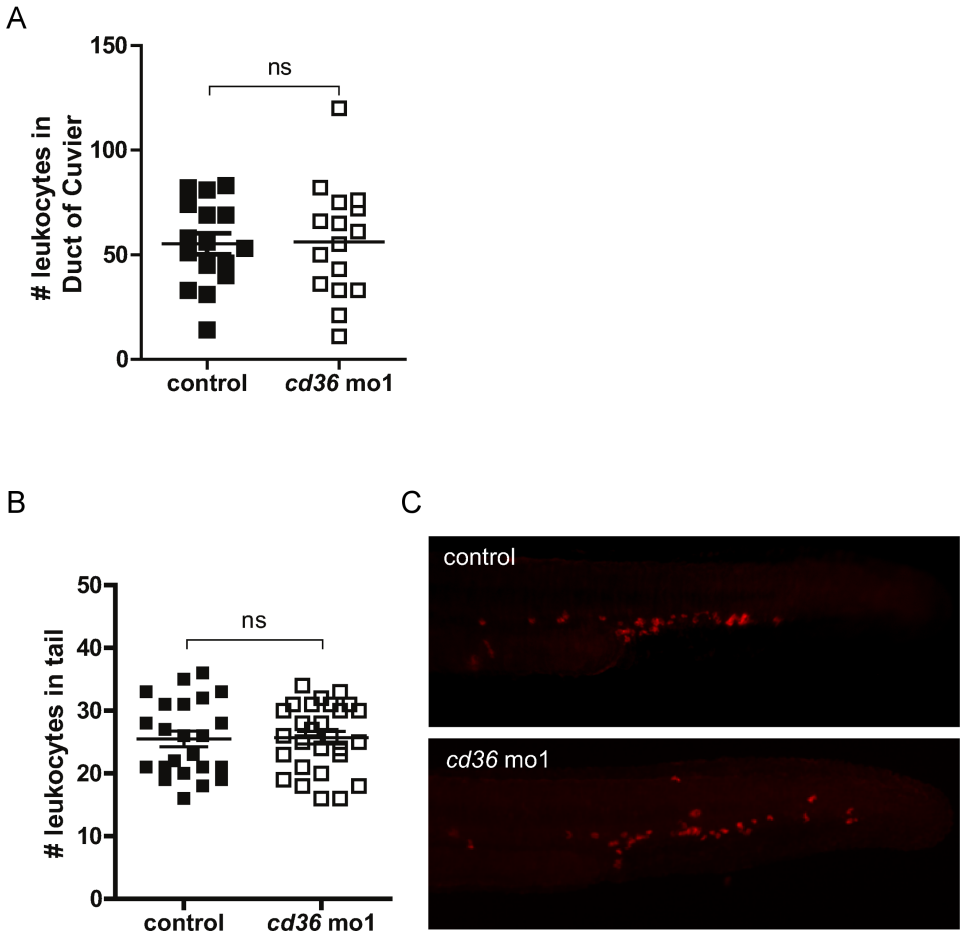
◂ **Supplementary Figure 1**. Transcription factor binding sites in the promoter regions of human, mouse, chicken and zebrafish *cd36* genes. MatInspector was used to predict the presence of vertebrate binding sites of the ETS family of transcription factors (including the Pu.1 transcription factor), NF-κB, p53 and the STAT family (including STAT1). (A) Human (*Homo sapiens*), (B) mouse (*Mus musculus*), (C) chicken (*Gallus gallus*), and (D) zebrafish (*Danio rerio*). Transcriptional start sites are indicated with arrows.



Supplementary Figure 2. Location of primers used for RT-qPCR in this study. The exon-intron structure of (A) zebrafish, and (B+C) carp *cd36* genes is indicated, with exons as boxes and introns as lines. The name and location of each primer pair is shown.



Supplementary Figure 3. Verification of morpholino knockdown. Locations are shown for morpholinos and RT-PCR primers used to verify knockdown. Agarose gel electrophoresis of RT-PCR products confirms zebrafish morpholino knockdown; the expected RT-PCR product size in MO1 knockdown is 252 bp, while MO2 gives a product of 268 bp. The control is 413 bp.



Supplementary Figure 4. Leukocyte development is not affected by morpholino knockdown of *drcd36*. Leukocyte numbers were assessed by L-plastin staining of zebrafish embryos at 28 hpf. (A) Number of leukocytes in the duct of Cuvier. (B) Number of leukocytes in the tail region. (C) Representative stereo fluorescence images of leukocytes in the tail region.

Chapter 6

Molecular and functional characterization of Toll-like receptor (Tlr)1 and Tlr2 in common carp *(Cyprinus carpio)*

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Abstract

Toll-like receptors (TLRs) are fundamental components of innate immunity that play significant roles in the defence against pathogen invasion. In this study, we present the molecular characterization of the full-length coding sequence of *tlr1*, *tlr2a* and *tlr2b* from common carp (*Cyprinus carpio)*. Each is encoded within a single exon and contains a conserved number of leucine-rich repeats, a transmembrane region and an intracellular TIR domain for signalling. Indeed, sequence, phylogenetic and synteny analysis of carp *tlr1*, *tlr2a* and *tlr2b* support that these genes are orthologues of mammalian TLR1 and TLR2. The *tlr* genes are expressed in various immune organs and cell types. Furthermore, the carp sequences exhibited a good three-dimensional fit with the heterodimer structure of human TLR1-TLR2, including the potential to bind to the ligand Pam_3CSK_4. This supports the possible formation of carp Tlr1-Tlr2 heterodimers. However, we were unable to demonstrate Tlr1/Tlr2-mediated ligand binding in transfected cell lines through NF-κB activation, despite showing the expression and co-localization of Tlr1 and Tlr2. We discuss possible limitations when studying ligand-specific activation of NF-κB after expression of Tlr1 and/or Tlr2 in human but also fish cell lines and we propose alternative future strategies for studying ligand-binding properties of fish Tlrs.

Introduction

Pattern recognition receptors recognize widely-conserved motifs of pathogens and are crucial for initiating immune responses against invading microorganisms. Toll-like receptors (TLRs) are a family of germline-encoded pattern recognition receptors and known to activate rapid inflammatory responses upon detection of their cognate ligands (Takeda et al., 2003). TLRs are type-I transmembrane proteins with numerous extracellular leucine-rich repeat (LRR) motifs which collectively form a horseshoe-shaped solenoid, which is responsible for ligand binding. The cytoplasmic part of each TLR contains a Toll/interleukin-1 receptor homology (TIR) domain which, upon dimerization of two TLRs, initiates a signalling cascade leading to activation of transcription factors such as NF-κB or AP-1, and subsequently to production of pro-inflammatory cytokines (Akira and Takeda, 2004).

Most vertebrate genomes are recognized to have at least one gene representing each of the six major TLR families (TLR1, TLR3, TLR4, TLR5, TLR7 and TLR11) (Roach et al., 2005). Also within the modern bony fish (Teleostei) the number of Tlr families generally is consistent with what is found for most (higher) vertebrates, although it is not unusual to find duplicated *tlr* genes due to several fish-specific whole-genome duplication events (Ohno, 1970; Jaillon et al., 2004; Kasahara et al., 2007; Allendorf and Thorgaard, 1984; David et al., 2003). In addition to these duplications, some novel Tlrs seem to be "fish-specific", such as a soluble form of Tlr5, and Tlrs 18-27 (Palti, 2011; Pietretti and Wiegertjes, 2014), indicating that an expansion of Tlrs has occurred during the evolution of teleosts. To date, little is known about the ligand specificities of individual Tlrs in fish, since the LRR ectodomains of Tlrs are not always highly conserved and sequence information alone cannot infer functional properties (Pietretti and Wiegertjes, 2014). In apparent contrast to the variation in the ectodomains, intracellular TIR domains of fish Tlrs appear highly conserved and downstream signalling via well-described molecules such as MyD88, Irak1 and Traf6 identified in several fish species, suggest a conserved mechanism of innate immune signalling could exist (Rebl et al., 2010). Yet, studies into ligand-binding properties of fish Tlrs are essential to characterize their exact function within the immune system of fish.

The mammalian TLR1 family consists of TLR1, 2, 6, and also includes TLR10. TLR2 recognizes a variety of microbial components including lipoproteins/lipopeptides, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, parasite glycosylphosphatidylinositol anchors, and fungal zymosan (reviewed by Takeda et al., 2003). TLR2 functions as a heterodimer with either TLR1 or TLR6; the TLR2/TLR1 heterodimer recognizes a variety of triacylated lipoproteins

(Takeuchi et al., 2002), whereas the TLR2/TLR6 recognizes mycoplasma-derived diacylated lipoproteins (Takeuchi et al., 2001). TLR6 and TLR10 seem to have arisen as paralogs of TLR1 in the mammalian lineage, with TLR10 found in humans. Neither TLR6 nor TLR10 have been identified in genomes of any lower vertebrate, including teleosts. In fish, Tlr1 and Tlr2 were first identified in fugu (Oshiumi et al., 2003) and zebrafish (Jault et al., 2004; Meijer et al., 2004). Subsequently, Tlr1 and/or Tlr2 have been described in several fish species; Japanese flounder (*Paralichthys olivaceus*) (Hirono et al., 2004), channel catfish (*Ictalurus punctatus*) (Baoprasertkul et al., 2007; Quiniou et al., 2013), rainbow trout (*Oncorhynchus mykiss*) (Palti et al., 2010; Brietzke et al., 2016), Tetraodon (*Tetraodon nigroviridis*) (Wu et al., 2008), orange-spotted grouper (*Epinephelus coioides*) (Wei et al., 2011), large yellow croaker (*Larimichthys crocea*) (Wang et al., 2013; Fan et al., 2015; Ao et al., 2016), and rohu (*Labeo rohita*) (Samanta et al., 2012). However, studies into ligand-binding properties of fish Tlr1 and/or Tlr2 molecules have been scarce.

We previously identified and characterized common carp (*Cyprinus carpio*) Tlr2 (Ribeiro et al., 2010a; Ribeiro et al., 2010b). Transfection of human HEK293 cells with carp *tlr2* suggested the ability to bind the prototypical TLR2 ligands LTA, PGN and Pam_3CSK_4. Stimulation of carp macrophages with PGN induced *tlr2* gene expression, MAPK-p38 phosphorylation and led to an increased production of nitrogen and oxygen radicals. Here, we present the identification of Tlr1 and molecular characterization of the mRNA and genomic structure of both *tlr1* and *tlr2* from common carp. We compare the gene expression of *tlr1* and *tlr2* in the same tissue samples and purified cell populations and describe our efforts to characterize the function of putative Tlr1/Tlr2 heterodimers by studying subcellular localization and ligand-binding properties. We discuss possible limitations when studying ligand-specific activation of NF-κB after over-expression of Tlr1 and/or Tlr2 in human but also fish cell lines and propose alternative future strategies for studying ligand-binding properties of fish Tlrs.

Materials and methods

Animals

European common carp (*Cyprinus carpio* L.) were reared in the central fish facility Carus, at Wageningen University, Wageningen, The Netherlands. Fish were kept at 23 °C in recirculating UV-treated tap water and fed pelleted dry food (Sniff, Soest, Germany) daily. R3xR8 carp are the hybrid offspring of a cross between fish of Polish origin (R3 strain) and Hungarian origin (R8 strain) (Irnazarow, 1995). Carp were between 9 and 11 months

old at the start of the experiments. All studies were performed with approval from the local animal welfare committee (DEC) of Wageningen University.

Organ isolation

Carp were euthanized with 0.3 g/L tricaine methane sulfonate (TMS, Crescent Research Chemicals, Phoenix, AZ, USA) buffered with 0.6 g/L $NaHCO_3$. Carp were bled from the caudal vein using a needle and syringe containing cRPMI medium (RPMI 1640 with 25 mM HEPES (Lonza, Basel, Switzerland) adjusted to an osmolality of 280 mOsm/kg with sterile water) containing 50 U/mL heparin (Leo Pharma, Ballerup, Denmark), 50 U/mL penicillin G (Sigma-Aldrich, St. Louis, MO, USA), and 50 µg/mL streptomycin sulphate (Sigma-Aldrich). For isolation of peripheral blood leukocytes (PBL), the heparinized blood was centrifuged at 100g for 5 min at 4 °C and then another 5 min at 300g. The buffy coat was collected, carefully layered on Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, UK) and centrifuged at 800g for 25 min at 4 °C without brake. The leukocyte layer was collected and washed twice with cRPMI. The obtained PBL were stored at -80 °C until used for RNA isolation. After bleeding the fish, the organs of interest were aseptically removed and immediately frozen in liquid nitrogen and stored at -80 °C until used for RNA isolation.

Isolation of leukocyte subtypes

Carp leukocyte subtypes were isolated by density gradient separation and/or magnetic cell sorting using specific antibodies as described before for thrombocytes (Rombout et al., 1996), granulocytes (Forlenza et al., 2008), B cells (Koumans-van Diepen et al., 1995; Secombes et al., 1983), and macrophages (Romano et al., 1998). In short, PBL or single-cell suspensions derived from carp organs were incubated with primary mouse monoclonal antibody: WCL-6 for thrombocytes (from blood), TCL-BE8 for neutrophils (from mid kidney), WCI-12 for B cells (from blood), and WCL-15 for monocytes/macrophages (from spleen). After incubation and washing, cells were stained with phycoerythrin (PE)-conjugated goat anti-mouse secondary antibody. After washing and counting of cells, magnetic beads (anti-PE MicroBeads, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added and allowed to bind, before washing and magnetic separation on LS Midi Columns using a MidiMACS Separator (Miltenyi Biotec). Head kidney-derived macrophages were isolated and cultured as described by Joerink et al., 2006.

RNA isolation

Total RNA from carp organs and leukocytes was extracted using the RNeasy Mini kit according to the manufacturer's protocol (Qiagen, Venlo, The Netherlands) including on-column DNase treatment with the RNase-free DNase set (Qiagen). Final elution was performed with 30 µL nuclease-free water. The integrity of the RNA was determined by agarose gel electrophoresis and the RNA quality and concentrations were assessed spectrophotometrically by measuring the absorbance at 260 nm and 280 nm (Nanodrop, Thermo Scientific, Waltham, MA, USA). RNA was stored at -80 °C until use.

cDNA synthesis

Prior to cDNA synthesis, 500 ng-1 µg of total RNA was subjected to an additional DNase treatment by using DNase I Amplification Grade (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNA was performed with Invitrogen's SuperScript III Reverse Transcriptase, according to the manufacturer's instructions. As control for genomic contamination, for each sample a reaction without SuperScript III Reverse Transcriptase was performed. cDNA samples were diluted 25 times in nuclease-free water before use as templates in real-time quantitative PCR experiments.

Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was performed in a Rotor-Gene 6000 with a 72-well rotor (Corbett Research, Qiagen) with the ABsolute QPCR SYBR Green Mix (Thermo Scientific) as detection chemistry as described previously (Forlenza et al., 2012). All primers were from Eurogentec (Liège, Belgium). RT-qPCR data were analysed by Rotor-Gene 6000 Series Software 1.7. The melting temperature and profile of the melting curves were used to validate the specificity of the amplification. The gene expression was analysed using the average amplification efficiency for each primer pair and the take-off value of each sample (as derived from the Comparative Quantitation Analysis of the Rotor-Gene 6000 Series Software 1.7). The relative gene expression was calculated as the average amplification efficiency to the power of the take-off value. The gene expression of the housekeeping gene *40s ribosomal protein s11* was used to normalize the data. The sequences of primers used in this study are given in Table 1.

Table 1. List of primers used in this study. Restriction sites are italicized, Kozak sequence is shown in bold, sequence encoding a FLAG-tag is single underlined, and His-tag is bolded and italicized.

Oligo name	Sequence 5' → 3'	Purpose
CycaTLR1_FL_FW1	ATCTACAGCAGACGGAAAG	Cloning of full-length *tlr1*
CycaTLR1_FL_RV4	TCTTGAAGCCCCTGTGAAAG	Cloning of full-length *tlr1*
TLR1_pcDNA3_FW1	ACACGTCTCCCATTCTGGCCGATTACAAGGATGACGATGACAAGATTAAGAGGATCATAGTGAA	Cloning of full-length *tlr1* with FLAG-tag
TLR1_pcDNA3_FW2	ACGA*TGATCA***AACATGG**AGCCGTTGGACTGGTGGCTG	Cloning of full-length *tlr1* with FLAG-tag
TLR1_ pcDNA3_FLAG	ACGA*TGATCA***AACATGG**AGCCGTTGGACTGGTGGCTGCTGTTTGTTTATGTCACATGTTTCCACACGTCTCCCATTCTGGCCGATTACAAGGATGACGATGACAAGATTAAGAGGATCATAGTGAA	Cloning of full-length *tlr1* with FLAG-tag
TLR1_pcDNA3_RV1	CAGT*CTCGAG*TTCTTCTCTCTCTGGGGGAC	Cloning of full-length *tlr1* with FLAG-tag
TLR2_pcDNA3_FW1	GCTTTCACTACTCCAGGACA***CATCATCACCATCACCAT***TGTGATTGTGACCAGCAATA	Cloning of full-length *tlr2a* with His-tag
TLR2_pcDNA3_FW2	ACGA*GGATCC***AACATGG**AATTCTTGGGAAGAGAGGCG	Cloning of full-length *tlr2a* with His-tag
TLR2_pcDNA3_His	ACGA*GGATCC***AACATGG**AATTCTTGGGAAGAGAGGCGTCCATAATTATTTTCATATTAATTTTGGCCCAAGGCTTTCACTACTCCAGGACA***CATCATCACCATCACCAT***TGTGATTGTGACCAGCAATA	Cloning of full-length *tlr2a* with His-tag
TLR2_pcDNA3_RV1	CATG*CTCGAG*ACATTCATCTCTCTGTAGAGCAGC	Cloning of full-length *tlr2a* with His-tag
CycaTLR1_qFW1	AAAAGCGACCTTGACATTGC	RT-qPCR of *tlr1*
CycaTLR1_qRV1	GCTAACGGTGCGTAGGATTC	RT-qPCR of *tlr1*
TLR2_qFW	TCAACA+CTCTTAATG+TGAGCCA[a]	RT-qPCR of *tlr2*
TLR2_qRV	TGTG+CTGGAAA+GGTTCAGAAA[a]	RT-qPCR of *tlr2*
40S_FW	CCGTGGGTGACATCGTTACA	RT-qPCR of carp *40S*
40S_RV	TCAGGACATTGAACCTCACTGTCT	RT-qPCR of carp *40S*

[a] + in front of a nucleotide indicates that it contains an LNA modification.

Cloning full-length *tlr1* from common carp

Carp head kidney leukocytes were obtained by density gradient separation. RNA was isolated as described above and then used as template in reverse transcription-PCR with degenerate primers designed on the basis of *tlr1* sequence information from other teleost fish. RACE (rapid amplification of cDNA ends) was then performed to obtain sequence at 5' and 3' ends. This allowed for design of carp primers in 5' and 3' untranslated regions (UTR), thus enabling the amplification of full-length carp *tlr1* sequence. For this, RNA from head kidney leukocytes was used as template in RT-PCR using the LongRange 2Step RT-PCR kit (Qiagen). Reverse transcription was done with an oligo-dT primer and the subsequent PCR was done with gene-specific primers located in 5' and 3' UTR (CycaTLR1_FL_FW1 and CycaTLR1_FL_RV4, see Table 1). The PCR product was subsequently cloned in pGEM-T Easy (Promega, Madison, WI, USA) and transformed into *Escherichia coli* JM109 competent cells (Promega). Clones were sequenced and sequence data were analysed with Sequencher version 4.10 (Gene Codes, Ann Arbor, MI, USA) and aligned with Clustal Omega (http://www.ebi. ac.uk/Tools/msa/clustalo/). A clone with the consensus sequence was used for further studies.

Bioinformatics

We obtained the coding sequence of the unique carp *tlr1* gene after cloning (our sequence is identical to the recently deposited GenBank accession number LHQP01021877, Contig21898, cypCar_00044472, from whole genome shotgun sequencing). Cloning of *tlr2* was previously reported by our group (Ribeiro et al., 2010a). Now referred to as *tlr2a*, the sequence has been updated in GenBank (accession number FJ858800) and the automatic annotation of the recently deposited LHQP01006764, Contig6769, cypCar_00005269 has been adjusted. A second *tlr2* sequence was identified in this study, predicted as genome sequence data (Bioproject PRJNA73579) (Henkel et al., 2012), and partially confirmed as RNAseq data (unpublished data) and will be referred to as *tlr2b* (GenBank accession number LHQP01045997, Contig46054, cypCar_00039549 has been adjusted to reflect our manual annotation). Exon-intron structure was studied by multiple alignments and open reading frame predictions (FGENESH at http://linux1.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind, and GENSCAN at http://genes.mit.edu/GENSCAN.html). Nucleotide sequences were translated into protein sequence using the EMBL-EBI tool (http://www.ebi.ac.uk/Tools/st/) and these were aligned with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Molecular weights were calculated with ProtParam (http://web.expasy.org/protparam/). The protein sequences were examined for the presence of a signal peptide using SignalP (http://www.

cbs.dtu.dk/services/SignalP/) and transmembrane regions were predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Individual LRRs were identified by LRRfinder (http://www.lrrfinder.com/) and manually according to previous descriptions (Bell et al., 2003; Matsushima et al., 2007). Synteny analysis was performed on the basis of sequence information from Ensembl release 82 (http://www.ensembl.org/). The multiple sequence alignment for the phylogenetic tree was made with ClustalX 2.1 (Larkin et al., 2007) and the tree was constructed using the Neighbour Joining method with the number of bootstrap trials set to 10000. The phylogenetic tree was visualized with MEGA6.

tlr1 and *tlr2* expression plasmids

Each of the genes encoding the fluorescent proteins GFP and mCherry were cloned into pcDNA3 using the *Eco*RI and *Xba*I sites. An *Xho*I site and a short linker sequence of 15 nucleotides encoding the amino acids GGSGG was placed upstream of the GFP and mCherry sequence. Subsequently, the full-length carp *tlr1* sequence was modified from the above-mentioned pGEM-T Easy construct to include sequence to encode a FLAG-tag at the N-terminus (after the leader peptide), and to remove the stop codon, using primers TLR1_pcDNA3_FW1, TLR1_pcDNA3_FW2, TLR1_pcDNA3_FLAG and TLR1_pcDNA3_RV1 (see Table 1). The modified *tlr1* was then cloned between the *Bam*HI site and the newly created *Xho*I site in pcDNA3, thus creating a fusion of *tlr1*-GFP and *tlr1*-mCherry each in pcDNA3. Given the high sequence similarity between *tlr2a* and *tlr2b*, for functional analysis, we proceeded with the previously characterized *tlr2a* construct (Ribeiro et al., 2010a). This gene was sub-cloned into mCherry-pcDNA3 using primers TLR2_pcDNA3_FW1, TLR2_pcDNA3_FW2, TLR2_pcDNA3_His and TLR2_pcDNA3_RV1 (see Table 1) thereby creating a His-tagged fusion of *tlr2a*-mCherry. Furthermore, *tlr1* was sub-cloned into pBI-CMV1, a plasmid with two multiple cloning sites (MCS), where *tlr1* was cloned in MCS1 between *Bam*HI and *Pvu*II sites, and the GFP was no longer fused to the *tlr* sequence but subcloned in the MCS2 between *Eco*RI and *Xba*I sites. A similar construct was made for *tlr2a*. Isolation of transfection-grade plasmid DNA was performed with the S.N.A.P. MidiPrep Kit (Invitrogen) according to the manufacturer's instructions.

Subcellular localization of carp Tlr1, and co-localization with Tlr2

Human embryonic kidney 293 (HEK) cells were cultured at 37 °C at 5% CO_2 in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (Gibco), 2 mM L-glutamine (Merck, Darmstadt, Germany), 50 U/mL penicillin G (Sigma-Aldrich), and 50 µg/mL streptomycin sulphate (Sigma-Aldrich). HEK cells were seeded on untreated glass cover slips placed in 6-well plates, 4.5 x 10^4 cells each. The following day, cells were transfected with 0.5 µg *tlr1*-mCherry-pcDNA3, *tlr2*-mCherry-pcDNA3, mCherry-pcDNA3, or a combination of *tlr1*-GFP-pcDNA3 with *tlr2*-mCherry-pcDNA3 using jetPRIME (Polyplus Transfection, Illkirch, France) according to the manufacturer's instructions at a 1:3 ratio of plasmid:transfection reagent. Three days after transfection, cover slips were carefully washed with HBSS, cells were fixed for 20 min at room temperature with 4% paraformaldehyde, washed with HBSS and stained with wheat germ agglutinin-Alexa Fluor 488 conjugate (Molecular Probes, Invitrogen) for 15 min at room temperature, before final washing with HBSS and mounting with Vectashield (Vector Laboratories, Burlingame, CA, USA).

