Stimulation of the innate immune system of carp:

role of Toll-like receptors

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Chapter 1

General Introduction

Pietretti Danilo

GENERAL INTRODUCTION

Beta-glucans as modulators of innate immunity in carp

Like all vertebrates also fish posses an immune system that is basically combining an innate (natural) and an acquired (adaptive) arm, each of which have unique features and their selective kinetics. However, both systems function in collaboration in order to provide the organism with effective protection against infections with rapid defence and long lasting immune memory. Innate (natural) immunity does not require prior exposure to an antigen (i.e. memory) to be effective. Thus, it can respond immediately to an invader. However, it recognizes mainly antigenic molecules that are broadly distributed rather than specific to one organism or cell. For recognition, the innate immune system employs receptors based on germline-encoded non-rearranged genes, while the adaptive immune system uniquely uses receptors based on rearranged receptor genes building T- and B-cell receptors that are specific for uniquely present epitopes on antigens. By their receptors, T- and B-cells can sense what kind of pathogen is present and they subsequently promote the development of the right kind of immune response by upregulation of certain activation markers and production of the appropriate cytokines. Phagocytic cells (neutrophils and monocytes in blood, macrophages in tissues) ingest and destroy invading antigens. Attack by phagocytic cells can be facilitated when antigens are coated with antibody (Ab), which is produced as part of acquired adaptive immunity. Antigen-presenting cells (macrophages, dendritic cells) present fragments of ingested antigens to T cells (which are part of acquired immunity). Natural killer cells kill virus-infected cells. Many molecular components (eg, complement, cytokines, acute phase proteins) participate in both innate and adaptive immunity.

Novel dietary based immunomodulation approaches can play a crucial role in preventing infections that cause economic losses in highly intensive aquaculture practices. However, there are still considerable gaps in our knowledge on how specific dietary components can modulate the immune system. This knowledge is essential in order to develop food products that modulate the immune system and confer protection against infections. Immuno-modulating properties of β-glucans in innate and adaptive immunity have been widely investigated over many years and β-glucans show their immunological effect by enhancing innate immunity through induction of cytokine production and phagocytosis.

Central to stimulating the innate immune system of fish are the macrophages with a widespread tissue distribution and responsiveness to many different stimuli. Macrophages, besides neutrophilic granulocytes, are phagocytic cells that can be activated after recognition of pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRRs) expressed by bacterial, viral and fungal parasites. Macrophages are important cells in the host resistance to fungal infections, and fungal recognition by macrophages triggers phagocytosis, intracellular killing, induction of inflammatory cytokines and chemokines, and initiation of the adaptive immune response [1]. The cell walls of fungi such as *Candida albicans* and baker's yeast *Saccharomyces cerevisiae* consist, for a large part, of glucose polymers or β-glucans (**Figure 1**), which are considered typical PAMPs. These polysaccharides are comprised of numerous repetitions of β1,3-D and β1,6-D glucans [2] (see Fig. 1) and can exhibit binding affinities affinities to different receptors. MacroGard® is a branched 1,3/1,6 β-glucan variant frequently used as feed ingredient for farmed animals, including fish [3, 4].

Macrophages of fish, especially salmonids, treated with β-glucans have been repeatedly shown

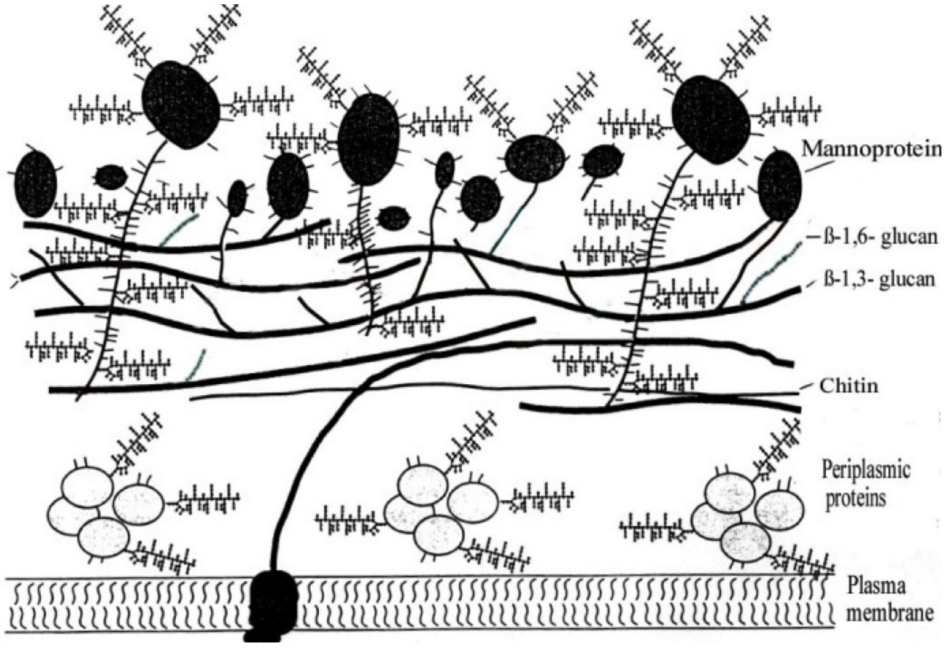

Figure 1. Composition and structure of yeast cell wall. The cell wall of yeast and other fungi determines the cell shape and integrity of the organism during growth and cell division. Three main groups of polysaccharides form the cell wall: polymers of mannose (mannoproteins around 40% of the cell dry mass), polymers of glucose (β-glucan around 60% of the cell wall dry mass) and polymers of N-acetylglucosamine (chitin around 2% of the cell wall dry mass). β-Glucan can be divided into two subtypes following the mode of glucose linkages: long chains of around 1500 β-1,3-glucose units which represents around 85% of total cell wall β-glucan, and short chain of around 150 β-1,6-glucose units that accounts for around 15% of the β-glucan [5]. Figure was adjusted from the original (http://www.guwsmedical.info/saccharomyces-cerevisiae/cell-wall-cell-surface-morphology-and-morphological-variation.html).

to have increased innate immune responses as shown by, for example, increases in respiratory burst activity and phagocytosis [6-8]. The ability of β-glucans to activate respiratory burst activity of Atlantic salmon (*Salmo salar*) macrophages has been shown in vitro [9], but also *in vivo*, with beneficial effects of administering β-glucans via feed [10]. *In vivo* screening models with zebrafish (*Danio rerio*) larvae have shown that immersion administration of β-glucan can induce expression of cytokines such as TNFα along with a temporal increase in resistance against *Vibrio anguillarum* [11]. Thus, several studies point at immune-modulating and often immune-activating properties of β-glucans in fish. Immunomodulatory effects of β-glucans are usually beneficial and thought to be mediated via the innate immune system and involve leucocytes with their associated β-glucan receptors [12]. β-glucans can not only stimulate and enhance immune function [13] but can even improve protection against several different pathogens [14, 15]. However, despite all evidence for immune-stimulating properties of β-glucans, also in fish [16, 17], the receptors on fish cells that can recognize β-glucans have never been identified.

Common carp (*Cyprinus carpio* L.) is worldwide the most cultured fish species for food consumption (FAO, 2009), with koi (*Cyprinus carpio koi*) as the ornamental variety kept for decorative purposes. Wageningen University breeds its own specific pathogen-free stock of carp at the CARUS animal facility, allowing for reliable studies on mature fish with a naïve immune status.

Few studies have addressed branched 1,3/1,6 β-glucan variants such as MacroGard® as immune stimulant for common carp.

The overall aim of this thesis is to study the immune-modulating effect of β-glucans, in particular MacroGard®, on the carp innate immune system, macrophages in particular. The research described in this thesis comprises an evaluation of how β-glucans affect the innate immune response of carp. Further, a molecular and functional characterization is made of candidate receptors on carp leukocytes sensing β-glucans. These studies will contribute to the valorisation and use of β-glucans as 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 measurement of modulation of innate immune responses by different forms of β-glucans including MacroGard® in carp (**chapters 2, 3**).
2. The characterization of candidate pattern recognition receptors on carp leukocytes that could sense β-glucans and initiate innate immune responses (**chapters 4, 5, 6 and 7**).

NEMO: a Marie Curie Intra-European Training Network

The intra-European traning network (ITN) on 'protective immune modulation in warm water fish by feeding glucans' (2008-2012) responded to a European and commercial need for scientists to be trained in scientific and generic skills in the areas of carp genetics, nutrition, health and immunology. The training network 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 use of fish diets supplemented with immune-stimulating compounds had already shown promising results. 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 aquaculture is a clear example of valorisation of 'waste' materials and can be produced in sufficient quantities to be economically used 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 improve fish health. The immune-stimulating effects of β-glucans depend on the position and configuration of the β-(1,6) side branches, which interact with leukocytes and their β-glucan receptors. 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 innate immune responses induced by β-glucans 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 assess oxygen or nitrogen radical production and induced (cytokine) gene

expression. Humoral studies can include the measurement of serum immunoglobulin levels, but also include the measurement of complement levels. Candidate receptors on carp leukocytes that can sense β-glucans and initiate innate immune responses can be studied in vitro by overexpression in cell lines transfected with plasmids carrying carp genes. All these measurements were included in the scientific program of NEMO to help ascertain how β-glucans modulates innate immune responses in carp. Thereby, the Network established optimum 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; Denmarks 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 2**). NEMO therefore provided training 'from producer to user'.

Figure 2. NEMO members enjoying a progress meeting in Split, Croatia prior to attending the 15th International Conference of the European Association of Fish Pathologists on Diseases of Fish and Shellfish; 12-16th September 2011.

Receptors that could play a role in the recognition of β-glucans

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. β-glucans are well-known immune stimulators, also applied in aquaculture and it is widely accepted that the health-promoting effect of β-glucans is based on modulation of the innate immune system, including macrophages. In this thesis we characterize, in detail, a number of pattern recognition

receptors (PRRs) considered to be important candidates for mediating the immune-stimulating effects of β-glucans in carp. Both Toll-like receptors as well as C-type lectin receptors are considered crucially important for optimal recognition of pathogen-derived molecules such as β-glucans. These receptors often complement each other when binding ligands and activating subsequent downstream intracellular responses.

Toll-like receptors (TLRs) are type I transmembrane proteins with an ectodomain containing interspersed leucine-rich repeat (LRR) motifs involved in recognition of PAMPs. The cytoplasmic domain is characterized by a Toll/IL-1 receptor (TIR) motif which is involved in signal transduction. Of the many TLR genes (>10), TLR2 may be the most important TLR for recognition of fungal cell wall components such as β-glucan. In mammals, TLR2 can form heterodimers with TLR1 and TLR6, whereas also TLR4 has been implicated in stimulations by fungal-derived PAMPs [18] (**Figure 3**). It recently became clear that distinct TLR2-containing receptor complexes allow for the accommodation of structurally diverse TLR2 ligands [19]. The ability of TLR2 to detect a relatively wide array of PAMPs has been attributed to a functional interaction with a number of other receptors [20, 21]. These not only include TLR1 and TLR6, but also the lipid scavenger receptor CD36 and the CD14 protein. The best described mode of action for TLR2 recognizing β-glucan is mediated by C-type lectin receptors [18].

C-type lectin receptors (CLRs) comprise a superfamily of proteins that were first characterised as proteins that possess a Ca^{++}-dependent carbohydrate recognition domain (CRD). Later it was discovered that other proteins with the same distinctive protein fold could bind non-carbohydrates in a Ca^{++}-independent manner, and this fold was named C-type lectin domain (CTLD). To date, the superfamily consists of all proteins that contain one or more structurally-related CTLDs and includes more than a thousand proteins. The family has been divided into fourteen groups (I-XIV), based on their domain architecture [22]. However, only five of them include proteins that play important roles in immunity, these are group: II, Asialoglycoprotein and DC receptors; III, Collectins; IV, Selectins; V, natural killer (NK) cell receptors; and VI, Multi-CTLD endocytic receptors. Of these groups, group II and V share overall structural similarity since they are both type II membrane receptors and have a singular extracellular CLTD [23]. However, 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^{++}. Maybe the best-known member of group V is Dectin-1, which is a non-classical natural killer (NK) receptor as it is not restricted to NK cells. Dectin-1 has specificity for β-1,3 and β-1,6 linked glucans.

Dectin-1 is frequently referred to as the primary membrane-bound PRR for exogenous β-glucan [24-28]. Dectin-1 is thought to act in conjunction with Toll-like receptor (TLR) 2, at least in murine and human dendritic cells [29]. Probably, Dectin-1 and TLR2 are involved in a receptor collaboration or synergy, enhancing intracellular transcription factor NF-kB-dependent signaling responses [30, 31]. Phagocytosis of β-glucan particles by for example Dectin-1, would permit destruction of the internalized particle by reactive oxygen species (ROS), nitric oxide and lytic enzymes in the acidic environment of the phagolysosome. TLRs are thought to be recruited to the phagosomes to detect β-glucan particles and induce the production of pro-inflammatory cytokines, but are not held responsible for phagocytosis. In general, candidates for phagocytic receptors not include the CLR superfamily member Dectin-1, but also the complement receptor or members of the scavanger receptor family. All these phagocytic receptors have been studied in detail in myeloid cells of humans and mice [32] [33], but the receptors that can phagocytose and/or sense β-glucan

have not been clearly identified in myeloid cells of common carp.

Numerous C-type lectin-like genes have been characterised in teleost species [34-36]. In a full-genome study of pufferfish (*Takifugu rubripes*), representatives were found for nearly all the CTLD groups, except for group V and VII [23]. Although in apparent conflict with the observation in pufferfish, two studies in cichlid fish identified group V receptors in a multi-gene family that resembles the mammalian NKC [37, 38]. However, phylogenetic analyses indicated that these receptors are actually group II receptors [23], which share overall structural similarities with group V receptors. This indicates that group V receptors may not be present in bony fish, supporting the idea that this group is probably the youngest and most rapidly evolving CTLD group, as can be seen from the large differences between rodent and human members of this group [23]. The absence in bony fish of group V CTLD receptors implies that a typical Dectin-1 receptor would not be present in common carp.

More recently, a multi-gene family of group II immune-related lectin-like receptors (*illrs*) was identified in the zebrafish genome [39]. The family consists of four genes: *illr1*, *illr2*, *illr3*, and *illrL*, which contain ITIM or ITIM-like sequences in their cytoplasmic tail, which is a key functional characteristic of group V CTLD receptors. Furthermore, *illr3* also encodes a positively charged residue in the transmembrane region, which is associated with activating forms of group V NK receptors. A cytotoxicity experiment confirmed this idea. Based on these results, the authors concluded that Illrs possess the structure of group II receptors, but the inhibitory/activating features of group V receptors [39]. All together, it is possible that group II and V share a common predecessor and that the lineage leading to group V receptors has lost the Ca^{++}-dependent carbohydrate-binding ability, which is still present in group II receptors. Of this group of CLRs, the Illrs could be candidate receptors for β-glucan (**Figure 3**).

As mentioned above, the specificity of TLR signaling is regulated, in part, through the association of TLRs with cell surface co-receptors that act to bind, concentrate, internalize, and deliver ligands to TLRs to initiate cell signaling. Both, the scavenger receptors CD36 and SCARF1 (scavenger receptor class F, member 1), have been implicated as co-receptors in TLR2 signaling (Figure 3). SCARF1, previously known as SREC-1 (scavenger receptor expressed by endothelial cell-1), is type 1 transmembrane protein with an extracellular domain that contains 5 epidermal growth factor (EGF)-like cysteine-rich repeats, likely important for ligand binding. CD36 also is a cell surface receptor with broad ligand specificity for endogenous (modified LDL, thrombospondin, apoptotic cells) and pathogenic (*Plasmodium falciparum*, mycobacterial lipopeptide, *Staphylococcus aureus*) ligands. In a study on the evolutionarily conservation of recognition of fungal pathogens in *Caenorhabditis elegans*, two receptors in *C. elegans* but also their mammalian orthologues CD36 and SCARF1 mediated the host defense against two prototypic fungal pathogens, *Cryptococcus neoformans* and *Candida albicans*. Both receptors in *C. elegans* mediated the production of antimicrobial peptides and were necessary for nematode survival after infection with *C. neoformans*. Both SCARF1 and CD36 mediated the production of cytokines and were required for macrophage binding to *C. neoformans* and control of the infection in mice. Binding of these pathogens to SCARF1 and CD36 was β-glucan dependent [1, 40]. Thus, the scavanger receptors SCARF1 and CD36 are candidate β-glucan binding receptors that may define an evolutionarily conserved pathway for the innate sensing of fungal pathogens.

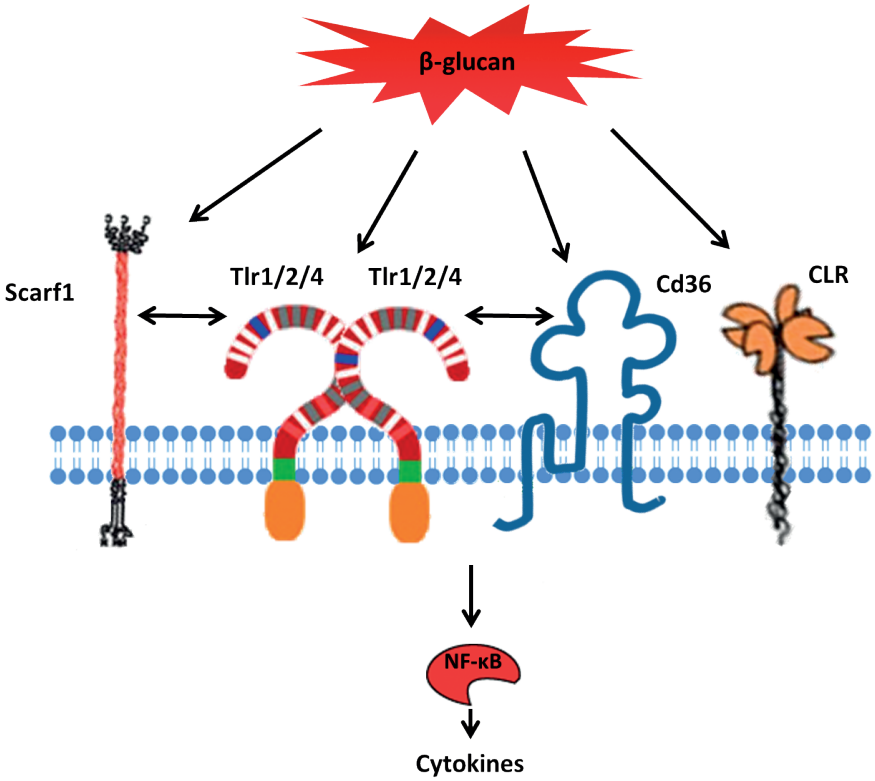

Figure 3. Putative receptors on carp macrophages that can play a role in the phagocytosis and/or signalling of β-glucans. Adjusted from Means el al. [1].

Outline of the thesis

A thorough analysis of the enhanced immunocompetence by β-glucans, 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 carp macrophages were lacking because of limited knowledge on the ligand-receptor interactions in fish macrophages. In this 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 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 Wageningen University, Cell Biology and Immunology group. Within NEMO, they worked as a team on a co-ordinated approach to the study of ligand-receptor interactions in common carp. Together they undertook the molecular and functional characterization of carp Tlr1 and Tlr2, in combination with the scavanger receptor Cd36 (Inge R. Fink), scavanger 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 Tlr4 and the non-mammalian Tlr20 are described in this thesis (Danilo Pietretti).The overall aim of this thesis is to study the immune-modulating effect of β-glucans, in particular MacroGard®, on the carp innate immune system, macrophages in particular.

To achieve the overall aim of this thesis, first, the modulation of innate immune responses by different forms of β-glucans, including MacroGard®, was studied in carp leukocytes, in particular macrophages (**chapters 2, 3**). Macrophages are considered important cells in the host resistance to fungal infections, and fungal recognition by macrophages is thought to trigger phagocytosis, intracellular killing, induction of inflammatory cytokines and chemokines, and initiation of the adaptive immune response.

Subsequently, the characterization of receptors on carp leukocytes sensing β-glucans is described. Thorough descriptions of candidate receptors contributes to the valorization and use of β-glucans as immunostimulants. The molecular and functional characterization of non-mammalian carp Tlr20 (**chapter 4**) and of Tlr4 (**chapter 5**) includes a description of their evolutionary conservation, sub-cellular localization and in vitro responses to different ligands. In vivo experiments provided additional information where in vitro ligand-binding studies were not always conclusive. We review (**chapter 6**) the presence of multiple TLR genes in fish, and summarize changes in their gene expression profiles induced by molecular patterns or by whole pathogens as part of infections. We conclude that induced changes of gene expression may provide (in)direct evidence for the involvement of a particular TLR in the reaction to a particular ligand. New findings with regard to the required presence of accessory molecules that may act in conjunction with TLR molecules (**chapter 7**), are discussed in the last chapter (**chapter 8**), along with a discussion on some of the 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 β-glucans affect the innate immune response of carp and will hopefully contribute to the valorisation and use of β-glucans as immunostimulants for sustainable aquaculture, aiming for a strategic improvement of fish health.

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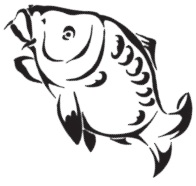

Chapter 2

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.)

N.I. Vera-Jimenez, D. Pietretti, G.F. Wiegertjes and M.E. Nielsen
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ABSTRACT

The respiratory burst is an important feature of the immune system. The increase in cellular oxygen uptake that marks the initiation of the respiratory burst is followed by the production of reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide which play a role in the clearance of pathogens and tissue regeneration processes. Therefore, the respiratory burst and associated ROS constitute important indicators of fish health status. This paper compares two methods for quantitation of ROS produced during the respiratory burst in common carp: the widely used, single-point measurement based on the intracellular reduction of nitroblue tetrazolium (NBT) and a real-time luminol-enhanced assay based on the detection of native chemiluminescence. Both assays allowed for detection of dose-dependent changes in magnitude of the respiratory burst response induced by β-glucans in head kidney cells of carp. However, whereas the NBT assay was shown to detect the production of only superoxide anions, the real-time luminol-enhanced assay could detect the production of both superoxide anions and hydrogen peroxide. Only the chemiluminescence assay could reliably record the production of ROS on a real-time scale at frequent and continual time intervals for time course experiments, providing more detailed information on the respiratory burst response. The real-time chemiluminescence assay was used to measure respiratory burst activity in macrophage and neutrophilic granulocyte-enriched head kidney cell fractions and total head kidney cell suspensions and proved to be a fast, reliable, automated multiwell microplate assay to quantitate fish health status modulated by β-glucans.

INTRODUCTION

Multicellular organisms mediate their early defence against pathogens based on their innate immune system, which provides them with the ability to recognize the presence of pathogens and react rapidly against them [1, 2].The common carp, *Cyprinus carpio*, has been intensive studied for many purposes. Common carp is worldwide the most cultured fish species for food consumption. It represents one of the most important species used in aquaculture and although many studies have focused on physiological aspects such as nutrition, farming conditions and infectious diseases [3-6], it is important to develop and improve reliable methods to monitor and control the health status of carp. The respiratory burst is regarded as one of the most important early defence mechanisms as it plays a crucial role in pathogen eradication, but has also been shown to be involved in tissue regeneration. Therefore, the respiratory burst is a significant mechanism that can be used to monitor health status in fish [7-9].

Several studies have dealt with the ability of phagocytes to recognize pathogens through the detection of pathogen-associated molecular patterns (PAMPs), which are highly conserved molecules not generally expressed in higher organisms [10, 11]. Phagocytes have also been related to the recognition of damage-associated molecular patterns (DAMPs), those being self signals of tissue damage and cell death [10, 12]. The recognition of all these molecules occurs using special receptors called pattern-recognition receptors (PRRs), and trigger a series of events including the respiratory burst [10, 13-15]. The initiation of the respiratory burst is marked by an increase in oxygen cellular uptake, followed by the one electron reduction of molecular oxygen (O_2) to superoxide anions (O_2^-). This reaction is catalysed by the membrane-associated enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, using NADPH as the electron donor [9, 16-19]. Further reduction of oxygen produces hydrogen peroxide (H_2O_2), which occurs either as a spontaneous dismutation,

especially at low pH, or as a catalyzed reaction by a family of enzymes called Superoxide dismutase (SOD). Additional reactions of O_2^- and H_2O_2 may lead to the formation of hydroxyl radicals (OH^-), especially in the presence of iron through the Fenton or Haber-Weiss reactions. The interaction of H_2O_2 with myeloperoxidase (MPO) can produce hypochlorous acid and other toxic metabolites if H_2O_2 is not dismutated to water and molecular oxygen by the enzyme catalase that can act as a natural scavenger [16, 20-24] (**See Figure 1**). Although different techniques for the quantiation of the respiratory burst have been developed through the years, comparisons of the accuracy and reliability to evaluate fish health status among those techniques are scarce.