The Epithelioma papulosum cyprini (EPC) cell line, which is an adherent cell type derived from fathead minnow, was cultured at 27 °C at 5% CO_2 in RPMI 1640 (Lonza) supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin G, and 50 µg/mL streptomycin sulphate. EPC cells were seeded in six-well plates, 8 x 10^5 cells/well, and allowed to adhere. EPC cells were transfected the following day with 2 µg *tlr1*-GFP-pcDNA3 using FuGENE HD Transfection Reagent (Promega) according to the manufacturer's instructions at a 1:3.5 ratio of plasmid:transfection reagent. Three days after transfection, EPC cells were detached with cold medium, fixed with 4% paraformaldehyde and washed with 1% BSA in PBS. Some samples were permeabilized with 0.1% Triton X-100 in 1%BSA/PBS while others were not permeabilized. Antibody staining was performed with mouse anti-FLAG primary antibody (1:200 dilution, Sigma-Aldrich) followed by Cy3-labelled donkey anti-mouse secondary antibody (1:100 dilution, Jackson ImmunoResearch, West Grove, PA, USA). Cells were mounted with Vectashield.

Cells were visualized with a Zeiss LSM-510 (Zeiss, Oberkochen, Germany) confocal laser scanning microscope with a Plan-Apochromat 63x /1.4 oil immersion objective. Green fluorescent signal (GFP or Alexa Fluor 488) was excited with a 488 nm argon laser and detected using a band-pass filter (505-550 nm). Red fluorescence (mCherry protein or Cy3) was excited with a 543 nm helium-neon laser and detected using a long-pass filter (585 nm). Image processing was performed with ImageJ (http://imagej.nih.gov/ij/).

Structural model of heterodimer formation

The sequences of carp Tlr1, carp Tlr2a/b, human TLR1 and human TLR2 were used for sequence alignment using ClustalX (Thompson et al., 1997). Using these alignments and the available structure of the human TLR1/TLR2 complex, including a tri-acylated lipopeptide moiety (Pam_3CSK_4) and all sugar chains and water molecules (PDB-id: 2z7x) as templates, structural models were obtained for the carp Tlr1-Tlr2a/b complexes using the Modeller program (version 9.12) (Eswar et al., 2007). Thirty comparative models were generated, after which the model with lowest corresponding DOPE score (Eswar et al., 2007) was selected for image generation.

Ligand binding

HeLa-57A cells (stably transfected with NF-κB luciferase reporter (Rodriguez et al., 1999)) were cultured in DMEM with 5% foetal bovine serum (Bodinco, Alkmaar, The Netherlands) at 37 °C in 10% CO_2 atmosphere. Cells were transfected in 6-well plates with a combination of carp *tlr1* and *tlr2* plasmids, or human *tlr1* and *tlr2* (Keestra et al., 2007) as positive control. All plasmids were transfected using FuGENE HD (Roche, Basel, Switzerland) in a DNA:FuGENE ratio of 1:3. A total of 2 µg plasmid DNA was transfected; when multiple plasmids were combined, equal amounts of each plasmid were used. To all combinations (except empty vector) a plasmid encoding human CD14 (Keestra et al., 2007) was added. Twenty-four hours after transfection cells were redistributed from 6-well plates to 48-well plates and left to attach for 24 h. Cells were then stimulated with the following ligands: Pam_3CSK_4 (100 ng/mL), peptidoglycan (PGN, 10 µg/mL), and lipoteichoic acid (LTA, 1 µg/mL) which were purchased from Invivogen, San Diego, CA, USA. Stimulation was performed for 5 h, after which the cells were washed twice with PBS and then lysed with 100 µL reporter lysis buffer (Promega) and frozen at -80 °C. After 1 h cells were thawed at room temperature and 20 µL cell lysate was mixed with 50 µL Luciferase-6-reagent (Promega). Luminescence was measured with a Turner Designs TD20/20 luminometer. Results are expressed as relative light units (RLU).

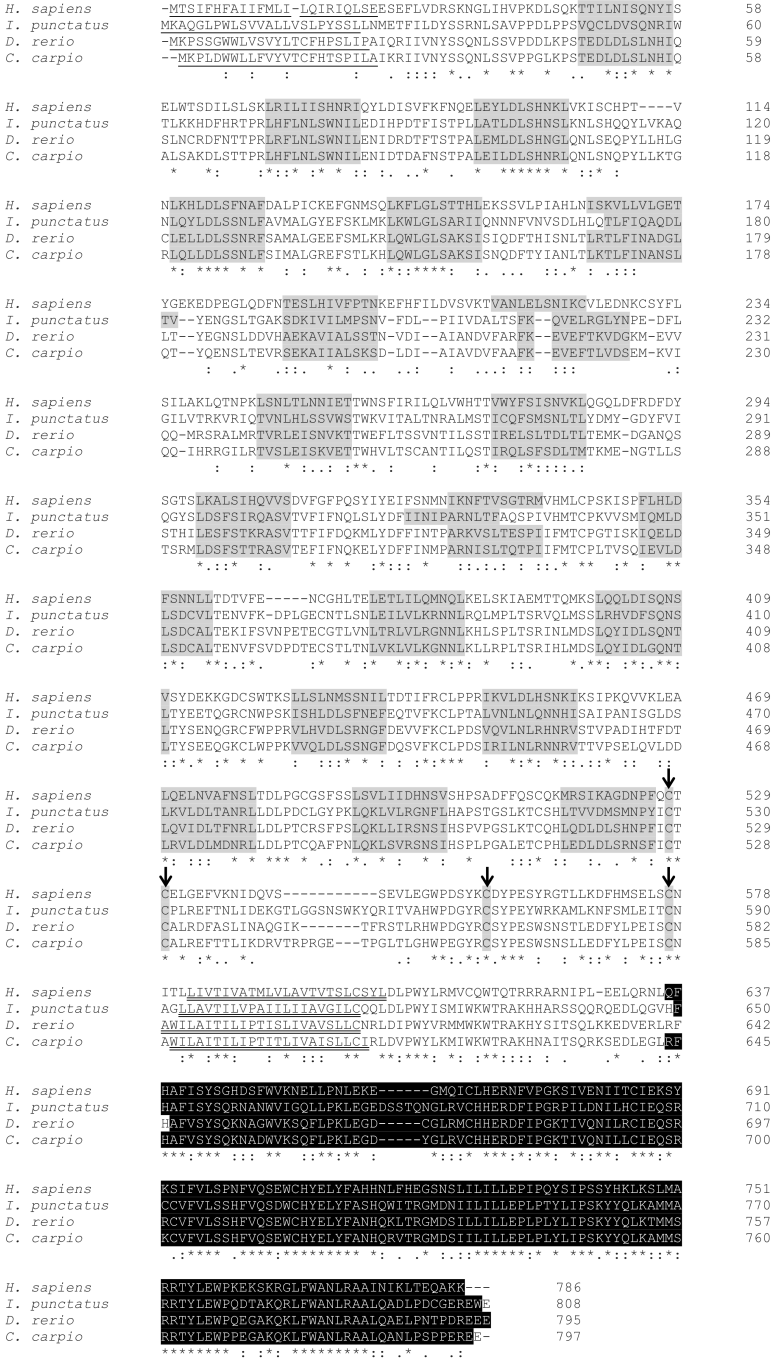
Results

Identification and characterization of carp *tlr1* and *tlr2* genes

Information on *tlr1* and *tlr2* genes is available for some fish species but *tlr1* orthologues had not yet been identified in carp. In the process of identifying new genes in carp, it is often useful to compare with the closely related zebrafish, a species which has a very well annotated genome. Usually, the presence of two genes in carp versus a single gene in the genome of zebrafish is expected based on an additional whole genome duplication (WGD) event that has taken place in carp (Henkel et al., 2012). Thus, although we previously reported on the presence of a *tlr2* orthologue in carp, given the additional WGD event in the carp lineage, a second copy of the *tlr2* gene was expected.

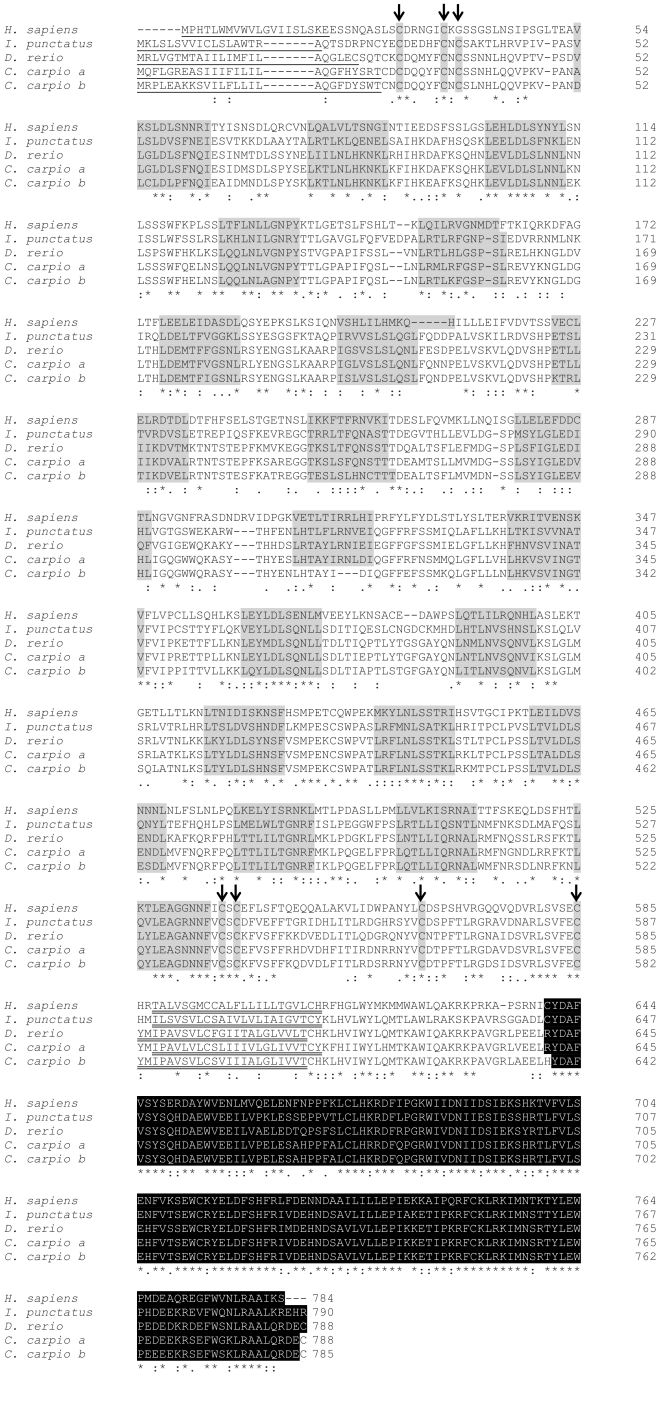
Making use of a conventional cloning approach combined with information from the recently annotated carp genome we identified a single complete *tlr1* gene (Figure 1) and an additional, but truncated, *tlr1* sequence in the carp genome (not shown). The full-length carp *tlr1* is composed of a single exon, similar to channel catfish, Tetraodon and fugu *tlr1* genes. Human and mouse *TLR1* are composed of four exons, but the entire coding region is contained in a single exon. Also for zebrafish, *tlr1* is divided over multiple (two) exons with the coding region contained in a single exon. The full-length coding sequence of carp *tlr1* is 2394 bp and translates into a Tlr1 protein of 797 aa with predicted molecular weight of 91 kDa. Tlr1 has 20 leucine-rich repeats (LRRs) and a C-terminal LRR (LRRCT), a transmembrane region and a highly conserved Toll/IL-1R (TIR) domain. Similar to other Tlr1 molecules (Quiniou et al., 2013), there is no N-terminal LRR (LRRNT).

▸ **Figure 1**. Alignment of Tlr1 protein sequences. The sequences from human, channel catfish, zebrafish and carp were aligned to show the conserved features and domains. The signal peptide is underlined, LRRs are highlighted in grey, transmembrane domain is indicated by a double underline, and the TIR domain is highlighted in black. Furthermore, LRRCT cysteines are indicated with arrows.


H. sapiens -MTSIFHFAIIFMLI-LQIRIQLSEESEFLVDRSKNGLIHVPKDLSQKTTILNISQNYIS 58
I. punctatus MKAQGLPWLSVVALLVSLPYSSLLNMETFILDYSSRNLSAVPPDLPPSVQCLDVSQNRIW 60
D. rerio -MKPSSGWWLVSVYLTCFHPSLIPAIQRIIVNYSSQNLSSVPDDLKPSTEDLDLSLNHIQ 59
C. carpio --MKPLDWWLLFVYVTCFHTSPILAIKRIIVNYSSQNLSSVPPGLKPSTEDLDLSLNHIQ 58
H. sapiens ELWTSDILSLSKLRILIISHNRIQYLDISVFKFNQELEYLDLSHNKLVKISCHPT----V 114
I. punctatus TLKKHDFHRTPRLHFLNLSWNILEDIHPDTFISTPLLATLDLSHNSLKNLSHQQYLVKAQ 120
D. rerio SLNCRDFNTTPRLRFLNLSWNILENIDRDTFTSTPALEMLDLSHNGLQNLSEQPYLLHLG 119
C. carpio ALSAKDLSTTPRLHFLNLSWNILENIDTDAFNSTPALEILDLSHNRLQNLSNQPYLLKTG 118
H. sapiens NLKHLDLSFNAFDALPICKEFGNMSQLKFLGLSTTHLEKSSVLPIAHLNISKVLLVLGET 174
I. punctatus NLQYLDLSSNLFAVMALGYEFSKLMKLKWLGLSARIIQNNNFVNVSDLHLQTLFIQAQDL 180
D. rerio CLELLDLSSNRFSAMALGEEFSMLKRLQWLGLSAKSISIQDFTHISNLTLRTLFINADGL 179
C. carpio RLQLLDLSSNLFSIMALGREFSTLKHLQWLGLSAKSISNQDFTYIANLTLKTLFINANSL 178
H. sapiens YGEKEDPEGLQDFNTESLHIVFPTNKEFHFILDVSVKTVANLELSNIKCVLEDNKCSYFL 234
I. punctatus TV--YENGSLTGAKSDKIVILMPSNV-FDL--PIIVDALTSFK--QVELRGLYNPE-DFL 232
D. rerio LT--YEGNSLDDVHAEKAVIALSSTN-VDI--AIANDVFARFK--EVEFTKVDGKM-EVV 231
C. carpio QT--YQENSLTEVRSEKAIIALSKSD-LDI--AIAVDVFAAFK--EVEFTLVDSEM-KVI 230
H. sapiens SILAKLQTNPKLSNLTLNNIETTWNSFIRILQLVWHTTVWYFSISNVKLQGQLDFRDFDY 294
I. punctatus GILVTRKVRIQTVNLHLSSVWSTWKVITALTNRALMSTICQFSMSNLTLYDMY-GDYFVI 291
D. rerio QQ-MRSRALMRTVRLEISNVKTTWEFLTSSVNTILSSTIRELSLTDLTLTEMK-DGANQS 289
C. carpio QQ-IHRRGILRTVSLEISKVETTWHVLTSCANTILQSTIRQLSFSDLTMTKME-NGTLLS 288
H. sapiens SGTSLKALSIHQVVSDVFGFPQSYIYEIFSNMNIKNFTVSGTRMVHMLCPSKISPFLHLD 354
I. punctatus QGYSLDSFSIRQASVTVFIFNQLSLYDFIINIPARNLTFAQSPIVHMTCPKVVSMIQMLD 351
D. rerio STHILESFSTKRASVTTFIFDQKMLYDFFINTPARKVSLTESPIIFMTCPGTISKIQELD 349
C. carpio TSRMLDSFSTTRASVTEFIFNQKELYDFFINMPARNISLTQTPIIFMTCPLTVSQIEVLD 348
H. sapiens FSNNLLTDTVFE-----NCGHLTELETLILQMNQLKELSKIAEMTTQMKSLQQLDISQNS 409
I. punctatus LSDCVLTENVFK-DPLGECNTLSNLEILVLKRNNLRQLMPLTSRVQLMSSLRHVDFSQNS 410
D. rerio LSDCALTEKIFSVNPETECGTLVNLTRLVLRGNNLKHLSPLTSRINLMDSLQYIDLSQNT 409
C. carpio LSDCALTENVFSVDPDTECSTLTNLVKLVLKGNNLKLLRPLTSRIHLMDSLQYIDLGQNT 408
H. sapiens VSYDEKKGDCSWTKSLLSLNMSSNILTDTIFRCLPPRIKVLDLHSNKIKSIPKQVVKLEA 469
I. punctatus LTYEETQGRCNWPSKISHLDLSFNEFEQTVFKCLPTALVNLNLQNNHISAIPANISGLDS 470
D. rerio LTYSENQGRCFWPPRVLHVDLSRNGFDEVVFKCLPDSVQVLNLRHNRVSTVPADIHTFDT 469
C. carpio LTYSEEQGKCLWPPKVVQLDLSSNGFDQSVFKCLPDSIRILNLRNNRVTTVPSELQVLDD 468
H. sapiens LQELNVAFNSLTDLPGCGSFSSLSVLIIDHNSVSHPSADFFQSCQKMRSIKAGDNPFQCT 529
I. punctatus LKVLDLTANRLLDLPDCLGYPKLQKLVLRGNFLHAPSTGSLKTCSHLTVVDMSMNPYICT 530
D. rerio LQVIDLTFNRLLDLPTCRSFPSLQKLLIRSNSIHSPVPGSLKTCQHLQDLDLSHNPFICT 529
C. carpio LRVLDLMDNRLLDLPTCQAFPNLQKLSVRSNSIHSPLPGALETCPHLEDLDLSRNSFICT 528
H. sapiens CELGEFVKNIDQVS-----------SEVLEGWPDSYKCDYPESYRGTLLKDFHMSELSCN 578
I. punctatus CPLREFTNLIDEKGTLGGSNSWKYQRITVAHWPDGYRCSYPEYWRKAMLKNFSMLEITCN 590
D. rerio CALRDFASLINAQGIK-------TFRSTLRHWPDGYRCSYPESWSNSTLEDFYLPEISCN 582
C. carpio CALREFTTLIKDRVTRPRGE---TPGLTLGHWPEGYRCSYPESWSNSLLEDFYLPEISCN 585
H. sapiens ITLLIVTIVATMLVLAVTVTSLCSYLDLPWYLRMVCQWTQTRRRARNIPL-EELQRNLQF 637
I. punctatus AGLLAVTILVPAIILIIAVGILCQQLDLPWYISMIWKWTRAKHHARSSQQRQEDLQGVHF 650
D. rerio AWILAITILIPTISLIVAVSLLCNRLDIPWYVRMMWKWTRAKHYSITSQLKKEDVERLRF 642
C. carpio AWILAITILIPTITLIVAISLLCIRLDVPWYLKMIWKWTRAKHNAITSQRKSEDLEGLRF 645
H. sapiens HAFISYSGHDSFWVKNELLPNLEKE------GMQICLHERNFVPGKSIVENIITCIEKSY 691
I. punctatus HAFISYSQRNANWVIGQLLPKLEGEDSSTQNGLRVCHHERDFIPGRPILDNILHCIEQSR 710
D. rerio HAFVSYSQKNAGWVKSQFLPKLEGD-----CGLRMCHHERDFIPGKTIVQNILRCIEQSR 697
C. carpio HAFVSYSQKNADWVKSQFLPKLEGD-----YGLRVCHHERDFIPGKTIVQNILLCIEQSR 700
H. sapiens KSIFVLSPNFVQSEWCHYELYFAHHNLFHEGSNSLILILLEPIPQYSIPSSYHKLKSLMA 751
I. punctatus CCVFVLSSHFVQSDWCHYELYFASHQWITRGMDNIILILLEPLPTYLIPSKYYQLKAMMA 770
D. rerio RCVFVLSSHFVQSEWCHYELYFANHQKLTRGMDSILLILLEPLPLYLIPSKYYQLKTMMS 757
C. carpio KCVFVLSSHFVQSEWCHYELYFANHQRVTRGMDSILILLEPLPLYLIPSKYYQLKAMMS 760
H. sapiens RRTYLEWPKEKSKRGLFWANLRAAINIKLTEQAKK--- 786
I. punctatus RRTYLEWPQDTAKQRLFWANLRAALQADLPDCGEREWE 808
D. rerio RRTYLEWPQEGAKQKLFWANLRAALQAELPNTPDREEE 795
C. carpio RRTYLEWPPEGAKQKLFWANLRAALQANLPSPPEREE- 797

We previously described a carp *tlr2* gene (Ribeiro et al., 2010a). Upon further investigation of the carp genome we now identified an additional *tlr2* gene in the carp genome (Figure 2). Both carp *tlr2* genes are composed of a single exon, similar to the human, zebrafish and channel catfish *tlr2* genes, whereas in some other fish species *tlr2* genes are encoded by multiple exons, e.g. Tetraodon and fugu *tlr2* each are comprised of 11 exons, confirming that the distribution and number of introns/exons among TLRs do not seem conserved across species (Quiniou et al., 2013). The full-length sequences of carp *tlr2a* and *tlr2b* are 2367 and 2358 bp encoding for Tlr2 proteins of 788 and 785 aa with predicted molecular weights of 91 and 90 kDa, respectively. They share 88% amino acid identity. Both Tlr2 molecules have 20 LRRs, and in addition an N-terminal (LRRNT) and a C-terminal LRR (LRRCT), a transmembrane region and a highly conserved TIR domain.

▸ **Figure 2**. Alignment of Tlr2 protein sequences. The sequences from human, channel catfish and zebrafish were aligned with both carp Tlr2 sequences to show the conserved features and domains. The signal peptide is underlined, LRRs are highlighted in grey, transmembrane domain is indicated by a double underline, and the TIR domain is highlighted in black. Furthermore, LRRNT and LRRCT cysteines are indicated with arrows.


H. sapiens
I. punctatus
D. rerio
C. carpio a
C. carpio b

Phylogenetic analyses on amino acid sequences of multiple Tlr1 and Tlr2 sequences (Figure 3), using carp Tlr3 as an outgroup to root the phylogenetic tree, showed an overall topology indicating clusters of Tlr sequences consistent with evolutionary distance between different fish families. Sequences for both Tlr1 and Tlr2, from Cyprinids (carp, zebrafish) and Siluriforms (catfish), which are their closest living relatives, clustered together with very high bootstrap values, away from other fish species belonging to the Salmonids, Tetraodontiforms and Perciforms. All fish Tlr1 as well as Tlr2 sequences form common clusters separate from mammalian TLR1 and TLR2 sequences.

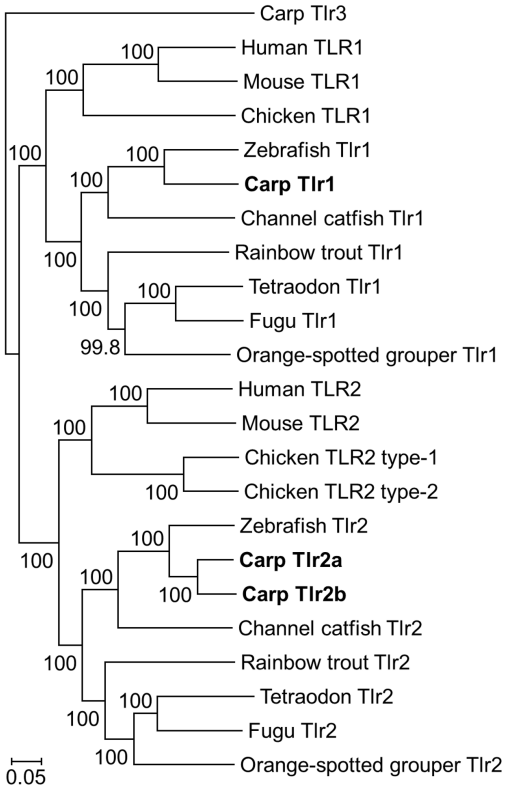


Figure 3. Phylogenetic tree of Tlr1 and Tlr2 protein sequences. The phylogenetic tree shows the evolutionary relationship between Tlr1 and Tlr2 proteins of various species. All branches have high bootstrap values. The sequence of carp Tlr3 was used as an outgroup to root the tree. Amino acid sequences used for Tlr1: Human (*Homo sapiens* NP_003254.2), mouse (*Mus musculus* NP_109607.1), chicken (*Gallus gallus* NP_001007489.4), zebrafish (*Danio rerio* AAI63271.1), carp (*Cyprinus carpio* cypCar_00044472), channel catfish (*Ictalurus punctatus* AEI59662.1), Tetraodon (*Tetraodon nigroviridis* ABO15772.1), fugu (*Takifugu rubripes* XP_003970412.2), orange-spotted grouper

(*Epinephelus coioides* AEB32452.1), rainbow trout (*Oncorhynchus mykiss* ACV92064.1). Amino acid sequences used for Tlr2: Human (*Homo sapiens* AAH33756.1), mouse (*Mus musculus* NP_036035.3), chicken type 1 (*Gallus gallus* NP_989609.1), chicken type 2 (*Gallus gallus* NP_001155122.1), zebrafish (*Danio rerio* NP_997977.1), carp a (*Cyprinus carpio* cypCar_00005269), carp b (*Cyprinus carpio* cypCar_00039549), channel catfish (*Ictalurus punctatus* AEI59663.1), Tetraodon (*Tetraodon nigroviridis* ENSTNIP00000005681), fugu (*Takifugu rubripes* XP_003976919.1), orange-spotted grouper (*Epinephelus coioides* AEB32453.1), rainbow trout (*Oncorhynchus mykiss* CCK73195.1). Amino acid sequence of carp Tlr3 (*Cyprinus carpio* AHE74141.1).

In the human genome, *TLR1*, *TLR6* and *TLR10* are organised as a conserved scaffold of genes. Clearly, in all fish genomes investigated so far, both *tlr6* and *tlr10* are missing, indicating a recent duplication of *TLR1* in the mammalian but not the teleost lineage. Conservation of synteny of teleost *tlr1* and *tlr2* was investigated by comparing the genomic regions immediately up- and down-stream of human *TLR1* (Figure 4) and human *TLR2* (Figure 5), with the genomic regions up- and down-stream of the corresponding homologue in the annotated genome of several teleost fish species, including zebrafish and common carp. For *tlr1*, the genomes of fish appear to have in common a block of 10 genes fairly conserved in the investigated fish species. For carp, although the scaffolds are limited in length, the analysis does confirm conservation of synteny with *ints10* downstream of *tlr1*, and *klb* upstream of the putative *tlr1* pseudogene. The fact that the carp *tlr1* pseudogene shows conservation of synteny, supports the whole genome duplication event from zebrafish to common carp, with loss of function associated with the second *tlr1* gene in carp. For *TLR2*, in the human genome a block of 8 genes fairly conserved in the investigated fish species could be identified. However, there is a gap in zebrafish between the *tlr2* region and the *rnf175/trim2* gene cluster further downstream. For carp, although the scaffolds are limited in length, the analysis does confirm conservation of synteny with zebrafish.

Altogether, sequence, phylogenetic and synteny analysis of carp *tlr1* and *tlr2* support that these genes are indeed orthologues of mammalian *TLR1* and *TLR2*.

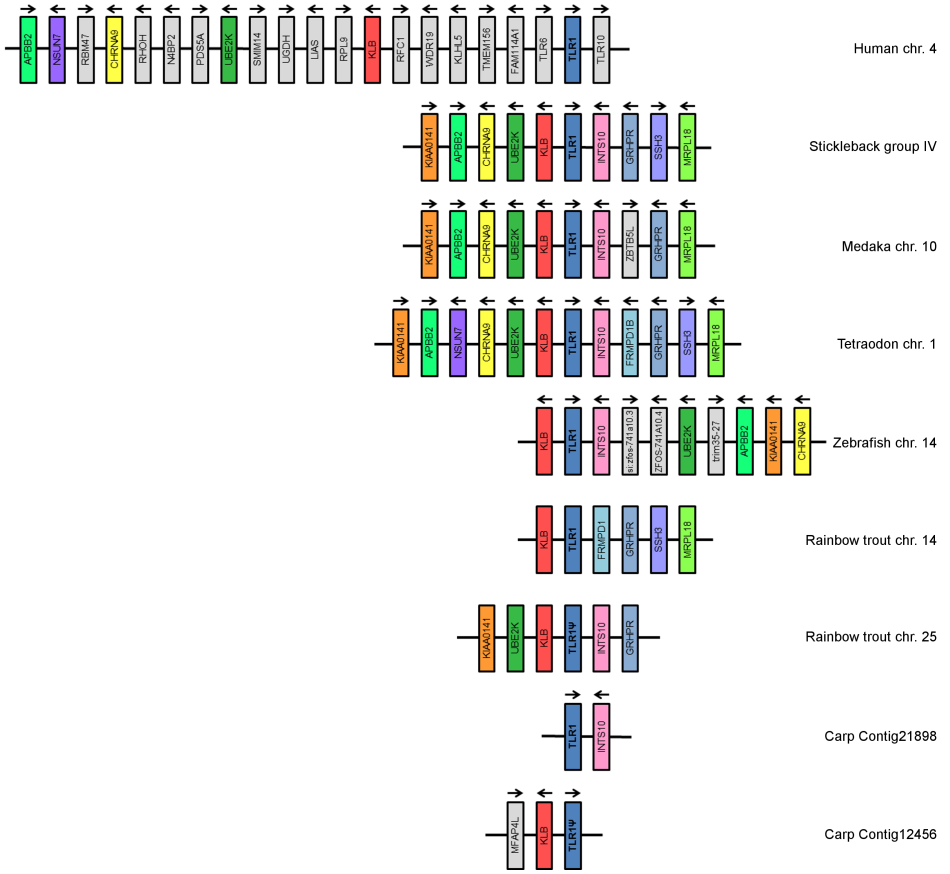


Figure 4. Synteny analysis of *tlr1*. Comparative gene organization map of the regions where human, stickleback, medaka, Tetraodon, zebrafish, rainbow trout and carp *tlr1* genes are found. Orthologous genes are indicated by the same colour, except unique genes which are all light grey. Gene direction is indicated with arrows. Rainbow trout and carp have a pseudogene (ψ) in addition to the full-length *tlr1* gene. The human genome assembly version GRCh38.p3, stickleback genome assembly BROAD S1, medaka genome assembly HdrR, Tetraodon genome assembly TETRAODON 8.0, zebrafish genome assembly GRCz10, and the carp genome (Henkel et al., 2012) were used for this analysis. Information on rainbow trout genomic organization is from Palti et al., 2010.

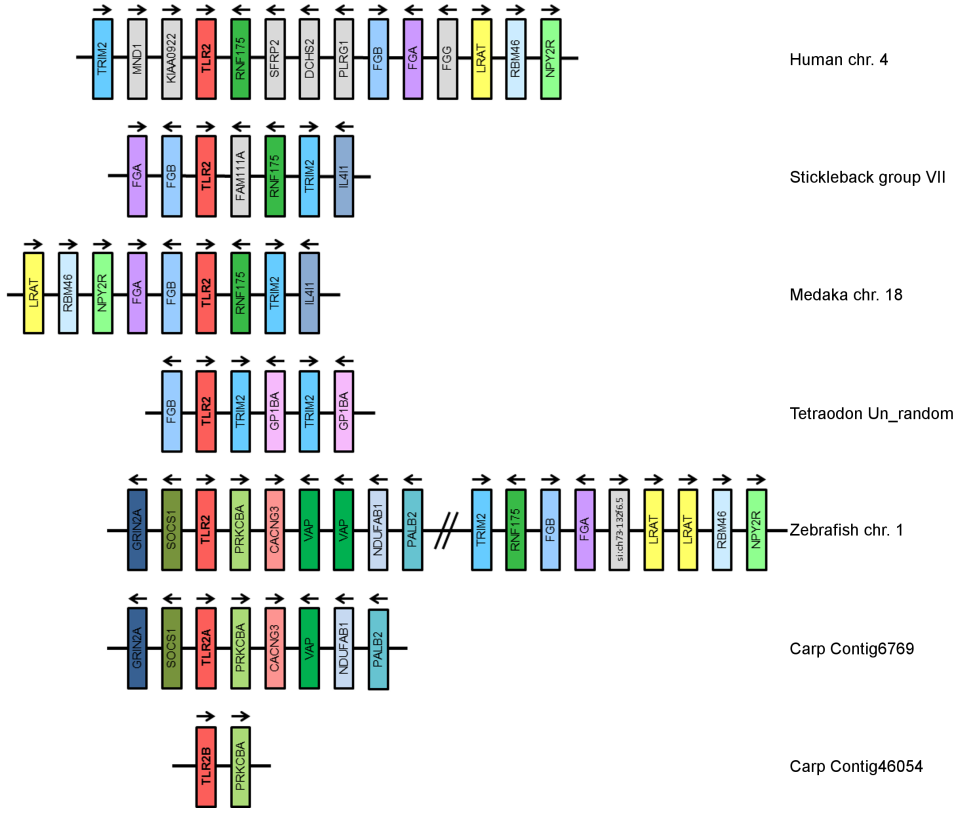


Figure 5. Synteny analysis of *tlr2*. Comparative gene organization map of the regions where human, stickleback, medaka, Tetraodon, zebrafish and carp *tlr2* genes are found. Orthologous genes are indicated by the same colour, except unique genes which are all light grey. Gene direction is indicated with arrows. The human genome assembly version GRCh38.p3, stickleback genome assembly BROAD S1, medaka genome assembly HdrR, Tetraodon genome assembly TETRAODON 8.0, zebrafish genome assembly GRCz10, and the carp genome (Henkel et al., 2012) were used for this analysis.

Differential expression of *tlr1* and *tlr2* genes

To investigate the relative gene expression of the newly identified carp *tlr1* and to determine whether the newly identified *tlr2b* would present an expression pattern similar to the previously reported *tlr2a* sequence, we measured the constitutive gene expression of *tlr1*, *tlr2a* and *tlr2b*, in organs of four healthy carp and in sorted leukocytes. The results from 15 organs are shown in Figure 6A. Significant differences were found between organs for all *tlrs* (one-way ANOVA, $P < 0.05$). Typically, highest *tlr* gene expression was found in head

kidney and mid kidney, peripheral blood leukocytes, spleen and gut. The relatively high gene expression in immune organs, and low to non-detectable expression in other tissues was confirmed by transcriptome analysis of the same organs from carp (unpublished data).

Detection of *tlr1*- and *tlr2*-specific gene expression in various immune cell types from carp (Figure 6B), showed significant differences between cell types (one-way ANOVA, $P < 0.05$). In general, among immune cell types B cells, in particular, express high levels of *tlr* genes. Of interest, granulocytes express *tlr1*>*tlr2*, whereas head kidney-derived macrophages express *tlr2a*>*tlr1*, suggesting high expression of *tlr1* and *tlr2* is not always restricted to the same immune cell type.

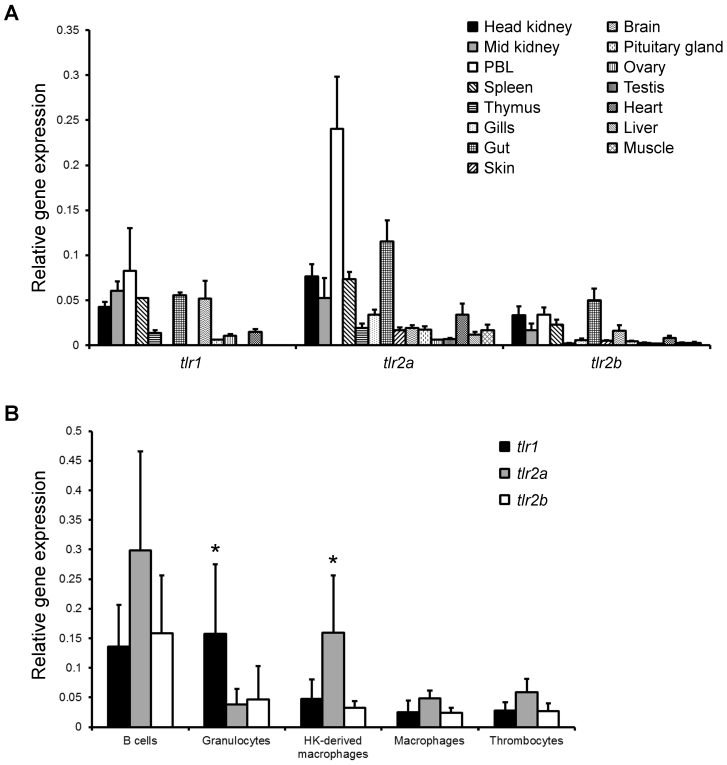


Figure 6. Basal gene expression levels of *tlr1*, *tlr2a* and *tlr2b* in carp organs and immune cell types. Real-time quantitative PCR was performed using primers specific for carp *tlr1*, *tlr2a* and *tlr2b* on cDNA from (A) organs or (B) immune cell types isolated from healthy fish. Gene expression is normalized to the expression of the *40s ribosomal protein s11* housekeeping gene. Bars indicate average gene expression of $n = 4$ adult fish and error bars indicate standard deviations. Asterisks denote significant differences in expression between *tlrs* within a cell type, $P < 0.05$. PBL = peripheral blood leukocytes. HK = head kidney.

Structural model of potential Tlr1/Tlr2 heterodimer

Mammalian TLR1 and TLR2 have been shown to heterodimerize and the crystal structure of the extracellular portion of human TLR1-TLR2 heterodimer with Pam_3CSK_4 as a ligand is known (PDB-id: 2z7x). Using the known human model, we set to investigate whether the identified carp Tlr1 and Tlr2 could potentially interact. In the human heterodimeric complex of TLR1-TLR2, the receptor multimer is arranged in a symmetrical manner. Three-dimensional modelling shows the typical horseshoe shape of each TLR with a well-positioned pocket to accommodate the tri-acylated lipopeptide ligand. Having noted that the number of LRRs is conserved between the human and carp molecules it was considered appropriate to model the carp Tlrs on their human counterparts. Thus, carp Tlr1 was modelled together with either carp Tlr2a (Figure 7) or Tlr2b (data not shown). Both Tlr1-Tlr2 combinations exhibited a good three-dimensional fit with the heterodimer structure of human TLR1-TLR2, including the potential to bind to Pam_3CSK_4. This three-dimensional fit supports the possible formation of carp Tlr1-Tlr2 heterodimers.

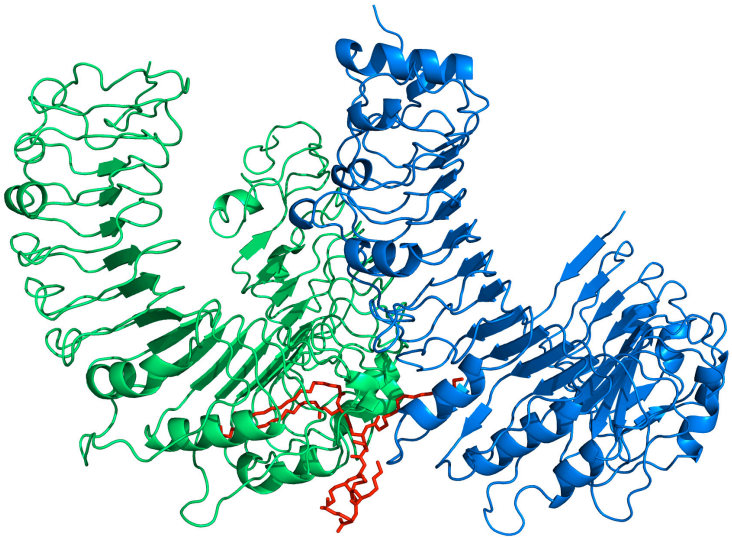
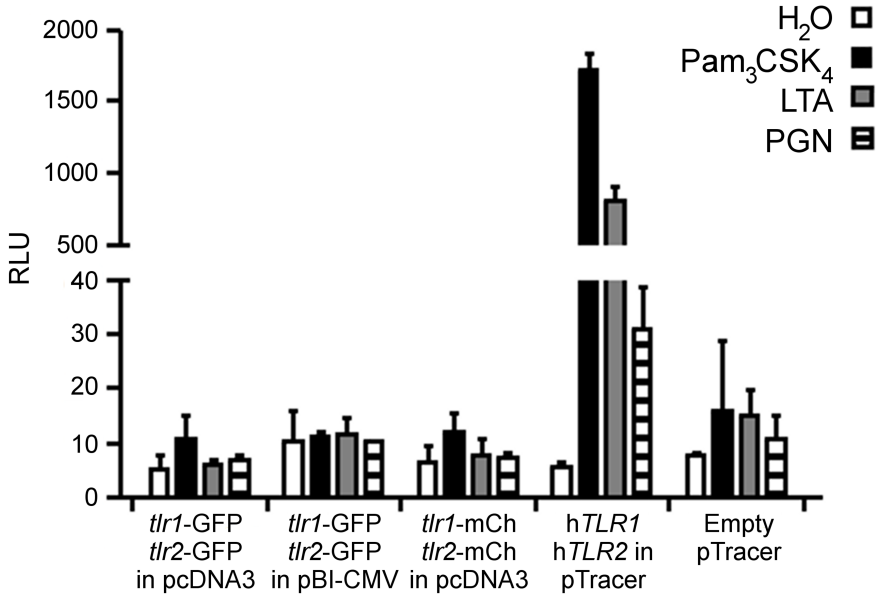


Figure 7. Heterodimer of carp Tlr1-Tlr2(a), modelled on human TLR1-TLR2 with Pam_3CSK_4 as ligand. The extracellular part of the human TLR1-TLR2 heterodimer, crystallized with Pam_3CSK_4 as a ligand (PDB-id: 2z7x) was used to create models of carp Tlr1 and Tlr2a using the Modeller program (version 9.12). Thirty comparative models were generated, after which the model with lowest corresponding DOPE score was selected for image generation. Carp Tlr1 is shown in green, Tlr2a in blue, Pam_3CSK_4 in red.

Ligand stimulation of Tlr1 and Tlr2 does not lead to NF-κB activation

To study the biological activity and ligand binding properties of possible Tlr1-Tlr2 heterodimers we first created human HEK and cyprinid EPC cells stably transfected with a NF-κB luciferase reporter (Pietretti et al., 2014). Both cell lines were subsequently transiently transfected with carp Tlr1 and Tlr2a, individually and combined. Each Tlr was cloned as a fusion to GFP/mCherry to monitor transfection efficiency. Stimulation with classical Tlr1-Tlr2 ligands including Pam_3CSK_4, LTA and PGN, however, did not lead to luciferase reporter activity (data not shown). Alternatively, we used HeLa-57A cells transfected with various *tlr1/tlr2* expression plasmids, which however gave a similar outcome (Figure 8): none of the tested ligands led to a significant increase in NF-κB activation via overexpressed carp Tlr1-Tlr2. To rule out any inhibitory effect of fusing GFP to the C-terminal end of the Tlr molecules, we also used pBI-CMV1 constructs in which GFP was placed in a cloning site separate from the Tlr. This still allowed for detection of transfection levels by GFP, whilst leaving the structure and function of the Tlr unaffected, but did not alter the negative outcome. Furthermore, in order to rule out an inhibitory effect of GFP itself (whether fused or not to the Tlrs), mCherry constructs were used instead of GFP. Again, no increase in NF-κB activity after ligand stimulation could be measured. Since the positive control; HeLa-57A cells transfected with human TLR1 and TLR2, did lead to high responses to Pam_3CSK_4, LTA and PGN (see Figure 8), we conclude that both the reporter system itself and the ligands used, were functional.



◂ **Figure 8**. Ligand stimulation of carp Tlr1 and Tlr2 in HeLa-57A cells does not lead to activation of NF-κB. HeLa-57A cells stably transfected with NF-κB luciferase reporter were transfected with carp or human TLR plasmids as indicated. Constructs in pcDNA3 encode for a fusion product of the Tlr and the fluorescent protein, whereas constructs in pBI-CMV express the two proteins separately. Cells were stimulated for 5 h with ligands Pam_3CSK_4 (100 ng/mL), LTA (1 µg/mL) or PGN (10 µg/mL), or water was added as negative control. Relative light units (RLU) are a measure of NF-κB activity. Bars indicate mean of technical duplicates and error bars indicate standard deviation.

Carp Tlr1 and Tlr2 are expressed and co-localize

Since we could not confirm NF-κB activation by over-expressed carp Tlr1 and Tlr2 stimulated with ligands, we next investigated whether this could be due to erroneous expression of these molecules. In a previous study, carp Tlr2 was successfully transfected into human HEK cells, so we continued with first transfecting this cell line with constructs expressing each carp Tlr fused to the fluorescent protein mCherry. The cell membrane was visualized by staining with wheat germ agglutinin-Alexa 488 conjugate which exhibits green fluorescence. Confocal microscopy confirmed expression of Tlr1, and Tlr2a, at least in the cytoplasm of the cells mostly concentrated in discrete speckles (Figure 9). For comparison, mCherry protein alone was expressed without being fused to a Tlr and red fluorescence was evenly distributed throughout the cells, indicating that the staining pattern observed when fused to a Tlr is determined by the Tlr itself. Upon further examination of subcellular co-localization of Tlr1 and Tlr2, using carp Tlr1 fused to GFP and carp Tlr2a fused to mCherry, it became clear that both carp Tlr1 and Tlr2a co-localized in the cytoplasm of HEK cells (Figure 10). To confirm the suspected cytoplasmic localization, carp Tlr1 fused to GFP was detected using anti-FLAG-specific antibody. Only in permeabilized cells, carp Tlr1 could be detected with antibody staining, confirming that Tlr1 was not expressed on the surface of HEK cells overexpressing Tlr1 (Figure 11). To exclude that the observed expression pattern was not an artefact due to the use of mammalian cells grown at 37 °C, we performed the same experiment using various fish cell lines grown at lower temperature. Similar results were obtained with EPC and CLC cells transfected with the same Tlr constructs (data not shown). Altogether, these data demonstrate that carp Tlr1 and Tlr2 can be both successfully (over)expressed *in vitro* in homologous and heterologous cell lines, and they appear to be present in the same subcellular compartments of these cells.

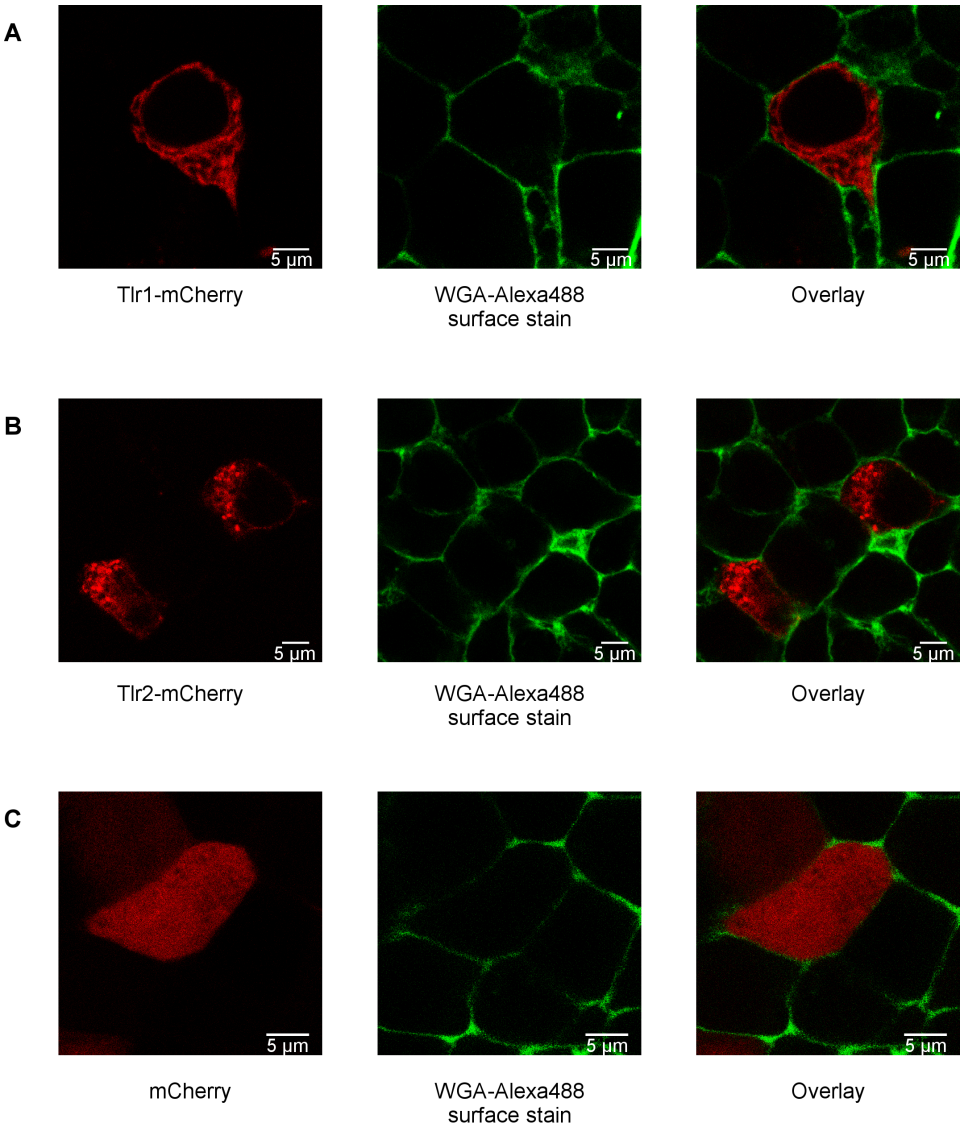


Figure 9. Carp Tlr1 and Tlr2 localize intracellularly in HEK cells. HEK cells were transfected with (A) *tlr1*-mCherry-pcDNA3, (B) *tlr2*-mCherry-pcDNA3, or (C) mCherry-pcDNA3 (red) and analysed after 3 days by confocal laser scanning microscopy. Wheat-germ agglutinin conjugated to Alexa488 (green) was used to stain the cell surface.