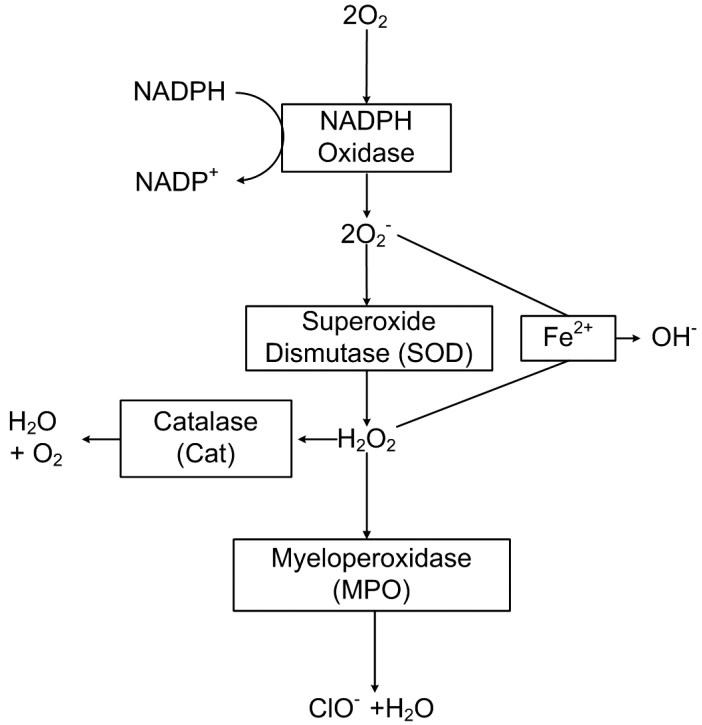


Figure 1. Schematic representation of the Respiratory burst main products. The membrane-associated enzyme nicotinamide adenine dinucleotide phosphate (NADPH) catalyzes the reduction of molecular oxygen (O_2) to superoxide anion (O_2^-), using NADPH as the electron donor. Further reduction of oxygen produces hydrogen peroxide (H_2O_2), which occurs either as a spontaneous dismutation, or as a catalyzed reaction by a family of enzymes called Superoxide dismutase (SOD). Additional reactions of O_2^- and H_2O_2 may lead to the formation of hydroxyl radicals (OH^-), especially in the presence of iron through the Fenton or Haber-Weiss reactions. The interaction of H_2O_2 with the enzyme myeloperoxidase (MPO) can produce hypochlorous acid and other toxic metabolities H_2O_2 is dismutated to water and molecular oxygen by the enzyme catalase.

To date, several methodologies for the measurement of respiratory burst have been described. Initially, Babior et al. (1973) assessed extracellular O2- based on its capability to reduce ferricytochrome c, reading absorbance at 550 nm. The main disadvantage of this methodology was its limitation to follow the kinetics of the reaction. This restrain was overcome by Cohen and Chovaniec (1978) by introducing the continuous recording of absorbance in a cell suspension, however both methods require large amounts of cells (≈2.5x106 cells/well) and reagents (≈950 µl/well) [19, 25-27]. In parallel, Root et al. (1975) formulated a new methodology for the calculation

of respiratory burst produced by human granulocytes; in this procedure the loss of fluorescence of scopoletin (7-OH-6-methoxycoumarin), a natural compound found in the root of plants in the genus *Scopolia*), was evaluated after exposure of H_2O_2 in the presence of horseradish peroxidase (HRP). This technique provided high detection sensitivity (as little as 0.2 nmoles H_2O_2/ml), but real-time measurements remained problematic due to the rapid diminution of scopoletin concentration in the samples. Furthermore, the technique cannot easily be applied to adherent cells, since it required the establishment of the cultures in flying coverslips which are then placed in the spectrofluorometer cuvette in a certain standard position [28, 29]. Pick and Keisari (1980) and Pick and Mizel (1981) established two detection methods based on the HRP-dependent oxidation of phenol sulfonephthalein (phenol red), and a combination of the phenol red and cytochrome c assay, respectively. These methodologies allowed them to measure respiratory burst in macrophage cultures of guinea pigs. However, the sensitivity of the H2O2 detection was reduced to 1 nmoles/ml [19, 29]. The most successful alternative was developed by Baehner and Nathan (1968) who introduced the use of nitroblue tetrazolium (NBT) in the detection of respiratory burst [30]. The NBT assay protocol has been optimized over the years but its principle has remained the same [17, 31-37]. NBT is a yellow, water soluble substance which is internalized by phagocytes, and then reduced intracellularly to formazan during the respiratory burst. For quantitation, the cell membrane is disrupted, the formazan is dissolved in KOH and the absorbance is read from 509 to 690 nm [30, 31, 33, 34, 37]. NBT has perhaps become the most popular method for monitoring biological responses to various stimuli through their influence on the respiratory burst [38]. However, inconveniences associated with the NBT assay such as the impossibility to measure real-time during the respiratory burst process and its laborious protocol which increases the risk of pipetting errors, therefore decreasing accuracy, have remained an issue.

Allen et al. described a different approach for the detection of respiratory burst for human polymorphonuclear leukocytes already in 1972. In this study, the authors describe the occurrence of electronically excited states during the production and transformation of free radicals in the respiratory burst. Furthermore, they observed that after electron relaxation to their initial ground state, energy was release in form of photons. This process is known now as native chemiluminescence and can be amplified for its detection using luminol [39, 40]. Different protocols for the luminol amplification of radical production have been used through the years in different species [41-44]. This paper, for the first time, compares the popular NBT method with the native chemiluminescence amplification method for use in carp (*Cyprinus carpio*). Using β-glucans to induce a respiratory burst response in head kidney leukocytes, the accuracy, sensitivity and adaptability of both methodologies are examined and compared, and their use to quantitate fish health status is discussed.

MATERIALS AND METHODS

Fish

European Common carp (*Cyprinus carpio carpio*) were obtained from the central fish facility 'De Haar-vissen' (Wageningen, The Netherlands). R3xR8 carp are the offspring of a cross between fish of Hungarian origin (R8 strain) and the Polish origin (R3) [45]. The fish used ranged between 50 to 100 g and were kept at 23°C (±1°C) with 12:12 h light: dark photoperiod.

Preparation of head kidney cell suspensions

During this study four different head kidney cell suspensions were used, they are referred

to throughout the paper as: total head kidney cell suspension (t-HK cells), head kidney leukocyte suspension (HK-Leukocyte), macrophage-enriched fraction cell suspension (MQ-f) and neutrophilic granulocyte-enriched fraction cell suspension (NG-f). To isolate the cells carps were euthanized using an overdose of MS-222 (100 mg/l). Fish were bled from the caudal vein, both head-kidneys were excised and placed in a 100 µm nylon cell strainer (BD Falcon). The purification processes used to obtain the different cell suspensions are explained below.

Total head-kidney cell suspensions (t-HK cells) were obtained by pressing the head-kidney with a plunger through the cell strainer, the cells collected were rinsed with phenol red-free Hank's balanced salt solution (HBSS, Sigma-Aldrich, Cat nr. H8264).

Head kidney leukocyte (HK-Leukocyte) suspensions were obtained using a non-continuous percoll (Sigma-Aldrich, Cat nr. P4937) gradient based on the protocol described by Kemenade et al. [46]. Shortly, percoll layers of 1,02 and 1,08 g/ml were used. After 25 minutes centrifugation at 800*g*, the cells present in the 1,02-1,08 interface were collected, washed three times and resuspended with HBSS. Cell viability was assessed by Trypan Blue exclusion (Sigma-Aldrich, Cat nr. T8154).

Macrophage and granulocyte enriched fractions, were obtained using non-continuous percoll gradient with percoll layer concentrations of 1.2; 1.06; 1.07 and 1.083 g/ml. Cells present in the 1.06-1.07 and the 1.07-1.083 interfaces were collected, representing the macrophage-enriched fraction (MQ-f) and the neutrophilic granulocyte-enriched fraction (NG-f) respectively. According to Kemenade et al., the expected yield of macrophages (plus some lymphocytes) for MQ-f is 90%. In addition, the expected yield of granulocytes in the NG-f is 64% [46]. As explained previously, the cell fractions were washed, resuspended in HBSS and cell viability was assessed.

Preparation of β-glucans

During this study two different β-glucans were used: MacroGard®, which is a bakers' yeast extract containing a 60% purified fraction of 1,3/1,6 β-glucan [47], and Zymosan a glucan molecule with repeating glucose units connected by β-1,3 glycosidic linkages. Both β-glucans have shown to trigger respiratory burst in different cell populations and several fish species [48, 49].

Stock solutions of MacroGard® (Biorigin) and Zymosan A (Sigma-Aldrich, Cat nr. Z4250) were prepared in milliQ water (PURELAB Ultra, Elga) at 20 mg/ml and 10 mg/ml respectively and sonicated twice during 30 seconds using power 6 of a Brandson sonifier 250. Subsequently, the sonicated solutions were pasteurized using a thermoblock at 80°C during 20 minutes.

Single point measurement of reactive oxygen species: Nitroblue Tetrazolium (NBT) assay

NBT is a water soluble yellow powder, when added to the phagocytes it is internalized and reduced to formazan during the respiratory burst. For quantitation of the respiratory burst, the formazan is dissolved and its concentration is determined spectrophotometrically. In this study, the NBT analysis was performed as previously described [36]. Briefly, cells were brought to $10X10^6$ cells/ml in RPMI medium (Sigma-Aldrich, Cat nr. R7509). Cell monolayers were prepared in a 96-well tissue culture plate (Corning®, Cat nr. 3300) by applying 100µl/well of the cell suspension. Cells were incubated during 1 hour at 26°C with 5% CO_2, after the incubation time, cells were washed twice with phenol red-free Hank's balanced salt solution (HBSS) and the respiratory burst was induced and measured. In general, 160µl of RPMI containing NBT (1 mg/ml. Sigma-Aldrich, Cat nr. N6876) were added to each well in a plate seeded with t-HK cells. To induce respiratory burst 10µl

of stimulus (either MacroGard® or Zymosan) were used. Plates were incubated at 26°C with 5% CO_2 during 60 minutes. After the incubation time was completed, the plates were washed once with 100 µl of RPMI medium and cells were fixated adding 100% methanol during 3 minutes. Subsequently, two washes in 70% ethanol were made and plates were allowed to air-dry. The reduced formazan was dissolved in 120 µl KOH (2M), and cells were lysed adding 140 µl dimethyl sulphoxide (DMSO Sigma-Aldrich, Cat nr. D2650). The reduction of NBT in each well was then measured at 690 nm with the reference filter 414nm using a multimode microplate reader (Synergy 2, Biotek).

To identify the type of reactive oxygen species measured by the NBT assay, total head kidney cells were first stimulated with 100 µg/ml MacroGard® and then treated with either catalase (Cat, 300 U/ml, Sigma-Aldrich, Cat nr. C1345) to provoke the dismutation of hydrogen peroxide to water and oxygen [24]or with superoxide dismutase (SOD, 250 U/ml, Sigma-Aldrich, Cat nr. S5395) to catalyze the dismutation of superoxide to hydrogen peroxide and oxygen [27].

To monitor the sensitivity and adaptability of the NBT assay to quantitate effects of different doses of β-glucans and different cell suspensions, monolayers of t-HK cells or HK-Leukocytes were seeded in 96-well plates. Cells were stimulated with 10, 50 or 100 µg/ml of MacroGard® or Zymosan and incubated during 30, 45, 90 or 180 minutes at 26°C with 5% CO_2. After the incubation time was completed the dissolution of formazan crystals was measured.

Real-time luminol-enhanced chemiluminescence assay (RT-luminol assay)

The RT-luminol assay is based on a protocol described by Allen et al. and later modified by Verho et al.; this method amplifies the native chemiluminescence produced during the respiratory burst using luminol [39, 50]. In general, white 96-well plates (Corning®, Cat nr. 3917) were prepared containing 40 µl of luminol (10mM, Sigma-Aldrich, Cat nr. A8511) in 0.2 M borate buffer (pH 9.0) and 100 µl of stimulus for the induction of respiratory burst (either MacroGard® or Zymosan), subsequently the volume of the wells was adjusted to 200 µl using HBSS. Head kidney cell suspensions were added at a concentration of $0.5X10^6$ cells/well in all the experiments performed, the final volume of each well was always 300 µl. The chemiluminescence emission of the cells was measured with a luminometer (synergy2, Biotek) every 3 minutes at 26°C. To identify the type of reactive oxygen species measured by the RT-luminol assay, total head kidney cells were first stimulated with 100 µg/ml MacroGard® and then treated with either catalase (Cat, 300 U/ml), to provoke the dismutation of hydrogen peroxide to water and oxygen, [24] or with superoxide dismutase (SOD, 250 U/ml) to catalyze the dismutation of superoxide to hydrogen peroxide and oxygen [27]. The chemiluminescence emission of the cells is expressed as the integral of the relative light units (Max RLU) recorded by the luminometer between 0 and 60 minutes.

To monitor dose-dependent sensitivity of the RT-luminol assay and the adaptability of this method to different cell suspensions, t-HK cells and HK-Leukocytes were stimulated with MacroGard® or Zymosan (10, 50 or 100 µg/ml). The chemiluminescence emissions are expressed as relative light units (RLU) recorded during 210 minutes. To quantitate ROS production by different phagocyte sub-populations, macrophage-enriched fractions (MQ-f) and neutrophilic granulocyte-enriched fractions (NG-f) from carp head kidney, were stimulated with MacroGard® or Zymosan (100 µg/ml). Results are expressed as RLU recorded during 210 minutes.

Statistical analysis

The software GraphPad Prism (version 4.03) was used for statistical work. Statistical comparison was performed by one-way analysis of variance (ANOVA) and futher Bonferroni post-tests $P < 0.05$ was

considered to be statistically significant.

RESULTS

The RT-luminol assay detects reactive oxygen species additional to those detected by the NBT assay

The NBT assay after stimulation of t-HK with MacroGard® showed an increase of oxygen radicals production. Cells treated with catalase did not indicate major changes to the production of oxygen radicals. Conversely, treatment with SOD, showed a markedly reduced magnitude of ROS. As expected, co-treatment with SOD and catalase also decreased of ROS production (see **figure 2A**). The RT-luminol assay showed an increase in the oxygen radicals produced by t-HK cells after stimulation with MacroGard®. Cells treatment with SOD or combination of SOD and catalase markedly decreased ROS production. In addition, treatment with catalase also showed a significant reduction of oxygen radical production (see **figure 2B**).

In conclusion, the NBT assay was shown to detect the production of only superoxide anions, the RT-luminol assay could detect the production of superoxide anions, hydrogen peroxide and related radicals.

Dose-effect sensitivity and adaptability of the methods.

The adaptability of the NBT and RT-luminol assays to different cell groups, and their sensitivity to dose-related changes in the respiratory burst response, were examined following stimulation of t-HK cells and HK-Leukocytes with different β-glucans doses (10, 50 and 100 μg/ml).

Measurements of the respiratory burst response of t-HK and HK-Leukocytes by NBT are plotted in **figure 3A** and **3B** respectively. Following stimulation with β-glucans, a higher oxygen radical production was elicited in HK-Leukocytes than in t-HK cells. Variations in the magnitude of oxygen radical production related to changes of the β-glucan doses were detectable using NBT in both cell groups.

The RT-luminol assay measurements of the respiratory burst response after β-glucan stimulation of t-HK cells and HK-Leukocytes are plotted in **figure 3C** and **figure 3D** respectively. The oxygen radical production elicited by β-glucans in t-HK cells, showed to be higher than the one from HK-Leukocytes. Differences in oxygen radical production due to the β-glucan doses were clearly identified in both cell groups. Furthermore, since the measurements of this method are made continuously, a peak of oxygen radical production was determined 36 post-stimulation for t-HK cells, and 57 minutes post-stimulation for HK-Leukocytes.

Summarizing, both methodologies studied showed the capacity to measure respiratory burst following stimulation with β-glucan. NBT and RT-luminol assay were responsive to changes of the β-glucan doses and correlated in the magnitude of the response. However, only RT-luminol offered measurements at frequent and continual time intervals during the course of the experiments, providing information on the stimulation peaks and the respiratory burst kinetics.

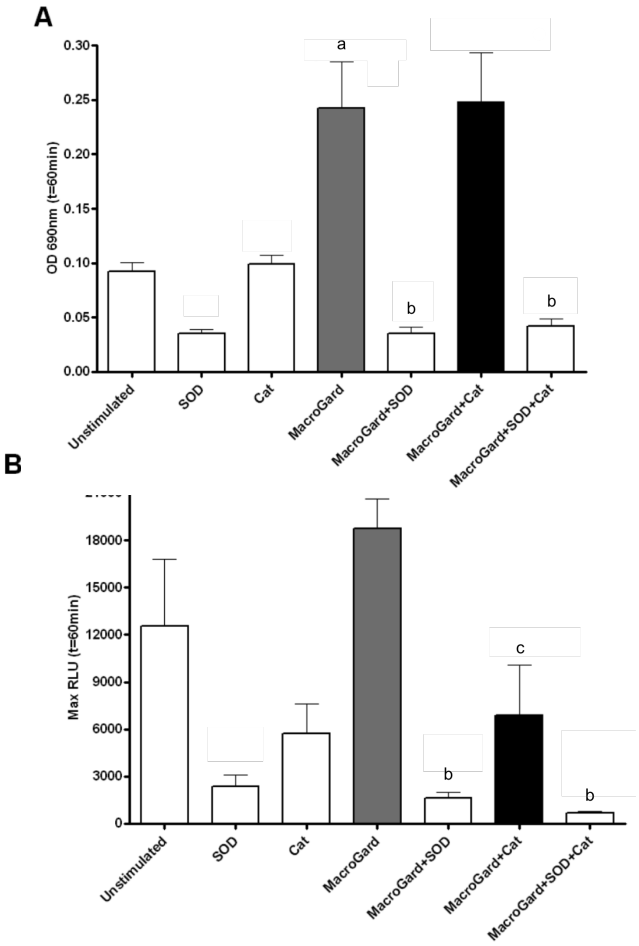
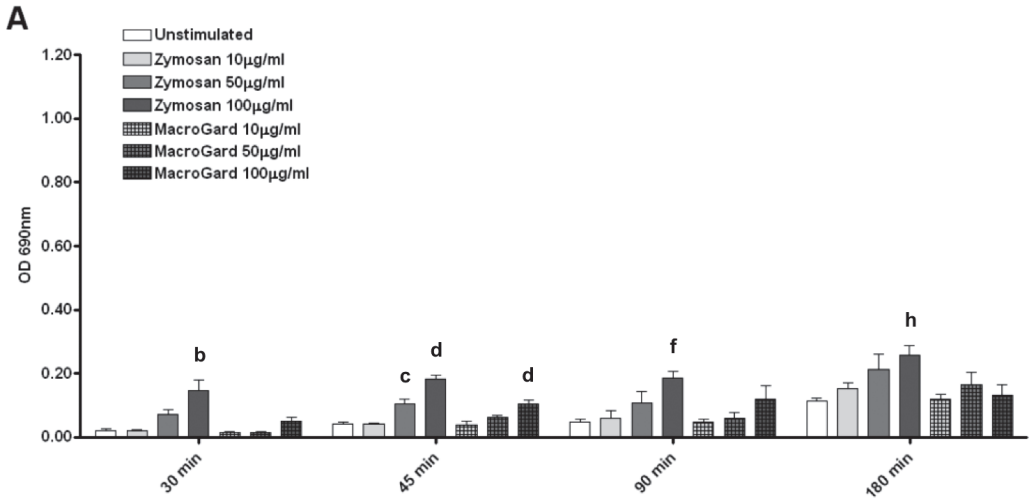


Figure 2. Identification of the oxygen radicals measured by NBT and RT-luminol assay. Comparison of the nitro blue tetrazolium NBT assay (A) and the Real-time luminol assay (B) in relation to the type of oxygen radicals being produce after stimulation of t-HK cells with MacroGard® (100µg/ml) and co-stimulation with SOD (250 U/ml) or catalase (300U/ml). The plots show the mean value of four independent studies for NBT and five for RT-luminol assays. Error bars represent standard error of the mean, a= significant difference to un-stimulated sample, b= significant difference to SOD, c= significant difference to Cat, d= significant difference to MacroGard®, e= significant difference to MacroGard®+SOD, f = significant difference to MacroGard®+Cat, g= significant difference to MacroGard®+SOD = Cat. All the difference a P value <0.05.

RT-luminol assay measurements of respiratory burst response in macrophage enriched and neutrophilic granulocyte -enriched fractions after β-glucan stimulation.

To evaluate the adaptability of the RT-luminol assay to further purified head-kidney phagocyte sub-populations, the respiratory burst response of MQ-f and NG-f was measured after stimulation with β-glucans.

The respiratory burst response of MQ-f and NG-f to β-glucans was detectable and is plotted in **figure 4**. Higher production of oxygen radicals was recorded from MQ-f than from NG-f after stimulation with β-glucans. The results also displayed a minor wave present between 15 and 35 minutes for both cell fractions.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1.20
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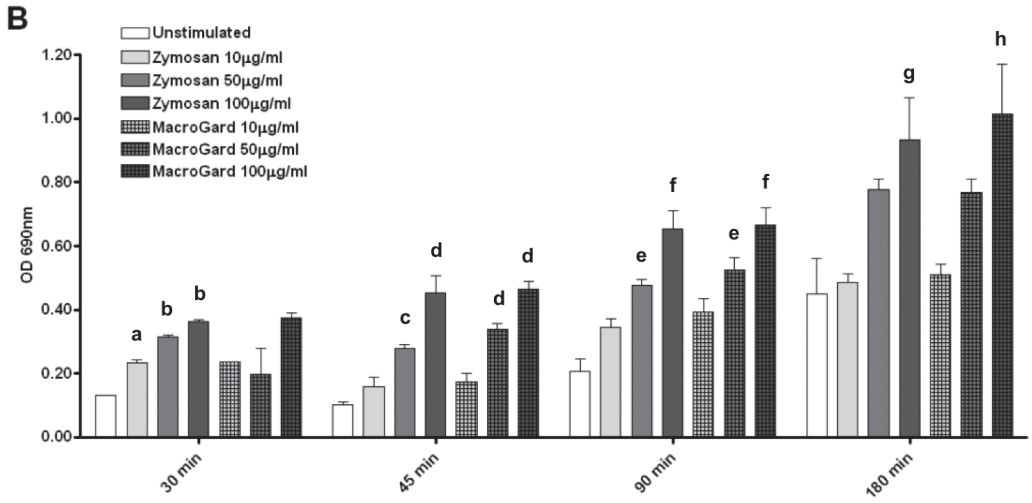
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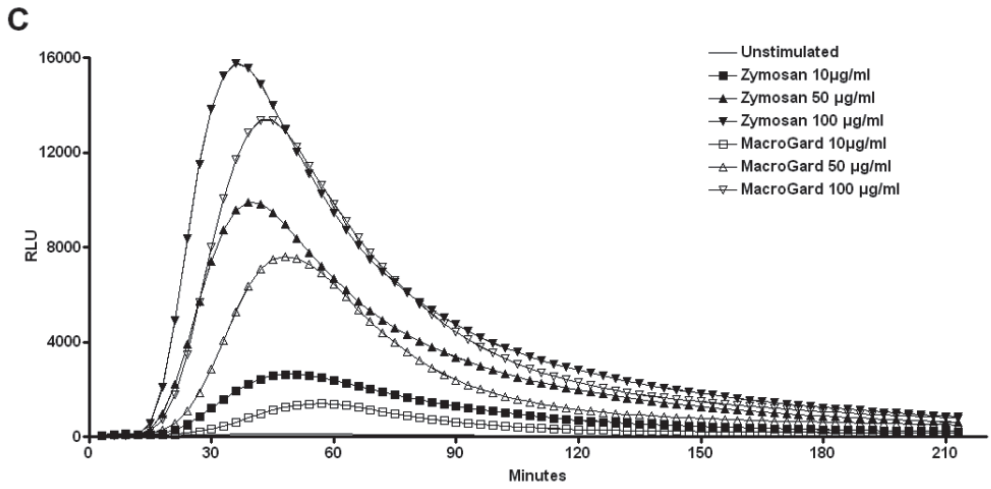
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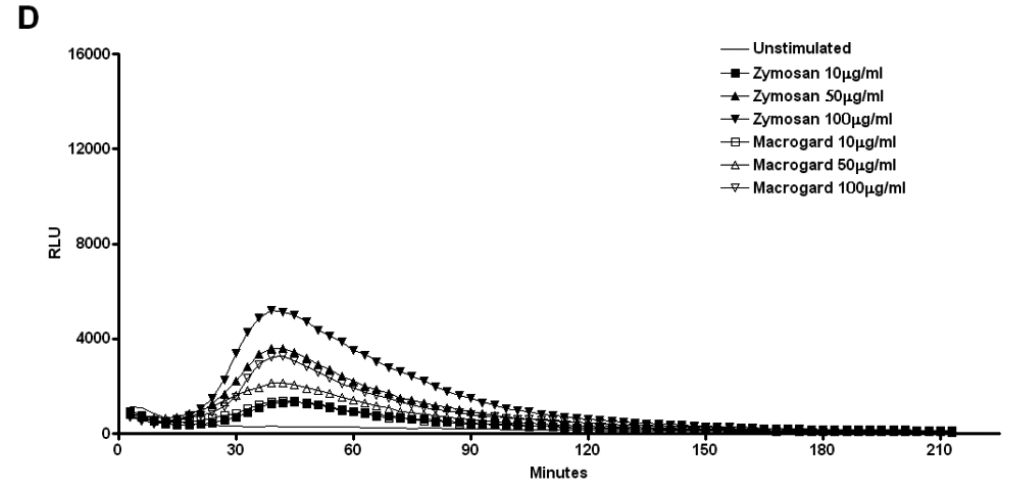

results of (A) t-HK cells and (B) HK-Leukocytes after stimulation with 10, 50 or 100 µg/ml of Zymosan or MacroGard®. NBT was measured at 30, 45, 90 and 180 minutes. The plots show the mean value of four independent studies for all the NBT time points, except for HK-Leukocytes 30 minutes where two independent studies are plotted. Continuous measurements of oxygen radical production by RT-luminol assay are shown in (C) for t-HK cells and (D) for HK-Leukocytes after stimulation with 10, 50 or 100 µg/ml of Zymosan or MacroGard®, chemiluminescence was monitored every three minutes during 210 minutes. The plots show the mean value of four independent RT-luminol assays in t-HK cells and 3 in HK-Leukocytes.