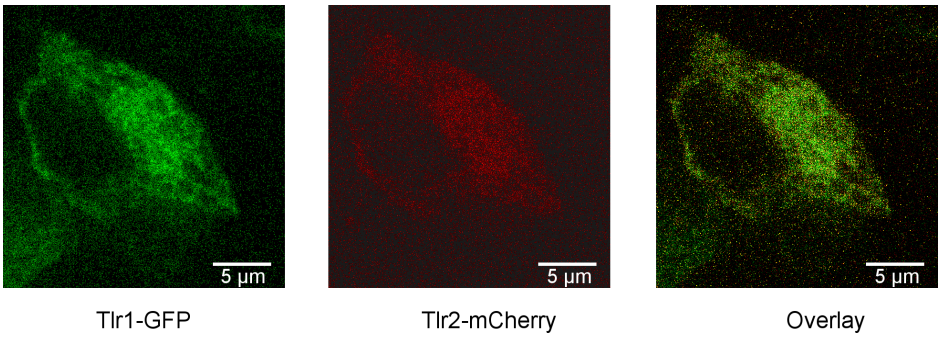


Figure 10. Carp Tlr1 and Tlr2 co-localize intracellularly in HEK cells. HEK cells were co-transfected with *tlr1*-GFP-pcDNA3 (green) and *tlr2*-mCherry-pcDNA3 (red) and analysed after 3 days by confocal laser scanning microscopy. The overlay shows extensive co-localization of the green and red signal.

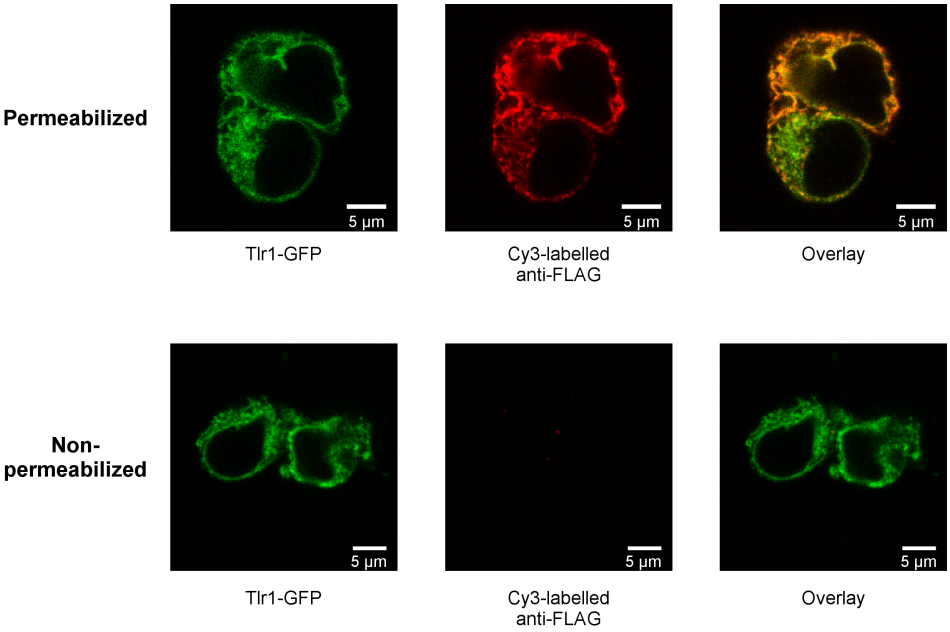


Figure 11. Carp Tlr1 is not expressed on the membrane of HEK cells. HEK cells were transfected with FLAG-tagged Tlr1-GFP (*tlr1*-GFP-pcDNA3) (green) and analysed after 3 days by confocal laser scanning microscopy. Antibody staining was performed with Cy3-labelled anti-FLAG antibody (red) in permeabilized (top row) and non-permeabilized cells (bottom row).

Discussion

Ever since TLRs were first described as important pattern recognition receptors for mice and humans, they have been studied extensively in an ever increasing number of animal species, including invertebrates such as sea urchin, where a great expansion of *tlr* genes has occurred (Hibino et al., 2006). The abundance of Tlrs in sea urchin suggests this class of receptors plays an important role in the innate immune defence also in lower animals. Often, the conserved nature of Tlrs is emphasized, with particular emphasis on the extracellular leucine rich repeat regions and the intracellular TIR domain. In this study, we present the molecular characterization of *tlr1* and *tlr2* from common carp and discuss our attempts to determine the ligand-binding properties of putative Tlr1/Tlr2 heterodimers.

Sometimes, after a WGD, gene duplication allows for partitioning of function or acquiring of new function, whereas in other cases the duplicated gene can lose its function. A single full-length carp *tlr1* gene could be identified; a second *tlr1* hit was truncated with a partial TIR domain only. Many of the orthologous relationships of TLRs can be confirmed by observations of conserved synteny, i.e. preservation between species of the order and orientation of orthologous genes (Roach et al., 2005). Based on the conserved order of the *klb* gene neighbouring the second *tlr1* hit we consider the truncated *tlr1* a true orthologue of *TLR1*, but one which has lost its function. In rainbow trout a similar situation exists; a single full-length *tlr1* gene is present on chromosome 14 and a *tlr1* pseudogene is located on chromosome 25 (Palti et al., 2010). Thus, it appears that only a single full-length *tlr1* gene remained in the genomes of rainbow trout and common carp. In contrast, two full-length genes for carp *tlr2* could be identified, both of which give rise to detectable transcripts. Also rainbow trout express two *tlr2* genes, one of which is contained within a single exon but the other segmented into multiple exons (Brietzke et al., 2016). Of interest, a similar situation has recently been reported for yellow croaker (Ao et al., 2016), suggesting more fish species might have *tlr2* genes with quite different intron-exon organisations. Zebrafish *tlr2* and also channel catfish (Quiniou et al., 2013), similar to human *TLR2*, is intronless. Likewise, we have not found evidence for the presence of a segmented *tlr2* gene in carp.

High expression of *tlr1* and both *tlr2* genes was found in immune organs such as head kidney and mid kidney, peripheral blood leukocytes, spleen and gut. Of the two *tlr2* genes present in carp, *tlr2a* was always higher expressed than *tlr2b* in all the investigated organs and cell types. Leukocytes of both myeloid and lymphoid origin expressed *tlr1* and *tlr2*; B cells, granulocytes, and head kidney-derived macrophages had the highest expression levels. Also rainbow trout *tlr2* is expressed in mononuclear cells/macrophages at levels similar to that in B cells (Brietzke et al., 2016), *tlr1* gene expression was not studied in rainbow trout. In mammals, B cells as well as cells of myeloid origin express

TLRs (Bekeredjian-Ding and Jego, 2009; Hua and Hou, 2013), suggesting a conserved expression pattern for carp *tlr1* and *tlr2* on phagocytes and B cells.

Our three-dimensional modelling of the carp Tlr1 and Tlr2 proteins suggests the possibility of formation of a heterodimer, with a pocket that can accommodate the tri-acylated lipopeptide ligand Pam_3CSK_4, similar to the heterodimer structure of human TLR1-TLR2 on which the model was built. Both Tlr2 proteins of carp appear structurally capable of heterodimer formation with carp Tlr1. The *in silico* modelling supports the potential formation of heterodimers of carp Tlr1-Tlr2 *in vitro* or *in vivo*. Indeed, in cell lines of human as well as fish origin overexpressing both proteins, confocal microscopy confirmed subcellular co-localization of carp Tlr1 and Tlr2. Further experimental evidence, such as co-immunoprecipitation or fluorescence resonance energy transfer (FRET) (Pollok and Heim, 1999; Sekar and Periasamy, 2003), would be needed to unequivocally prove molecular interaction between the two Tlr proteins. The work by Sandor et al., 2003, demonstrates that human TLR1 and TLR2 co-localize in double-transfected (TLR1-YFP + TLR2-CFP) HEK cells in a ligand-independent manner, similar to our observations for carp Tlr1 and Tlr2. The pre-assembled human TLR1-TLR2 heterodimers are present inside as well as on the surface of cells.

Possibly because over-expression affects the natural molecular distribution, or because only minute and therefore difficult to detect amounts of Tlrs are required at the cell surface, we were unable to pinpoint sub-cellular localization to the cell surface. This may also be true for human TLR1 when over-expressed in HEK cells, because TLR1 is hard to detect at the cell membrane and is found mainly localized inside the cells with a diffuse pattern of distribution (Sandor et al., 2003). Further, the endogenous cytoplasmic pool of TLR1 in HEK cells can be detected in permeabilized cells only, and human TLR2 is not found exclusively at the cell membrane either (Sandor et al., 2003). Also chicken TLRs cannot easily be detected on the cell surface after transfection (unpublished data). Yet, both human and chicken TLRs, when over-expressed, can lead to NF-κB activation upon stimulation with appropriate ligands. These collective findings suggest that, although it is not easy to detect TLR molecules at the cell surface after transfection, intracellular localization of carp Tlr1 and Tlr2 may not necessarily explain the absence of NF-κB activation in our experiments.

In chicken, both TLR1 and TLR2 exist as duplicated copies; each pair of genes is found as tandem array genes, suggesting that they arose from local gene duplication events (Temperley et al., 2008), rather than from a whole-genome duplication, as is the case for carp Tlrs. Chicken TLR1 and TLR2 are not functional as homodimers whereas they do recognize bacterial lipoproteins (di- as well as tri-acylated) as heterodimers in certain

combinations (Keestra et al., 2007; Higuchi et al., 2008). Although the three-dimensional model of carp Tlr1-Tlr2 does not suggest this would be the case, it is difficult to fully exclude the possibility that Tlr2b rather than Tlr2a could be the natural partner of a heterodimeric Tlr1-Tlr2, an option we did not explore experimentally.

In mammals, TLR2 does not only form heterodimers with TLR1, but also with TLR6 (and TLR10 in humans). In fish genomes, *tlr6* or *tlr10* genes are absent but instead a number of fish-specific Tlrs have been recognized as members of the Tlr1 family. It is interesting to speculate that Tlr2 in fish might heterodimerize with some of these members, including Tlr14, Tlr18, Tlr25 and Tlr27 (Palti, 2011; Quiniou et al., 2013; Wang et al., 2015). So far we could not identify *tlr14*, *tlr25*, or *tlr27* in the common carp genome. However, a screen of the carp genome did confirm the presence of two *tlr18* genes (data not shown), as could be expected based on the presence of *tlr18* in the zebrafish genome (Meijer et al., 2004). Molecular and functional characterization of new, fish-specific, Tlr1 family members should provide insight in the number of different heterodimer combinations possible with Tlr2.

In previous work, we have described ligand-specific activation of carp Tlr2 via measurement of increased phosphorylation levels of the MAP kinase p38 in human HEK cells by Western blot (Ribeiro et al., 2010a). Instead of this semi-quantitative method, we now used a quantitative read-out system based on NF-κB activation and subsequent luminescence measurements. We could not confirm our initial ligand binding studies based on phosphorylation of p38, neither using the human HEK or HeLa-57A cell lines, nor using a cyprinid fish cell line (EPC), from fathead minnow (Winton et al., 2010). Of interest, the HeLa-57A cells have successfully been used to study ligand binding of several chicken TLRs (Keestra et al., 2007) and even reptilian TLR5 (Voogdt et al., 2016), confirming that human cells can be used to study ligand binding by Tlrs from non-mammalian species. There are several possible explanations for the inability of carp Trl1 and/or Tlr2 to induce activation in our NF-κB reporter assay; i) carp Tlrs may not have been functioning optimally because they did not display the correct sub-cellular localization on the cell surface, e.g. due to over-expression, ii) carp Tlrs were not properly folded e.g. due to high temperature conditions for human cells, although this would not be the case for the fish cell line grown at 27 °C, iii) the carp TIR domains may not have been able to interact with human MYD88 (at least in HEK and HeLa-57A cells), thus initiating no signalling cascade, iv) carp Tlrs may need a co-receptor or other co-factor (Pietretti et al., 2013), absent from the cell lines we tested, v) different ligands are recognized by the carp Tlrs, vi) the present NF-κB read-out system (although appropriate for mammalian, chicken and amphibian TLRs) is not suitable for studying Tlrs of fish. Indeed, ligand-dependent NF-κB activation mediated through fish-encoded Tlrs is not easily shown *in vitro* (as discussed

in Brietzke et al., 2016). We do believe that bacterial PAMPs including lipoproteins could be potential activators of Tlr signalling in carp, as several of the components of the signalling pathway including MYD88 and TRAF6 (Kongchum et al., 2011), IRAK1 (Shan et al., 2015), and several accessory molecules for Tlrs (Pietretti et al., 2013) are all present. Indeed, carp macrophages respond to classical TLR2 ligands such as Pam_3CSK_4 and MALP-2 by altering gene expression of pro-inflammatory cytokines (Ribeiro et al., 2010a). The latter study on carp Tlr2 using phosphorylation of p38 as a read-out is not in contradiction with the present study, the two studies rather suggest that several TLR signalling pathways and transcription factors should be scrutinized. Future studies could include measuring activation of transcription factors other than NF-κB, such as AP-1 or IRF5, measuring caspase activity, or measuring downstream effects such as the production of pro-inflammatory cytokines. In other words, given the present difficulties detecting NF-κB activation downstream of Tlr1-Tlr2 heterodimerization, it may be worthwhile in future work to refocus on signalling pathways other than NF-κB as important activators of inflammatory cytokines.

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Abbreviations

BSA, bovine serum albumin; GFP, green fluorescent protein; IRAK1, interleukin-1 receptor-associated kinase 1; LRR, leucine-rich repeat; LTA, lipoteichoic acid; MYD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa B; PAMP, pathogen-associated molecular pattern; Pam_3CSK_4, *N*-palmitoyl-*S*-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-(*R*)-Cys-(*S*)-Ser-(*S*)-Lys_4; PBL, peripheral blood leukocytes; PBS, phosphate buffered saline; PE, phycoerythrin; PGN, peptidoglycan; RLU, relative light units; RT-qPCR, real-time quantitative polymerase chain reaction; TIR, toll/interleukin-1 receptor; TLR, Toll-like receptor; TRAF6, TNF receptor-associated factor 6; UTR, untranslated region; WGD, whole genome duplication.

References

S. Akira, K. Takeda, Toll-like receptor signalling, Nat. Rev. Immunol. 4 (7) (2004) 499-511.

F.W. Allendorf, G.H. Thorgaard, Tetraploidy and the evolution of salmonid fishes, in: B.J. Turner (Ed.), Evolutionary Genetics of Fishes, Springer, 1984, pp. 1-53.

J. Ao, Y. Mu, K. Wang, M. Sun, X. Wang, X. Chen, Identification and characterization of a novel Toll-like receptor 2 homologue in the large yellow croaker *Larimichthys crocea*, Fish Shellfish Immunol. 48 (2016) 221-227.

P. Baoprasertkul, E. Peatman, J. Abernathy, Z. Liu, Structural characterisation and expression analysis of Toll-like receptor 2 gene from catfish, Fish Shellfish Immunol. 22 (4) (2007) 418-426.

I. Bekeredjian-Ding, G. Jego, Toll-like receptors - sentries in the B-cell response, Immunology 128 (3) (2009) 311-323.

J.K. Bell, G.E.D. Mullen, C.A. Leifer, A. Mazzoni, D.R. Davies, D.M. Segal, Leucine-rich repeats and pathogen recognition in Toll-like receptors, Trends Immunol. 24 (10) (2003) 528-533.

A. Brietzke, M. Arnemo, T. Gjoen, H. Rebl, T. Korytar, T. Goldammer, A. Rebl, H.M. Seyfert, Structurally diverse genes encode Tlr2 in rainbow trout: the conserved receptor cannot be stimulated by classical ligands to activate NF-κB in vitro, Dev. Comp. Immunol. 54 (1) (2016) 75-88.

L. David, S. Blum, M.W. Feldman, U. Lavi, J. Hillel, Recent duplication of the common carp (*Cyprinus carpio* L.) genome as revealed by analyses of microsatellite loci, Mol. Biol. Evol. 20 (9) (2003) 1425-1434.

N. Eswar, B. Webb, M.A. Marti-Renom, M. Madhusudhan, D. Eramian, M.-y. Shen, U. Pieper, A. Sali, Comparative protein structure modeling using MODELLER, Curr. Protoc. Protein Sci. (2007), 50:2.9:2.9.1-2.9.31.

Z.-J. Fan, Q.-J. Jia, C.-L. Yao, Characterization and expression analysis of Toll-like receptor 2 gene in large yellow croaker, *Larimichthys crocea*, Fish Shellfish Immunol. 44 (1) (2015) 129-137.

M. Forlenza, T. Kaiser, H.F.J. Savelkoul, G.F. Wiegertjes, The use of real-time quantitative PCR for the analysis of cytokine mRNA levels, Methods Mol. Biol. 820 (2012) 7-23.

M. Forlenza, J.P. Scharsack, N.M. Kachamakova, A.J. Taverne-Thiele, J.H. Rombout, G.F. Wiegertjes, Differential contribution of neutrophilic granulocytes and macrophages to nitrosative stress in a host-parasite animal model, Mol. Immunol. 45 (11) (2008) 3178-3189.

C.V. Henkel, R.P. Dirks, H.J. Jansen, M. Forlenza, G.F. Wiegertjes, K. Howe, G.E. van den Thillart, H.P. Spaink, Comparison of the exomes of common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*), Zebrafish 9 (2) (2012) 59-67.

T. Hibino, M. Loza-Coll, C. Messier, A.J. Majeske, A.H. Cohen, D.P. Terwilliger, K.M. Buckley, V. Brockton, S.V. Nair, K. Berney, S.D. Fugmann, M.K. Anderson, Z. Pancer, R.A. Cameron, L.C. Smith, J.P. Rast, The immune gene repertoire encoded in the purple sea urchin genome, Dev. Biol. 300 (1) (2006) 349-365.

M. Higuchi, A. Matsuo, M. Shingai, K. Shida, A. Ishii, K. Funami, Y. Suzuki, H. Oshiumi, M. Matsumoto, T. Seya, Combinational recognition of bacterial lipoproteins and peptidoglycan by chicken Toll-like receptor 2 subfamily, Dev. Comp. Immunol. 32 (2) (2008) 147-155.

I. Hirono, M. Takami, M. Miyata, T. Miyazaki, H.J. Han, T. Takano, M. Endo, T. Aoki, Characterization of gene structure and expression of two toll-like receptors from Japanese flounder, *Paralichthys olivaceus*, Immunogenetics 56 (1) (2004) 38-46.

Z. Hua, B. Hou, TLR signaling in B-cell development and activation, Cell. Mol. Immunol. 10 (2) (2013) 103-106.

I. Irnazarow, Genetic variability of Polish and Hungarian carp lines, Aquaculture 129 (1995) 215-219.

O. Jaillon, J.-M. Aury, F. Brunet, J.-L. Petit, N. Stange-Thomann, E. Mauceli, L. Bouneau, C. Fischer, C. Ozouf-Costaz, A. Bernot, S. Nicaud, D. Jaffe, S. Fisher, G. Lutfalla, C. Dossat, B. Segurens, C. Dasilva, M. Salanoubat, M. Levy, N. Boudet, S. Castellano, V. Anthouard, C. Jubin, V. Castelli, M. Katinka, B. Vacherie, C. Biemont, Z. Skalli, L. Cattolico, J. Poulain, V. de Berardinis, C. Cruaud, S. Duprat, P. Brottier, J.-P. Coutanceau, J. Gouzy, G. Parra, G. Lardier, C. Chapple, K.J. McKernan, P. McEwan, S. Bosak, M. Kellis, J.-N. Volff, R. Guigo, M.C. Zody, J. Mesirov, K. Lindblad-Toh, B. Birren, C. Nusbaum, D. Kahn, M. Robinson-Rechavi, V. Laudet, V. Schachter, F. Quetier, W. Saurin, C. Scarpelli, P. Wincker, E.S. Lander, J. Weissenbach, H. Roest Crollius, Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype, Nature 431 (7011) (2004) 946-957.

C. Jault, L. Pichon, J. Chluba, Toll-like receptor gene family and TIR-domain adapters in *Danio rerio*, Mol. Immunol. 40 (11) (2004) 759-771.

M. Joerink, C.M.S. Ribeiro, R.J.M. Stet, T. Hermsen, H.F.J. Savelkoul, G.F. Wiegertjes, Head kidney-derived macrophages of common carp (*Cyprinus carpio* L.) show plasticity and functional polarization upon differential stimulation, J. Immunol. 177 (2006) 61-69.

M. Kasahara, K. Naruse, S. Sasaki, Y. Nakatani, W. Qu, B. Ahsan, T. Yamada, Y. Nagayasu, K. Doi, Y. Kasai, T. Jindo, D. Kobayashi, A. Shimada, A. Toyoda, Y. Kuroki, A. Fujiyama, T. Sasaki, A. Shimizu, S. Asakawa, N. Shimizu, S. Hashimoto, J. Yang, Y. Lee, K. Matsushima, S. Sugano, M. Sakaizumi, T. Narita, K. Ohishi, S. Haga, F. Ohta, H. Nomoto, K. Nogata, T. Morishita, T. Endo, I.T. Shin, H. Takeda, S. Morishita, Y. Kohara, The medaka draft genome and insights into vertebrate genome evolution, Nature 447 (7145) (2007) 714-719.

A.M. Keestra, M.R. De Zoete, R.A.M.H. van Aubel, J.P.M. van Putten, The central leucine-rich repeat region of chicken TLR16 dictates unique ligand specificity and species-specific interaction with TLR2, J. Immunol. 178 (11) (2007) 7110-7119.

P. Kongchum, E.M. Hallerman, G. Hulata, L. David, Y. Palti, Molecular cloning, characterization and expression analysis of TLR9, MyD88 and TRAF6 genes in common carp (*Cyprinus carpio*), Fish Shellfish Immunol. 30 (1) (2011) 361-371.

J.C.E. Koumans-van Diepen, E. Egberts, B.R. Peixoto, N. Taverne, J.H.W.M. Rombout, B cell and immunoglobulin heterogeneity in carp (*Cyprinus carpio* L.): an immuno(cyto)chemical study, Dev. Comp. Immunol. 19 (1) (1995) 97-108.

M.A. Larkin, G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J. Gibson, D.G. Higgins, Clustal W and clustal X version 2.0, Bioinformatics 23 (21) (2007) 2947-2948.

N. Matsushima, T. Tanaka, P. Enkhbayar, T. Mikami, M. Taga, K. Yamada, Y. Kuroki, Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors, BMC Genom. 8 (2007) 124.

A.H. Meijer, S.F. Gabby Krens, I.A. Medina Rodriguez, S. He, W. Bitter, B. Ewa Snaar-Jagalska, H.P. Spaink, Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish, Mol. Immunol. 40 (11) (2004) 773-783.

S. Ohno, Evolution by Gene Duplication, Springer-Verlag, Berlin Heidelberg, 1970.

H. Oshiumi, T. Tsujita, K. Shida, M. Matsumoto, K. Ikeo, T. Seya, Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, *Fugu rubripes*, genome, Immunogenetics 54 (11) (2003) 791-800.

Y. Palti, Toll-like receptors in bony fish: from genomics to function, Dev. Comp. Immunol. 35 (12) (2011) 1263-1272.

Y. Palti, M.F. Rodriguez, S.A. Gahr, M.K. Purcell, C.E. Rexroad 3rd, G.D. Wiens, Identification, characterization and genetic mapping of *TLR1* loci in rainbow trout (*Oncorhynchus mykiss*), Fish Shellfish Immunol. 28 (5-6) (2010) 918-926.

D. Pietretti, M. Scheer, I.R. Fink, N. Taverne, H.F. Savelkoul, H.P. Spaink, M. Forlenza, G.F. Wiegertjes, Identification and functional characterization of nonmammalian Toll-like receptor 20, Immunogenetics 66 (2) (2014) 123-141.