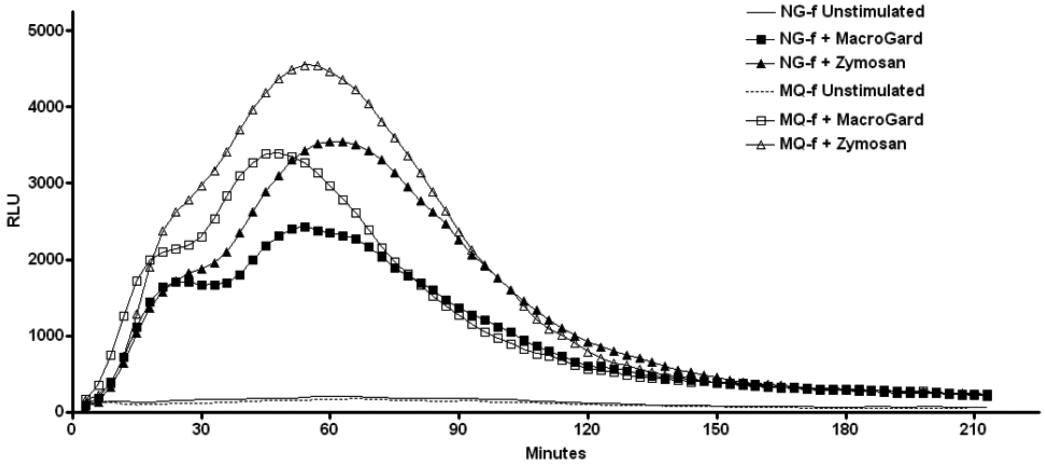

Figure 4. RT-luminol assay on further purified head kidney fractions. Comparison of the respiratory burst measurements by RT-luminol assay in two different cell fractions of carp head kidney cells. Macrophage-enriched fraction (MQ-f) and neutrophil granulocyte-enriched fraction (NG-f) were stimulated with either MacroGard® 100 µg/ml, Zymosan 100 µg/ml or left untreated. Luminescence was continuously monitored for 210 minutes. The graph shows the mean value of two independent studies.

DISCUSSION

The present study compares the use of NBT and RT-luminol assays for the assessment of oxygen radical production in carp after stimulation with β-glucans, a PAMP known to stimulate the respiratory burst in fish and mammalian systems [48, 51]. Both methods were able to detect the production of oxygen radicals after stimulation with MacroGard® and Zymosan, and allowed the detection of dose-dependent changes on the respiratory burst magnitude. On this basis, both methods can be used not only to study the respiratory burst responses during microbicidal events, but also to study the effect of immune-stimulants, vaccines and pharmacological agents on the immune-system [50, 52, 53]. However, one of the major differences between the methods compared in this study consisted on the possibility to follow the kinetics of the respiratory burst response. Since the NBT assay is based on the intracellular reduction of the nitroblue tetrazolium salt by the superoxide anion (O_2^-) [31], the cells had to be lysed to perform the measurements, becoming a one time-point measurement method, and expressed by the accumulative value of oxygen radicals produced intracellularly during a set period of time. On the other hand, the RT luminol assay amplifies the native chemiluminescence produced during the respiratory burst process at any given instant, this allows the tracking of the reaction kinetics, making possible the identification of oxygen radical production peaks. Furthermore, due to its chemical structure luminol can cross biological membranes, allowing the detection of extracellular and intracellular production of oxygen radicals [54]. Although in this study several time-points were measured using NBT to produce a kinetic profile of the respiratory burst, due to the accumulative nature of its data, peaks of oxygen radical production could not be identified. Besides, the amount of cells required for the experiment was at least 4 times higher than the one used with the RT-luminol assay, the lab-work was considerable more time consuming and the increase of sample manipulations increases the risk of pipetting mistakes.

The reduction in the magnitude of the respiratory burst response after addition of SOD, a scavenger of superoxide anion (O_2^-) [27], showed that NBT and RT-luminol assay detect O_2^-. The use of catalase, an enzyme which catalyses the dismutation of hydrogen peroxide to water and oxygen [24], evidenced the incapability of NBT to detect other radicals than O_2^-, suggesting the NBT as a semi quantitative method. On the contrary, the RT-luminol assay successfully detected the variation on the respiratory burst from carp head-kidney cells after the addition of catalase, therefore detecting hydrogen peroxide and related radicals such as hypochlorous acid and hydroxyl radical. The type of radicals being measured by this two methods had not been compared using fish cells, however the results of this study are in agreement with Cheson et al., [55] who postulated that, the light emitted by human phagocytosing granulocytes came from the oxidative capacity of any of the oxidizing agents released by the cells, as well as with the work of Schopf et al., [37] in human monocytes and polymorphonuclear leukocytes, who attributed the luminescence directly to the oxidizing properties of superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals.

Both, the NBT and the RT-luminol assays measured the respiratory burst response triggered by β-glucans independently of the cell suspension used, indicating the adaptability of the methods to different degrees of cell purification. However, higher values of respiratory burst response of HK-Leukocytes were measured by NBT when compared to t-HK cells, such difference in the magnitude of the response might be related to the activation of the leukocytes due to the purification process. Interestingly the RT-luminol assay evidenced higher production of oxygen radicals of t-HK cells when compared to HK-Leukocytes, this augment in the respiratory burst response of t-HK cells might be explained by the presence

of damage associated molecular patterns (DAMPs). Different studies have examined the ability of DAMPs to trigger immune responses [40]and their synergism with pathogen-associated molecular patterns (PAMPs) [56]. Furthermore, non-immune cells such as epithelial cells, fibroblasts and erythrocytes have been shown to produce reactive oxygen species [57-59], and to interact with different cell types to enhance the magnitude of the respiratory burst response [60, 61]. Since the t-HK cell suspensions used in this study did not have any purification process, it contained healthy head kidney cells (not only leukocytes) mixed with necrotic, damaged cells, and debris from the tissue. The synergistic effect of PAMPs and DAMPs, added to the collaboration of different cell types in the cell suspension could cause the higher respiratory burst response evidenced with the RT-luminol assay. The fact that t-HK cell suspensions did not show an increase of the respiratory burst response when measured by NBT could be explained by the limitation of this method to detect hydrogen peroxide. Cell proliferation and tissue regeneration processes have been linked to the presence of H_2O_2 in mammalian models [62, 63], furthermore a gradient of hydrogen peroxide has been reported in zebra fish after tissue injure [8]. Therefore, it would be logical to think that the DAMPs present in the cell suspensions, could trigger the production of messengers for tissue regeneration such as hydrogen peroxide, which was detected by RT-luminol assay but not by NBT.

Finally, the minor biphasic response evidenced during the oxygen radical production of MQ-f and NG-f after stimulation with β-glucans, could be associated to the adhesion and ingestion phases of the phagocytosis. Nikoskelainen et.al [64] described these two phases during the respiratory burst induced by *Aeromonas salmonicida* in rainbow trout phagocytes, although they claimed that those phases cannot be distinguished in rainbow trout when the number of head kidney cells exceeds $5x10^4$ cells/well because the peaks are merged. The fact that a similar response was observed from carp MQ-f and NG-f even though the cell number used in the experiment was higher ($5x10^5$ cells/well) may be due to the difference in fish species, since it has already been discussed the occurrence of variations among fish immune responses [1, 65].

CONCLUSIONS

Both of the methods compared during this study, showed the capacity to detect and measure the respiratory burst response of carp head kidney cells after stimulation with β-glucans, therefore constitute an indicator of the general fish health status. However, only the RT-luminol assay allowed the tracking of kinetics during the respiratory burst response, offering information about peaks of oxygen radical production. Furthermore, only the RT-luminol assay detected the production of hydrogen peroxide and oxygen related radicals, becoming an important tool to monitor production of oxygen radicals involved in tissue regeneration processes. The RT-luminol assay also proved to be a simple and fast protocol which reduces sample manipulation, requires fewer amounts of cells per experiment, and can be used to evaluate the respiratory burst responses from mixed cell populations to highly purified subpopulations.

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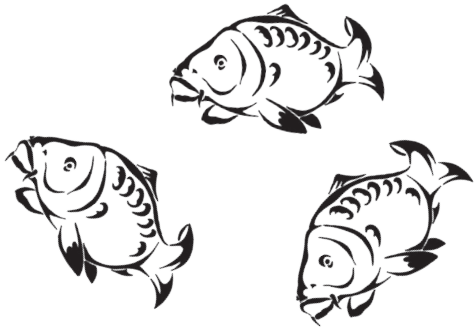

Chapter 3

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

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ABSTRACT

β-glucans are glucose polymers that are found in the cell walls of plants, bacteria, certain fungi, mushrooms and the cell wall of baker's yeast. In mammals, myeloid cells express several receptors capable of recognizing β-glucans, with the C-type lectin receptor dectin-1 in conjunction with Toll-like receptor 2 (TLR2), considered key receptors for recognition of β-glucan. In our studies to determine the possible involvement of these receptors on carp macrophages a range of sources of β-glucans were utilised including particulate β-glucan preparations of baker's yeast such as zymosan, which is composed of insoluble β-glucan and mannan, and MacroGard®, a β-glucan-based feed ingredient for farmed animals including several fish species. Both preparations were confirmed TLR2 ligands by measuring activation of HEK293 cells transfected with human TLR2 and CD14, co-transfected with a secreted embryonic alkaline phosphatase (SEAP) reporter gene. In addition, dectin-1-specific ligands in mammals i.e. zymosan treated to deplete the TLR-stimulating properties and curdlan, were monitored for their effects on carp macrophages by measuring reactive oxygen and nitrogen radicals production, as well as cytokine gene expression by real time PCR. Results clearly show the ability of carp macrophages to strongly react to particulate β-glucans with an increase in the production of reactive oxygen and nitrogen radicals and increase in cytokine gene expression, in particular *il-1β, il-6* and *il-11*. We identified carp *il-6*, that was previously unknown. In addition, carp macrophages are less, but not unresponsive to selective dectin-1 agonists, suggesting recognition of β-glucans by multiple pattern recognition receptors that could include TLR but also non-TLR receptors. Candidate receptors for recognition of β-glucans are discussed.

INTRODUCTION

β-glucans, glucose polymers that are found in the cell walls of fungi such as *Candida albicans* and baker's yeast *Saccharomyces cerevisiae*, possess differences in molecular weights and in degree of branching. For example, β-glucan can occur in forms containing different, often repeating branches with β-D-glucosidic linkages at position three or six. Branched 1,3/1,6 β-glucan variants such as MacroGard® are frequently used as a feed ingredient for farmed animals including several fish species [1, 2]. Zymosan, obtained from baker's yeast cell walls, is composed of insoluble β-glucan and mannan, and has been utilized in numerous experiments owing to its ability to activate macrophages [3, 4]. In purified form, exogenous β-glucan can stimulate and enhance immune function [5] and improve protection against infection with a variety of pathogens [6, 7]. However, despite clear indications for immune-modulating properties of β-glucans on fish cells [8, 9], the receptors on fish cells that can recognize the β-glucan have not been identified.

Both Toll-like receptors (TLRs) as well as C-type lectin receptors (CLRs) are considered crucially important for optimal recognition of pathogen-derived molecules such as β-glucan. These receptors often complement each other when binding ligands and activating subsequent downstream intracellular responses. One CLR in particular, known as dectin-1, is the primary membrane-bound PRR for exogenous β-glucan [10-12] acting in conjunction with TLR2, at least in murine and human dendritic cells [13]. Phagocytosis of particles such as zymosan by for example dectin-1, induces the activation of different mechanisms including reactive oxygen species, nitric oxide and lytic enzymes in the acidic enviroment of the phagolysosome in order to destroy the phagocytosed particles. TLRs which are thought to be recruited to the phagosomes detect zymosan

and induce the production of pro-inflammatory cytokines, but are not thought to be responsible for phagocytosis of zymosan particles. Treatment of zymosan with hot alkali or organic solvents abrogates the TLR-dependent response leaving 'depleted zymosan' that activates cells via dectin-1 but not TLR2. Similarly, curdlan, a high molecular weight linear polymer consisting of β-1,3-linked glucose residues, is a water-insoluble polysaccharide recognized in mammals by the dectin-1 receptor only [14]. The interplay between phagocytic receptors such as the CLR superfamily member dectin-1, the complement receptor or members of the scavanger receptor family, and sensing receptors such as TLRs has been studied in detail in myeloid cells of humans and mice [15, 16], but not fish. Not only is the presence of the Dectin-1 receptor limited to mammalian genomes [17], there is a general lack of knowledge on the receptors on myeloid cells of fish that can phagocytose and/or sense β-glucans.

We used head kidney leukocytes and head kidney-derived carp macrophages to study induction of reactive oxygen species (ROS), nitric oxide (NO) and modulation of cytokine gene expression on exposure to different preparations of β-glucan. Zymosan depleted of TLR-stimulating activity and curdlan, both dectin-1-specific ligands, were compared to stimulation induced by untreated zymosan and branched 1,3/1,6 β-glucan (MacroGard®). The specificity of the applied ligands was confirmed by measuring activation of HEK293 cells transfected with human TLR2 and CD14, co-transfected with a secreted embryonic alkaline phosphatase (SEAP) reporter gene. Both zymosan and MacroGard®, but not alkali-depleted zymosan nor curdlan activated the transfected HEK293 cells. In addition, carp macrophages reacted with a higher oxidative burst and nitric oxide response to the particulate β-glucans zymosan and MacroGard® than to the selective dectin-1 agonists i.e. depleted-zymosan and curdlan. Furthermore, zymosan induced a higher *il-1β*, *il-6* and *il-11* cytokine gene expression in carp macrophages than did depleted-zymosan. Also curdlan induced a high gene expression of *il-1β* and *il-11*, which suggests that these cytokines may be good indicators of macrophage activation by β-glucans. Our data suggest recognition of β-glucans by multiple pattern recognition receptors on carp macrophages.

MATERIALS AND METHODS

Fish

European common carp (*Cyprinus carpio carpio*) were reared in the central fish facility of Wageningen University, The Netherlands, at 23±2°C in recirculating UV-treated tap water and fed pelleted dry food devoid of additional β-glucans (Ssniff, Soest, Germany) daily. R3 x R8 heterozygous carp are the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) [18]. Carp were between 9 and 11 months old at sampling. All studies were performed with approval from the Animal Experimental Committee of Wageningen University.

β-glucan preparations

Zymosan (insoluble cell wall of *Saccharomyces cerevisae*; tlrl-zyn), depleted-zymosan (hot alkali treated cell wall from *Saccharomyces cerevisae*, tlrl-dzn) and curdlan (a high molecular weight linear polymer consisting of β-1-3-linked glucose residues from *Alcaligenes feacalis*, tlrl-cura) were all purchased from InvivoGen (Cayla SAS, France) and dissolved in milliQ water as per manufacturer's instructions. Laminarin, a linear β-glucan from *Laminaria digitata*, was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in milliQ water as per manufacturer's instructions. MacroGard®, a cell wall preparation of *Saccharomyces cerevisae* comprising 60% β-glucan, was purchased from

Biorigin (São Paulo, Brazil). MacroGard® was suspended in milliQ water, sonicated twice for 30 sec at power 6 with a Branson sonifier 250 (Danbury, CT, USA), and then pasteurized at 80°C for 20 minutes.

Isolation of head kidney leukocytes (HKL)

Fish were euthanized with 0.3 g/l Tricaine Methane Sulfonate (TMS) (Crescent Research Chemicals, Phoenix, USA) in aquarium water buffered with 0.6 g/l sodium bicarbonate and exsanguinated via the caudal vein. Head kidneys were removed aseptically, gently disrupted through a 100 µm sterile nylon mesh and the cell suspension washed with RPMI 1640 medium (Invitrogen, CA, USA) adjusted to 280 mOsmol/kg and supplemented with 1.5% (v/v) heat inactivated-pooled carp serum, 2 mM L-glutamine, 100 U/ml penicillin G and 50 mg/ml streptomycin sulphate (cRPMI). Cell separation was performed with a non-continuous Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) gradient as described previously [19]. Briefly, to obtain the Head Kidney Leucocytes (HKL) suspension, Percoll layers of 1,02 and 1,08 g/ml were first collected as two separate layers and then mixed, washed three times and resuspended in cRPMI. Cell viability was assessed by Trypan blue exclusion.

Head kidney-derived macrophages

Carp head kidney-derived macrophages were cultured as described previously [20]**.** Head kidneys were gently disrupted through a 100 µm sterile nylon mesh and rinsed with homogenization buffer comprising incomplete-NMGFL-15 medium containing 50 U/ml penicillin, 50 µg/ml streptomycin, and 20 U/ml heparin (Leo Pharma BV, Breda, The Netherlands) [21].

Cell suspensions were layered on a 1.071 g/ml Percoll suspension and centrifuged at 450x *g* for 25 min at 4˚C without brakes. Cells at the medium/Percoll interface were removed and washed twice. Cell cultures were initiated by seeding $1.75x10^7$ cells in a 75 cm^2 culture flask containing 20 ml complete-NMGFL-15 medium comprising incomplete-NMGFL-15 medium supplemented with 5% heat-inactivated pooled carp serum and 10% bovine calf serum (Invitrogen, Breda, The Netherlands). Cells were incubated at 27˚C and head kidney-derived macrophages, named macrophages throughout the manuscript, were harvested after 6 days by placing the flask on ice for 10 min and collecting adherent cells by gentle scraping.

Real time luminol-enhanced chemiluminescence (RT-luminol assay)

To detect the native chemiluminescence produced during activation of cells a real time luminol-enhanced chemiluminescence assay was performed as described before [22-24]. Briefly, white 96-well plates (Corning®, Cat nr. 3917) were prepared with 40 µl luminol (10 mM, Sigma-Aldrich, Cat nr. A8511) in 0.2 M borate buffer (pH 9.0) and 100 µl volume of β-glucan stimulus. Cell suspensions were added at concentration of $1.0x10^6$ cells/well for HKL and $0.5x10^6$ cells/well for macrophages up to a final volume of 300 µl. Chemiluminescence emission was measured with a multimode microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA) every 3 minutes at 27°C and expressed as integral of relative light units (Max RLU) recorded between 0 and 150 minutes.

NitroBlue Tetrazolium (NBT) assay

To measure oxygen radical production due to the respiratory burst in cells activated by β-glucan, a NitroBlue Tetrazolium (NBT, Sigma-Aldrich, Cat nr. N6876) assay was performed as previously described for carp leukocytes [19]. HKL or macrophages were seeded at concentration

of $1.0x10^6$ cells/well and $0.5x10^6$ cells/well respectively in 96-well tissue culture plates (Corning®, Cat nr. 3300), and incubated for 1 h at 27°C with 5% CO_2. Subsequently, cells were stimulated with β-glucan in presence of NBT at concentration of 1 mg/ml and incubated at 27°C with 5% CO_2 for 90 minutes. Reduced formazan was dissolved in KOH (2M) and 140 µl dimethyl sulphoxide (DMSO, Sigma-Aldrich, Cat nr. D2650). The reduction of NBT in each well was measured at 690 nm with the reference filter 414 nm using a multimode microplate reader (SpectraMax M5, Molecular Devices).

Nitric oxide assay

Nitrite production by carp HKL or macrophages after stimulation with β-glucans was measured as described before [25]. A volume of 75 µl of cell culture supernatant, 100 µl of 1% sulphanilamide in 2.5% (v/v) phosphoric acid and 100 µl of 0.1% (w/v) N-naphthly-ethylenediamine in 2.5% (v/v) phosphoric acid were added in a 96-well flat bottom plate. The absorbance was read at 540 nm (with 690 nm as a reference) and nitrite concentration (µM) was calculated by comparison with a sodium nitrite standard curve.

RNA isolation and cDNA synthesis

Total RNA was isolated from carp macrophages using the RNeasy Mini Kit according to the Manufacturer's (Qiagen) instructions, including on-column treatment with RNAse-free DNase and stored at -80˚C. Prior to cDNA synthesis from 0.25-1 µg total RNA, a second DNase treatment was performed using DNase I, Amplification Grade (Invitrogen). Synthesis of cDNA was performed using random primers (300 ng) and Superscript™ II First Strand Synthesis for RT-PCR (Invitrogen). A non-reverse transcriptase control was included for each sample. cDNA samples were further diluted in nuclease-free water prior to real time PCR analysis.

Gene expression analysis by real-time quantitative PCR (RT-qPCR)

Immune gene expression in carp macrophages following stimulation with different β-glucan preparations was analysed with a Rotor-Gene™ 6000 (Corbett Research) using Brilliant® SYBR® Green (Stratagene) as detection chemistry for real time quantitative PCR analysis, as previously described [26]. Primers used for RT-qPCR (see Table 1) were designed with Primer Express software.

Fluorescence data from RT-qPCR experiments were analysed using Rotor-Gene Analysis software version 1.7. The cycle threshold C_t for each sample and the reaction efficiencies (E) for each primer set were obtained upon Comparative Quantitation Analisys from Rotor Gene software. The relative expression ratio (R) of a target gene was calculated based on the E and the C_t deviation of sample versus control, and expressed relative to the S11 protein of the 40S subunit as reference gene.

Activating properties of β-glucan preparations for human TLR2

All β-glucan preparations were tested for their TLR-activating properties by measuring activation of HEK293 cells transfected with human TLR2 and CD14, co-transfected with a secreted embryonic alkaline phosphatase (SEAP) reporter gene (HEK-Blue™-hTLR2; InvivoGen, Cayla SAS, France). HEK-Blue™-hTLR2 stably express an optimized alkaline phosphatase gene engineered to be secreted and placed under the control of a promoter inducible by several transcription factors, such as NF-κB and alkaline phosphatase-1. HEK-Blue™-hTLR2 were seeded at a concentration of 2×10^5 cells/ml in 96-well flat-bottom tissue culture plates and incubated with Dulbecco's modified

Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO2 for 3 days. Cells were stimulated with different β-glucan preparations at a concentration of 100 µg/ml, using as positive control purified lipotheichoic acid from *Staphylococcus aureus* (tlrl-psLTA; InvivoGen). After 24 h, activation was measured by determining the levels of SEAP in the supernatant using QUANTI-Blue™ (InvivoGen), according to the manufacturer's instructions.