D. Pietretti, H.P. Spaink, A. Falco, M. Forlenza, G.F. Wiegertjes, Accessory molecules for Toll-like receptors in teleost fish. Identification of TLR4 interactor with leucine-rich repeats (TRIL), Mol. Immunol. 56 (4) (2013) 745-756.

D. Pietretti, G.F. Wiegertjes, Ligand specificities of Toll-like receptors in fish: indications from infection studies, Dev. Comp. Immunol. 43 (2) (2014) 205-222.

B.A. Pollok, R. Heim, Using GFP in FRET-based applications, Trends Cell Biol. 9 (2) (1999) 57-60.

S.M. Quiniou, P. Boudinot, E. Bengten, Comprehensive survey and genomic characterization of Toll-like receptors (TLRs) in channel catfish, *Ictalurus punctatus*: identification of novel fish TLRs, Immunogenetics 65 (7) (2013) 511-530.

A. Rebl, T. Goldammer, H.M. Seyfert, Toll-like receptor signaling in bony fish, Veterinary Immunol. Immunopathol. 134 (3-4) (2010) 139-150.

C.M. Ribeiro, T. Hermsen, A.J. Taverne-Thiele, H.F. Savelkoul, G.F. Wiegertjes, Evolution of recognition of ligands from Gram-positive bacteria: similarities and differences in the TLR2-mediated response between mammalian vertebrates and teleost fish, J. Immunol. 184 (5) (2010a) 2355-2368.

C.M. Ribeiro, M.J. Pontes, S. Bird, M. Chadzinska, M. Scheer, B.M. Verburg-van Kemenade, H.F. Savelkoul, G.F. Wiegertjes, Trypanosomiasis-induced Th17-like immune responses in carp, PLoS One 5 (9) (2010b) e13012.

J.C. Roach, G. Glusman, L. Rowen, A. Kaur, M.K. Purcell, K.D. Smith, L.E. Hood, A. Aderem, The evolution of vertebrate Toll-like receptors, Proc. Natl. Acad. Sci. U. S. A. 102 (27) (2005) 9577-9582.

M.S. Rodriguez, J. Thompson, R.T. Hay, C. Dargemont, Nuclear retention of IκBα protects it from signal-induced degradation and inhibits Nuclear Factor κB transcriptional activation, J. Biol. Chem. 274 (13) (1999) 9108-9115.

N. Romano, S. Picchietti, J.J. Taverne-Thiele, N. Taverne, L. Abelli, L. Mastrolia, B.M.L. Verburg-van Kemenade, J.H.W.M. Rombout, Distribution of macrophages during fish development: an immunohistochemical study in carp (*Cyprinus carpio, L.*), Anat. Embryol. 198 (1998) 31-41.

J.H.W.M. Rombout, J.C.E. Koumans-van Diepen, P.M. Emmer, J.J. Taverne-Thiele, N. Taverne, Characterization of carp thrombocytes with specific monoclonal antibodies, J. Fish Biol. 49 (1996) 521-531.

M. Samanta, B. Swain, M. Basu, P. Panda, G.B. Mohapatra, B.R. Sahoo, N.K. Maiti, Molecular characterization of toll-like receptor 2 (TLR2), analysis of its inductive expression and associated down-stream signaling molecules following ligands exposure and bacterial infection in the Indian major carp, rohu (*Labeo rohita*), Fish Shellfish Immunol. 32 (3) (2012) 411-425.

F. Sandor, E. Latz, F. Re, L. Mandell, G. Repik, D.T. Golenbock, T. Espevik, E.A. Kurt-Jones, R.W. Finberg, Importance of extra- and intracellular domains of TLR1 and TLR2 in NFκB signaling, J. Cell Biol. 162 (6) (2003) 1099-1110.

C.J. Secombes, J.J.M. van Groningen, E. Egberts, Separation of lymphocyte subpopulations in carp *Cyprinus carpio* L. by monoclonal antibodies: immunohistochemical studies, Immunology 48 (1983) 165-175.

R.B. Sekar, A. Periasamy, Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations, J. Cell Biol. 160 (5) (2003) 629-633.

S.J. Shan, D.Z. Liu, L. Wang, Y.Y. Zhu, F.M. Zhang, T. Li, L.G. An, G.W. Yang, Identification and expression analysis of *irak1* gene in common carp *Cyprinus carpio* L.: indications for a role of antibacterial and antiviral immunity, J. Fish. Biol. 87 (2) (2015) 241-255.

K. Takeda, T. Kaisho, S. Akira, Toll-like receptors, Annu. Rev. Immunol. 21 (2003) 335-376.

O. Takeuchi, T. Kawai, P.F. Mühlradt, M. Morr, J.D. Radolf, A. Zychlinsky, K. Takeda, S. Akira, Discrimination of bacterial lipoproteins by toll-like receptor 6, Int. Immunol. 13 (7) (2001) 933-940.

O. Takeuchi, S. Sato, T. Horiuchi, K. Hoshino, K. Takeda, Z. Dong, R.L. Modlin, S. Akira, Cutting edge: role of toll-like receptor 1 in mediating immune response to microbial lipoproteins, J. Immunol. 169 (1) (2002) 10-14.

N.D. Temperley, S. Berlin, I.R. Paton, D.K. Griffin, D.W. Burt, Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss, BMC Genom. 9 (2008) 62.

J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, Nucleic Acids Res. 25 (24) (1997) 4876-4882.

C.G.P. Voogdt, L.I. Bouwman, M.J.L. Kik, J.A. Wagenaar, J.P.M. van Putten, Reptile Toll-like receptor 5 unveils adaptive evolution of bacterial flagellin recognition, Sci. Rep. 6 (2016) 19046.

J. Wang, Z. Zhang, J. Liu, F. Li, F. Chang, H. Fu, J. Zhao, D. Yin, Structural characterization and evolutionary analysis of fish-specific TLR27, Fish Shellfish Immunol. 45 (2) (2015) 940-945.

K. Wang, Y. Mu, T. Qian, J. Ao, X. Chen, Molecular characterization and expression analysis of Toll-like receptor 1 from large yellow croaker (*Pseudosciaena crocea*), Fish Shellfish Immunol. 35 (6) (2013) 2046-2050.

Y.C. Wei, T.S. Pan, M.X. Chang, B. Huang, Z. Xu, T.R. Luo, P. Nie, Cloning and expression of Toll-like receptors 1 and 2 from a teleost fish, the orange-spotted grouper *Epinephelus coioides*, Veterinary Immunol. Immunopathol. 141 (3-4) (2011) 173-182.

J. Winton, W. Batts, P. deKinkelin, M. LeBerre, M. Bremont, N. Fijan, Current lineages of the *epithelioma papulosum cyprini* (EPC) cell line are contaminated with fathead minnow, *Pimephales promelas*, cells, J. Fish Dis. 33 (8) (2010) 701-704.

X.Y. Wu, L.X. Xiang, L. Huang, Y. Jin, J.Z. Shao, Characterization, expression and evolution analysis of Toll-like receptor 1 gene in pufferfish (*Tetraodon nigroviridis*), Int. J. Immunogenet. 35 (3) (2008) 215-225.

Chapter 7

General Discussion

Inge Rosenbek Fink

Discussion

The innate immune system is an ancient collection of defence mechanisms against infections that rapidly responds to pathogen-associated molecular patterns (PAMPs). Recognition of pathogens is mediated by several families of innate immune receptors that collectively sample the extracellular space, endolysosomal compartment and the cytoplasm for signs of infection or tissue damage. The overall aim of this thesis was to perform a molecular and functional characterization of how PAMPs, such as β-glucans, affect the innate immune response of carp and which receptors on carp leukocytes are likely candidates to play a role in sensing such PAMPs.

Having found NEMO, next step is finding DORY

The NEMO network formed a nucleus of 15 young scientists, of which one more PhD student (Danilo Pietretti) and one post-doctoral fellow (Anders Østergaard) were appointed at Wageningen University. Next to the work described in this thesis, they examined the modulation of innate immune responses (oxidative burst and nitric oxide responses) of carp leukocytes to various β-glucans (D. Pietretti), as well as receptors on carp leukocytes that were candidates for β-glucan recognition (Tlr4 and Tlr20 by D. Pietretti, and Scarf1 and Illrs by A. Østergaard). The results of these studies are relevant to the research described in this thesis and shortly discussed below.

Various β-glucans were able to induce oxidative burst and nitric oxide responses in total head kidney leukocytes as well as purified populations of macrophages and neutrophilic granulocytes (Vera-Jimenez et al., 2013). β-glucans also induced the production of pro-inflammatory cytokines such as *il1β*, *il6*, and *il11* (Pietretti et al., 2013). However, identification of the actual β-glucan receptor appeared difficult and remains a challenge for the future. In mammals, the prototypical β-glucan receptor is Dectin-1, which can synergize with TLR2 and with TLR4 to enhance the production of pro-inflammatory cytokines in response to stimulation with β-glucans (Ferwerda et al., 2008). However, a *dectin-1* gene appears absent from fish genomes and none of the fish Tlrs we studied could be conclusively linked to β-glucan recognition. Carp Tlr4 may instead be involved in virus recognition (Pietretti, 2013), while Tlr20 may play a role in the immune response to protozoan parasites (Pietretti et al., 2014). The scavenger receptor Scarf1 in carp has all the hallmarks of the mammalian ortholog, including conserved structural domain architecture, synteny and gene expression pattern. The Illrs were previously identified in zebrafish as belonging to the C-type lectin receptor-family (Panagos et al., 2006), and orthologs were identified in carp. It remains to be clarified whether carp Scarf1 or any of

the Illrs are involved in β-glucan recognition. In conclusion, receptors on carp leukocytes that play a determining role in β-glucan recognition remain to be identified.

Other young scientists of the NEMO consortium have studied various effects of oral administration of β-glucans on complement activity (Pionnier et al., 2013), (Pionnier et al., 2014), on (immune) gene expression (Falco et al., 2012), (Falco et al., 2014), (Miest et al., 2012), (van der Marel et al., 2012) and on diversity of the microbial communities in the gut (Kuhlwein et al., 2013). These studies confirmed the immunomodulatory role of β-glucans on the carp immune system when administered by feeding. β-glucan administration was also studied as immersion treatment. Although it appeared to have only a local effect on the immune system, β-glucans can significantly improve wound healing of damaged carp skin when applied to the water (Przybylska-Diaz et al., 2013). In vitro studies have shown that β-glucans applied to carp leukocytes can induce the formation of neutrophil extracellular traps (NETs) and modulate the response to *Aeromonas hydrophila*, an important fish pathogen (Brogden et al., 2012), (Brogden et al., 2014). Thus, it may be clear that, even though definite β-glucan receptors remain to be identified, administration of β-glucans does stimulate the innate immune system of carp. Overall, figuratively speaking, we conclude we may have succeeded in finding NEMO, but should continue looking for DORY (Detailed Observations of Receptors for Yeast-derived β-glucans).

In light of the above, there are several studies that provide indications that the stimulatory effects of β-glucans could in fact sustain over a longer timespan than initially anticipated. For example, various innate immune parameters such as oxidative burst of phagocytes and complement activity were still increased several weeks after β-glucan administration (either orally or by intraperitoneal injection) (Samuel et al., 1996), (Paredes et al., 2013), (Bagni et al., 2000) as reviewed by Petit and Wiegertjes (Petit and Wiegertjes, 2016). Innate immunity is traditionally viewed as rapid but short-lived (occurring within hours to days), while adaptive immunity is credited for the slow but long-lasting protective effects. So how does a long-lasting innate response fit into this dogma? It might be explained by the phenomenon of trained immunity – the notion that even innate immunity exhibits a form of immunological memory induced by a first infection and conferring 'non-specific' but improved protection against a secondary infection (see Figure 1), through mechanisms independent of adaptive responses regulated by T and B lymphocytes (Netea et al., 2011). Rather, trained immunity may rely on changes in the number and phenotype of innate immune cell subpopulations, presumably natural killer (NK) cells and macrophages. The phenotypic changes may include differential expression of pattern recognition receptors (PRRs) or cytokines, or may include differential ability to phagocytose. The molecular mechanisms behind such changes may involve epigenetic modifications (based on histone modification and DNA methylation) leading to transcriptional reprogramming

(Kleinnijenhuis et al., 2012), (Saeed et al., 2014), (Álvarez-Errico et al., 2015). The 'memory' characteristics of trained immunity are therefore fundamentally different from the characteristics of the classic adaptive immunological memory, which relies on gene rearrangements leading to irreversible changes in the DNA of lymphocytes. Furthermore, while memory of the adaptive immune system is highly specific for the pathogen that caused the first infection, trained immunity may also confer cross-protection to a broad range of secondary infectious agents, since the changes in number and/or phenotype of the innate immune cell types involved may affect the control of several pathogens.

Although immune training is a relatively new and emerging concept, several studies have already, sometimes indirectly, demonstrated the existence of the phenomenon. Bistoni *et al.* observed that inoculating mice with an attenuated strain of the fungus *Candida albicans* not only protects animals against re-infection with a virulent *Candida* strain but also against infection with the bacteria *Staphylococcus aureus* (Bistoni et al., 1986). Importantly, the mechanism of protection proved independent of T lymphocytes, since protection was also induced in athymic mice (Bistoni et al., 1988), but dependent on macrophages (Bistoni et al., 1986) and inflammatory cytokines (Vecchiarelli et al., 1989). Similarly, vaccination of mice with Bacillus Calmette–Guérin (BCG) protects against tuberculosis as intended, but also offers cross-protection to secondary infections with *C. albicans* or *Schistosoma mansoni*, involving activated tissue macrophages (van 't Wout et al., 1992). More recently, it was demonstrated that exposure to a low dose of *C. albicans* was able to induce protection against re-infection with *C. albicans* in mice deficient in T and B cells. The protective effect was found to be dependent on monocytes (Quintin et al., 2012). Thus, the phenomenon of trained immunity can provide mammals, but also organisms lacking adaptive immunity, with enhanced resistance to re-infection. The conceptual discovery of trained immunity may pave the way to novel vaccine approaches through harnessing both innate immunity and classical T/B lymphocyte-mediated immunological memory. Other exciting prospects of trained immunity may include its application to devise new therapeutic strategies for immune-related diseases such as immunodeficiencies or autoinflammatory diseases, since modulating innate immune cells has the potential to mitigate the effects of under- or over-activation of the immune system as a whole. Trained immunity could provide the conceptual framework within which the immune-stimulating ability of compounds such as β-glucans could be explained. Such a property of innate immunity, and stimulation thereof, may therefore be highly relevant for future studies of immunostimulants. For example, it could be of interest to study prolonged effects of immunostimulants on purified populations of innate immune cell types, especially macrophages, as in vitro models for trained immunity in fish. Such analyses should examine expression patterns of pro-inflammatory cytokines and PRRs (among which the TLRs) as well as epigenetic modifications of relevant promoter regions. Of particular interest would be the immune

training properties of β-glucans, since their application to fish feed is common practise. Although the receptors that recognize β-glucans are not yet characterized, there is ample evidence to support that β-glucans do stimulate the immune system of carp, thus future searches will continue from finding NEMO to being on the look-out for DORY.

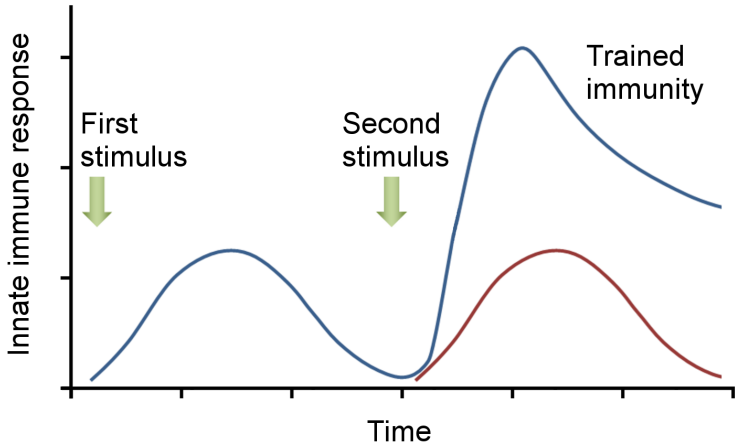

Figure 1. Schematic representation of trained immunity. In the traditional understanding of the innate immune system, repeated stimulation always gives a similar outcome; a second stimulation is regarded as an independent event (red line) and as such is independent from the first stimulation. In contrast, trained immunity (blue line) implies that the innate immune response to a second stimulus is dependent on the first stimulus. The second stimulus now leads to an amplified immune response, albeit independent of adaptive responses regulated by T and B lymphocytes, and presumably involving innate cell types such as macrophages. Figure adapted from (Álvarez-Errico et al., 2015) and (Petit and Wiegertjes, 2016).

Recent advances in the field of TLR research

TLRs are central to pathogen recognition and activation of inflammatory responses, in fish as well as in mammals. With more and more fish genomes becoming available, many studies report identification of *tlr* sequences based on conserved features, e.g. presence of leucine-rich repeats, a TIR domain, and the topology of type-I transmembrane proteins. Two very recent examples of the outcome of genome studies are the identification of a suite of 14 *tlr* genes in maraena whitefish (*Coregonus maraena*), a salmonid species (Altmann et al., 2016), and an analysis of the *tlr* repertoire of Atlantic cod (*Gadus morhua* L.) (Solbakken et al., 2016). The case of maraena whitefish appears to be a classic example with seven orthologs of mammalian *TLRs* (*TLR1*, *-2*, *-3*, *-5*, *-7*, *-8*, *-9*) and three fish-specific *tlrs* (*tlr19*, *-21*, and *-22*), some of which are duplicated and some of which are only partial sequences (Altmann et al., 2016). In contrast, the case of Atlantic cod clearly is a non-classical example: the genome of Atlantic cod has undergone dramatic changes compared to other vertebrate genomes; while most PRR families (collectin, pentraxin, retinoic acid-inducible (RIG) 1-like, and nucleotide-binding oligomerization domain (NOD)-like families) are well conserved, there are major differences in the Tlr family. *tlr1/6*, *tlr2* and *tlr5* appear to have been lost from the genome, making Atlantic cod the only known species lacking *tlr1/6* and *tlr2* which could be confirmed by thorough gene synteny analysis. In contrast, cod *tlr7*, *-8*, *-9*, *-22* and *-25* have expanded up to as many as 12 copies each, with gene copies displaying both tandem and non-tandem organization. The authors of this study found proof for diversifying selection within the *tlr* expansions which, most likely, have led to an increase of the detectable ligand repertoire through neo- and subfunctionalization (Solbakken et al., 2016). Intriguingly, analyses of the Atlantic cod genome revealed a complete loss of the Major Histocompatibility Complex (MHC) class II pathway including CD4 and invariant chain (Ii), accompanied by a gene expansion of MHC-I. The loss of MHC-II function and lack of a CD4 T-cell response represent a fundamental change in how the adaptive immune system is initiated and regulated in Atlantic cod (Star et al., 2011). How this may be linked to the changes in the innate immune system as evidenced in the unique *tlr* repertoire remains to be evaluated. It may be evident that Atlantic cod relies more heavily on the innate part of the immune system, as exemplified by the high level of natural antibodies present and only poor acquired antibody responses to immunization and pathogenic challenge (Magnadottir et al., 2001), (Magnadottir et al., 2009). Special attention should therefore be given to the study of trained immunity in this interesting fish species, where training of the innate immune system could prove especially valuable. Furthermore, we trust that the increasing number of genomic and transcriptomic studies will shed light on those *tlrs* which have remained in the dark so far.

True understanding of function of fish Tlrs still has a long way to go, because one cannot simply assume that ligand-binding properties are conserved between fish and mammals. At present, however, the majority of conclusions on the putative functions of fish Tlrs and their potential ligands are still based on studies of *tlr* gene expression. Of course this is not completely without meaning; the up-regulation of a *tlr* gene after stimulation with a certain ligand may (in)directly be linked to function. However, many of these studies show a bias in their choice of ligands, testing only those that correspond to the dogma described for mammalian TLRs, or testing ligands known to cross-react with a variety of receptors (e.g. C-type lectin receptors and scavenger receptors) and therefore difficult to link uniquely to Tlr activation specifically. More precise information might be obtained by including the complete range of ligands used for mammalian TLR studies, and even better would be to include ligands purified from fish pathogens. Examination of activation by PAMPs derived from fish pathogens is especially relevant for studies on fish-specific Tlrs – currently ranging from Tlr18 to Tlr28 but continuously expanding – where no mammalian orthologs exist and no prototypical ligand can be designated. In the context of this thesis, it would be desirable to also include β-glucans in future ligand-binding studies of Tlrs.

Current methods for studying ligand binding include overexpressing the Tlr of interest in a cell line that does not already express the receptor, and adding various ligands. When a particular ligand would be recognized by the Tlr, the subsequent activation of Tlr signalling can then be determined by measuring luciferase activity via a reporter construct usually based on activation of the highly conserved transcription factor NF-κB. Several inherent difficulties of this method have already been discussed (Chapter 6) and these include the use of cell lines whose signalling molecules are incompatible with the Tlrs of interest due to species differences, or cell lines which lack appropriate accessory molecules. We have used HEK and HeLa-57A cells, both of which are human cell lines and therefore may fail to propagate signals from the TIR domain of fish Tlrs to the human MyD88 in a natural manner. As an alternative to human cell lines we have also used EPC cells, an epithelial cell line from fathead minnow, a cyprinid fish species and close relative of carp. The use of EPC cells did not lead to reporter activity either, which may be because of a natural absence in epithelial cells of co-receptors or other co-factors required for signal transduction. One cell line that might lead to the future development of a reporter assay for Tlr activation, is the grass carp *Ctenopharyngodon idella* kidney (CIK) cell line, which has already been used to study Tlr5 (Xu et al., 2016), Tlr7 (Yang et al., 2012), Tlr8 (Chen et al., 2013), Tlr18 (Huang et al., 2015), and Tlr22 (Su et al., 2012). Similar to fathead minnow, grass carp is phylogenetically close to common carp but in contrast to the EPC, the CIK cell line may naturally express the appropriate accessory and signalling molecules needed to develop a reporter function. Regretfully, although Huang *et al*. (Huang et al., 2015) succeeded in co-transfecting the CIK cell line with *tlr18* and a NF-κB reporter

plasmid and observed increased NF-κB activity upon over-expression of *tlr18*, they did not study presumed stimulating effects of adding potential ligands on their read-out system. Future technical developments such as the establishment of a CIK cell line stably expressing a NF-κB reporter system may assist in obtaining the much-needed functional knowledge of fish Tlrs.

Another possible new approach for Tlr studies would be to use fish embryos or a fish cell line that naturally expresses (several) Tlrs and use either a knock-out (CRISPR/Cas9) or a knock-down (siRNA silencing) method to effectively inactivate one specific Tlr. Subsequently, the response of the modified embryos or cell line to various Tlr ligands could be measured to ascertain the importance of the Tlr of interest for ligand sensing. The CIK cell line mentioned above could be of interest with this respect, but first would have to be analysed further for natural Tlr expression. The CRISPR/Cas9 approach was recently used to create a *tlr22* knock-out in embryos of rohu (*Labeo rohita*), thus establishing the method of targeted gene disruption in this species (Chakrapani et al., 2016), however further studies are warranted to ascertain the functional consequences for Tlr22 in terms of ligand recognition and susceptibility to infections.

In addition to the above-described knock-in or knock-out approaches, recent advances in Tlr research increasingly include large-scale transcriptome studies. These can give a global and unbiased picture of up- and down-regulated Tlr genes, for example during infections or as a response to immunostimulants. As argued above, even though conclusions on the putative functions of fish Tlrs and their potential ligands cannot easily be extracted from studies of *tlr* gene expression, the up-regulation of *tlr* genes after stimulation with a certain ligand may still be of value in the understanding of *tlr* regulation and the importance of *tlrs* for various types of immune responses. Table 1 shows recent, preliminary transcriptome data of *tlr* gene expression in carp macrophages stimulated with β-glucans. Several interesting although preliminary conclusions can be drawn on these data.

Table 1. Transcriptome data of *tlr* gene expression levels in carp macrophages stimulated with β-glucan. The data shown is the average of n=2, and counts have been normalized to take into account the variation in sequencing depth and gene size. The colour gradient indicates the expression levels of each *tlr*. (Unpublished data, J. Petit).

	Control	β-glucan
tlr1	6	3
tlr2	85	40
tlr3	5	4
tlr4	2	17*
tlr5	31	32
tlr7	944	1434
tlr8	1	2
tlr9	3	0
tlr18	4	4
tlr19	1	2
tlr20	0	0
tlr21	0	1
tlr22	1000	2409*

*Significantly up-regulated in β-glucan stimulated cells compared to control.

First of all, some comments on the *tlrs* in Table 1 may be relevant. For most carp *tlrs*, several paralogs exist, as described for *tlr2* in Chapter 6 of this thesis. Meanwhile, the data in Table 1 were obtained from RNA-Seq experiments, which have been automatically annotated mainly based on the zebrafish genome (Kolder et al., 2016). This automatic annotation is not perfect and awaits manual curation. Thus, for the purpose of clarity only the counts for the highest expressed paralogs have been included in Table 1. When more details become available, further analyses of *tlr* gene expression can be undertaken and these may be extended to include the entire Tlr signalling pathways. This may reveal further similarities as well as differences between mammalian and fish Tlrs.

Secondly, the basal gene expression level of *tlrs* in carp macrophages varies greatly. Several *tlr* genes are hardly expressed at all, while especially *tlr7* and *tlr22* are very highly expressed. Both of these latter *tlrs* are suspected of functioning in nucleotide recognition, at least in other fish species, with *tlr7* being specific for single-stranded RNA and *tlr22* being specific for double-stranded RNA. As yet, no studies have been published on the function of these *tlrs* in common carp, and many questions remain, e.g. why transcription of these particular *tlrs* is needed by macrophages and why *tlr3* – which presumably also recognizes dsRNA – is not regulated in the same manner. As mentioned earlier, *tlr7* and

tlr22 are among the highly duplicated genes in Atlantic cod, which may reflect their importance in fish species.

Finally, the effect of adding β-glucans to carp macrophages is not overwhelming in terms of changes in *tlr* expression and characterized by a few fold differences. Only *tlr4* and *tlr22* are significantly up-regulated by β-glucans. Although fold change in gene expression was highest for *tlr4*, the total expression level of *tlr4* remains relatively low. It appears that the immune-modulating effects of β-glucans should not primarily be sought within regulation of *tlr* gene expression.

Evolving views on macrophage polarization

Macrophages are versatile cells, which play key roles in homeostasis as well as immunity. They are primary sensors of danger of both endogenous and exogenous origin. In order to emulate the well-described polarization of helper T cells, macrophages have been classified as M1 (classically activated) or M2 (alternatively activated) cells. However, this simplified classification of macrophages is evolving continuously with more and more unique macrophage phenotypes being described; it is emerging from in vitro studies that each single stimulus can cause a distinct macrophage response. Also in vivo, a macrophage is surrounded by numerous stimuli and its phenotype therefore reflects an integrated response to the particular circumstances. Instead of the one-dimensional M1/M2 classification, it may therefore be more appropriate to adopt a multi-dimensional understanding of macrophage polarization where M1 and M2 are well-described extremes and therefore can serve as landmarks which are useful when navigating this expanding field. Another reason to be careful with the M1/M2 nomenclature is the misunderstanding it often creates, in which the polarization of macrophages is seen as secondary to T cell fate, i.e. once the adaptive immune response has "chosen" either the Th1 or the Th2 route, the polarization of macrophages is subsequently steered in the corresponding direction. This traditional view on polarized immune responses can be fine-tuned by recognizing that macrophages, rather than being responders, most often are the initiators of an immune response and subsequently direct T lymphocytes to produce Th1 or Th2 cytokines. Only then does the adaptive immunity come into play, with T cell-derived cytokines acting on macrophages in feedback loops serving to sustain the polarized immune response. Not only do macrophages act before T cells in responding to infections, they also pre-date adaptive immunity as a whole, when looking at the evolution of the components of the immune system. In particular from an evolutionary perspective, it therefore makes sense to take polarized immune responses from a 'macrophage first' point of view as shown in Figure 2 (Martinez and Gordon, 2014), (Mills and Ley, 2014), (Wiegertjes et al., 2016). This view

of macrophages highlights their pleiotropic functions as both sensors and effectors. Their omnipotent ability presumably reflects the evolutionary history of macrophages where they can also function in simpler pathways without the involvement of lymphocytes (Iwasaki and Medzhitov, 2015). Teleost fish are excellently positioned close to the root of the vertebrate lineage to study the role of macrophages. These studies may be complemented by future studies on macrophage-like cell types from lower vertebrates and invertebrates to shed further light on the evolutionarily conserved functions of macrophages.

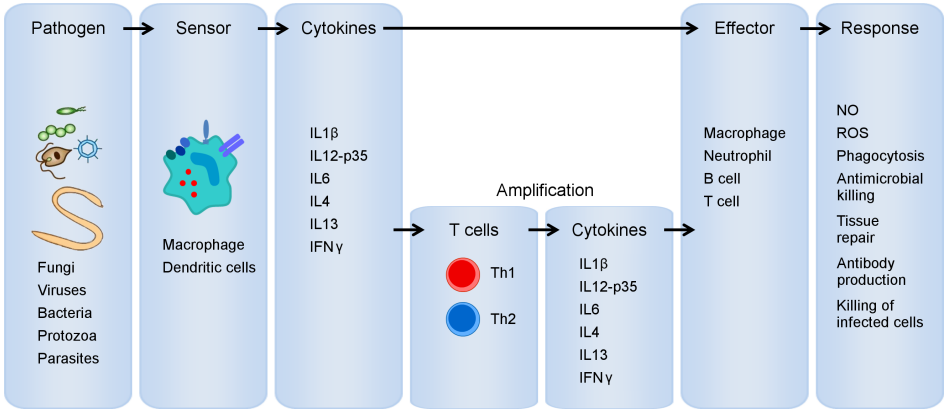

Figure 2. The 'macrophage first' point of view. Immune responses are orchestrated as a sequence of events. Macrophages (and dendritic cells) are typical sensors of pathogens, and respond by producing pro- or anti-inflammatory cytokines depending on the type of pathogen. The cytokines can act in an autocrine and paracrine manner to enhance effector functions of the cell itself or enhance effector functions of neighbouring cells, e.g. macrophages, neutrophils, B and T cells. In addition, the cytokine production can direct T cells to differentiate into Th1 or Th2 cells, producing even more cytokines and thereby amplifying the stimulation initiated by macrophages. Ultimately, effector functions are carried out as response to the pathogenic attack, and include the production of nitric oxide (NO) and radical oxygen species (ROS), phagocytosis and, ultimately, antibody production and cytotoxicity. Adapted from (Iwasaki and Medzhitov, 2015).

Along with a further characterization of fish macrophages, an increasing number of fish cytokines are being characterized which will be useful to decipher the above-described communication between macrophages and lymphocytes in fish. Until recently, pro-inflammatory cytokines such as Ifnγ and Tnfα were receiving most attention, simply because they were first identified. With fish genomes increasingly becoming available in great(er) detail, more paralogs of these cytokines are being identified, the existence of whom often can be ascribed to whole genome duplication (WGD) events. Common carp is notable among vertebrates for its very recent fourth WGD, and analyses have revealed a pattern of slow gene loss, which means that most of the recently duplicated

genes were retained. However, most of the gene pairs did evolve and do show differences in expression levels, whereas some have undergone neo- or sub-functionalization (Li et al., 2015); the process in which one paralog retains the original function while the other can attain a new function, or the process in which each of two paralogs retains a subset of their original function. Clearly, WGD and the resulting effects on gene pairs multiplies the work required to study in detail the functions of cytokine paralogs relevant to macrophage polarization.

In addition to the above-described pro-inflammatory cytokines, also attention for the, initially more difficult to identify, (presumed) anti-inflammatory cytokines is rapidly increasing – for an overview of the current knowledge see Zou and Secombes (Zou and Secombes, 2016) and Piazzon *et al*. (Piazzon et al., 2016). Highly relevant to the polarization of macrophages are not only the cytokine Ifnγ for M1 macrophages but also the cytokine Il4/13 for M2 macrophages. The effect of Ifnγ on macrophage polarization was presented in Chapter 4 of this thesis while the effect of the presumed anti-inflammatory Il4/13 is presently being investigated. Of interest, two paralogs of Il4/13 have recently been characterized in goldfish (*Carassius auratus* L.). Recombinants of both paralogs appear to enhance arginase activity and increase *arginase-2* gene expression of goldfish primary kidney macrophages, and furthermore counteract the Ifnγ-induced nitric oxide production (Hodgkinson et al., 2016). In contrast to *arginase-2*, gene expression of *arginase-1* was not regulated by recombinant Il4/13, confirming our findings in carp (Wiegertjes et al., 2016). Although the latter finding, at first sight, may appear contradictory to the situation in mammalian M2 macrophages, where *arginase-1* is upregulated and *arginase-2* is unaltered (Munder et al., 1999), it may not be illogical. Mammalian arginase is found in a cytosolic (ARG-1) and a mitochondrial form (ARG-2). Where ARG-2 is expressed in most organs, ARG-1 is expressed almost exclusively in the liver where it is a cornerstone of the urea cycle, ensuring the conversion of ammonia to urea which can be excreted through the kidney (Jenkinson et al., 1996). Fish, in contrast, are able to excrete ammonia directly to their aquatic environment, mainly through the gills, and thus do not rely on the urea cycle for ammonia detoxification. Furthermore, both arginase genes in fish encode a mitochondrial targeting sequence (Joerink et al., 2006b), meaning fish do not seem to express a cytosolic form of arginase. Thus, it may not be surprising that these inherent differences between mammalian and fish arginases are reflected in their differential regulation of gene expression in M2 macrophages. Ultimately, no matter which arginase gene of macrophages is activated by Il4/13, the end result is that arginine is converted into urea and ornithine, which can be used to produce polyamines, proline and glutamate, important for cell proliferation and collagen production during extracellular matrix regeneration, one of the hallmarks of M2 macrophages. At the same time, the Il4/13-mediated arginase activation results in the depletion of intracellular arginine levels, so less

is available for the competing enzyme inducible nitric oxide synthase (iNOS) to convert into nitric oxide and therefore the formation of M1 macrophages is suppressed (Rath et al., 2014). This mechanism presumably holds true for both mammalian and fish macrophages.

The induction of arginase activity by goldfish Il4/13 is in line with our preliminary data (I.R. Fink, unpublished data) where recombinant carp Il4/13 induces arginase activity of macrophages in a dose-dependent manner. It thus appears that Il4/13 polarizes macrophages into an M2 phenotype similar to the effect of cAMP, confirming that cAMP is a useful M2 regulator (Joerink et al., 2006b), (Joerink et al., 2006a), (Hodgkinson et al., 2016). The polarizing effect of cAMP alone provides evidence suggesting cytokine-independent M2 polarization does occur, and possibly other effectors besides cAMP may prove to have the same capacity. It remains to be seen whether Il4/13 can also augment the effect of cAMP on M2 macrophages, in a manner similar to the way Ifnγ augments the effect of LPS on M1 macrophages. No matter the exact stimulus, we present in this thesis a range of gene signatures (Chapter 4) to aid the identification of polarized states of macrophages in fish, allowing for more detailed studies on these fascinating cells.

Our efforts to characterize polarized macrophages of fish included transcriptome analyses of LPS-, LPS+Ifnγ-, and cAMP-stimulated macrophages. In the context of this thesis, it is interesting to analyse these datasets for gene expression of *tlrs* (Table 2). It is evident that the gene expression of *tlr4*, *tlr5*, *tlr18* and *tlr22* is up-regulated by LPS and by LPS+Ifnγ. On the other hand, cAMP induces a partly overlapping and partly unique profile with a significant increase in gene expression of *tlr1*, *tlr2*, *tlr5* and *tlr22*. Interestingly, *tlr5* and *tlr22* are up-regulated in both M1 and M2 cells, highlighting the fact that M1 and M2 cells may have common characteristics.

Table 2. Transcriptome data of *tlr* gene expression levels in carp macrophages stimulated with cAMP, LPS, and LPS+Ifnγ. The data shown is the average of n=2, and counts have been normalized to take into account the variation in sequencing depth. The colour gradient indicates the expression levels of each *tlr*.

	Control	LPS	LPS+Ifnγ	Control	cAMP
tlr1	87	83	66	82	208*
tlr2	381	324	554	283	629*
tlr3	12	19	11	7	20
tlr4	17	153*	257*	9	31
tlr5	204	695*	864*	160	302*
tlr7	65	43	45	47	15
tlr8	9	9	7	13	2
tlr9	2	2	4	1	7
tlr18	26	195*	260*	55	72
tlr19	3	5	4	5	4
tlr20	0	0	1	0	0
tlr21	1	2	3	8	3
tlr22	1222	4801*	3845*	2343	5013*

*Significantly up-regulated in stimulated cells compared to control.

Another interesting observation is that the *tlrs* presumably involved in recognition of nucleic acids (*tlrs 3*, *7*, *8*, and *9*, with the prominent exception of *tlr22*) are hardly expressed in these head kidney-derived in vitro cultures of macrophages, neither when in control state nor in polarized state. Furthermore, the expression of *tlr18* (which is a member of the TLR1 family) is up-regulated by LPS and LPS+Ifnγ but not by cAMP, whereas for *tlr1* and *tlr2* (which are members of the same family) the opposite is true. While *tlr18* appears to be linked to the pro-inflammatory M1 macrophages, it is not up-regulated by β-glucan (see Table 1). This observation supports the unique profile of β-glucan versus LPS-stimulated macrophages and indicates that β-glucan-stimulated cells do not assume an M1 profile.

Further comparing the data on M1/M2 macrophages to the β-glucan stimulated macrophages shown in Table 1, the difference in *tlr7* expression of the control macrophages stands

out. At the moment, we have no obvious explanation for this difference. As mentioned previously, the annotation of *tlrs* in the transcriptome is awaiting further scrutiny, so for the time being, we only included the highest expressed paralogs in Table 2. There are however quite substantial differences in expression between some of the paralogs, so further analyses are warranted. No matter what challenges remain after these preliminary findings, the recent availability of transcriptomes of polarized carp macrophages not only allows for expression studies of *tlr* genes, but also of other PRRs, members of signalling pathways, cytokines and other molecules involved in macrophage effector functions. Thus, future analyses will undoubtedly lead to the characterization of more genes relevant to macrophage polarization and recognition of immunostimulants.

Immunometabolism – a novel paradigm

A central point in macrophage polarization is the different fate of the amino acid arginine in M1 versus M2 macrophages, described as the arginine fork, and as such the two pathways of arginine metabolism are vital for the immune functions of macrophages. The pathway associated with inflammatory M1 macrophages involves conversion of arginine by the enzyme iNOS leading to NO and citrulline formation. On the other hand, arginine conversion to urea and ornithine, carried out by the enzyme arginase, is associated with alternatively activated M2 macrophages. Importantly, like arginine metabolism, there are several other pathways of cellular metabolism that are also deeply intertwined with the immune system; a research field collectively defined as immunometabolism (O'Neill et al., 2016). Of course, metabolic pathways regulate the generation of key metabolic products to support growth and survival needs of all cell types, including leukocytes. Pertaining to immunity, certain metabolic pathways can be coupled to immune effector functions such as production of distinct sets of cytokines. Of relevance to this thesis, M1 macrophages are typically associated with glycolysis and pentose phosphate pathway usage, while M2 macrophages rely on the tricarboxylic acid (TCA) cycle and fatty acid oxidation. This will be elaborated further in the following paragraphs while an overview of the relevant pathways is given in Figure 3.

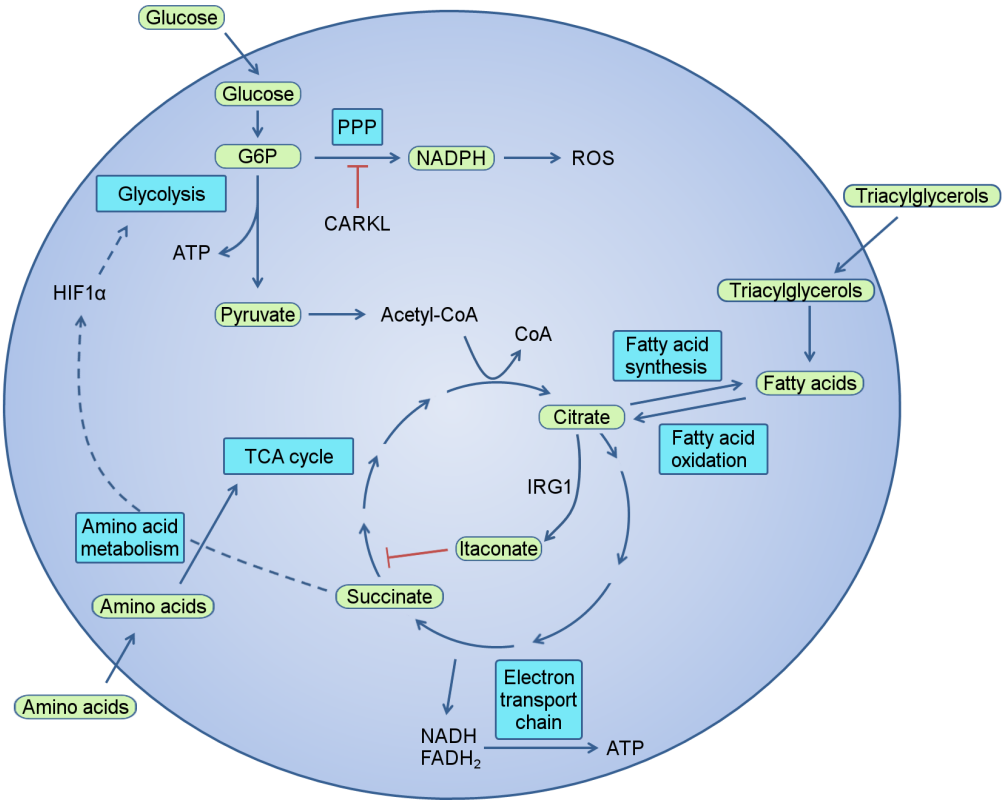

Figure 3. Major metabolic pathways of relevance to macrophage polarization. Glycolysis produces ATP while converting glucose into pyruvate, which can then be funnelled into the tricarboxylic acid (TCA) cycle. The glycolysis intermediate glucose-6-phosphate (G6P) also feeds into the pentose phosphate pathway (PPP), which generates NADPH required for production of reactive oxygen species (ROS). Glycolysis and an active PPP are hallmarks of M1 macrophages, along with fatty acid synthesis. In M1 macrophages, the TCA cycle is 'broken' and citrate accumulates. It can be used for fatty acid synthesis, or it can be converted by the enzyme IRG1 into itaconate, a metabolite which inhibits the conversion of succinate. Succinate in turn stabilizes the transcription factor HIF1α, which has a positive effect on glycolysis. In M2 macrophages, emphasis is placed on a fully functioning TCA cycle, yielding NADH and $FADH_2$, resulting in generation of ATP through use of the electron transport chain. M2 macrophages rely on uptake of triacylglycerols as a source for fatty acids, which are oxidized and enter the TCA cycle. M2 macrophages shut down the PPP through the inhibitor CARKL. Furthermore, M1 and M2 macrophages have different ways of metabolizing amino acids, especially arginine. Some amino acids can feed into the TCA cycle thereby contributing to energy production.

In M1 macrophages, glycolysis is the metabolic pathway used to generate ATP and biosynthetic intermediates needed to carry out effector functions such as phagocytosis and pro-inflammatory cytokine production. Although glycolysis is not the most efficient way of utilizing glucose for ATP production, it can be strongly up-regulated and thereby generate more ATP per unit time, enough for the surge in energy consumption typical of M1 cells. Glycolysis is triggered through, for example, LPS-induced activation of hypoxia-inducible factor 1α (HIF1α), a transcription factor that is required for the induction of several enzymes involved in glycolysis (Tannahill et al., 2013). One of the glycolytic enzymes (hexokinase 1) has an extra function, in that it regulates the NLRP3 inflammasome, which is important for the generation of the mature form of the pro-inflammatory cytokine IL1β. Another outcome of glycolysis is the production of the biosynthetic intermediate glucose-6-phosphate, which subsequently feeds into the pentose phosphate pathway, resulting in NADPH production. NADPH is used to generate reactive oxygen species (ROS), another of the characteristics of M1 macrophages (O'Neill et al., 2016). Besides glycolysis and the pentose phosphate pathway, another pivotal metabolic module is the TCA cycle. In M1 macrophages the TCA cycle is 'broken' in two places: after citrate and after succinate, leading to the accumulation of these two metabolites (Jha et al., 2015), (Tannahill et al., 2013). The citrate is subsequently funnelled into fatty acid synthesis or into pathways leading to ROS, nitric oxide and prostaglandin synthesis (Infantino et al., 2011). Citrate is also converted by an *IRG1*-encoded enzyme into itaconate, one of the most prominent metabolites of M1 macrophages. Of interest, expression of *irg1* is highly up-regulated in the transcriptome datasets of LPS- and LPS+Ifnγ-stimulated carp macrophages and is among the highest expressed genes overall (Chapter 4). The resulting itaconate is responsible for the next block in the TCA cycle, by inhibiting succinate dehydrogenase and thereby leading to accumulation of succinate (Cordes et al., 2016), (Lampropoulou et al., 2016). Succinate accumulation plays an important role during inflammation as it contributes to the induction of IL1β expression via stabilization of the above-mentioned transcription factor HIF1α (Tannahill et al., 2013).

In M2 macrophages the TCA cycle is intact and produces NADH and $FADH_2$, which can transfer electrons to the electron transport chain to support oxidative phosphorylation, providing ATP for energy. Furthermore, there is an up-regulation of the synthesis of UDP-GlcNAc intermediates that are necessary for the N-glycosylation of M2-associated receptors, such as the mannose receptor (Jha et al., 2015). In contrast to the situation in M1 macrophages, the pentose phosphate pathway is inhibited in M2 macrophages,

mainly due to high expression levels of the enzyme CARKL that limits the flux through this pathway (Haschemi et al., 2012), another example of the opposing nature of M1 vs M2 macrophages. This is also the case with regards to the fatty acid metabolism: M1 macrophages drive fatty acid synthesis (although it is unclear how this is coupled to immune function), while M2 macrophages drive fatty acid oxidation, i.e. breakdown, which is required for the inhibition of pro-inflammatory cytokine production, leading to immune tolerance and the inhibition of inflammation (Vats et al., 2006). Interestingly, the source of fatty acids for M2 macrophage fatty acid oxidation is triacylglycerol substrates taken up by the scavenger receptor CD36, at least in mammals (Huang et al., 2014). Our studies of Cd36 did not reveal a similar role for this molecule in carp, as no expression could be detected in macrophages by RT-qPCR (Chapter 5), neither by RNA-Seq of cAMP-stimulated macrophages (Chapter 4). Other members of the scavenger receptor class B family have been linked to fatty acid translocation/metabolism in Atlantic cod and rainbow trout (Sanchez-Gurmaches et al., 2011), (Sanchez-Gurmaches et al., 2012), (Torstensen et al., 2011), and indeed one of the *scarb2* paralogs (cypCar_00041717) is significantly up-regulated in the transcriptome of our M2 macrophages, albeit only 2-fold.

Besides playing a role in M1 versus M2 macrophage polarization, shifts in metabolic pathways are also associated with the concept of trained immunity. One example of this is the metabolic shift that is created in β-glucan-trained monocytes, which display elevated glycolysis rates and increased glucose consumption among other biochemical changes. Central to the metabolic reprogramming that occurs during trained immunity is the transcription factor HIF1α (Cheng et al., 2014), reminiscent of the situation in M1 macrophages.

These metabolic pathways, although diverse in terms of their end products, are closely connected as a consequence of shared energy inputs, and a reliance on products from one pathway that feed into other pathways. Furthermore, there is crosstalk between pathways as the activity of some enzymes can be regulated by metabolites from other pathways. Future investigations into these intricate networks of metabolism are underway in several fish species and hold great potential for boosting (or inhibiting) the immune system through modulating the cellular metabolism of immune cells. If this could be achieved, e.g. via modulation of fish feed, it could open up new ways to steer innate immunity via feed ingredients. In this thesis, we have been viewing macrophages from the perspective of immunity and therefore studies have involved stimulating these cells with e.g. PAMPs

and cytokines. Given the recent realization of the importance of cellular metabolism as discussed above, future studies can be extended towards understanding the roles of various metabolic pathways involving fatty acid, amino acid and glucose usage in macrophage polarization also in fish.

From a bird's eye view, we should of course consider not only the metabolites that are available to cells of the immune system but look for the source of these metabolites. That brings into play the gut microbiota which has a significant influence on feed digestion and the uptake of amino acids, fatty acids etc. Manipulating the microbial communities of the gut e.g. through use of probiotics or immunostimulants such as β-glucans therefore has potential to indirectly steer host immune responses. At the moment, more knowledge is needed to understand the principles governing microbial community assembly and the persistence of specific populations within the gut, and this issue is being addressed for several fish species (Giatsis et al., 2016). Possibly, in the future, through enhancing or suppressing various gut microorganisms, certain 'polarizing' metabolites can be favoured which will influence the immune responses to subsequent encounters with infectious agents.