Table 1. Primers used for Real-Time quantitative PCR

Primer	Sequence (5'-3')	GenBank Accession No.
Il-1β Fw	**AAGGAGGCCAGTGGCTCTGT**	**AJ245635**
Il-1β Rv	**CCTGAAGAAGAGGAGGAGGCTGTCA**	
Il-6a Fw	**CAGATAGCGGACGGAGGGGC**	**KC858890**
Il-6a Rv	**GCGGGTCTCTTCGTGTCTT**	
Il-6b Fw	**GGCGTATGAAGGAGCGAAGA**	**KC858889**
Il-6b Rv	**ATCTGACCGATAGAGGAGCG**	
cxcl8_l2 Fw	**TCACTTCACTGGTGTTGCTC**	**AB470924**
cxcl8_l2 Rv	**GGAATTGCTGGCTCTGAATG**	
Il-10 Fw	**CGCCAGCATAAAGAACTCGT**	**AB110780**
Il-10 Rv	**TGCCAAATACTGCTCGATGT**	
Il-11 Fw	**CAGCAGCACAGCTCAGTACCA**	**AJ632159**
Il-11 Rv	**AGCCTCTGCTCGGGTCATCT**	
Il12-p35 Fw	**TGCTTCTCTGTCTCTGTGATGGA**	**AJ580354**
Il12-p35 Rv	**CACAGCTGCAGTCGTTCTTGA**	
Il12-p40a Fw	**GAGCGCATCAACCTGACCAT**	**AJ621425**
Il12-p40a Rv	**AGGATCGTGGATATGTGACCTCTAC**	
Il12-p40b Fw	**TCTTGCACCGCAAGAAACTATG**	**AJ628699**
Il12-p40b Rv	**TGCAGTTGATGAGACTAGAGTTTCG**	
Il12-p40c Fw	**TGGTGATAAGGTTCACCCTTCTC**	**AJ628700**
Il12-p40c Rv	**TATCTGTTACAGGTCAGGGTAACG**	
tnf-α1 Fw	**GAGCTTCACGAGGACTAATAGACAGT**	**AJ311800**
tnf-α1 Rv	**CTGCGGTAAGGGCAGCAATC**	
tnf-α2 Fw	**CGGCACGAGGAAGAAACCGAGC**	**AJ311801**
tnf-α2 Rv	**CATCGTTGTGTCTGTTAGTAAGTTC**	
ifnγ1 Fw	**TGCACTTGTCAGTCTCTGCT**	**AM261214**
ifnγ1 RV	**TGTACTTGTCCCTCAGTATTT**	
40s Fw	**CCGTGGGTGACATCGTTACA**	**AB012087**
40s Rv	**TCAGGACATTGAACCTCACTGTCT**	

Bioinformatics interleukin (il)-6

Interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpa (TNFα) are typical examples of multifunctional cytokines involved in the regulation of immune responses with overlapping

functions but also with their own characteristic properties. Previous to our study, the sequence of the IL-6 gene had not been described for common carp. Carp *il-6* (accession numbers *il-6a* KC858890 and *il-6b* KC858889) was identified blasting the zebrafish *il-6* sequence [27] against the draft carp genome (Bioproject PRJNA73579) [28]. The nucleotide sequence was translated using the ExPASy translate tool (http://us.expasy.org/tools/dna.html) and aligned with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The signal peptide cleavage site was predicted with SignalIP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) [29]. Known protein domains were defined using Interpro (http://www.ebi.ac.uk/interpro/) and SMART (http://smart.embl-heidelberg.de/). The secondary structure of the protein was predicted using PSIPRED Server (http://bioinf.cs.ucl.ac.uk/psipred/) [30]. A multiple sequence alignment with other IL-6 sequences *Homo sapiens* (NP_000591), *Mus musculus* (NP_112445), *Oncorhynchus mykiss* (NP_001118129) and *Danio rerio* (JN698962) was generated using ClustalW2.

Statistical Analysis

Significant ($P<0.05$) differences in the production of oxygen, nitrogen radicals or gene expression induced by different β-glucan preparations were determined by a two-way ANOVA, followed by Bonferroni post-hoc tests. For the gene expression analysis, relative expression ratios were first transformed to LN(R) values. Statistical analysis was performed using GraphPad Prism (version 5.03) software.

RESULTS

TLR-activating properties of the different β-glucan preparations

We used a human cell line stably transfected with TLR2 (HEK-Blue-TLR2) to study the ligand specifity of our β-glucan preparations. Whilst HEK-Blue-TLR2 cells were activated with MacroGard® and with zymosan, as shown by spectrophotometric analysis of a blue colour linked with alkaline phosphatase activity (**Figure 1**), they were not affected by treatment with depleted zymosan (DPZ), curdlan or laminarin. This confirmed the assumption that treatment of zymosan with hot alkali or organic solvents abrogates the TLR2-dependent response. It also highlighted that the high molecular weight linear polymer curdlan, an assumed ligand for the dectin-1 receptor, could not activate HEK-Blue cells via TLR2. Neither could the soluble β-glucan laminarin activate HEK-Blue cells via TLR2. These data show that depleted zymosan and curdlan as β-glucan preparations are particularly valuable for studies on the specificity of receptors on fish cells. MacroGard® clearly activated HEK-Blue-TLR2 cells, indicating this branched 1,3/1,6 β-glucan could be recognized by at least human TLR2.

Particulate β-glucans induce the production of oxygen and nitrogen radicals in head kidney leukocytes

Carp head kidney leukocytes (HKL) could be activated by β-glucans to produce reactive oxygen species detected by both, the RT-luminol assay (**Figure 2A**) and reduction of the tetrazolium salt NBT (**Figure 2B**). Whereas the NBT assay detects the production of only superoxide anions, the real-time luminol-enhanced assay detects the production of both superoxide anions and hydrogen peroxide [22]. The greatest increase in the production of oxygen radicals was observed after stimulation with MacroGard® and zymosan. The maximum response, expressed as relative light units in the RT-luminol assay, was observed approximately 30 min after application of the β-glucan

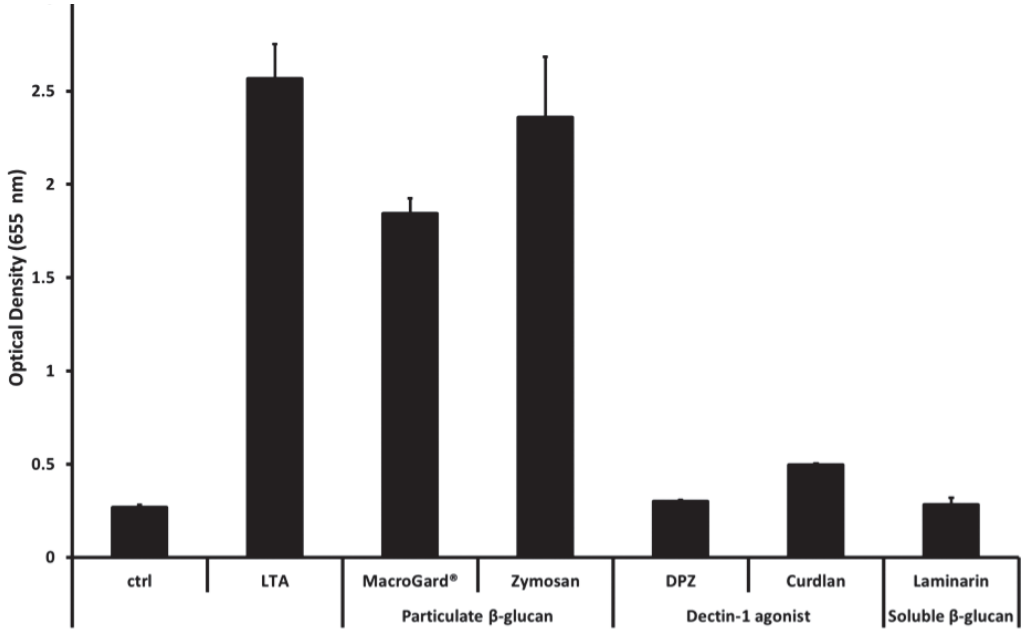

Figure 1. Activation of a human cell line stably transfected with TLR2 by different β-glucan preparations. HEK-Blue-TLR2 cells were stimulated with the TLR2 ligand lipoteichoic acid LTA (50 μg/ml) as positive control or with different preparations of β-glucans, including MacroGard®, zymosan, depleted-zymosan (DPZ), curdlan and laminarin (all 100 μg/ml). Cells were seeded at $2x10^5$ cells/well and optical density was read at 655 nm after 24 h. Values represent mean +/- S.D. of triplicate wells of one representative experiment out of three independent experiments with similar

results

preparations. This response falls well within the total period of 90 minutes allowed for NBT reduction. The soluble β-glucan, laminarin, induced a much lower response in head kidney leukocytes, and the dectin-1 specific ligands i.e. depleted zymosan and curdlan, also induced lower production of oxygen radicals. Interestingly, MacroGard® and zymosan also induced the greatest production of nitric oxide as determined by measuring nitrite, in comparison with the other β-glucan preparations (**Figure 2C**). For example, depleted zymosan induced a lower nitric oxide response than untreated zymosan, and curdlan and laminarin induced the lowest NO response (**Figure 2C**). This suggests that carp leukocytes can be activated by the particulate β-glucans MacroGard® and zymosan and are less responsive to selective dectin-1 agonists.

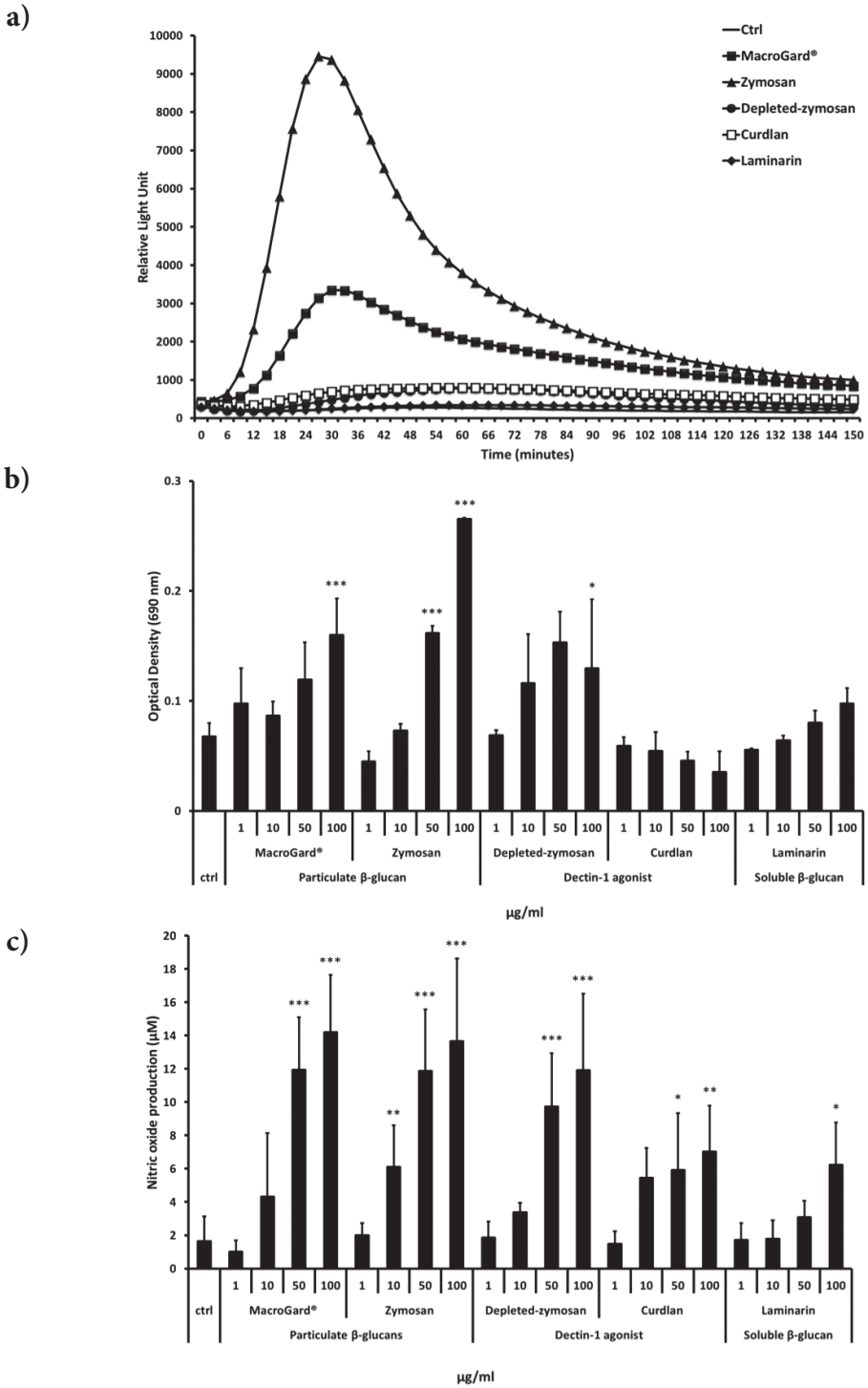

Figure 2. β-glucans induce production of oxygen radicals and nitric oxide in total head kidney leukocytes (HKL) of carp. HKL suspensions were seeded at 1x106 cells/well, in triplicate, and stimulated with different concentrations (1, 10, 50 and 100 µg/ml) of MacroGard®, zymosan, depleted-zymosan, curdlan or laminarin. Oxygen radicals were measured by RT-luminol (A) or reduction of nitroblue

tetrazolium (NBT) (B). Nitrogen radicals were measured by determining nitrite (C). Assays were performed on at least n=3 fish. Bars show mean ± S.D. Statistical analysis was performed by two-way ANOVA and Bonferroni post-hoc tests. *** = significant difference to control sample with $P < 0.0001$, **= significant difference to control sample with $P < 0.001$, *= significant difference to control sample with $P < 0.05$.

A) RT-luminol assay to determine oxygen radicals. Values are expressed as Relative Light Units (RLU). This is one representative experiment out of three independent experiments.

B) Reduction of NBT to determine oxygen radicals. Values are expressed as Optical Density values at 690 nm.

C) Nitric oxide assay to determine nitrite. Nitrite concentrations were measured in cell culture supernatants after 72 hours. Concentration of nitrite in μM was calculated using a sodium nitrite standard curve.

Particulate β-glucans induce the production of oxygen and nitrogen radicals in carp macrophages

Head kidney-derived macrophages were activated by different preparations of β-glucans. Similar to our observations on HKL, both MacroGard® and zymosan induced the greatest production of oxygen radicals and nitrogen radicals. The maximum response, expressed as relative light units in the RT-luminol assay (**Figure 3A**), was seen approximately 40 min after application of the β-glucan preparations, slightly later than observed for HKL but well within the total period of 90 minutes allowed for NBT reduction (**Figure 3B**). Overall responses were much higher than those noted with HKL suspensions perhaps reflecting the purity of cultures of head kidney-derived macrophages in comparison with the former (**cf. Figure 2 and Figure 3**). Depleted zymosan also induced a lower response than untreated zymosan, whereas curdlan in comparison to HKL was able to induce a relatively high production of NO in macrophages (**Figure 3C**). The soluble β-glucan, laminarin, however did not stimulate macrophages. Our data therefore suggests that carp macrophages are more sensitive to β-glucans than the HKL suspension, although both cell preparations are activated by the particulate β-glucans such as MacroGard® and zymosan, and less stimulated by dectin-1 agonists.

Interleukin-6 in common carp

IL-6 is a typical example of a multifunctional cytokine involved in the regulation of inflammatory immune responses, but had not been described for carp. There are several cytokines that belong to the IL-6 family, including IL-6, IL-11, oncostatin M (OSM), cardiotrophin-1 (CT-1), ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF). *M17* is one member of the IL-6 family that has been identified previously in, amongst others, carp [31], goldfish [32] and zebrafish [33] with highest sequence identity with CNTFs. But we realized M-17 is not IL-6. Using a predicted sequence for *il-6* from zebrafish (JN698962) we identified two putative *il-6* molecules (*il-6a*, *il-6b*) in the draft genome of common carp [28]. We obtained full-length sequences from scaffolds 126953, 153956 and 82770 (*il-6a*) and scaffolds 132438, 132934 and 119195 (*il-6b*), with a predicted open reading frame of 699 bp for both carp *il-6a* and *il-6b*, encoding for a protein of 232 aa with a predicted molecular weight of 30.44 and 30.29 KDa for IL-6a and Il-6b, respectively. Carp *il-6* has the same genomic organization as *il-6* from other fish species, containing five exons and four introns (**Figure 4**). The intron-exon splicing consensus (GT/AG) was found to be conserved. Carp IL-6 has a signal peptide, an IL-6 domain a core domain with 4 helices and 4 predicted glycosylation sites (**Figure 5a and 5b**). A multiple alignment of carp IL-6 with carp M-17 and other IL-6 sequences confirms conservation of the structural features in IL-6, but not M-17 (**Figure 6**).

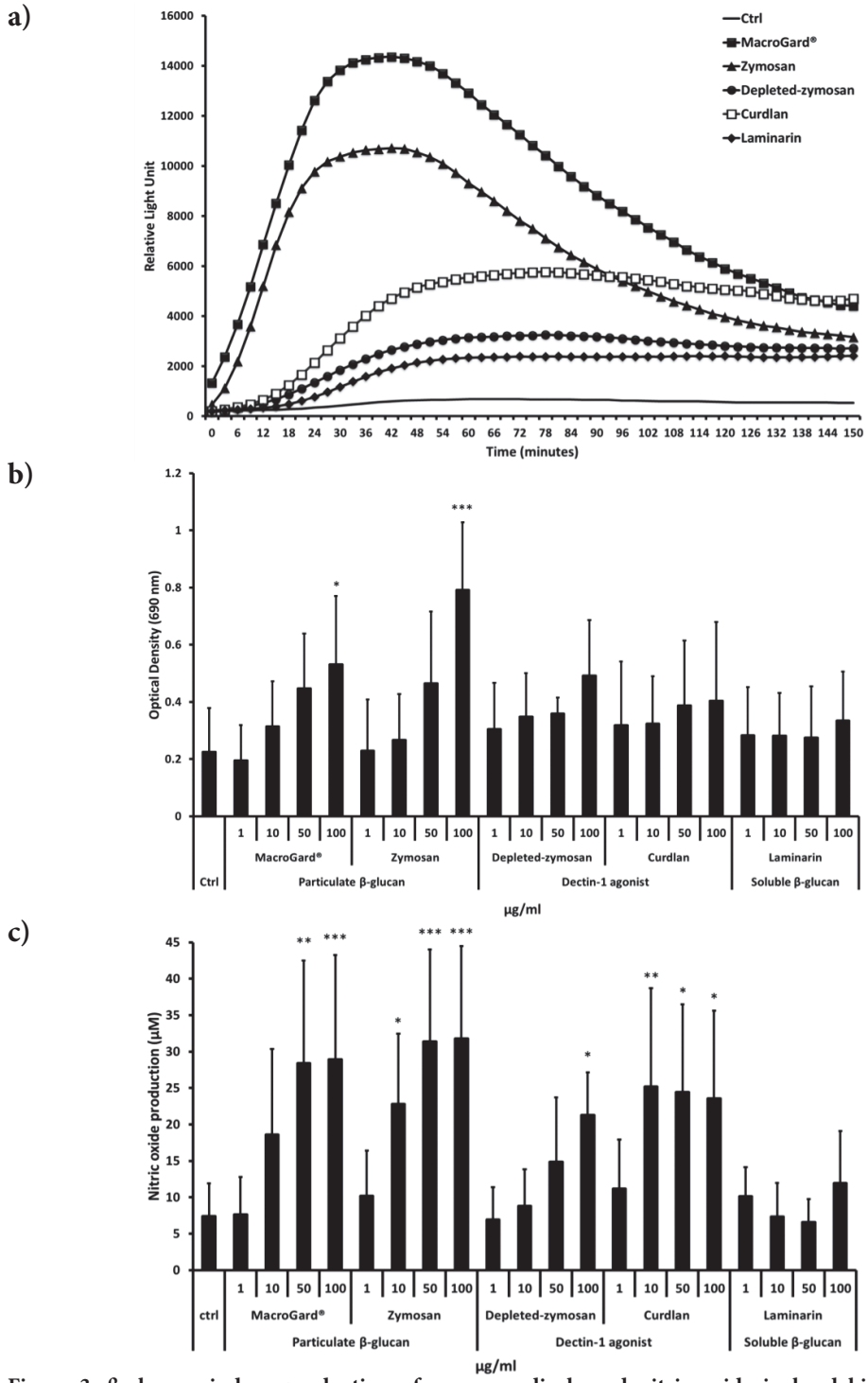

Figure 3. β-glucans induce production of oxygen radicals and nitric oxide in head kidney derived macrophages. Head kidney derived macrophages suspensions were seeded at $0.5x10^6$ cells/well, in triplicate, and stimulated with different concentrations (1, 10, 50 and 100 µg/ml) of MacroGard®, zymosan, depleted-zymosan, curdlan or laminarin. Oxygen radicals were measured by RT-luminol (A) or reduction of nitroblue

tetrazolium (NBT) (B). Nitrogen radicals were measured by determining nitrite (C). Assays were performed on at least n=3 fish. Bars show mean ± S.D. Statistical comparison was performed by two-way ANOVA. *** = significant difference to control sample with $P < 0.0001$, **= significant difference to control sample with $P < 0.001$, *= significant difference to control sample with $P < 0.05$.

A) RT-luminol assay to determine oxygen radicals. Values are expressed as Relative Light Units (RLU). This is one representative experiment out of three independent experiments.

B) Reduction of NBT to determine oxygen radicals. Values are expressed as Optical Density values at 690 nm.

C) Nitric oxide assay to determine nitrite. Nitrite concentrations were measured in cell culture supernatants after 72 hours. Concentration of nitrite in μM was calculated using a sodium nitrite standard curve.

Cytokine gene expression upon stimulation with particulate β-glucan in carp macrophages

Recognition of pathogen-associated patterns, such as β-glucans, by pattern recognition receptors (PRRs) generally lead to the upregulation of transcription of genes involved in inflammatory responses. These genes can include (pro)-inflammatory cytokines but also chemokines and other antimicrobial proteins. Induced expression patterns of these genes may differ among activated PRRs and thus may represent 'typical' profiles of activation. We measured cytokine gene expression in carp head kidney-derived macrophages, induced by stimulation with different β-glucan preparations. We examined the existence of profiles 'typical' for stimulation with β-glucans presumed to be preferentially recognized by TLRs and CLRs (MacroGard® and zymosan) and β-glucans presumed to be preferentially recognized by CLRs only, such as dectin-1 (depleted zymosan and curdlan). To this end, changes in gene expression of *il-1β*, *il-6*, *il-8*, *il-10*, *il-11*, *il-12*, *tnfα* and *ifn-γ* were measured (**Table 2**).

In general, both MacroGard® and zymosan, as well as dectin-1 specific ligands, were able to induce gene expression of several cytokines, with up-regulation of *il-1β*, *il-6* and *il-11* forming good markers for macrophage activation by β-glucans. Our data indicate that 'typical' profiles of modulated cytokine gene expression may exist for β-glucan-stimulated carp macrophages and could include upregulation of *il-1β*, *il-6* and *il-11*. Whereas *il-1β* belongs to the IL-1 cytokine family, both *il-6* and *il-11* are members of the IL-6 family. 'Typical' profiles for stimulation with β-glucans presumed to be preferentially recognized by TLRs and CLRs (MacroGard® and zymosan) on one hand and β-glucans presumed to be preferentially recognized by CLRs such as dectin-1 (depleted zymosan and curdlan) on the other hand, could not be distinguished, however. Although it does not seem possible to assign cytokine profiles to a particular group of PRR, our data does suggest that expression patterns of cytokine genes can indeed differ depending on the ligand.

a)Interleukin-6a

```
atgccgtcagctcagaacgcagctctcttcctgtctgccgtactggcagtgttcatcagt
 M  P  S  A  Q  N  A  A  L  F  L  S  A  V  L  A  V  F  I  S
ctggtggatgcggtgcctgtctacagcggtctggccgaactatccgaaacatctggggac
 L  V  D  A  V  P  V  Y  S  G  L  A  E  L  S  E  T  S  G  D
gaggttcaggacgtggatggaaagagtcctctgagcgaccggcagaaatggcatctgatg
 E  V  Q  D  V  D  G  K  S  P  L  S  D  R  Q  K  W  H  L  M
gccagagatctgcacagagacgtcaagacgctgcgagaccagcagtttgagagggacttc
 A  R  D  L  H  R  D  V  K  T  L  R  D  Q  Q  F  E  R  D  F
agagagatggtgaacatgacggcgtatgaaggagtgagggtcaaaaccctctcctcaaa
 R  E  M  V  N  M  T  A  Y  E  G  V  R  V  K  T  P  L  L  K
ccctctgatggctgtctgtccagaaacttcagctcagaaaggtgtctgagccgcatttac
 P  S  D  G  C  L  S  R  N  F  S  S  E  R  C  L  S  R  I  Y
agcgtcctgacctggtacagagagaactggagcttcattgagaaggaaaacctgacctcg
 S  V  L  T  W  Y  R  E  N  W  S  F  I  E  K  E  N  L  T  S
agcctggtgaaggacatcaaacacgcgagcaaacgtctgctggaggccctcaacagccag
 S  L  V  K  D  I  K  H  A  S  K  R  L  L  E  A  L  N  S  Q
ctgcagatagcggacggaggggcggatcagatctccagtgctcctctctcggtcagatcc
 L  Q  I  A  D  G  G  A  D  Q  I  S  S  A  P  L  S  V  R  S
gcgtggacacgcaagaccacggtgcattcgatcctgttcaacttcaccagcgtgatgatc
 A  W  T  R  K  T  T  V  H  S  I  L  F  N  F  T  S  V  M  I
gacacgtgcagagccatcaattacatgagcaaacgcaaacctgcgtatcgagcaaaagac
 D  T  C  R  A  I  N  Y  M  S  K  R  K  P  A  Y  R  A  K  D
acgaagagacccgcagactggagcgccgacaagaactaa
 T  K  R  P  A  D  W  S  A  D  K  N  *
```

b)Interlukin-6b

```
atgtcgtcagctcagaacgcagcgctcttcctgtctgccgtactggcagtgttcatcagt
 M  S  S  A  Q  N  A  A  L  F  L  S  A  V  L  A  V  F  I  S
ctggtggatgcggtgcctgcctacagcggtctgaccgaactatccgaaacatctggagac
 L  V  D  A  V  P  A  Y  S  G  L  T  E  L  S  E  T  S  G  D
gaggttcaggacgtggagggaaagagtcctctgagcgagcggcagaaatggtatctgatg
 E  V  Q  D  V  E  G  K  S  P  L  S  E  R  Q  K  W  Y  L  M
gccagagatctgcacagagacgtcaagacgctgcgagaccagcagtttgagagggacttc
 A  R  D  L  H  R  D  V  K  T  L  R  D  Q  Q  F  E  R  D  F
agagagacggtgaacatgacggcgtatgaaggagcgaagatcaaaaccctctcctcaga
 R  E  T  V  N  M  T  A  Y  E  G  A  K  I  K  T  P  L  L  R
ccctccgacggctgtgtgtccagaaacttcagcgcagatttgtgcctgagccgcatttac
 P  S  D  G  C  V  S  R  N  F  S  A  D  L  C  L  S  R  I  Y
agcgtcctgacctggtataaagagaactggagcttcatcgagaacgaaaacctgacctcc
 S  V  L  T  W  Y  K  E  N  W  S  F  I  E  N  E  N  L  T  S
agcctggtgaacgacatcaaacacgggaccaaacgcctgctggaggccattaacagccag
 S  L  V  N  D  I  K  H  G  T  K  R  L  L  E  A  I  N  S  Q
ctgcagatagcggacggacagcaggatcagatctccagcgctcctctatcggtcagatcc
 L  Q  I  A  D  G  Q  Q  D  Q  I  S  S  A  P  L  S  V  R  S
gcatggacacgcaagatcacgacgcattcgatcctgttcaacttcagcagcgtgatgatc
 A  W  T  R  K  I  T  T  H  S  I  L  F  N  F  S  S  V  M  I
gactcgtgcagagccctccattacatcagcaaacgcaaagctgcgcaccgagcaaaagac
 D  S  C  R  A  L  H  Y  I  S  K  R  K  A  A  H  R  A  K  D
accaagagacccgcagaccggagctccggcaagaactga
 T  K  R  P  A  D  R  S  S  G  K  N  *
```

Figure 5. **Nucleotide and predicted amino acid sequences of carp *il-6*.** Nucleotide sequences for carp *il-6a* (A) and carp *il-6b* (B) were derived from the draft carp genome [28]. The predicted signal peptide is underlined with a dashed line. The IL-6 domain is indicated with a grey shade. The IL-6 family signature (C–X(9)–C–X(6)–G–L–X(2)–Y–X(3)–L) is indicated with boxes. Predicted N-glycosylation sites are underlined in bold.

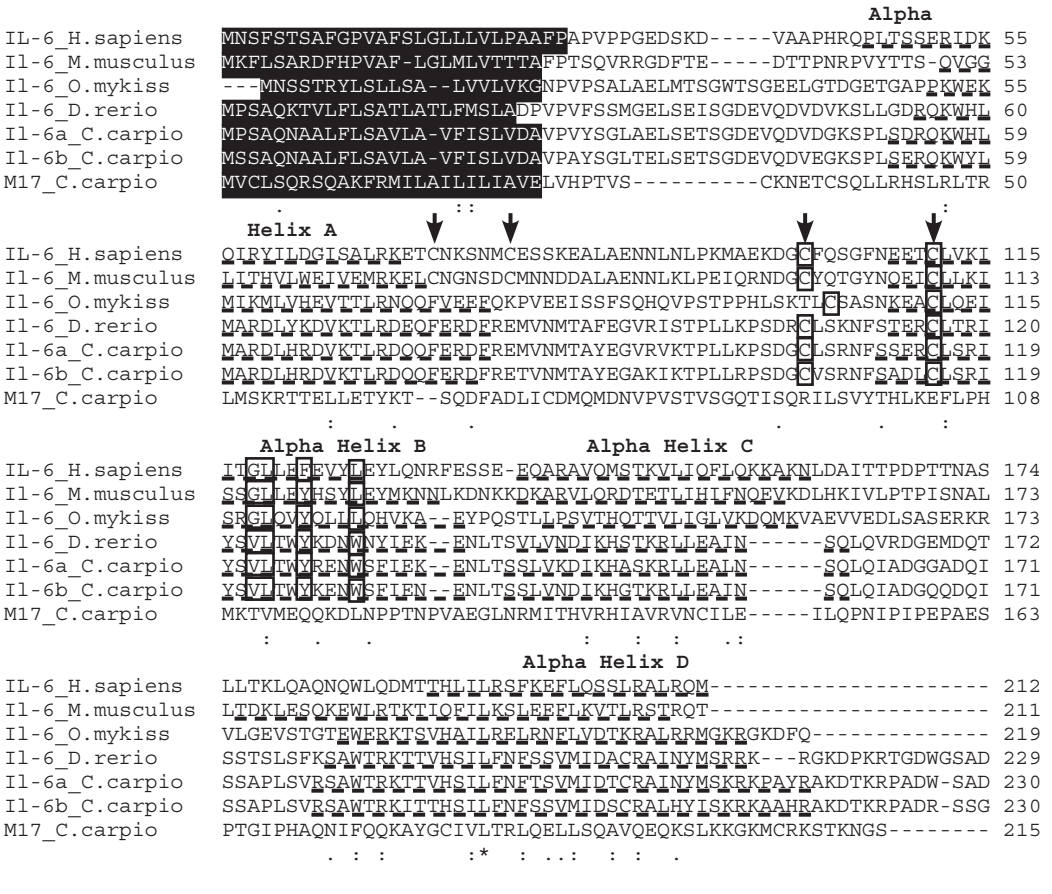

Figure 6. **Multiple alignment of IL-6 sequences with M-17.** Multiple alignment of carp Il-6a and carp Il-6b with IL-6 from *Homo sapiens* (NP_000591), *Mus musculus* (NP_112445), *Oncorhynchus mykiss* (NP_001118129), *Danio rerio* (JN698962) and with carp M17 (*Cyprinus carpio*; AAM52338). The signal peptide is highlighted in black. Alpha-helices are indicated with a dashed line underlining the sequence. The IL-6 family signature is indicated with bold letters and shaded in grey, whereas arrows indicate cysteine residues involved in disulfide formation.

Table 2. β-glucans induce gene expression of cytokines in carp macrophages. Gene expression was measured in control (unstimulated macrophages) and macrophages stimulated for 6 h with MacroGard®, zymosan, depleted-zymosan (DPZ) or curdlan at concentrations of 100 and 10 μg/ml. mRNA levels of the analysed genes were first normalized against the house keeping gene. Fold change was determined by real-time quantitative PCR and values (mean values of n=2 fish) expressed relative to the values in control cells. Statistical analysis was performed by two-way ANOVA and post-hoc Bonferroni. *Significant difference compared to control (P <0.05).

Gene	MacroGard®		Zymosan		DPZ		Curdlan	
	10 μg/ml	100 μg/ml	10 μg/ml	100 μg/ml	10 μg/ml	100 μg/ml	10 μg/ml	100 μg/ml
il-1β	2.2	7.9	14.2*	10.9*	2.5	5.3	12.2*	13.6*
il-6[a]	2.0	2.6	6.8*	8.5*	2.6	1.6	2.4	3.2
il-8[b]	0.8	0.4	1.0	0.5	1.1	0.5	0.9	0.6
il-10	1.1	0.7	0.9	5.4	0.7	1.1	0.7	0.7
il-11	1.6	3.5	8.8*	12.7*	1.7	8.9*	13.7*	21.2*
il-12 p35	1.0	1.1	1.3	1.6	0.7	0.7	1.1	1.2
il-12 p40[c]	1.4	3.3	1.8	1.3	1.3	2.3	2.4	2.1
tnfα[d]	1.8	4.6	2.3	1.7	1.7	4.7	3.8	4.9
ifn-γ[e]	0.9	1.0	1.6	1.9	1.0	0.8	1.4	1.6

a Only values for *il-6a* are shown. Both *il-6a* and *il-6b* show the same trend.

b *il-8 = cxcl8* [34]

c Only values for *il-12p40b* are shown. *il-12p40a* and *il-12p40c* [35] were not induced

d Only values for *tnfα2* are shown. tnfα1 and *tnfα2* [36] showed the same trend

e Only values for *ifnγ1* are shown. *ifnγ2* [37] was not included

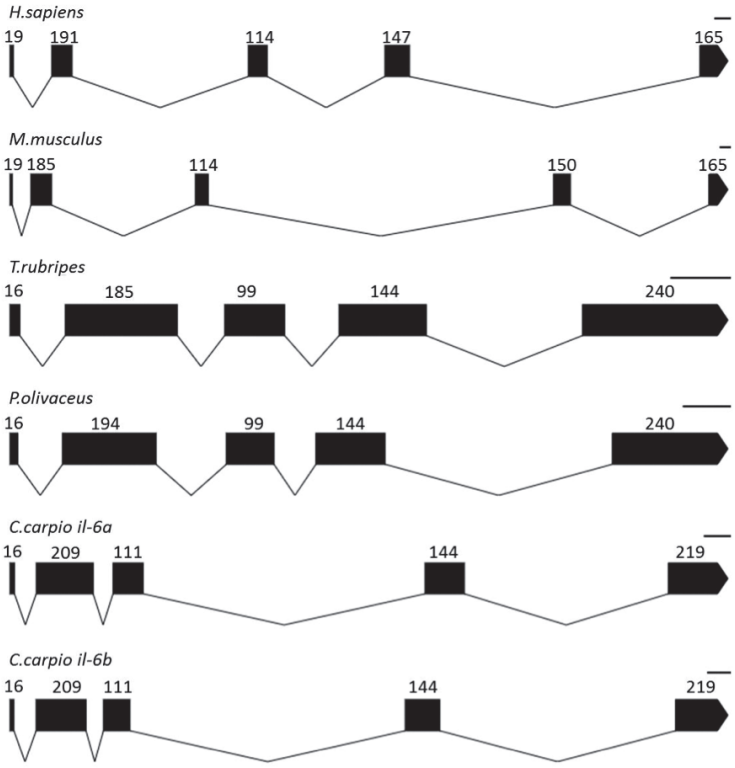

Figure 4. Predicted genomic organization of IL-6 genes. Comparison of the intron-exon organization of carp il-6a and il-6b with other IL-6 genes of Homo sapiens (NP_000591), Mus musculus (NP_112445), Takifugu rubripes (NP_001027894) and Paralichtys olivaceus (ABJ53333).

DISCUSSION

In the present study we examined the activation of carp macrophages by different β-glucan preparations including MacroGard® and zymosan as particulate stimulants and depleted zymosan and curdlan as dectin-1-specific ligands. Our results show that β-glucans stimulate carp macrophages to increase the production of reactive oxygen and nitrogen radicals and affect the expression patterns of cytokine genes that can differ among activated pattern recognition receptors. Carp macrophages although less responsive to selective dectin-1 agonists than other forms of β-glucans utilised were stimulated by curdlan and depleted zymosan despite the presumed absence of the dectin-1 receptor from fish genomes. It is therefore suggested that recognition of β-glucans occurs by multiple pattern recognition receptors on carp macrophages that could include both phagocytic receptors and TLRs as sensing receptors.

Our observations support previous reports on immune modulatory effects of β-glucans on the fish immune system [38]. The ability of β-glucans to activate respiratory burst activity of Atlantic salmon (*Salmo salar*) macrophages *in vitro* is well-known [39]. *In vivo* studies have shown beneficial effects of administering β-glucans via feed, on disease resistance of Atlantic salmon [40] and sea bass (*Dicentrarchus labrax*) [41]. Also for carp, several immunomodulatory effects of β-glucans have been shown. *In vivo* injection of common carp with zymosan induces peritonitis with associated up-regulation of several cytokines [42]. Dietary β-glucan or microbial-derived levan, a natural polymer of fructose, showed positive effects on the immune response and disease resistance

of carp to *Aeromonas hydrophila*. In these studies blood parameters such as total red or white blood cell counts, neutrophil NBT activity and serum lysozyme activity were measured [43], [44] but also phagocytic capacity and NBT respiratory burst activity of head kidney phagocytes [45]. Although in one study intraperitoneal injection, but not dietary β-glucan or bath administration of β-glucan, had a positive effect on resistance of carp to A. hydrophila, recent studies indicate β-glucans can promote wound healing *in vitro* [46] but also *in vivo* after bath application [47]. β-glucans seems to help protect carp neutrophil extracellular traps against degradation by *A. hydrophila* [48], which could maybe explain positive effects of β-glucans on disease resistance. *In vivo* screening models with zebrafish (*Danio rerio*) larvae have shown that immersion administration of β-glucan can induce expression of cytokines such as *tnf-α* along with a temporal increase in resistance against *Vibrio anguillarum* [49]. In conclusion, most *in vitro* and several *in vivo* studies in fish, including common carp, point at immune-modulating and often immune-activating properties of β-glucans, including positive effects on disease resistance. None of these studies, however, made any attempt at studying the receptors on myeloid cells important for β-glucan recognition.

Recognition of β-glucans can lead to the upregulation of transcription of genes involved in inflammatory responses. We examined changes in gene expression of *il-1β*, *il-6*, *il-8*, *il-10*, *il-11*, *il-12*, *tnfα* and *ifn-γ* following stimulation with different preparations of β-glucans. Both MacroGard® and zymosan, as well as dectin-1 specific ligands, were able to induce up-regulation of gene expression of *il-1β*, *il-6* and *il-11*. Until present, *il-6* had not been identified in carp and, instead, gene expression of the IL-6 family member M-17 has been measured [31, 50, 51]. M-17 is one of several cytokines that belong to the interleukin-6 family that includes besides IL-6, IL-11, oncostatin M (OSM), cardiotrophin-1 (CT-1), ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF). M17 has been identified in, amongst others, carp [31], goldfish [32] and zebrafish [33] with highest sequence identity with CNTFs, at least in goldfish [32]. As may be clear from our data, *m-17* should not be mistaken for carp *il-6*. Carp macrophages showed a clear immune response to several β-glucan preparations by up-regulated gene expression of *il-1β, il-6* and *il-11*. *Interleukin-11* has previously been cloned in carp [52] but its function in fish is not well described. In humans, IL-11 plays a significant role in the synthesis and maturation of hematopoietic cells, amongst others [53], which could maybe explain its upregulation in our study. The cytokine IL-1β is well-known for its involvement in pro-inflammatory immune responses, including those of carp [54]. Overall, up-regulation of IL-1β, IL-6 and IL-11 seem to form good markers for macrophage activation by β-glucans.

Professional phagocytes play an important role in the clearance of microbial pathogens, for example via the production of oxygen and nitrogen radicals. TLRs are thought to influence the phagosome maturation associated with this process. It is the interplay between phagocytic receptors such as dectin-1, or other phagocytic receptors, and TLRs as sensing receptors that is thought to result in the recruitment of TLRs to the phagosomes where they detect β-glucans and induce the production of pro-inflammatory cytokines. However, the presence of sequences in genomes, of receptors which can be considered clear orthologs to specific sequence receptors of the dectin-1 cluster, seem to be limited to mammalian genomes [17, 55]. This would suggest that fish may not express dectin-1 receptors and indeed, a search for dectin-1 in the genome of common carp [28] did not identify a clear ortholog (Pietretti, data not shown). Of course, there are additional pattern recognition receptors beside dectin-1 that could play a key role in the recognition of β-glucans, either or not in conjunction with TLR2. Phagocytic receptors in mammals can include i) members of the C-type lectin superfamily, ii) receptors involved in the uptake of opsonised pathogens such

as the FcγR [56] or the complement receptor 3 (CD11b/CD18), and iii) members of the scavanger receptor (SR) family. The possibility that these receptors could be β-glucans receptors in fish is shortly discussed below.

(i) The C-type lectin superfamily comprises a large group of functionally diverse proteins characterised by the presence of one or more C-type lectin-like domains (CTLDs). DC specific ICAM grabbing non-integrin (DC-SIGN, CD209) is a group II receptor recognising mannose, SIGNR1 is a homologue of DC-SIGN that captures *C. albicans* and zymosan and increase TLR2-dependent TNF-α production in mice [57]. Studies on zebrafish DC-SIGN showed no participation in phagocytosis of keyhole limpet haemocyanin [58], however. Dectin-1, the major ß-glucan receptor in mammals [10], is a group V member of the C-type lectin superfamily absent from fish genomes. Functional evidence on the group VI mannose receptor in fish is based only on mannan-inhibitable uptake [59]. Overall, considering the activating properties of depleted zymosan and curdlan on carp macrophages, C-type lectin superfamily members are likely candidate receptors for phagocytosis of particulate β-glucans by fish macrophages.

(ii) Both, the Fc receptor of immunoglobulin (Ig) FcγR and the complement receptor 3 (CR3), are involved in the uptake of opsonised pathogens. Although a soluble FcRI of catfish binds linear epitopes on Ig heavy and light chains [60], and a homolog of the polymeric Ig receptor has been identified in carp [61], and maybe can transport secretory Ig [62], true FcγRs able to bind Ig-opsonised particles have not been conclusively identified in fish. In mammals, CR3 is composed of a common β subunit (CD18) and a unique but most prevalent α subunit (CD11) β2 integrin. CD11b-like/CD18 integrins have been identified in common carp [63] and rainbow trout [64]. Despite the presence of potential opsonising factors in carp serum in our assays, the absence of a true FcγR and the absence of CR3-mediated induction of respiratory burst activity [65], makes it unlikely that opsonising phagocytic receptors play a major role in the uptake by carp macrophages of particulate β-glucans.

(iii) Scavanger receptors (SCAR) can act as phagocytic receptors or as co-receptors to TLRs, in particular TLR2 [66]. Most of the eight (A-H) classes display low-affinity and bind many polyanionic and modified substances [67]. In mammals, MARCO (macrophage receptor with collageneous structure), a member of the SCAR-A class, is constitutively expressed only on macrophage subpopulations but can be upregulated in a TLR-dependent manner. Although gene ontology frequently identifies MARCO-like transcripts in fish studies [68], functional studies on SCAR-A receptors are scarce [69]. CD36 is a SCAR-B receptor also expressed on macrophages that can act as a co-receptor for TLR2. Although SCAR-B receptors may bind anionic ß-glucan, they are not considered involved in ß-glucan binding and internalization, at least in mammal [70]. Yet, CD36 was recently shown involved in antifungal defence binding ß-glucan in *Caenorhabditis elegans* [71], and thus CD36 should not be ruled out for ß-glucan recognition.

ß-glucan activation of carp macrophages induced both oxygen and nitrogen radicals as well as increased cytokine gene expression. In mammals, the production of reactive oxygen species is triggered by phagocytic receptors, whereas TLRs induce the production of pro-inflammatory cytokines, especially. It thus seems likely that ß-glucans activate carp macrophages via both phagocytic and sensing, Toll-like receptors. Future studies on pattern recognition receptors in, for example, knock-out studies zebrafish lines or cell lines overexpressing these molecules could help identify the pattern recognition receptors crucial to recognition of β-glucan in fish.

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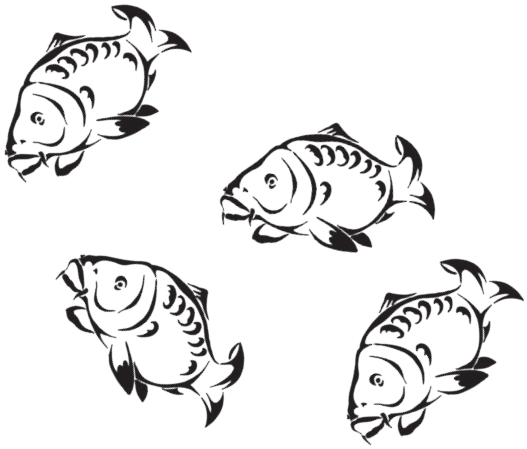

Chapter 4

Identification and functional characterization of non-mammalian Toll-like receptor 20

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Submitted paper

ABSTRACT

Alike other vertebrate TLRs, the Toll-like receptors of teleost fish can be subdivided into six major families, each of which recognize a general class of molecular patterns. However, there also are a number of Tlrs with unknown function which presence seems unique to the modern bony fish, among which is Tlr20. We identified full-length cDNA sequences for *tlr20* of zebrafish and common carp, two closely-related cyprinid fish species. Zebrafish has six copies of *tlr20*, whereas carp express only a single copy. Both zebrafish Tlr20 (at least Tlr20-a, Tlr20-b, Tlr20-c and Tlr20-d) and carp Tlr20 have 26 discernible leucine-rich repeats (LRR). Three-dimensional modelling indicates a best fit to the crystal of human TLR8. Although phylogenetic analyses place Tlr20 in the TLR11 family closest to Tlr11 and Tlr12, two TLRs that sense ligands from protozoan parasites in the mouse, analysis of the genes adjacent to zebrafish *tlr20* do not indicate conserved synteny between Tlr20 and murine members of the TLR11 family. Confocal microscopy suggests a sub-cellular localization of Tlr20 at the endoplasmatic reticulum. Although *in vitro* reporter assays could not identify a ligand unique to Tlr20, *in vivo* infection experiments indicate a role for Tlr20 in the immune response of carp to protozoan parasites (*Trypanoplasma borreli*). Carp *tlr20* is mainly expressed in peripheral blood leukocytes (PBL) with B lymphocytes, in particular, expressing relatively high levels of *Tlr20*. *In vitro* stimulation of PBL with *T. borreli* induces an upregulation of *tlr20*, confirming a role for Tlr20 in the immune response to protozoan parasites.

INTRODUCTION

Toll-Like receptors (TLRs) play an important role in innate immune mechanisms that form the first line of defense against invading pathogens. TLRs are a group of Pattern-Recognition Receptors (PRRs) recognizing conserved molecular motifs also named pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs by TLRs not only activates the innate immune system but also activates pathways important for acquired immunity [1]. TLRs typically are type I transmembrane proteins composed of three different domains; an extracellular domain (ECD) characterized by a horseshoe shape based on a large number of leucin-rich repeats (LRRs) of 20-30 amino acids important for the recognition of PAMPs, a transmembrane domain and an intracellular Toll/interleukin-1 receptor (TIR) domain that initiates intracellular signalling [2].