An integrated view

Where the innate immune system could be viewed by some as less advanced than – and lacking the specificity and memory of – the adaptive parts of the immune system, it is safe to conclude that main players of the innate immune system such as macrophages are highly complex leukocyte cell types, harbouring a multitude of pattern recognition receptors each with a high specificity for its corresponding pathogen-associated molecular pattern. New concepts such as trained immunity have helped to underline this complexity by introducing to innate immunity features such as 'memory'. We characterized Tlr1 and Tlr2 and discussed several methods for studying ligand-binding properties of fish Tlrs, and identified by transcriptome analyses fish-specific Tlrs such as Tlr22 as of great interest for future studies which should build on our improved knowledge on knock-in approaches while keeping an open eye for novel knock-out approaches. Cd36 as scavenger receptor remains a PRR of interest despite the fact that *cd36* could not be detected in immune organs or immune cell types of carp; a recent study shows that a recombinant protein corresponding to the extracellular part of zebrafish Cd36 is capable of binding both Gram-negative and Gram-positive bacteria (Liu et al., 2016). Still, the exact role of Cd36 in

fish immunity is unclear. We characterized carp thrombocytes as a leukocyte cell type which expresses a large number of immune-relevant genes, and their relevance to the immune system was recently reinforced by a study of chicken thrombocytes, in which transcriptome profiles reveal conserved responses to LPS-stimulation (Ferdous et al., 2016). We studied carp macrophages as cell types pivotal to (innate) immune responses, characterizing polarized phenotypes in terms of function and in terms of gene expression profiles. Transcriptome analyses have provided us with a range of potential molecular markers for fish M1 and M2 macrophages that will be paramount in future studies of polarization of fish macrophages. All studies described in this thesis should be seen in the larger context of the NEMO project, in which several academic and industry partners contributed to the increased understanding of immunostimulation in general, and more specifically of immune-stimulating effects of β-glucans on carp leukocytes. Thereby the NEMO project contributed to the valorisation of β-glucans as immunostimulant for aquaculture and improved fish health.

References

Altmann, S., Korytář, T., Kaczmarzyk, D., Nipkow, M., Kühn, C., Goldammer, T., Rebl, A., 2016. Toll-like receptors in maraena whitefish: Evolutionary relationship among salmonid fishes and patterns of response to *Aeromonas salmonicida*. Fish & shellfish immunology 54, 391-401.

Álvarez-Errico, D., Vento-Tormo, R., Sieweke, M., Ballestar, E., 2015. Epigenetic control of myeloid cell differentiation, identity and function. Nature reviews. Immunology 15, 7-17.

Bagni, M., Archetti, L., Amadori, M., Marino, G., 2000. Effect of long-term oral administration of an immunostimulant diet on innate immunity in sea bass (*Dicentrarchus labrax*). J Vet Med B 47, 745-751.

Bistoni, F., Vecchiarelli, A., Cenci, E., Puccetti, P., Marconi, P., Cassone, A., 1986. Evidence for macrophage-mediated protection against lethal *Candida albicans* infection. Infection and immunity 51, 668-674.

Bistoni, F., Verducci, G., Perito, S., Vecchiarelli, A., Puccetti, P., Marconi, P., Cassone, A., 1988. Immunomodulation by a low-virulence, agerminative variant of *Candida albicans*. Further evidence for macrophage activation as one of the effector mechanisms of nonspecific anti-infectious protection. J Med Vet Mycol. 26, 285-299.

Brogden, G., Krimmling, T., Adamek, M., Naim, H.Y., Steinhagen, D., von Köckritz-Blickwede, M., 2014. The effect of β-glucan on formation and functionality of neutrophil extracellular traps in carp (*Cyprinus carpio* L.). Developmental and comparative immunology 44, 280-285.

Brogden, G., von Köckritz-Blickwede, M., Adamek, M., Reuner, F., Jung-Schroers, V., Naim, H.Y., Steinhagen, D., 2012. β-Glucan protects neutrophil extracellular traps against degradation by *Aeromonas hydrophila* in carp (*Cyprinus carpio*). Fish & shellfish immunology 33, 1060-1064.

Chakrapani, V., Patra, S.K., Panda, R.P., Rasal, K.D., Jayasankar, P., Barman, H.K., 2016. Establishing targeted carp TLR22 gene disruption via homologous recombination using CRISPR/Cas9. Developmental and comparative immunology 61, 242-247.

Chen, X., Wang, Q., Yang, C., Rao, Y., Li, Q., Wan, Q., Peng, L., Wu, S., Su, J., 2013. Identification, expression profiling of a grass carp *TLR8* and its inhibition leading to the resistance to reovirus in CIK cells. Developmental and comparative immunology 41, 82-93.

Cheng, S.C., Quintin, J., Cramer, R.A., Shepardson, K.M., Saeed, S., Kumar, V., Giamarellos-Bourboulis, E.J., Martens, J.H.A., Rao, N.A., Aghajanirefah, A., Manjeri, G.R., Li, Y., Ifrim, D.C., Arts, R.J.W., van der Veer, B.M.J.W., Deen, P.M.T., Logie, C., O'Neill, L.A., Willems, P., van de Veerdonk, F.L., van der Meer, J.W.M., Ng, A., Joosten, L.A.B., Wijmenga, C., Stunnenberg, H.G., Xavier, R.J., Netea, M.G., 2014. mTOR- and HIF-1a-mediated aerobic glycolysis as metabolic basis for trained immunity. Science 345, 1250684.

Cordes, T., Wallace, M., Michelucci, A., Divakaruni, A.S., Sapcariu, S.C., Sousa, C., Koseki, H., Cabrales, P., Murphy, A.N., Hiller, K., Metallo, C.M., 2016. Immunoresponsive gene 1 and itaconate inhibit succinate dehydrogenase to modulate intracellular succinate levels. The Journal of biological chemistry 291, 14274-14284.

Falco, A., Frost, P., Miest, J., Pionnier, N., Irnazarow, I., Hoole, D., 2012. Reduced inflammatory response to *Aeromonas salmonicida* infection in common carp (*Cyprinus carpio* L.) fed with β-glucan supplements. Fish & shellfish immunology 32, 1051-1057.

Falco, A., Miest, J.J., Pionnier, N., Pietretti, D., Forlenza, M., Wiegertjes, G.F., Hoole, D., 2014. β-Glucan-supplemented diets increase poly(I:C)-induced gene expression of Mx, possibly via Tlr3-mediated recognition mechanism in common carp (*Cyprinus carpio*). Fish & shellfish immunology 36, 494-502.

Ferdous, F., Saski, C., Bridges, W., Burns, M., Dunn, H., Elliott, K., Scott, T.R., 2016. Transcriptome profile of the chicken thrombocyte: New implications as an advanced immune effector cell. PloS one 11, e0163890.

Ferwerda, G., Meyer-Wentrup, F., Kullberg, B.J., Netea, M.G., Adema, G.J., 2008. Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. Cellular microbiology 10, 2058-2066.

Giatsis, C., Sipkema, D., Ramiro-Garcia, J., Bacanu, G.M., Abernathy, J., Verreth, J., Smidt, H., Verdegem, M., 2016. Probiotic legacy effects on gut microbial assembly in tilapia larvae. Scientific reports 6, 33965.

Haschemi, A., Kosma, P., Gille, L., Evans, C.R., Burant, C.F., Starkl, P., Knapp, B., Haas, R., Schmid, J.A., Jandl, C., Amir, S., Lubec, G., Park, J., Esterbauer, H., Bilban, M., Brizuela, L., Pospisilik, J.A., Otterbein, L.E., Wagner, O., 2012. The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism. Cell metabolism 15, 813-826.

Hodgkinson, J.W., Fibke, C., Belosevic, M., 2016. Recombinant IL-4/13A and IL-4/13B induce arginase activity and down-regulate nitric oxide response of primary goldfish (*Carassius auratus* L.) macrophages. Developmental and comparative immunology.

Huang, S.C.C., Everts, B., Ivanova, Y., O'Sullivan, D., Nascimento, M., Smith, A.M., Beatty, W., Love-Gregory, L., Lam, W.Y., O'Neill, C.M., Yan, C., Du, H., Abumrad, N.A., Urban, J.F., Jr., Artyomov, M.N., Pearce, E.L., Pearce, E.J., 2014. Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. Nature immunology 15, 846-855.

Huang, W.J., Shen, Y., Xu, X.Y., Hu, M.Y., Li, J.L., 2015. Identification and characterization of the TLR18 gene in grass carp (*Ctenopharyngodon idella*). Fish & shellfish immunology 47, 681-688.

Infantino, V., Convertini, P., Cucci, L., Panaro, M.A., Di Noia, M.A., Calvello, R., Palmieri, F., Iacobazzi, V., 2011. The mitochondrial citrate carrier: a new player in inflammation. The Biochemical journal 438, 433-436.

Iwasaki, A., Medzhitov, R., 2015. Control of adaptive immunity by the innate immune system. Nature immunology 16, 343-353.

Jenkinson, C.P., Grody, W.W., Cederbaum, S.D., 1996. Comparative properties of arginases. Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology 114, 107-132.

Jha, A.K., Huang, S.C.C., Sergushichev, A., Lampropoulou, V., Ivanova, Y., Loginicheva, E., Chmielewski, K., Stewart, K.M., Ashall, J., Everts, B., Pearce, E.J., Driggers, E.M., Artyomov, M.N., 2015. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. Immunity 42, 419-430.

Joerink, M., Ribeiro, C.M.S., Stet, R.J.M., Hermsen, T., Savelkoul, H.F.J., Wiegertjes, G.F., 2006a. Head kidney-derived macrophages of common carp (*Cyprinus carpio* L.) show plasticity and functional polarization upon differential stimulation. Journal of immunology 177, 61-69.

Joerink, M., Savelkoul, H.F., Wiegertjes, G.F., 2006b. Evolutionary conservation of alternative activation of macrophages: Structural and functional characterization of arginase 1 and 2 in carp (*Cyprinus carpio* L.). Molecular immunology 43, 1116-1128.

Kleinnijenhuis, J., Quintin, J., Preijers, F., Joosten, L.A.B., Ifrim, D.C., Saeed, S., Jacobs, C., van Loenhout, J., de Jong, D., Stunnenberg, H.G., Xavier, R.J., van der Meer, J.W.M., van Crevel, R., Netea, M.G., 2012. Bacille Calmette-Guérin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proceedings of the National Academy of Sciences of the United States of America 109, 17537-17542.

Kolder, I.C.R.M., van der Plas-Duivesteijn, S.J., Tan, G., Wiegertjes, G.F., Forlenza, M., Guler, A.T., Travin, D.Y., Nakao, M., Moritomo, T., Irnazarow, I., den Dunnen, J.T., Anvar, S.Y., Jansen, H.J., Dirks, R.P., Palmblad, M., Lenhard, B., Henkel, C.V., Spaink, H.P., 2016. A full-body transcriptome and proteome resource for the European common carp. BMC genomics 17, 701.

Kuhlwein, H., Emery, M.J., Rawling, M.D., Harper, G.M., Merrifield, D.L., Davies, S.J., 2013. Effects of a dietary b-(1,3)(1,6)-D-glucan supplementation on intestinal microbial communities and intestinal ultrastructure of mirror carp (*Cyprinus carpio* L.). Journal of applied microbiology 115, 1091-1106.

Lampropoulou, V., Sergushichev, A., Bambouskova, M., Nair, S., Vincent, E.E., Loginicheva, E., Cervantes-Barragan, L., Ma, X., Huang, S.C.C., Griss, T., Weinheimer, C.J., Khader, S., Randolph, G.J., Pearce, E.J., Jones, R.G., Diwan, A., Diamond, M.S., Artyomov, M.N., 2016. Itaconate links inhibition of succinate dehydrogenase with macrophage metabolic remodeling and regulation of inflammation. Cell metabolism 24, 158-166.

Li, J.T., Hou, G.Y., Kong, X.F., Li, C.Y., Zeng, J.M., Li, H.D., Xiao, G.B., Li, X.M., Sun, X.W., 2015. The fate of recent duplicated genes following a fourth-round whole genome duplication in a tetraploid fish, common carp (*Cyprinus carpio*). Scientific reports 5, 8199.

Liu, K., Xu, Y., Wang, Y., Wei, S., Feng, D., Huang, Q., Zhang, S., Liu, Z., 2016. Developmental expression and immune role of the class B scavenger receptor *cd36* in zebrafish. Developmental and comparative immunology 60, 91-95.

Magnadottir, B., Gudmundsdottir, S., Gudmundsdottir, B.K., Helgason, S., 2009. Natural antibodies of cod (*Gadus morhua* L.): specificity, activity and affinity. Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology 154, 309-316.

Magnadottir, B., Jonsdottir, H., Helgason, S., Bjornsson, B., Solem, S.T., Pilstrom, L., 2001. Immune parameters of immunised cod (*Gadus morhua* L.). Fish & shellfish immunology 11, 75-89.

Martinez, F.O., Gordon, S., 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000prime reports 6, 13.

Miest, J.J., Falco, A., Pionnier, N.P.M., Frost, P., Irnazarow, I., Williams, G.T., Hoole, D., 2012. The influence of dietary β-glucan, PAMP exposure and *Aeromonas salmonicida* on apoptosis modulation in common carp (*Cyprinus carpio*). Fish & shellfish immunology 33, 846-856.

Mills, C.D., Ley, K., 2014. M1 and M2 macrophages: the chicken and the egg of immunity. Journal of innate immunity 6, 716-726.

Munder, M., Eichmann, K., Morán, J.M., Centeno, F., Soler, G., Modolell, M., 1999. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. Journal of immunology 163, 3771-3777.

Netea, M.G., Quintin, J., van der Meer, J.W.M., 2011. Trained immunity: a memory for innate host defense. Cell host & microbe 9, 355-361.

O'Neill, L.A.J., Kishton, R.J., Rathmell, J., 2016. A guide to immunometabolism for immunologists. Nature reviews. Immunology 16, 553-565.

Panagos, P.G., Dobrinski, K.P., Chen, X., Grant, A.W., Traver, D., Djeu, J.Y., Wei, S., Yoder, J.A., 2006. Immune-related, lectin-like receptors are differentially expressed in the myeloid and lymphoid lineages of zebrafish. Immunogenetics 58, 31-40.

Paredes, M., Gonzalez, K., Figueroa, J., Montiel-Eulefi, E., 2013. Immunomodulatory effect of prolactin on Atlantic salmon (*Salmo salar*) macrophage function. Fish physiology and biochemistry 39, 1215-1221.

Petit, J., Wiegertjes, G.F., 2016. Long-lived effects of administering β-glucans: Indications for trained immunity in fish. Developmental and comparative immunology 64, 93-102.

Piazzon, M.C., Lutfalla, G., Forlenza, M., 2016. IL10, a tale of an evolutionarily conserved cytokine across vertebrates. Crit Rev Immunol 36, 99-129.

Pietretti, D., 2013. Stimulation of the innate immune system of carp: role of Toll-like receptors. Wageningen University, Wageningen, The Netherlands.

Pietretti, D., Scheer, M., Fink, I.R., Taverne, N., Savelkoul, H.F.J., Spaink, H.P., Forlenza, M., Wiegertjes, G.F., 2014. Identification and functional characterization of nonmammalian Toll-like receptor 20. Immunogenetics 66, 123-141.

Pietretti, D., Vera-Jimenez, N.I., Hoole, D., Wiegertjes, G.F., 2013. Oxidative burst and nitric oxide responses in carp macrophages induced by zymosan, MacroGard and selective dectin-1 agonists suggest recognition by multiple pattern recognition receptors. Fish & shellfish immunology 35, 847-857.

Pionnier, N., Falco, A., Miest, J., Frost, P., Irnazarow, I., Shrive, A., Hoole, D., 2013. Dietary β-glucan stimulate complement and C-reactive protein acute phase responses in common carp (*Cyprinus carpio*) during an *Aeromonas salmonicida* infection. Fish & shellfish immunology 34, 819-831.

Pionnier, N., Falco, A., Miest, J.J., Shrive, A.K., Hoole, D., 2014. Feeding common carp *Cyprinus carpio* with β-glucan supplemented diet stimulates C-reactive protein and complement immune acute phase responses following PAMPs injection. Fish & shellfish immunology 39, 285-295.

Przybylska-Diaz, D.A., Schmidt, J.G., Vera-Jimenez, N.I., Steinhagen, D., Nielsen, M.E., 2013. β-glucan enriched bath directly stimulates the wound healing process in common carp (*Cyprinus carpio* L.). Fish & shellfish immunology 35, 998-1006.

Quintin, J., Saeed, S., Martens, J.H.A., Giamarellos-Bourboulis, E.J., Ifrim, D.C., Logie, C., Jacobs, L., Jansen, T., Kullberg, B.J., Wijmenga, C., Joosten, L.A.B., Xavier, R.J., van der Meer, J.W.M., Stunnenberg, H.G., Netea, M.G., 2012. *Candida albicans* infection affords protection against reinfection via functional reprogramming of monocytes. Cell host & microbe 12, 223-232.

Rath, M., Müller, I., Kropf, P., Closs, E.I., Munder, M., 2014. Metabolism via arginase or nitric oxide synthase: two competing arginine pathways in macrophages. Frontiers in immunology 5, 532.

Saeed, S., Quintin, J., Kerstens, H.H.D., Rao, N.A., Aghajanirefah, A., Matarese, F., Cheng, S.C., Ratter, J., Berentsen, K., van der Ent, M.A., Sharifi, N., Janssen-Megens, E.M., Ter Huurne, M., Mandoli, A., van Schaik, T., Ng, A., Burden, F., Downes, K., Frontini, M., Kumar, V., Giamarellos-Bourboulis, E.J., Ouwehand, W.H., van der Meer, J.W.M., Joosten, L.A.B., Wijmenga, C., Martens, J.H.A., Xavier, R.J., Logie, C., Netea, M.G., Stunnenberg, H.G., 2014. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. Science 345.

Samuel, M., Lam, T.J., Sin, Y.M., 1996. Effect of Laminaran [β(1,3)-D-Glucan] on the protective immunity of blue gourami,*Trichogaster trichopterus* against *Aeromonas hydrophila*. Fish & shellfish immunology 6, 443-454.

Sanchez-Gurmaches, J., Cruz-Garcia, L., Gutierrez, J., Navarro, I., 2012. mRNA expression of fatty acid transporters in rainbow trout: *in vivo* and *in vitro* regulation by insulin, fasting and inflammation and infection mediators. Comparative biochemistry and physiology. Part A, Molecular & integrative physiology 163, 177-188.

Sanchez-Gurmaches, J., Østbye, T.-K., Navarro, I., Torgersen, J., Hevrøy, E.M., Ruyter, B., Torstensen, B.E., 2011. In vivo and in vitro insulin and fasting control of the transmembrane fatty acid transport proteins in Atlantic salmon (*Salmo salar*). Am J Physiol Regul Integr Comp Physiol 301, R947-957.

Solbakken, M.H., Tørresen, O.K., Nederbragt, A.J., Seppola, M., Gregers, T.F., Jakobsen, K.S., Jentoft, S., 2016. Evolutionary redesign of the Atlantic cod (*Gadus morhua* L.) Toll-like receptor repertoire by gene losses and expansions. Scientific reports 6, 25211.

Star, B., Nederbragt, A.J., Jentoft, S., Grimholt, U., Malmstrom, M., Gregers, T.F., Rounge, T.B., Paulsen, J., Solbakken, M.H., Sharma, A., Wetten, O.F., Lanzen, A., Winer, R., Knight, J., Vogel, J.H., Aken, B., Andersen, O., Lagesen, K., Tooming-Klunderud, A., Edvardsen, R.B., Tina, K.G., Espelund, M., Nepal, C., Previti, C., Karlsen, B.O., Moum, T., Skage, M., Berg, P.R., Gjoen, T., Kuhl, H., Thorsen, J., Malde, K., Reinhardt, R., Du, L., Johansen, S.D., Searle, S., Lien, S., Nilsen, F., Jonassen, I., Omholt, S.W., Stenseth, N.C., Jakobsen, K.S., 2011. The genome sequence of Atlantic cod reveals a unique immune system. Nature 477, 207-210.

Su, J., Heng, J., Huang, T., Peng, L., Yang, C., Li, Q., 2012. Identification, mRNA expression and genomic structure of *TLR22* and its association with GCRV susceptibility/resistance in grass carp (*Ctenopharyngodon idella*). Developmental and comparative immunology 36, 450-462.

Tannahill, G.M., Curtis, A.M., Adamik, J., Palsson-McDermott, E.M., McGettrick, A.F., Goel, G., Frezza, C., Bernard, N.J., Kelly, B., Foley, N.H., Zheng, L., Gardet, A., Tong, Z., Jany, S.S., Corr, S.C., Haneklaus, M., Caffrey, B.E., Pierce, K., Walmsley, S., Beasley, F.C., Cummins, E., Nizet, V., Whyte, M., Taylor, C.T., Lin, H., Masters, S.L., Gottlieb, E., Kelly, V.P., Clish, C., Auron, P.E., Xavier, R.J., O'Neill, L.A.J., 2013. Succinate is an inflammatory signal that induces IL-1b through HIF-1a. Nature 496, 238-242.

Torstensen, B.E., Espe, M., Stubhaug, I., Lie, O., 2011. Dietary plant proteins and vegetable oil blends increase adiposity and plasma lipids in Atlantic salmon (*Salmo salar* L.). The British journal of nutrition 106, 633-647.

van 't Wout, J.W., Poell, R., van Furth, R., 1992. The role of BCG/PPD-activated macrophages in resistance against systemic candidiasis in mice. Scand J Immunol 36, 713-719.

van der Marel, M., Adamek, M., Gonzalez, S.F., Frost, P., Rombout, J.H.W.M., Wiegertjes, G.F., Savelkoul, H.F.J., Steinhagen, D., 2012. Molecular cloning and expression of two b-defensin and two mucin genes in common carp (*Cyprinus carpio* L.) and their up-regulation after β-glucan feeding. Fish & shellfish immunology 32, 494-501.

Vats, D., Mukundan, L., Odegaard, J.I., Zhang, L., Smith, K.L., Morel, C.R., Wagner, R.A., Greaves, D.R., Murray, P.J., Chawla, A., 2006. Oxidative metabolism and PGC-1b attenuate macrophage-mediated inflammation. Cell metabolism 4, 13-24.

Vecchiarelli, A., Cenci, E., Puliti, M., Blasi, E., Puccetti, P., Cassone, A., Bistoni, F., 1989. Protective immunity induced by low-virulence *Candida albicans*: cytokine production in the development of the anti-infectious state. Cellular immunology 124, 334-344.

Vera-Jimenez, N.I., Pietretti, D., Wiegertjes, G.F., Nielsen, M.E., 2013. Comparative study of β-glucan induced respiratory burst measured by nitroblue tetrazolium assay and real-time luminol-

enhanced chemiluminescence assay in common carp (*Cyprinus carpio* L.). Fish & shellfish immunology 34, 1216-1222.

Wiegertjes, G.F., Wentzel, A.S., Spaink, H.P., Elks, P.M., Fink, I.R., 2016. Polarization of immune responses in fish: The 'macrophages first' point of view. Molecular immunology 69, 146-156.

Xu, X.Y., Shen, Y.B., Fu, J.J., Yu, H.Y., Huang, W.J., Lu, L.Q., Li, J.L., 2016. MicroRNA-induced negative regulation of TLR-5 in grass carp, *Ctenopharyngodon idella*. Scientific reports 6, 18595.

Yang, C., Su, J., Zhang, R., Peng, L., Li, Q., 2012. Identification and expression profiles of grass carp *Ctenopharyngodon idella TLR7* in responses to double-stranded RNA and virus infection. J Fish Biol 80, 2605-2622.

Zou, J., Secombes, C.J., 2016. The function of fish cytokines. Biology 5, 23.

Appendix

Summary

Samenvatting

Acknowledgements

Summary

Recent decades have seen a significant intensification of aquaculture leading to increased risk of infections with several pathogenic organisms. On economical and ethical grounds it is more appropriate to improve general welfare conditions and prevent infections rather than treating disease outbreaks once they have occurred. Immunostimulation through feed can provide more efficient and sustainable control of diseases in aquaculture through enhancing the immunocompetence of fish; however, the underlying mechanisms are poorly characterized. The overall aim of this thesis was to perform a molecular and functional characterization of how pathogen-associated molecular patterns (PAMPs), such as β-glucans, affect the innate immune response of carp and which receptors on carp leukocytes are likely candidates to play a role in sensing such PAMPs.

In **chapter 1** we provide a framework for this thesis by introducing different classes of PAMPs, including β-glucans. These molecules were the centrepiece of an intra-European training network called NEMO (Protective immune modulation in warm water fish by feeding glucans), which this PhD project was part of. The scientific aim of the NEMO network was to develop a sustainable and cost-effective use of β-glucans as immunostimulants for aquaculture, using common carp as the model fish species, since on a global scale common carp is the most cultured fish species for food consumption. Our aims within the NEMO project entailed both the characterization of carp leukocytes and the characterization of candidate pattern recognition receptors (PRRs) that could play a role in sensing PAMPs and initiating immune responses. Chapter 1 therefore introduces the thrombocytes and macrophages pertinent to this thesis, as well as important classes of PRRs.