Most vertebrate genomes have at least one gene representing each of six major TLR families (TLR1, TLR3, TLR4, TLR5, TLR7 and TLR11), each of which recognize a general class of molecular patterns (Roach et al., 2005). The ECD of TLRs, important for ligand recognition, can consist of 16-28 LRRs; the total number of LRRs can often be linked with one of the six major families [3]. Although most vertebrate genomes have TLR genes for each of six major families, not all vertebrates express the exact same repertoire. For example, the human genome contains 10 functional TLRs whereas the mouse genome contains 12 Tlrs, with *tlr10* being a pseudogene and *Tlr11, Tlr12 and Tlr13* being mouse-specific (Ariffin and Sweet, 2013). Murine Tlr11 and Tlr12 sense profillin from *Toxoplasma gondii* [4, 5], whereas Tlr13 was recently described as a sensor of bacterial 23S rRNA [6]. The latter Tlrs (Tlr11, Tlr12 and Tlr13) are the best-described members of the TLR11 family which also includes a number of non-mammalian TLRs, among which Tlr20 the subject of the

present study.

To date, a total number of 26 Tlrs have been identified across different vertebrate species based on teleost, amphibian and avian genomes [7-11]. At present, the TLR11 family not only contains Tlr11-13 from the mouse but also non-mammalian Tlr15, Tlr16, Tlr17, Tlr19, Tlr20, Tlr21, Tlr22, Tlr23 and Tlr24 [11, 12]. Of these TLR11 family members, full sequences have been reported for Tlr15 and Tlr16 in chicken [13, 14];, Tlr19 and Tlr20 in zebrafish and channel catfish [11, 15]; Tlr21 in several fish species and in chicken [11, 15-17]; Tlr22 in several fish species [7, 11, 15, 16, 18] and Tlr23 in pufferfish and Atlantic cod [7, 18]. Ligand recognition and exact function of the non-mammalian TLR11 family members remain undefined, with the one exception of Tlr22 that has been reported to sense long-sized dsRNA on the cell surface [19]. With regard to Tlr20, already in one of the first studies on teleost TLRs, multiple but partial Tlr20 sequences (*tlr20a-f*) were identified in the zebrafish genome [15], followed by partial sequences for Tlr20 in rainbow trout [20] and channel catfish [21]. Only very recently, a full-length *tlr20* sequence was described in channel catfish [11]. Nevertheless, functional studies on teleost TLR20 have not been reported so far.

We characterize for the first time in detail Tlr20 of zebrafish and common carp, two cyprinid fish species that are closely related [22]. Teleost Tlr20 has the conserved features of mammalian TLRs with an ECD containing 24-26 LRRs, a conserved intracellular TIR domain and an N-terminal LRR (LRRNT) and C-terminal LRR (LRRCT). Leucine-rich repeats are often flanked by N-terminal and C-terminal cysteine-rich domains (LRRNT and LRRCT) [23]. Teleost Tlr20s cluster with mouse Tlr11 and Tlr12, both members of the TLR11 family. We used a three-dimensional modelling approach to find a best fit for teleost Tlr20 to known TLR crystal structures and used a synteny approach to examine the conservation of genomic organization of the genes adjacent to zebrafish Tlr20. Confocal microscopy was used to study sub-cellular localization in human and fish cell lines transfected with carp Tlr20. *In vitro* reporter assays based on NF-κB activation following stimulation of a human cell line overexpressing carp Tlr20 were used to identify putative ligands of Tlr20. *In vivo* infection experiments allowed for an investigation of *tlr20* gene expression induced by protozoan parasites (*Trypanoplasma borreli*) of carp. Screening of a cDNA library of carp tissues and leukocyte subtypes indicated that carp *tlr20* is highly expressed in peripheral blood leukocytes (PBL), in particular B lymphocytes, relative to other leukocyte cell types. *In vitro* stimulation of carp PBL and re-stimulation of PBL from fish that survived a *T. borreli* infection with parasite lysate induced an upregulation of *tlr20*, suggesting a role for Tlr20 in the immune response of carp to protozoan parasites.

MATERIALS AND METHODS

Animals

European common carp (*Cyprinus carpio carpio*) were reared in the central fish facility of Wageningen University at 23±2°C in recirculating UV-treated water and fed pelleted dry food (Sniff, Soest, Germany) daily. R3xR8 heterozygous carp (9-11 months old) were the offspring of a cross between fish of Hungarian (R8 strain) and of Polish (R3 strain) origin [24]. All studies on carp were performed with approval from the animal experimental committee of Wageningen University. Zebrafish (*Danio rerio*) were reared in the central fish facility of Leiden University at 28± 2°C in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org) in recirculating UV-treated water and fed flakes (Tetra, Melle, Germany) daily.

Isolation of immune organs and purification of leukocyte sub-types

Total RNA was isolated from different carp organs [25], and from different leukocyte sub-types purified by magnetic cell sorting using specific antibodies as described before for head kidney-derived macrophages [26], thrombocytes [27], thymocytes [28], granulocytes [29] and B cells [30, 31].

Peripheral blood leukocytes (PBL) were obtained from carp blood first centrifuged for 5 min at 100 x *g* and then for 10 min at 600 x *g* to obtain the buffy coat. The buffy coat was layered on 3 ml Ficoll-Paque™ Plus (Amersham Biosciences) and centrifuged at 800 x *g* for 25 min. PBL were collected, washed three times in culture medium (RPMI 1640 adjusted to 270 mOsmol kg^{-1}) (Cambrex) and counted. For stimulation assays, PBL were seeded at a concentration of $0.5x10^6$ cells/well in 96-well culture plates and stimulated with *T. borreli* lysate (equivalent of 1:2 parasites:cells) or with culture medium alone as negative control for 3, 6 and 24 hours. After incubation, cells were collected for RNA isolation.

RNA isolation and cDNA synthesis

RNA was isolated using Trizol® (Invitrogen, Breda, The Netherlands) according to the manufacturer's protocol and stored a -80˚C until use. RNA concentration was measured spectophotometrically (GeneQuant, Pharmacia Biotech) at OD_{260nm} and the purity determined as the OD_{260nm}/OD_{280nm} ratio with expected values between 1.8 and 2.0. The integrity of RNA was determined by gel electrophoresis on 1% agarose gel containing 0.1% of SYBR® Safe DNA Gel Stain (Invitrogen™). cDNA synthesis was performed with 1 µg total RNA using DNase I amplification grade (Invitrogen) according to the manufacturer's instructions. Synthesis of cDNA was performed with Invitrogen's SuperScript™ III First Strand Synthesis Systems for RT-PCR, according to the manufacturer's instructions. A non-reverse transcriptase control was included for each sample. Before use as template in real time-quantitative PCR (RT-qPCR) analysis, the cDNA was further diluted 25-50 times in nuclease-free water.

Molecular cloning of zebrafish *tlr20*

The initial *in silico* prediction of six zebrafish *tlr20* genes in the genome of zebrafish [15] was used to detect *tlr20* in the most recent zebrafish genome assembly Zv9 (GCA_000002035.2) using Genomics Workbench version 4.9 (CLC Bio, Aarhus, Denmark). The putative coding regions within the genomic DNA were identified using FGENESH and the predicted amino acid sequences were confirmed by using these sequences as template in BLAST [32] and FAST [33].

Molecular cloning of *carp tlr20*

Carp *tlr20* was first identified in the draft genome of common carp (Bioproject PRJNA73579) [22] using zebrafish *tlr20a* (accession number AAI63786) as reference sequence for the BLAST search. We identified one contig within the carp genome (scaffold 28896) with a region coding for a single Tlr20 sequence. Gene-specific primers to amplify the full-length coding sequence (CDS) were designed using the Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). As template we used cDNA synthesized using a LongRange 2Step RT-PCR kit (Qiagen) from RNA collected from head kidney tissue taken from carp three weeks after infection with the parasite *Trypanosoma carassii* [26]. A first PCR to obtain the full-length carp *tlr20* CDS was performed using the Expand High Fidelity Plus PCR System (Roche) followed by a second PCR using tlr20Fw- and tlr20Rv-specific primers (see Table 1). The product was cloned in JM109 competent *E. coli* cells

using the pGEM-TEasy kit (Promega) and both strands of eight positive clones were sequenced using the ABI Prism-Bigdye Terminator Cycle Sequencing Ready Reaction kit, and analysed using an ABI 3730 sequencer. Nucleotide sequence data were analysed for identity to other sequences using the GenBank database [34].

Bioinformatics and synteny analysis

Nucleotide sequences of *tlr20* were translated using the ExPASy *Translate* tool (http://us.expasy.org/tolls/dna.html) [35] and aligned with Multiple Sequence Alignment by CLUSTALW (http://www.genome.jp/tools/clustalw/). The predicted amino acid sequences were examined for the presence of a signal peptide using the SignalP (http://www.cbs.dtu.dk/services/SignalP-2.0/), Predisi (http://www.predisi.de/) [36] and TMHMM2.0 programs (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Identification of protein domains was done with SMART [37] (http://smart.embl-heidelberg.de/) and LRRfinder (http://www.lrrfinder.com/), whereas individual LRRs were identified manually according to prior definitions [3, 38] and three-dimensional modelling. A phylogenetic tree based on the TIR intracellular domains was constructed using the Neighbor-Joining method [39] using MEGA5 software [40]. Evolutionary distances were computed using the Poisson correction method [41], all positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were done with 10,000 bootstrap replicates. Genome synteny of the loci harbouring *tlr20* was examined by comparing the genomes of mouse (GCA_000001635.3), carp (PRJNA73579) and zebrafish (GCA_000002035.2) retrieved from the Ensemble Genome Browser [42] (http://www.ensembl.org/index.html).

Three-dimensional modelling

Structural models were obtained using the amino acid sequence alignment of carp Tlr20 and human TLR8, and the available dimer structure of human TLR8 (PDB-id: 3w3g) as template using the Modeller program (version 9.12) [43]. In addition, the N-acetylglucosamine (NAG), β-D-mannose (BMA) and water molecules present in the crystal structure were included in the modelling procedure. Thirty comparative models were generated, after which the model with lowest corresponding DOPE score [44] was selected for image generation with Pymol, an OpenGL based molecular visualization system.

HA-Tlr20-GFP expression plasmid

The PCR product amplifying the complete *tlr20* CDS was used as template for a PCR using the cyca-tlr20-HA-Fw1 in combination with cyca-tlr20-XhoI-Rv1 (primers listed in Table 1) followed by a second PCR using the cyca-HA-tlr20-BamHI-Fw2 in combination with cyca-tlr20-XhoI-Rv1. The PCR products were purified and used as template for a final PCR using cyca-tlr20-BamHI-Fw3 and cyca-tlr20-XhoI-Rv1. Primers were designed to add a BamHI site at the 5'end, upstream of the signal peptide and the hemagglutinin (HA)-tag sequence, and an XhoI site at the 3' end, excluding the *tlr20* stop codon. Subsequently, this product was ligated into the BamHI and XhoI sites of a pcDNA3.1 plasmid (Promega) in frame with the sequence of the Green Fluorescent Protein (GFP) that was already inserted in the vector, to obtain the HA-Tlr20-GFP fusion product.

Table 1. Primers used

Primer	Sequence (5'-3')	Used
tlr20Fw	ATTGAAGATGGTGCCTCTGTTC	Cloning
tlr20Rv	TAGAGAAATGAAGTTTAGTTGG	Cloning
q40SFw	CCGTGGGTGACATCGTTACA	RT-qPCR
q40SRv	TCAGGACATTGAACCTCACTGTCT	RT-qPCR
qtlr20Fw	ATTATGTGACCGTTGAGGGCTGC	RT-qPCR
qtLR20Rv	TCCAGATTGACGACCGATCTTAC	RT-qPCR
cyca-tlr20-HA-Fw1	TTGTTCTTGGCTTGCTTACCCATACGATGTTC CAGATTACGCTGATAAATGCCTTTTCTACAGT GATG	HA-Tlr20-GFP
cyca-HA-tlr20-BamHI-Fw2	TGAGGGATCCAACATGGTGCCTCTGTTCTCG CTCTTCATACTGTTTCTGAAGACTTCATGCAT TTGTTCTTGGCTTGCTTACCCATACGATGTTC CAGATTACGCTGATAAATGCCTTTTCTACAGT GATG	HA-Tlr20-GFP
cyca-tlr20-BamHI-Fw3	TGAGGGATCCAACATGGTGCCTCTGTTCTCG CTCT	HA-Tlr20-GFP
cyca-tlr20-XhoI-Rv1	GGAACTATACAATATAGAGAAATGACTCGAG GTTGGT	HA-Tlr20-GFP

Highlighted sequence indicates the HA tag (TACCCATACGATGTTCCAGATTACGCT); underlined sequence indicates the BamHI restriction site (GGATCC); double underlined sequence indicates the XhoI restrictio site (CTCGAG).

Sub-cellular localization of Tlr20

Computational prediction of the subcellular localization of zebrafish and carp Tlr20 was performed with the TargetP program (http://www.cbs.dtu.dk/services/TargetP/) ([36]. Experimental determination of the sub-cellular localization was performed using the carp HA-Tlr20-GFP construct. To this end, a human cell line (human embryonic kidney cells, HEK 293) and three fish cell lines; EPC (epithelioma papulosum cyprini) [45]; CLC (carp leukocyte culture) [46] and ZF4 (zebrafish embryonic fibroblast 4) [47] were used. Both HEK 293 and ZF4 cells were cultured in DMEM F12 (Gibco®) medium supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamin and 1% streptomycin/penicillin at 37˚C, or at 27˚C with 5% CO_2. Both EPC and CLC cells were cultured in RPMI 1640 (Cambrex) supplemented with 10% FBS, 1% L-glutamin and 1% streptomycin/penicillin at 27˚C with 5% CO_2.

HEK 293, EPC and CLC cells were seeded in 6-well plates (0.5 x 10^6 cells/well [HEK 293] and 1 x 10^6 cells/well [EPC and CLC]) 24 h prior to transfection. Cells were transfected with 2 µg of carp HA-Tlr20-GFP plasmid using jetPRIME (Polyplus; HEK 293) or FuGENE 6 (Roche Molecular

Biochemicals; EPC or CLC) all according to the manufacturer's instructions.

ZF4 cells were seeded 24 hours prior to transfection on glass bottom culture dishes (P35G-0-14-C, MatTek corporation Ashland) at 2 x 10^4 cells/dish in a volume of 500 µl medium. ZF4 cells were (co)-transfected using jetPRIME with 0.3 µg HA-Tlr20-GFP plasmid and 0.2 µg red fluorescent protein Vector-Endoplasmic Reticulum (ER) plasmid (RFP-KDEL, catalog number 558725 BD Pharmingen™); the latter containing a KDEL sequence that specifically targets the endoplasmic reticulum (ER). For ER localization ZF4 cells were used because they are particularly suitable for live imaging of cell compartments, becuase they adhere by stretching, allowing for a good view on the intracellular compartment.

Four hours post-transfection, medium was replaced with 3 ml complete medium. Sub-cellular localization was determined two-three days after transfection with the help of a Zeiss LSM-510 laser scanning microscope. Green fluorescent signal (rhodamine or green-fluorescent protein) was excited with a 488 nm argon laser and detected using a band-pass filter (505-530 nm). Red-fluorescent signal (propidium iodide) was excited with a 543 nm helium-neon laser and detected using a long-pass filter (560 nm).

To distinguish between intra- and extracellular localization of carp HA-Tlr20-GFP, cells (HEK 293, EPC, CLC) were harvested 72 h post-transfection and fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature (RT), followed by a washing step with PBS containing 1% (w/v) bovine serum albumin (PBS-BSA). Cells were incubated with mouse anti-HA antibody (Cell Signaling Technology™) for 1h at RT and washed with PBS-BSA followed by incubation with donkey anti-mouse-Cy3 antibody (Merck Millipore) for 1 h at RT in the dark and a washing-step. For intracellular localization, prior to incubation with the anti-HA antibody, cells were permeabilized by re-suspension in 100 µl of 0.05% (v/v) Triton X-100 in PBS-BSA and incubation for 20 min at RT followed by a washing step with PBS-BSA. Nuclei were stained with VECTASHIELD® Mounting Media containing propidium iodide (Vector Laboratories) after overnight incubation.

In vitro ligand studies

HEK 293 cells were transfected with 3.5 µg of pNiFty-Luc, a plasmid encoding for the luciferase reporter gene under the control of the NF-κB-inducible ELAM-1 composite promoter (InvivoGen). Stably transfected cells (HEK-NFκB-Luc) were selected using 250 µg/ml Zeocin (Life technologies™). For transient transfection of the HA-Tlr20-GFP vector, stably-transfected HEK-NFκB-Luc cells were first plated at 5 x 10^4 cells/well in a 96 well plate and incubated for 24 h, followed by transfection with 0.125 µg of carp HA-Tlr20-GFP vector. Alternatively, cells were transfected with the same amount of a pcDNA3-GFP plasmid as negative control, or with pcDNA3-TLR2-YFP (Addgene plasmid 13016 encoding for human TLR2) as positive control and incubated for 72 h. After this incubation period, cells were stimulated with different ligands for 5 h, medium was replaced with Bright glow (Promega), the suspension transferred to a white 96 well plate with opaque bottom (Corning®, Cat nr. 3300) and luminescence measured (Filtermax 5, Molecular Devices).

Cells were stimulated with recombinant human tumour necrosis factor alpha (rhTNFα) or with one of the following TLR ligands (all InvivoGen); synthetic diacylated lipopeptide Pam_2CSK_4 (tlrl-pm2s), ultra-pure LPS from *Escherichia coli* O111:B4 (tlrl-eblps), ultrapure lipopolysaccharide from *Porphyromonas gingivalis* (tlrl-pgLPS), purified lipoteichoic acid from *Staphylococcus aureus* (tlrl-psLTA), ultrapure endotoxin-free single-stranded DNA from *E. coli* (tlrl-ssec), CpG ODNs 1668 (tlrl-1668), polyinosinic-polycytidylic acid (tlrl-pic), flagellin from *S. typhimurium* (tlrl-stfla),

23S rRNA (ORN Sa19) from *S. aureus* (tlrl-orn19). Parasite lysates of *Trypanoplasma borreli* were made by washing column-purified parasites (1×10^8 parasites/ml) [48] in carp RPMI, and lysing by sonication. Lysates were stored at −80 ˚C until use. Profillin from *Toxoplasma gondii* (ALX-522-093-C010) was purchased from Enzo® Life Sciences.

Real-time quantitative polymerase chain reaction (RT-qPCR)

To investigate gene expression of *tlr20*, RT-qPCR was performed using ABsolute QPCR SYBR Green Mix (no Rox) (Thermoscientific) with a Rotor-Gene™ 6000 (Corbett Reasearch) as previously described [49]. Primers used for RT-qPCR were designed to amplify the S11 protein of the 40S subunit (40S) as a reference gene or carp tlr20 (see Table 1) using OligoAnalyser 3.1 IDT (Integrated DNA Technologies) (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx). To 7 µl SYBR Green master mix containing forward and reverse primers (300 nM each), 5 µl of 50 times-diluted cDNA, was added. The following cycling conditions were used: one holding step of 15 min at 95˚C; followed by 40 cycles of 15 sec at 95˚C for denaturation, 20 sec at 60˚C for annealing and 20 sec at 72˚C for elongation, followed by a final holding step of 1 min at 60˚C. A melting curve was then created with continuous fluorescence acquisition starting at 60˚C with a rate of 0.5˚C/5 sec up to 90˚C to determine the amplification specificity. In all cases, amplification was specific and no amplification was observed in negative control samples (non-template control and non-reverse transcriptase control). Fluorescence data from RT-qPCR experiments were analysed using Rotor-Gene software version 1.7 (built 87) and exported to Microsoft Excel. Relative expression ratios were obtained using the Pfaffl method [50], using average efficiencies per run, per gene. Gene expression of the house keeping gene was highly constant as determined by the BestKeeper software [51] and used to normalize the data. Each PCR product was checked at least once by sequencing.

In vivo infection with *Trypanoplasma borreli*

Infections with extracellular blood parasites *Trypanoplasma borreli* were performed as described previously [52]. Briefly, *T. borreli* was maintained by syringe passage through carp following intra-peritoneal (i.p.) injections with 1×10^4 parasites per fish. Before infection with *T. borreli*, carp were anaesthetised in 0.3 g/l TricaineMethane Sulfonate (TMS, Crescent Research Chemicals, Phoenix, USA) and i.p. injected with *T. borreli* or with PBS as non-infected control. At various time points after infection, fish were euthanized with an overdose of anaesthetic.

Statistical analysis

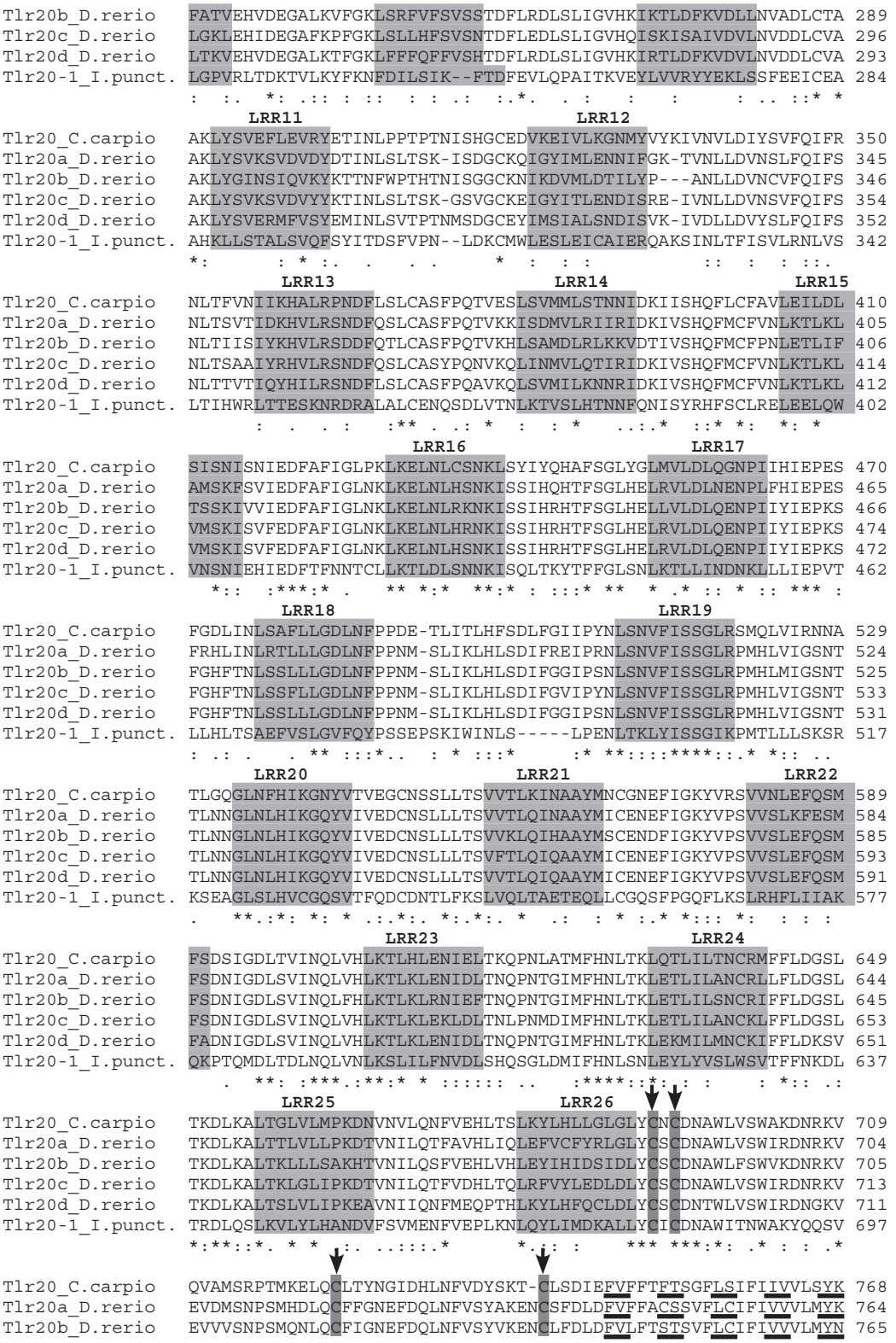
Relative expression ratios (R) were calculated as described above. Transformed (LN(R)) values were used for statistical analysis in GraphPad prism version 5. For all tests, homogeneity of variance was assessed using Levene's test. Significant differences ($P<0.05$) were determined by two-way ANOVA followed by Sidak test. In case of unequal variances between groups, a two-way ANOVA was performed followed by a Games-Howell test for the infection studies with parasites. For studies on constitutive gene expression levels, significance of differences were determined by one-way ANOVA in comparison with values in organs or cells with lowest expressed values.