In our first experimental study, described in **chapter 2**, we investigated the relevance of thrombocytes for the immune system of carp. We found that thrombocytes from healthy carp express a large number of immune-relevant genes, among which several cytokines and Toll-like receptors (Tlrs). Furthermore, we dissected the role of thrombocytes during infections with two different, albeit related, protozoan parasites, *Trypanoplasma borreli* and *Trypanosoma carassii*, and found thrombocytes were massively depleted from blood and spleen of fish infected with *T. borreli*. The pathology of this infection is associated with elevated levels of tissue nitration, prompting us to investigate, ex vivo, the effect of nitric oxide on thrombocytes. Our studies revealed that nitric oxide can induce a clear and rapid apoptosis of thrombocytes from healthy carp, supporting a role for nitric oxide-mediated

control of immune-relevant thrombocytes during infection with *T. borreli*. Thereby, this particular study provided an excellent example of interplay between pathogen and the innate immune system of carp.

We reviewed in **chapter 3** another cell type central to innate immunity: the macrophage. We focused on the heterogeneity of macrophage activation states as these cells, at least in humans and mice, have the ability to polarize in several directions during an immune response. Based on the signals that lead to activation and the effector functions and cytokine profile as a result thereof, macrophages can be broadly divided into two types: classically activated macrophages induced in a T helper 1 (T_H1) cytokine environment, and alternatively activated macrophages, induced in a T_H2 cytokine environment. Mirroring the T_H1–T_H2 dichotomy, classically activated macrophages have also been termed M1, whereas alternatively activated macrophages have been termed M2. Classically activated macrophages are typically induced by stimulation with microbial ligands such as LPS in combination with pro-inflammatory cytokines such as IFNγ, and can be viewed as an extension of innate activated macrophages which are induced by microbial ligands only, thus are independent of cytokines. Alternatively activated macrophages are generated in the presence of IL4 and/or IL13. In addition to M1 and M2, one can distinguish regulatory macrophages, which are associated with the presence of the cytokine IL10. In this chapter, we reviewed the evidence of existence of polarized macrophages in teleost fish, among other things based on observations of the fundamentally different immune responses elicited by the parasites *T. borreli* and *T. carassii*.

We further investigated the polarization of carp macrophages in **chapter 4**, where we obtained gene signature profiles of carp macrophages via a transcriptome approach. Independently of cytokines, carp macrophages showed the ability to differentiate into cells with functional characteristics highly comparable to those of mammalian M1 and M2, consistent with a conserved ability of macrophages to polarize into distinct subsets. In addition to obtaining a global view of gene expression, our transcriptome approach identified gene signatures for M1 and M2 macrophages which appear conserved from fish to mammals. We selected a number of these interesting genes that were differentially regulated between M1 and M2 macrophages and discussed in detail five potential M1 markers; *il1β*, *ptx3a*, *saa*, *nos2b*, and *il12a* – as well as five potential M2 markers; *cyr61*, *inhba*, *timp2*, *tgm2*, and *arg2*. These transcriptome studies may pave the way for future studies of polarized macrophages during immune responses in fish. Furthermore,

additional analyses of the datasets described in this chapter will undoubtedly lead to the characterization of more genes relevant to macrophage polarization and recognition of immunostimulants.

As part of the characterization of candidate PRRs that could play a role in sensing PAMPs and initiating immune responses, we studied the scavenger receptor Cd36 (**chapter 5**), which in mammals is expressed by many different (immune) cell types and plays a role in highly diverse processes, both homeostatic and pathologic. Among other things, it is often found associated with sensing of β-glucans and also with M2 macrophage activation, sparking our interest in this molecule in fish. We studied Cd36 in common carp as well as in zebrafish, a closely related cyprinid fish species. Whereas a single *cd36* gene is present in zebrafish, carp was shown to have two paralogs of *cd36*. Although all genes show conserved synteny compared to mammalian CD36, unexpectedly we could not detect gene expression of cyprinid *cd36* in macrophages or any other immune cell type or immune organ. Yet, because gene expression of *cd36* was down-regulated during *Mycobacterium marinum* infection of zebrafish, and knockdown of *cd36* in zebrafish embryos led to higher bacterial burden upon such infection, our data imply a role for Cd36 in immune responses of fish. Future studies are needed to clarify the exact mechanisms involved.

As characterization of candidate PRRs we also examined the Toll-like receptors Tlr1 and Tlr2 (**chapter 6**). We identified a full-length, expressed *tlr1* gene, a *tlr1* pseudogene, and a second *tlr2* gene next to the *tlr2* which had been described previously. Sequence, phylogenetic and synteny analyses supported the conserved nature of these genes, and three-dimensional modelling showed a good fit with the mammalian TLR1/TLR2 heterodimer including the potential to bind to the prototypical ligand Pam_3CSK_4. However, we were unable to demonstrate Tlr1/Tlr2-mediated ligand binding in transfected cell lines through NFκB activation, despite showing the expression and co-localization of Tlr1 and Tlr2. This prompted a discussion of methods available for studying ligand-binding properties of fish Tlrs.

Finally, we discuss in **chapter 7** the findings of this thesis in the context of the NEMO project. We present the concept of trained immunity, which could provide the conceptual framework within which the immune-stimulating ability of compounds such as β-glucans could be explained. We discuss recent advances in the field of TLR research as well as that of macrophage polarization, and highlight immunometabolism as a new area of interest which may help to illuminate the molecular events occurring in immune cells

during health and disease. In conclusion, we found that carp leukocytes, along with their pattern recognition receptors, are central players of the innate immune system of carp. Our findings contribute to the understanding of mechanisms of immunostimulation, and expect this will enable the valorisation and use of immunostimulants for sustainable aquaculture and improvement of fish health.

Samenvatting

De afgelopen decennia heeft er een sterke intensivering plaatsgevonden binnen de aquacultuur sector, die heeft geleidt tot een verhoogde infectiekans met verscheidene ziekteverwekkers. In plaats van het behandelen van dieren tijdens een ziekte uitbraak is het, op basis van economische én ethische gronden beter om infecties te voorkómen en daarmee hun algemene welzijn te verbeteren. Hoewel immuun stimulatie via het voer kan bijdragen aan een efficiënte en duurzame controle van ziektes in de aquacultuur middels het verhogen van de immuun competentie van vissen, zijn de onderliggende mechanismen van immuun competentie tot nu toe slecht gekarakteriseerd. Het overkoepelende doel van dit proefschrift betreft daarom een moleculaire en functionele karakterisering hoe ziekteverwekker-gerelateerde patronen (ENG: pathogen-associated molecular patterns, PAMPs), zoals β-glucaan, het aangeboren afweersysteem van de karper beïnvloeden en welke receptoren aanwezig op karper leukocyten deze PAMPs mogelijk zou kunnen herkennen.

In **hoofdstuk 1** beschrijven wij een kader voor dit proefschrift door het introduceren van de verschillende groepen PAMPs, inclusief de β-glucanen. Deze moleculen vormden het middelpunt van een intra-Europees trainingsnetwerk met titel “Beschermende immuun modulatie van warmwater vissen door middel van het voeren van β-glucanen”, kortweg NEMO, waar ook deze studie deel van uitmaakte. Het wetenschappelijke doel van NEMO was het ontwikkelen van een duurzaam en kosteneffectief gebruik van β-glucanen als immuun modulator binnen de aquacultuur sector, gericht op de karper. Op wereldwijde schaal wordt vooral deze vissoort het meest gekweekt voor consumptie. Onze doelen binnen het NEMO netwerk waren het karakteriseren van karper leukocyten en van kandidaat receptoren voor de herkenning van patronen (ENG: pattern recognition receptors, PRRs) die een rol zouden kunnen spelen in het herkennen van PAMPs en een afweerreactie op gang zouden kunnen brengen. Om deze redenen en vanwege hun centrale rol in dit proefschrift, introduceert hoofdstuk 1 trombocyten en macrofagen, evenals een aantal belangrijke groepen kandidaat receptoren.

In onze eerste experimentele studie, beschreven in **hoofdstuk 2**, hebben we de relevantie van trombocyten onderzocht voor afweerreacties van de karper. We vonden dat trombocyten van gezonde karpers een groot aantal immuun relevante genen tot expressie brengen, waaronder verschillende cytokinen en ‘Toll-like receptoren’ (TLRs). Verder hebben we de rol van trombocyten tijdens een infectie met twee verschillende, maar

gerelateerde, protozoïsche parasieten, *Trypanoplasma borreli* en *Trypanosoma carassii* onderzocht. In deze studie vonden we dat het aantal trombocyten in bloed en milt van met *T. borreli*-geïnfecteerde karpers drastisch verlaagd was. Het ziektebeeld van deze infectie is gerelateerd aan verhoogde nitrificering van het weefsel en daarom hebben wij het effect van stikstofoxide op trombocyten bestudeerd in een *ex vivo* experiment. Onze studie toont aan dat in gezonde karpers, stikstofoxide voor een sterke en snelle geprogrammeerde celdood van trombocyten kan zorgen. Dit resultaat ondersteunt de rol voor een stikstofoxide-gemedieerde controle van immuun relevante trombocyten tijdens een infectie met *T. borreli*. Daarmee geeft deze studie een uitstekend voorbeeld van het samenspel tussen ziekteverwekker en het aangeboren afweersysteem van de karper.

In **hoofdstuk 3** behandelen we een ander celtype dat een centrale rol speelt in het aangeboren afweersysteem: de macrofaag. Hier hebben we ons vooral gericht op de heterogeniteit in activeringstatus van macrofagen omdat deze cellen, tenminste in mens en muis, in staat zijn verschillend te polariseren tijdens een immuunrespons. Gebaseerd op signalen die normaliter leiden tot activering en bijbehorende effectorfuncties en cytokineprofielen, kunnen macrofagen ingedeeld worden in (tenminste) twee types: de klassiek-geactiveerde macrofaag, geïnduceerd door een T helper 1 (T_h1) cytokine milieu, en de alternatief-geactiveerde macrofaag, geïnduceerd door een T helper 2 (T_h2) cytokine milieu. Gespiegeld aan de tweedeling T_h1- T_h2 worden de klassiek-geactiveerde macrofagen ook wel M1 genoemd, en de alternatief-geactiveerde macrofagen M2. Klassiek-geactiveerde macrofagen worden geactiveerd door stimulatie met microbiële stimuli zoals LPS, in combinatie met pro-inflammatoire cytokinen zoals IFNγ, en kunnen worden gezien als een actievere versie van macrofagen met 'aangeboren' activiteit die worden geïnduceerd door enkel en alleen een microbiële ligand en hierdoor onafhankelijk zijn van de aanwezigheid van cytokinen. Alternatief-geactiveerde macrofagen worden gegenereerd in de aanwezigheid van IL4 en/of IL13. Naast M1 en M2 macrofagen kunnen nog 'regulerende' macrofagen onderscheiden worden; deze worden geassocieerd met de aanwezigheid van de cytokine IL10. In dit hoofdstuk behandelen we het bewijs voor de aanwezigheid van gepolariseerde macrofagen in beenvissen dat, onder andere, gesteund wordt door observaties dat *T. borreli* en *T. carassi* parasieten een fundamenteel-verschillende immuunrespons induceren.

De polarisatie van karper macrofagen hebben we verder onderzocht in **hoofdstuk 4**, waar we genexpressie profielen van karper macrofagen hebben verkregen door middel van

een transcriptoom benadering. Onafhankelijk van de aanwezigheid van cytokinen zijn karper macrofagen in staat te differentiëren in cellen met karakteristieken vergelijkbaar met de M1 en M2 macrofagen van zoogdieren, gelijk aan het evolutionair-geconserveerde vermogen van macrofagen om te polariseren in verschillende onderscheidende subtypen. Naast het verkrijgen van een globaal overzicht van genexpressie hebben wij door middel van een transcriptoom benadering verschillende genprofielen voor M1 en M2 macrofagen geïdentificeerd die inderdaad geconserveerd lijken tussen vissen en zoogdieren. Vervolgens hebben we een aantal interessante genen geselecteerd die verschillend gereguleerd zijn in M1 en M2 macrofagen en bediscussiëren vijf mogelijke M1 markers; *il1*β, *ptx3a*, *saa*, *nos2b* en *il12a*, en vijf mogelijke M2 markers; *cyr61, inhba, timp2, tgm2* en *arg2*. Deze transcriptoom studies kunnen leiden tot nieuwe studies naar de rol van gepolariseerde macrofagen tijdens afweerreacties in vissen. Verder zullen verdere analyses van de datasets beschreven in dit hoofdstuk ongetwijfeld leiden tot de karakterisering van nog meer relevante genen voor macrofaag polarisatie en herkenning van immuun stimulanten.

Als onderdeel van de karakterisering van kandidaat receptoren voor de herkenning van patronen (PRRs) die een rol zouden kunnen spelen in detecteren van ziekteverwekker-gerelateerde patronen (PAMPs) en het initiëren van afweerreacties, hebben we de 'scavenger' receptor Cd36 bestudeerd (**hoofdstuk 5**). Deze receptor komt in zoogdieren tot expressie in vele verschillende (immuun-)cellen en speelt een rol in een grote diversiteit aan processen, zowel homeostatisch als pathologisch. Cd36 wordt vaak geassocieerd, onder andere, met het herkennen van β-glucanen maar ook met M2 macrofaag activatie, en daarom trok met name dit molecuul onze aandacht. We bestudeerden Cd36 in karper en in zebravis; een nauwverwante cyprinide vissoort. Waar de zebravis slechts één *cd36* gen heeft, heeft de karper twee *cd36* paralogen. Hoewel al deze *cd36* genen dezelfde volgorde op het genoom vertonen als die in zoogdieren konden we, geheel onverwacht, geen genexpressie aantonen van karper *cd36* in macrofagen of welk ander immuun gerelateerd celtype of orgaan dan ook. Desondanks; de verlaagde genexpressie van *cd36* in zebravis tijdens infectie met *Mycobacterium marinum,* en het feit dat tijdelijke uitschakeling van dit gen in zebravis embryo's leidde tot hogere infectiedruk, suggereert een rol voor Cd36 in afweerreacties in vissen. Meer onderzoek is nodig om de exacte mechanismen betrokken bij deze rol verder op te helderen.

Als kandidaat PRRs hebben we ook de 'Toll-like' receptoren Tlr1 en Tlr2 van de karper bestudeerd **(hoofdstuk 6)**. We hebben het volledige, tot expressie komende, *tlr1* gen

geïdentificeerd, evenals een *tlr1* pseudogen en een tweede *tlr2* gen naast het al beschreven *tlr2* gen. De sequentie, fylogenetische en ook de genvolgorde op het genoom ondersteunen de geconserveerde aard van deze genen, terwijl de 3D modellering een goede overeenkomst laat zien met de zoogdier TLR1/TLR2 heterodimeer, inclusief de potentie om te binden aan de prototypische ligand Pam_3CSK_4. Desondanks waren we niet in staat, ondanks de expressie en co-lokalisatie van Tlr1 en Tlr2, om de Tlr1/Tlr2-gemedieerde ligandbinding door activatie van NFκB te demonstreren in getransfecteerde cellijnen. Dit vraagt om een discussie over de beschikbare methoden om ligandbindende eigenschappen van vissen Tlr's te bestuderen.

Tot slot bediscussiëren we in **hoofdstuk 7** de bevindingen beschreven in dit proefschrift in het kader van het NEMO netwerk. We presenteren een conceptueel kader waarin de immuun stimulerende eigenschappen van stoffen zoals β-glucanen uitgelegd kunnen worden aan de hand van een nieuw concept gebaseerd op training van het aangeboren afweersysteem. We bediscussiëren de recente vooruitgangen in het veld van TLR onderzoek en dat van macrofaag activering, met nadruk op immuunmetabolisme aan als een nieuw interesseveld dat kan helpen om de moleculaire stappen in leukocyten gedurende gezondheid en ziekte te kunnen belichten. Het mag duidelijk zijn dat karper leukocyten en hun herkenningsreceptoren een centrale rol spelen in de aangeboren afweer van de karper. Onze bevindingen dragen bij aan het begrip van de mechanismen van immuun stimulatie. Naar verwachting zullen deze resultaten de valorisatie en het gebruik van immuunstimulante middelen en daarmee een duurzame viskweek mogelijk maken en dus bijdragen aan de verbetering van visgezondheid.

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My "new" office mates, the ladies of CBI :-) **Carmen**, **Christine**, **Nathalie** and **Lieke G**. Thanks for creating a supportive, caring and warm atmosphere (feeling always cold, I really needed that) with lots of tea, cake and dropjes. Taking part in the pub quiz with you was also great fun. Lieke, I admire your ability to get things done, for example it meant that organizing the lab uitje with you was a lot of fun! I miss you ladies :-)

Carla R, some of the work in this thesis is a continuation of your work, especially the macrophage cultures which you taught me. We share the passion for these beautiful cells, and now they continue with new people. **Annelieke** and **Jules** are now carrying the torch of my work with even more macrophages, receptors and β-glucans. Good luck to both of you!

Carla P, thanks for your help with analysing transcriptome data, genome searches and all things fishy. You are so efficient and hardworking! I hope life in Spain is great :-)

Joeri, the lonely chicken guy with a bunch of fish people, thanks for your optimism and ideas, and especially your great sense of humour! We have spent a lot of time together at meetings, courses, in the lab – it is always good to know there is also someone else checking cells or collecting samples at 9 pm because of some strange time points in an experiment.

Mark, **Carla** and the boys – thank you for your kind hospitality, like inviting us to family birthday parties. It was nice to have another family in the neighbourhood, who could understand the daily life with small kids (now they are suddenly not so small any longer!).

Many thanks to all people from CBI: **Gerco**, **Yvonne**, **Joop**, **Tosca**, **Eva**, **Martin**, **Alberto**, **Adriaan**, **Marloes**, **Hans**, **Jacobien**, **Ruth**, **Gosia**, **Makoto**, **Lieke vd A.** I have shared many experiences with each of you and I have good memories of our time together. **Magda**, special thanks for letting me stay with you the last night in Japan, when every hotel in Fukuoka was fully booked!

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Anders, jeg elsker dig, min eneste ene. You are always there for me, for better and for worse. I would never have ended up in this world of fish if it hadn't been for you :-) You can always make me smile! Together we are stronger from what we have been through, and I look forward to new endeavours with you by my side.

About the author

List of publications

Curriculum vitae

Training activities

List of publications

Fink IR, Pietretti D, Voogdt CGP, Westphal AH, Savelkoul HFJ, Forlenza M, Wiegertjes GF.
Molecular and functional characterization of Toll-like receptor (Tlr)1 and Tlr2 in common carp (*Cyprinus carpio*).
Fish & Shellfish Immunology 56 (2016) 70-83
doi: 10.1016/j.fsi.2016.06.049

Wiegertjes GF, Wentzel AS, Spaink HP, Elks PM, **Fink IR**.
Polarization of immune responses in fish: The 'macrophages first' point of view.
Molecular Immunology 69 (2016) 146–156
doi: 10.1016/j.molimm.2015.09.026

Fink IR, Ribeiro CMS, Forlenza M, Taverne-Thiele A, Rombout JHWM, Savelkoul HFJ, Wiegertjes GF.
Immune-relevant thrombocytes of common carp undergo parasite-induced nitric oxide-mediated apoptosis.
Developmental and Comparative Immunology 50 (2015) 146–154
doi: 10.1016/j.dci.2015.02.008

Fink IR, Benard EL, Hermsen T, Meijer AH, Forlenza M, Wiegertjes GF.
Molecular and functional characterization of the scavenger receptor CD36 in zebrafish and common carp.
Molecular Immunology 63 (2015) 381–393
doi:10.1016/j.molimm.2014.09.010

Pietretti D, Scheer M, **Fink IR**, Taverne N, Savelkoul HFJ, Spaink HP, Forlenza M, Wiegertjes GF.
Identification and functional characterization of nonmammalian Toll-like receptor 20.
Immunogenetics 66 (2014) 123-141
doi:10.1007/s00251-013-0751-4

Forlenza M, **Fink IR**, Raes G, Wiegertjes GF.
Heterogeneity of macrophage activation in fish.
Developmental and Comparative Immunology 35 (2011) 1246–1255
doi:10.1016/j.dci.2011.03.008

Curriculum vitae

Inge Rosenbek Fink was born on the 27th of December 1977 in Herning, Denmark. She grew up in Denmark and in Zambia. She attended high school in Holstebro, Denmark, before enrolling at the University of Southern Denmark. Here she completed her Master's degree in Molecular and Cell Biology in 2006. Subsequently, she worked as a research assistant at the University of Aberdeen, Scotland. Her project was aimed at understanding the autoimmune mechanisms involved in multiple sclerosis, and she used rat and mouse models of the disease. In 2009 she entered the world of fish, when she joined the Cell Biology and Immunology group at Wageningen University to undertake her PhD project under the supervision of prof. dr. Geert Wiegertjes and prof. dr. Huub Savelkoul. The PhD was part of an intra-European Marie Curie training network called NEMO. She has presented the results of her PhD at several international conferences, and now also in this thesis.

Training activities

The Basic Package (3 credits)	
WIAS Introduction Course	2009
Ethics and Philosophy in Life Sciences	2011
Scientific Exposure (15 credits)	
International conferences	
Nijmegen Centre for Molecular Life Sciences (NCMLS): New Frontiers in Pattern Recognition, Nijmegen	2009
Dutch Society for Immunology (NVvI), Noordwijkerhout	2010
European Association of Fish Pathologists (EAFP), Split, Croatia	2011
European Macrophage and Dendritic Cell Society (EMDS), Brussels, Belgium	2011
Nijmegen Centre for Molecular Life Sciences (NCMLS): Summer Frontiers - Training the innate immunity, Nijmegen	2012
International Society for Development & Comparative Immunology (ISDCI), Fukuoka, Japan	2012
Seminars and workshops	
16th Benelux Congress of Zoology, Wageningen	2009
WIAS seminar: Of fish and men: curiosities of the immune system	2009
WIAS seminar: It makes sense to know your enemy	2010
WIAS seminar: Allergenicity in food allergy: Influence of food processing and immunomodulation by lactic acid bacteria	2011
WIAS seminar: Infection and Inflammation: Tracking the evolution of the immune system	2012
WIAS seminar: Mucosal factors regulating allergy	2013
WIAS Science Day	2013
Presentations	
Dutch Society for Immunology (NVvI), Noordwijkerhout (poster)	2010
Fish Immunology Workshop, Wageningen (poster)	2010
Fish Immunology Workshop, Wageningen (oral)	2011
European Association of Fish Pathologists (EAFP), Split, Croatia (oral)	2011
European Macrophage and Dendritic Cell Society (EMDS), Brussels, Belgium (poster)	2011
Fish Immunology Workshop, Wageningen (oral)	2012
International Society for Developmental & Comparative Immunology (ISDCI), Fukuoka, Japan (oral)	2012
International Society for Fish and Shellfish Immunology (ISFSI), Vigo, Spain (poster)	2013

In-Depth Studies (6.6 credits)	
Disciplinary and interdisciplinary courses	
Advanced Immunology, Utrecht	2010
ELISA course, Wageningen	2010
Fish Immunology Workshop, Wageningen	2010
Bioinformatics and synteny analysis, Aberdeen, Scotland	2011
Danish Fish Immunology Research Center & Network (DAFINET), Copenhagen, Denmark	2011
Flow cytometry, BD Biosciences, Wageningen	2012
NVvI, APC's Revisited - The function of antigen presenting cells in health and disease, Lunteren	2012
Advanced statistics courses	
Design of Experiments, Wageningen	2011
Professional Skills Support Courses (4.8 credits)	
Project management NEMO I, Copenhagen, Denmark	2009
Project management NEMO II, Wageningen	2010
Intercultural awareness NEMO, Wageningen	2010
Writing grant proposals NEMO, Krakow, Poland	2011
Time management, assertion skills and effective decision making, NEMO, Split, Croatia	2011
Techniques for Writing and Presenting a Scientific Paper, Wageningen	2012
Training in Research Presentations at NEMO meetings	2009-2012
Didactic Skills Training (9.1 credits)	
Supervising practicals and excursions	
Supervising practicals Fish Workshop, Wageningen	2011-2012
Supervising practicals Human and Veterinary Immunology, Wageningen	2011-2012
Supervising theses	
3 MSc and 1 BSc	2011-2013
Management Skills Training (1 credit)	
Membership of boards and committees	
NEMO Young Scientists' board	2010

Total number of credits: 39.5 ECTS

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