RESULTS

Identification of multiple zebrafish *Toll-like receptors 20 genes*

In an earlier genome analysis of the Toll-like receptor families of zebrafish, the presence of six zebrafish *tlr20* genes was predicted *in silico* [15]. More detailed analysis of the latest zebrafish genome assembly Zv9 confirmed the presence of six *tlr20* genes. Zebrafish *tlr20b*, *tlr20a*, *tlr20c* and *tlr20d* are located (in this order) adjacent to each other on one part of chromosome 9 (region: 28388211-28414876), whereas *tlr20f* and *tlr20e* are also located next to each other but on a different part (region: 31239519-31278948) of chromosome 9, distant from the other *tlr20 genes* (see also **Figure 4**). However, the latter two copies (*tlr20f* and *tlr20e*) contain mutations in the reading frame that are not leading to the expected products, in one case leading to a premature stop codon. This further supports the prediction that two of the six copies of zebrafish *tlr20* are pseudogenes. The full-length sequences (*tlr20b*, *tlr20a*, *tlr20c* and *tlr20d*) have open reading frames ranging from 2826-2853 bp encoding for proteins of 942-951 aa (**Figure 1**) with predicted molecular weights of 124.7-126.0 kDa. Leucine-rich repeats are often flanked by N-terminal and C-terminal cysteine-rich domains (LRRNT and LRRCT). The four full-length zebrafish Tlr20 molecules have an N-terminal leucine rich repeat (LRRNT) ($Cx_{14}Cx_8C$ or $Cx_{23}C$), a C-terminal LRR (LRRCT) ($CxC_{28}Cx_{18}C$) and 26 additional LRR, a transmembrane domain and Toll/IL-1R domain (TIR) (see also **Tables 2** and **3**). Zebrafish Tlr20 molecules (*tlr20a-d*) have a signal peptide of 19-25 aa indicating that they are targeted to the secretory pathway.


```
                                                                                                  LRR1
Tlr20_C.carpio   --------MVPLFSLFILFLKTSCICSWLADKCLFYSDVQEFPVATTCAGKTNIAECHHV 52
Tlr20a_D.rerio   --------MGLFFAFFILFLKTSYVCSWLAGKCHIESDGEEFSFSTSSPRKRNTTVCRSV 52
Tlr20b_D.rerio   --------MLLLFGFVILFLKTSCVCSWLNEKCRVYSDGEEFPVKASSASKLNITVCRSV 52
Tlr20c_D.rerio   MKIPEKLKMVLLFALFILCPKTCYVCSWLAEKCHFFSDVEGLNIASFYHGKTSIAECLSV 60
Tlr20d_D.rerio   ---MAMLKRVLLFALLLLFLKTYFVCSWLAGKCFVYGDVKEFPLTTYCPGNINGADCKHV 57
Tlr20-1_I.punct.  --------MQFFSFVVFKNLLTCVLCWVSEKCFVYEDYLDVKLVGDCPKSNLTGYCRGV 51
                            :*.:.::      : .*:  ** .  *   . .      .     *  *
                                      LRR2                          LRR3
Tlr20_C.carpio   TNIKEDLRGLPSNLLNLCIQMERDFHGALAPDSFSRFASLEYLEIVGCFSEIPPEAFNEL 112
Tlr20a_D.rerio   TDMKEDLRGLPANLQNLIVQTDLGYHGVLAPNSFLRFGSLENLEIVGCLSEVPPEAFNGL 112
Tlr20b_D.rerio   TDIKEDLQGLPANLLNLFVHMDGGCHGVLAPNSFSSFASLEQLTISGCFSEIPPEAFNGL 112
Tlr20c_D.rerio   TDIKEDLRGLPTNLLNLLVQMDLHFHGVLAPNSFSRFGSLENLKIAGCFSEIPPEAFNGL 120
Tlr20d_D.rerio   TDIKEDLRGLPSSLQSVCIQMDGGSDGVLAPNSFSSFASLKQLTIAGCFSQIPPEAFNGL 117
Tlr20-1_I.punct. SNVALDLAGIPPHLEVLCVDLMKDSS--LHPYSFSRFQKLMKLKIFGEISTIHPGAFKNL 109
                 :::  ** *:*. *  : :.     .. * * **  * .*  * * * :* : * **: *
                     LRR4                          LRR5                          LRR6
Tlr20_C.carpio   TNVTSLKLSFLSTAICCEVALDFSRLSSLNHLSLSQYSLSSLAPNVFEMIPQLQKLKILN 172
Tlr20a_D.rerio   TNVTTLTLSCS--QQCTEVAFDFSRLTSLTSLSLSDYSLSLLPSNVFEKIPQLRWLHLCS 170
Tlr20b_D.rerio   TNVTSLTISYNFSENCSKVALDFSHLPSLTSLSISDYSLSLLASNVFETIPLLQNLILAN 172
Tlr20c_D.rerio   TNVTSLTITALDSKNCCEVALDFSHLPSLTSLFISHHDLSLLALNVFEKIPHLQWLYLDS 180
Tlr20d_D.rerio   TNVTSLSISSSNSEKCCEVALDFSRLPSLTRLFISYYDLSLFKLNVFDKIPHLEELHLGN 177
Tlr20-1_I.punct. SSLQRLEIYSLQSLNLSLSSEILNDLPNATTLLFINCRLSSMAIDVFKGMKKLEQLGFIN 169
                 :.:  * :           :  :. *.. . * :    ** :  :**. :  *. * : .
                                LRR7                                  LRR8
Tlr20_C.carpio   VCLKDISEVVCRLAKAKSLKLFKLEEYDLNRLHYPNCSVFNISDGYISTEFD-IEEVHFY 231
Tlr20a_D.rerio   ECLKDLSEVLCRLKNVKSLKYLGLGNTMLTRLQYPNCSVFNVSE--ISTKFN-IERADLL 227
Tlr20b_D.rerio   VCLRDMSEVLCRLSKVKTLKHFTLKEFLLKRLQYPNCSVFNTSD--ISTEFN-IEEVNLH 229
Tlr20c_D.rerio   VCLKDLSDVLCRLANVKLLKLFSL-DDWNIRLQYQNCSVFNTTD--ISTEFS-IETVDLL 236
Tlr20d_D.rerio   VCLNNISEVLCRLANVKSLKQFNL-DGVLHKLQHSNCSVFNTSN--ISTEFN-IEKVNLT 233
Tlr20-1_I.punct. -STEDFSELLCRLTFVSSSLNYLYVESESLVIDRPNCTFSNGTS--FDVMLHGIEEVSLI 226
                  . .::*:::***  ..        :     :.  **:. * :.  :.. :  ** . :
                                LRR9                          LRR10
Tlr20_C.carpio   LGIVEHVDEGALEVLGNLFSLRFV-SNTDFLRDLSLFGVHKIHNLRVRMAVLNVDDLCTA 290
Tlr20a_D.rerio   LGKLEHVDEGALKVFGKLSRLYFTVSSKNFLRDLSLIGVHQISVIIATVDVLNVDDLCVA 287
```


LRR11
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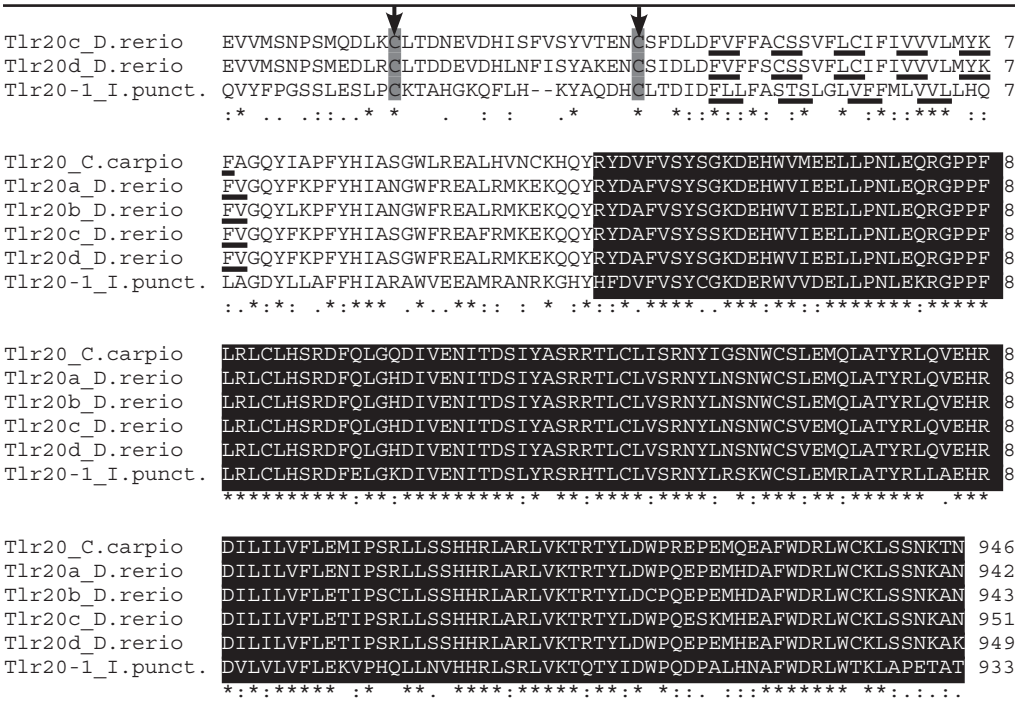


Figure 1. Multiple amino acid alignment of Tlr20 shows conservation of leucine rich repeat (LRR), transmembrane (TM) and toll/interleukin-1 (TIR) domains. Multiple alignment of amino acid sequences from common carp (*Cyprinus carpio*) Tlr20 (KF482527), zebrafish (*Danio rerio*) Tlr20a (ENSDARG00000094411), Tlr20b (ENSDARG00000092668), Tlr20c (ENSDARG00000041164), Tlr20d (ENSDARG00000088701) and channel catfish (*Ictalurus punctatus*) Tlr20-1 (AEI59676). Alignment was performed using ClustalW v2.0. The putative signal peptide is underlined. Predicted leucine rich repeat domains are highlighted in grey and numbered LRR1-LRR26. Conserved cysteine residues important for the LRRNT and LRRCT domains are indicated by arrows above the sequence alignment. The transmembrane region is underlined, whereas the TIR domain is highlighted in black. Asterisks (*) indicate identities, double dots (:) indicate conserved substitutions, single dots (.) indicate semi-conserved substitutions and dashes (-) denote gaps used to maximize the alignment. A predicted, but not yet confirmed Tlr20 (AGKD01003001) from the genome of Atlantic salmon (*Salmo salar*) and an incomplete Tlr20-2 (AEI59677) from channel catfish [11] were not included in the alignment.

Identification of a single *Toll-like receptor 20 (tlr20)* in carp

A putative *tlr20* sequence was identified in the draft genome of common carp based on ORF prediction and BLAST alignment with zebrafish *tlr20a*. A single exon containing carp *tlr20* was predicted from scaffold 28896 and this sequence was used to clone the full-length carp *tlr20* cDNA (GenBank accession number KF482527). We obtained a complete cDNA sequence with open reading frame of 2841 bp, encoding for a protein of 946 aa with a predicted molecular weight of 124.97 kDa. Carp Tlr20 is predicted to contain a signal peptide of 22 aa, an N-terminal and a C-terminal leucine rich repeat (LRRNT, LRRCT) and 26 additional LRRs, a transmembrane domain and a TIR domain (see **Figure 1**). Multiple sequence alignments of carp Tlr20 with the four full-length zebrafish Tlr20 and with channel catfish (*Ictalurus punctatus*) Tlr20-1 showed a high degree of conservation.

Table 2. Comparison of amino acid identity of extracellular and TIR domains of Tlr20

Name		*C.car.*	*D.rerio*				*I.pun.*	*M.musculus*		
		Tlr20	Tlr20 a	Tlr20 b	Tlr20 c	Tlr20 d	Tlr20 -1	Tlr11	Tlr12	Tlr13
C.car.	Tlr20		63.7	63.9	63.9	64.7	38.4	14.9	16.3	14.3
D.rerio	Tlr20a	86.0		72.2	76.9	71.8	34.4	18.3	14.9	16.6
	Tlr20b	84.9	95.5		71.9	71.6	37.1	16.5	17.5	13.9
	Tlr20c	84.5	96	92.5		74.4	34.4	15.3	16.3	14.0
	Tlr20d	86.0	97	64.0	97.0		36.4	17.4	14.2	17.1
I.pun.	Tlr20-1	64.1	65.7	66.7	64.1	59.1		21.2	19.2	19.9
M.mus.	Tlr11	32.5	33.5	33.7	33.0	21.1	35.3		33.0	17.0
	Tlr12	44.9	45.6	44.9	44.2	29.2	47.6	51.0		18.7
	Tlr13	44.0	45.3	44.6	44.6	26.4	42.8	32.7	40.1	

Numbers (top right triangle) indicate percentage identity of the extracellular domains (ECD). Highlighted numbers (lower left triangle) indicate percentage identity of the intracellular TIR domain. Abbreviations: *C.car = Cyprinus carpio; D.rerio = Danio rerio; I.pun.= Ictalurus punctatus; M.mus.=Mus musculus*.

Tlr20 three-dimensional modelling and phylogeny

The three-dimensional structure of carp Tlr20 was modelled using as best fit the crystal structure of human TLR8 (PDB-id: 3w3g). Carp Tlr20 fit well the dimer structure of human TLR8 composed of two copies arranged in a symmetrical fashion (**Figure 2**). Mammalian TLR8 is known to contain 27 LRRs; a characteristic of the TLR7 family that includes TLR7, TLR8 and TLR9. Although Tlr20 has (only) 26 LRR, the human TLR8 model showed the best possible fit. TLRs from the TLR7 family have a 58-73 residue loop between LRR15 and LRR16 [3], whereas carp Tlr20 has a shorter, 13 residue loop between LRR15 and LRR16. The biological consequence, if any, of these slight differences is unknown.

Phylogenetic analysis was conducted based on the amino acid sequences of the TIR domain of known Tlrs from common carp, zebrafish and channel catfish in comparison with Tlrs from the mouse (**Figure 3**). Phylogenetic analysis supported previous observations that the fish species have at least one gene representing each of six major TLR families (TLR1, TLR3, TLR4, TLR5, TLR7 and TLR11). The TLR11 family presently includes Tlr11, Tlr12 and Tlr13 (mouse) and Tlr19, Tlr20, Tlr21, Tlr22 and Tlr26 (non-mammalian Tlrs). Within the Tlr11 family, Tlr19, but also Tlr21 and Tlr22 branched off close to Tlr13 from the mouse. Tlr20 and Tlr26 (catfish) branched off close to

Tlr11 and Tlr12 (mouse), suggesting that teleost Tlr20 may share a common ancestor with Tlr11/ Tlr12.

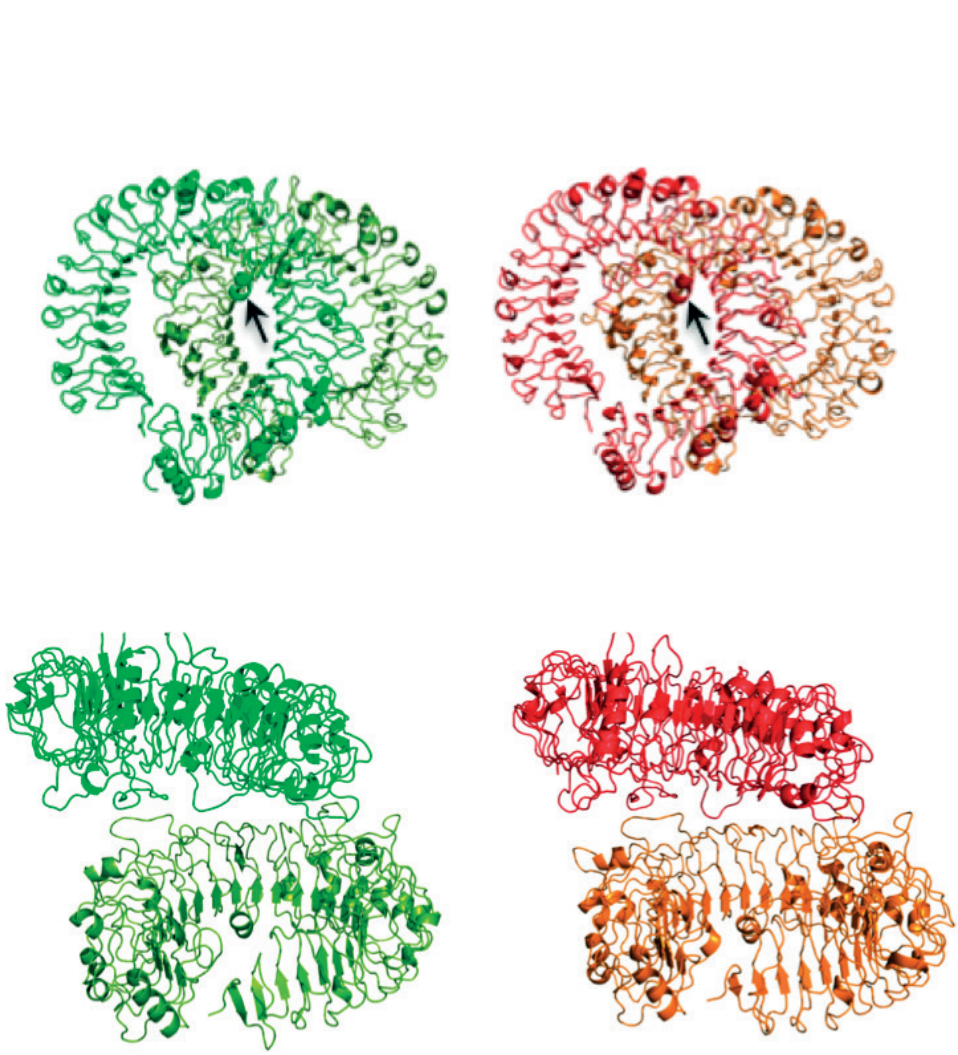


Figure 2. Three-dimensional structure of Tlr20 shows a best fit to human TLR8. a) Frontal view and b) top view of carp Tlr20 (green) and human TLR8 (red). Three-dimensional model for carp Tlr20 based on the crystal structure of human TLR8 (PDB-id: 3w3g). Carp Tlr20 is shown as a homodimer on the left panels (bright and dark green for each monomer); human TLR8 as a homodimer on the right panels (bright and dark red for each monomer). The loop between LLR15 and LRR16 is indicatedwith black arrow.

Sequence and synteny analysis of Tlr20

In general, sequence similarity was high comparing carp and zebrafish, especially between TIR domains (**Table 2**), but lower comparing carp or zebrafish with channel catfish. Sequence analysis confirmed conservation of structural features of TLR20s including (the number of) n=26

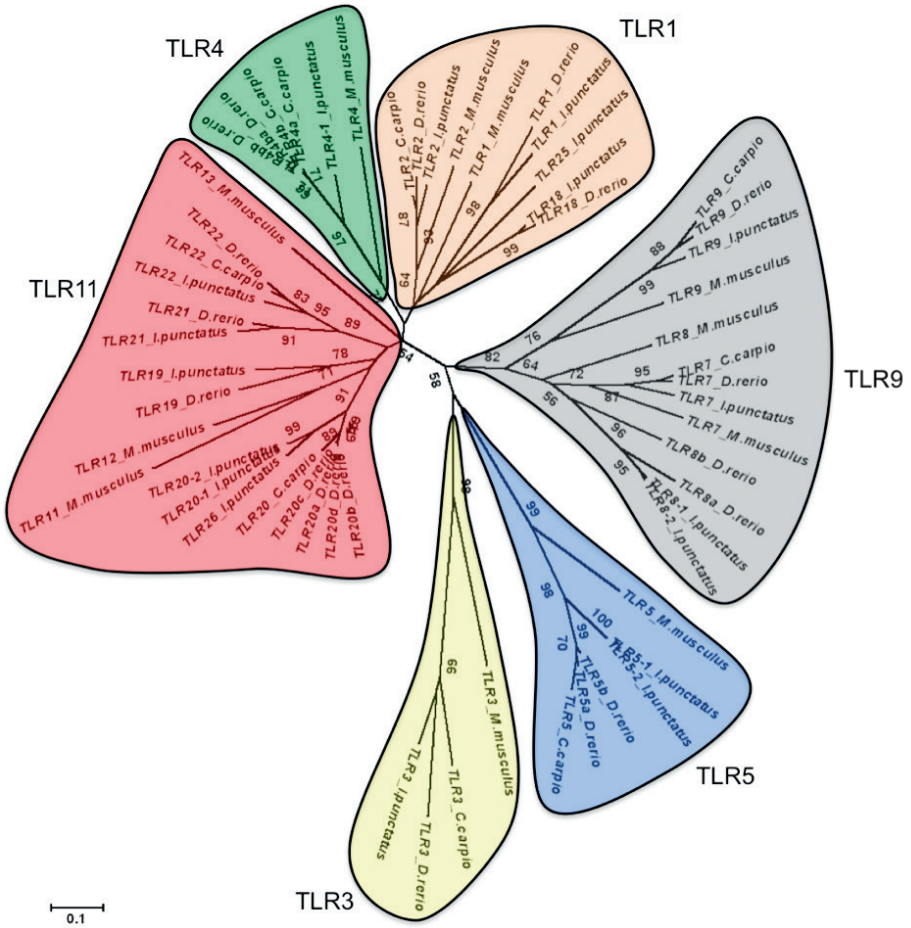


Figure 3. Phylogenetic analysis of Tlr20 shows clustering with Tlr11 family members Tlr11 and Tlr12. Neighbor-Joining tree based on amino acid identities in the TIR domains of Tlrs from carp, zebrafish, channel catfish and mouse. Phylogenetic analysis was conducted with 10,000 bootstrap replicates; bootstrap values at major branching points are shown as percentages. The sequences were derived from *Cyprinus carpio* (Tlr2: ACP20793, Tlr3: ABL11473, Tlr4a: ADC45015, Tlr4b: ADY76945, Tlr5: AGH15501, Tlr7: BAJ19518, Tlr9: ADC45018, Tlr20: KF482527, Tlr22: ADR66025); *Danio rerio* (Tlr1: AAI63271, Tlr2: AAQ90474, Tlr3: NP_001013287, Tlr4ba: ACE74929, Tlr4bb: NP_997978, Tlr5a: XP_001919052, Tlr5b: NP_001124067, Tlr7: XP_003199309, Tlr8a: XP_001920594, Tlr8b: XP_001340186, Tlr9: NP_001124066, Tlr18: AAI63840, Tlr19: XP_002664892, Tlr20a: ENSDARG00000094411; Tlr20b: ENSDARG00000092668; Tlr20c: ENSDARG00000041164; Tlr20d: ENSDARG00000088701; Tlr21: NP_001186264, TLR22: AAI63527); *Ictalurus punctatus* (Tlr1: AEI59662, Tlr2: AEI59663, Tlr3: AEI59664, Tlr4-1: AEI59665, TLR5S: AEI59667, Tlr5-1: AEI59668, Tlr5-2: AEI59669, Tlr7: AEI59670, Tlr8-1: AEI59671, Tlr8-2: AEI59672, Tlr9: AEI59673, Tlr18: AEI59674, Tlr19: AEI59675, Tlr20-1: AEI59676, Tlr20-2: AEI59677, Tlr21: AEI59678, Tlr22: AEI59679, Tlr25: AEI59680, Tlr26: AEI59681); *Mus musculus* (Tlr1: NP_001263374, Tlr2: AAD46481, Tlr3: NP_569054, Tlr4: AAD29272, Tlr5: NP_058624, Tlr7: AAL73191, Tlr8: AAK62677, Tlr9: AAK28488, Tlr11: AAS37672, Tlr12: AAS37673, Tlr13: AAS37674).

LRRs, presence of LRRNT and LRRCT, transmembrane domain and, in particular, the intracellular TIR domain (aa identity > 60%) (**Table 2**). Comparison of aa identity between fish *tlr20* and mouse *tlr11*, *tlr12* and *tlr13* generally showed a low degree of conservation. The extracellular domain of TLRs, important for the recognition of PAMPs, can consist of 16-28 LRRs. The TLR11

family members have 20-28 LRR [3]. LRRs are sometimes difficult to predict using programs such as SMART or LRR-finder; therefore we predicted LRRs manually and by three-dimensional modelling of carp Tlr20. The number of LRRs for teleost Tlr20 and murine Tlr11-13, including LRRNT and LRRCT, are listed in **Table 3**. All Tlr20 molecules from carp, zebrafish and catfish except the channel catfish Tlr4-2 have an identical number of 26 LRRs. In mouse, Tlr11, Tlr12 and Tlr13 have 25, 24 or 27 LRRs, respectively. The $Cx_{14}Cx_{8}C$ LRRNT motif is shared among teleost Tlr20, with the exceptions of zebrafish Tlr20b and Tlr20c ($Cx_{23}C$). The LRRCT motif of the Tlr20 molecules is comparable among the different teleosts ($CxCx_{28}Cx_{16}C$ (catfish) or $CxCx_{28}Cx_{17}C$ (carp) or $CxCx_{28}Cx_{18}C$ (zebrafish)), but different from the LRRCT motifs of mouse Tlr11-13 ($CxCx_{24}Cx_{16-19}C$).

Conservation of synteny was investigated by comparing the genomic regions immediately up-and down-stream of mouse *tlr11* on chromosome 14, mouse *tlr12* on chromosome 4 and mouse *tlr13* on the x chromosome with the genomic regions up- and down-stream of the zebrafish *tlr20* genes on two regions of chromosome 9. In catfish, genome information on the immediate areas around *tlr20* is scarce [53] and could not be used to investigate synteny. In carp, although limited in length, genomic regions up-and down-stream of *tlr20* (scaffold 28896; size 20020 bp) confirmed conservation of synteny with two genes (genes *slc10a2* and *gtpbp8*) in the region upstream of zebrafish *tlr20*. Synteny analysis of zebrafish *tlr20* did not reveal any conservation with genes flanking mouse *tlr11*, *tlr12* or *tlr13* (**Figure 4**). Moreover, the two genes found in close proximity of zebrafish and carp *tlr20*; *slc10a2* and *gtbp8,* in mouse are located on two different chromosomes, *slc10a2* is located on chromosome 16 (region 44736768-44746363) and *gtbp8* is located on chromosome 8 (region 5085623-5105232). Thus, analysis of the genes adjacent to zebrafish *tlr20* do not indicate conserved synteny between teleost Tlr20 and murine members of the TLR11 family.

Table 3. Molecular characteristics of teleost Tlr20 and mouse Tlr11, Tlr12 and Tlr13. List of open reading frame (aa length), signal peptide, number of leucine rich repeats (LRR) and signature of leucine rich N-terminal (LRRNT) and C-terminal (LRRCT) domains in common carp (*Cyprinus carpio*), zebrafish (*Danio rerio*) and channel catfish (*Ictalurus punctatus*) Tlr20 and mouse (*Mus musculus*) Tlr11, Tlr12 and Tlr13. Predicted Atalntic salmon (*Salmo salar*) Tlr20 was not include.

Name	aa lenght	Signal peptide	LRR	LRRNT	LRRCT
CcTlr20	946	22	26	$Cx_{14}Cx_{8}C$	$CxCx_{28}Cx_{17}C$
DrTlr20a	942	19	26	$Cx_{14}Cx_{8}C$	$CxCx_{28}Cx_{18}C$
DrTlr20b	943	19	26	$Cx_{23}C$	$CxCx_{28}Cx_{18}C$
DrTlr20c	951	25	26	$Cx_{23}C$	$CxCx_{28}Cx_{18}C$
DrTlr20d	949	24	26	$Cx_{14}Cx_{8}C$	$CxCx_{28}Cx_{18}C$
IpTlr20-1	933	18	26	$Cx_{14}Cx_{8}C$	$CxCx_{28}Cx_{16}C$
IpTlr20-2	351	NA	4	NA	$CxCx_{28}Cx_{16}C$
MmTlr11	931	35	25	$Cx_{17}Cx_{11}C$	$CxCx_{24}Cx_{19}C$
MmTlr12	906	19	24	$Cx_{17}Cx_{10}C$	$CxCx_{24}Cx_{19}C$
MmTlr13	991	NA	27	$Cx_{11}C$	$CxCx_{24}Cx_{16}C$

Abbreviation: Tlr, Toll-like receptor; Cc, *Cyprinus carpio*; Dr: *Danio rerio*, Ip: *Ictalurus punctatus,* Mm*: Mus musculus.* NA= not applicable.

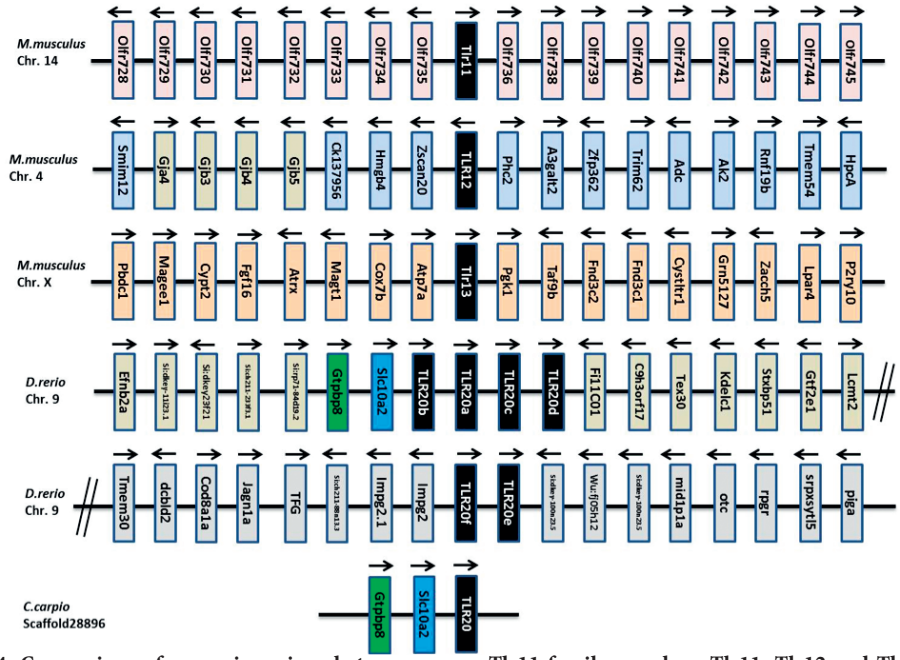


Figure 4. Comparison of genomic regions between mouse Tlr11 family members Tlr11, Tlr12 and Tlr13 and teleost Tlr20 does not show conservation of synteny. Comparative maps of the regions locating Tlr11, Tlr12 and Tlr13 genes on mouse chromosomes (genome GRCm38), Tlr20 genes on zebrafish chromosome 9 (genome assembly GCA_000002035) and Tlr20 on carp scaffold 28896 (genome assembly PRJNA73579).

Sub-cellular localization of Tlr20

To investigate the sub-cellular localization of Tlr20, we transfected human HEK 293 and fish EPC and CLC cell lines with HA-tagged carp Tlr20-GFP (HA-Tlr20-GFP; **Figure 5**). Only in permeabilized cells the presence of the HA-Tlr20 could be visualized (**Figure 5b**), suggesting a preferential expression of Tlr20 in intracellular compartments in all three cell lines studied. To further investigate the sub-cellular localization of Tlr20, we co-transfected HA-Tlr20-GFP-transfected zebrafish ZF4 cells with a plasmid encoding for KDEL-RFP protein for specific localization to the ER (**Figure 5c**). Co-localization of KDEL-RFP with Tlr20-GFP confirmed a preferential expression of Tlr20 in intracellular compartments and suggested a sub-cellular localization to the ER.

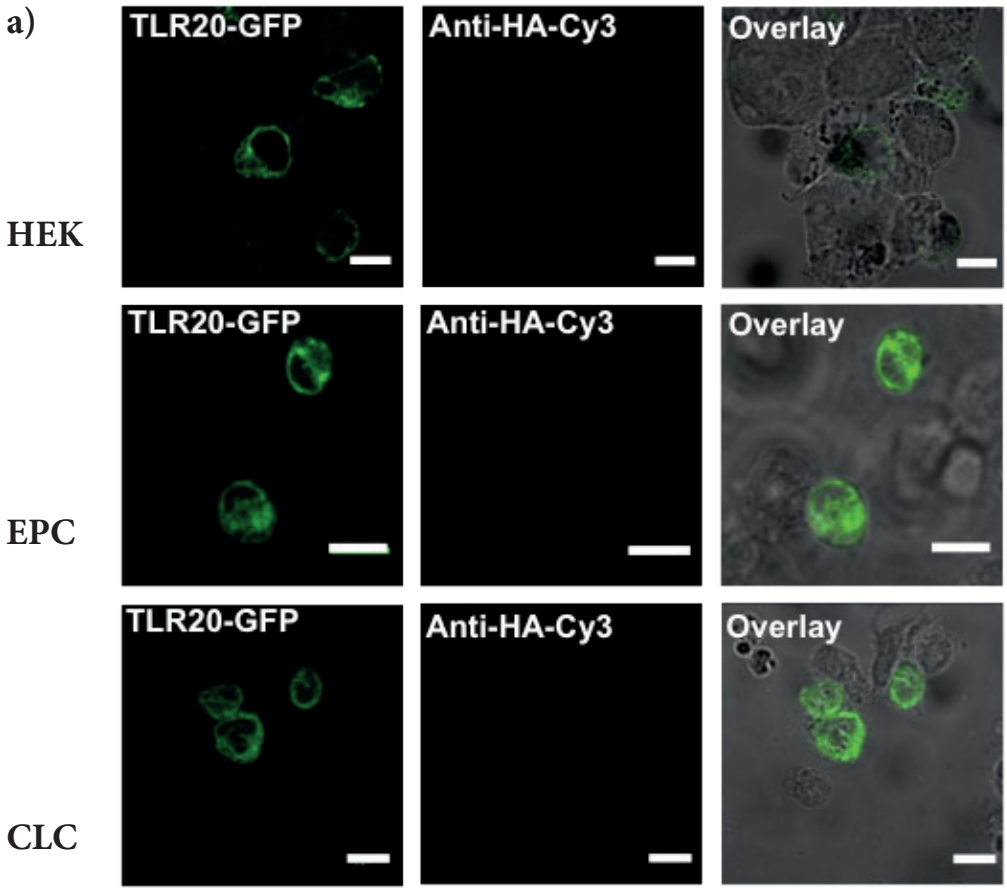
In vitro ligand binding of Tlr20

To investigate the putative ligands of Tlr20 we developed a reporter assay based on a human (HEK 293) cell line stably transfected with a NF-κB luciferase reporter construct (HEK-pNiFty-Luc) and transiently transfected with carp Tlr20. We used transient transfection with human TLR2 as positive control. Successful transfection was confirmed by microscopy, evaluating the percentage of fluorescent cells (40-50% approximately) by visualizing GFP for carp Tlr20 or YFP for human TLR2. Responses to ligands were measured as luminescence and expressed as relative light units. Stimulation with human TNFα induced a very high response in HEK-NFκB-Luc cells, either transiently transfected with empty plasmid, human TLR2 or carp Tlr20. Overexpression of human TLR2 and stimulation with a prototypical TLR2 ligand, Pam_2CSK_4, induced a very high and specific luminescence response. However, stimulation with profillin (*Toxoplasma gondii*-derived ligand of murine Tlr11 and Tlr12) did not lead to activation of the NFκB promotor (**Figure 6**).

Stimulation with other prototypical TLR ligands (LPS-PG, LPS-EB, LTA, E. coli ssDNA, CpG, Poly (I:C), flagellin and 23S rRNA (ORN Sa19) also did not lead to cell activation (data not shown).

In vivo modulation of tlr20 gene expression after parasitic infection

In vitro studies could not clearly identify a ligand for carp Tlr20 therefore we examined biological sample collections from both zebrafish and carp for *tlr20* gene expression during infection with bacterum, virus or parasite. Using existing biological sample collections [54, 55] we mapped the reads of RNAseq experiments on the zebrafish *tlr20* transcripts. Zebrafish *tlr20a-d* are transcribed during both larval and adult stages but at very low levels, close to the detection limit. Based on reads linked to the polymorphic regions we can conclude that all full length copies have a detectable, although low transcription level. Since the *tlr20* copies are extremely similar to each other, a majority of the mapped reads could not be assigned to a particular *tlr20* transcript and thus a specific induction of one of the copies of *tlr20* could not be discerned and is technically not possible with the current standards of sequencing depth. We could not obtain evidence for an induction of any of the *tlr20* copies by infection with *Mycobacterium marinum* nor *Staphylococcus epidermis* bacterial infection (data not shown). *Tlr20* was also not significantly modulated in biological sample collections taken after viral infection of carp with spring viraemia of carp virus (SVCV) [52]. In contrast, infection of carp with the blood parasite *T. borreli* [29], induced a clear 2-6 fold upregulation of *tlr20* gene expression 6 weeks after infection (late stage of infection),



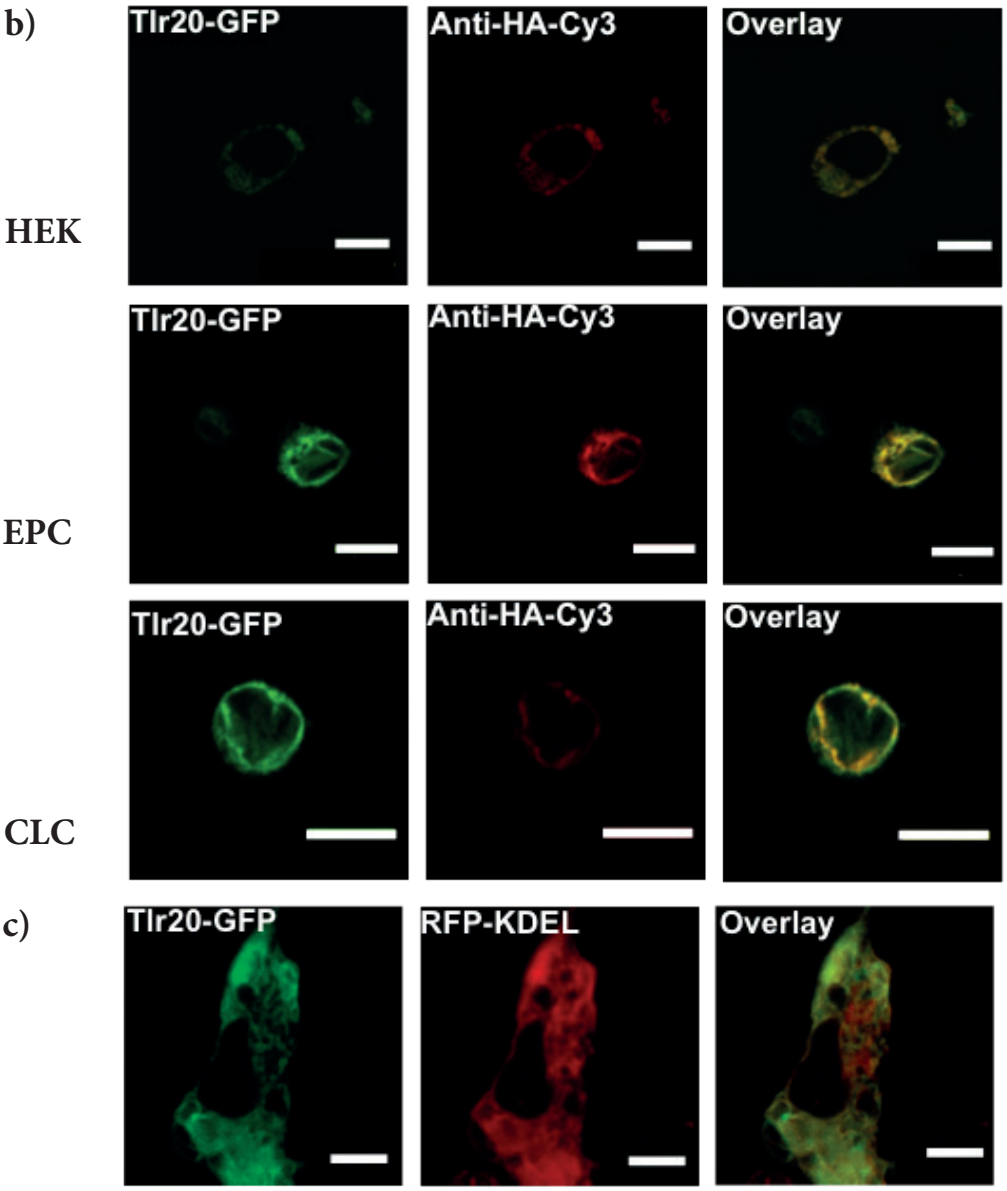


Figure 5. Tlr20 preferentially locates intracellularly at the endoplasmatic reticulum (ER). a) non-permeabilized cells, **b)** permeabilized cells, **c)** cell co-transfected with KDEL-RFP for localization to the ER. **A** and **B**: Confocal microscopy of HEK 293, EPC, CLC cells overexpressing carp Tlr20. Cells were seeded 24 h prior to transfection with HA-Tlr20-GFP. Three days later, cells were either permeabilized or not and stained for microscopy using mouse anti-HA and donkey anti-mouse Cy3 antibodies. Left panels: Tlr20-GFP (green); middle panels: recognition of HA-tagged proteins (red); right panels: overlay (yellow-orange).
C: Confocal microscopy of ZF4 cells overexpressing carp Tlr20. Sub-cellular localization to the ER was examined in live cells 2 days after transfection with KDEL-RFP. Left: Tlr20-GFP (green); middle: KDEL-RFP (red); right: overlay (yellow-orange). Bar= 10μm.

at least in head kidney, spleen and in peripheral blood leukocytes (PBL) (**Figure 7**). Similarly, infection with *Trypanosoma carassii* [26] a related blood parasite of carp, induced a clear 2-4 fold upregulation of *tlr20* gene expression at 6-8 weeks after infection (data not shown). Altogether, these data suggest that *tlr20* plays a in the immune response to parasitic rather than bacterial or viral infections.

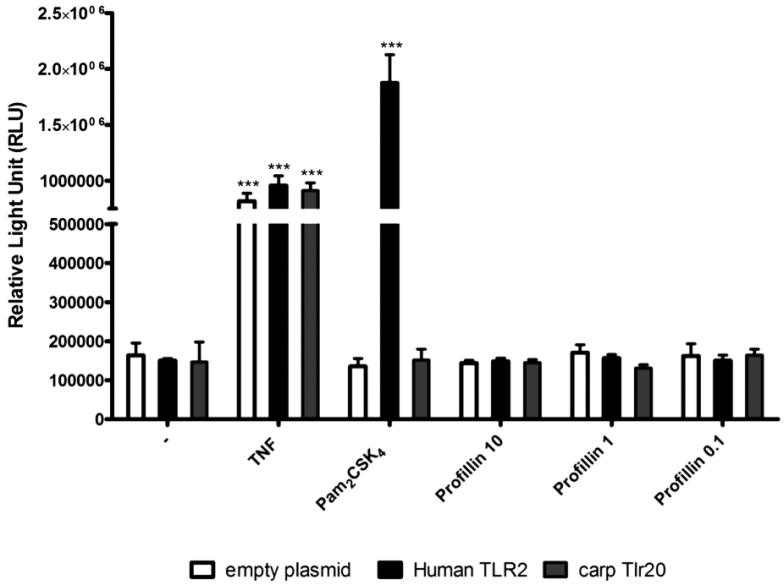


Figure 6. **In vitro ligand-binding studies do not clearly identify a ligand for Tlr20.** Reporter HEK-NFκB-Luc cells were transiently transfected with empty plasmid (pcDNA3-GFP) or with plasmid coding for human TLR2-YFP or carp HA-Tlr20-GFP. After 72 h, transiently transfected cells were stimulated with PBS, human TNF (200 ng/ml), Pam_2CSK_4 (20 μg/ml) or profillin (10, 1, 0.1 μg/ml) from *Toxoplasma gondii* for 5 h. After stimulation, luminescence was measured in cell lysates and expressed as relative light units. Values represent mean ± S.D. of triplicate wells of one representative experiment out of three independent experiments. Significant differences with respect the control (empty plasmid) are indicated with an asterisk (***) (P<0.001).

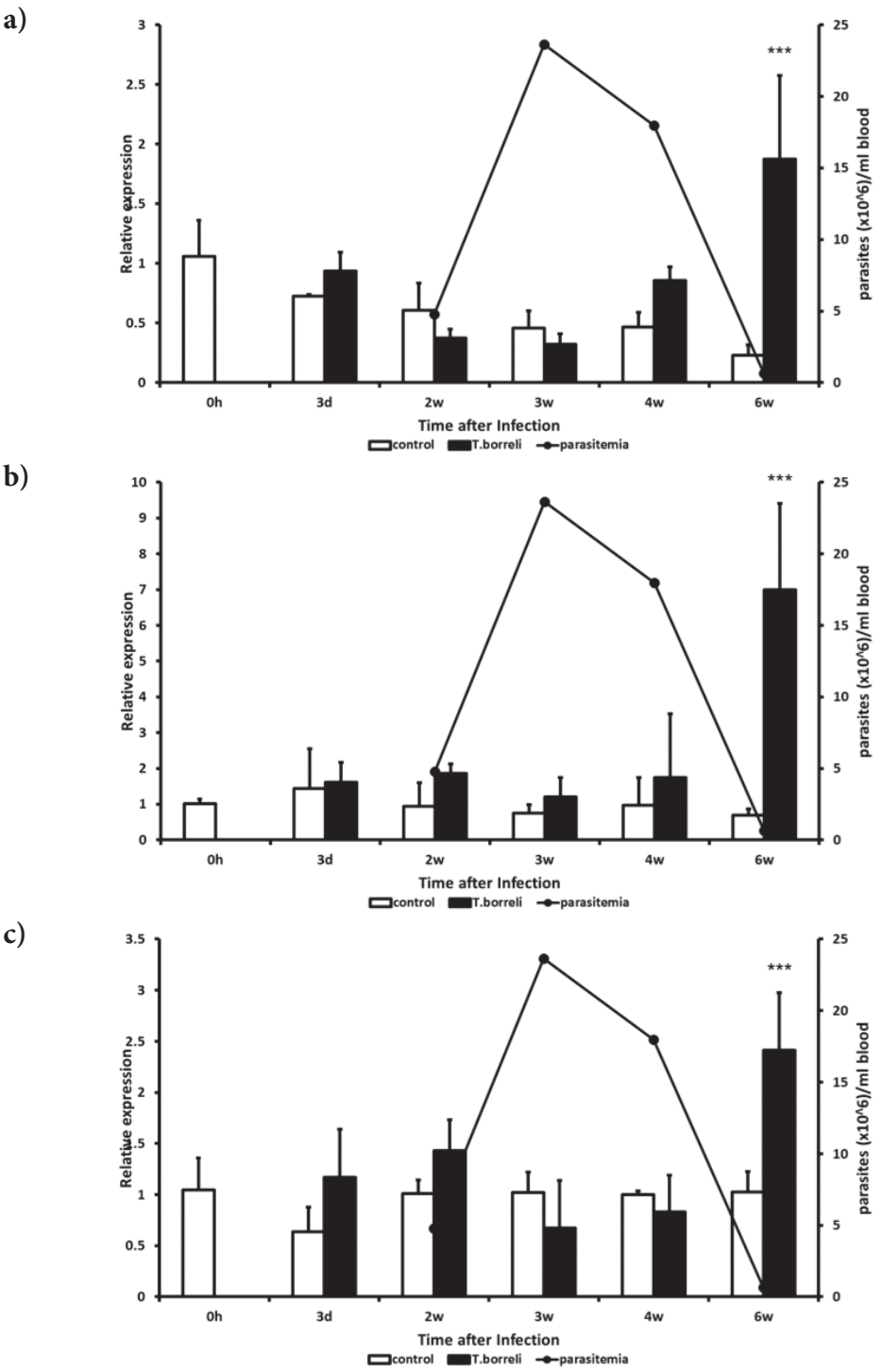


Figure 7. In vivo infection with parasites induces upregulation of *tlr20* gene expression in carp. Gene expression profiles after infection with *T. borreli* in a) head kidney, b) spleen and c) peripheral blood leukocytes (PBL). Carp were injected with 1 x 10^4 *Trypanoplasma borreli* parasites per fish, or with PBS (negative control). Organs were collected from *n*=5 infected and *n*=3 non-infected fish at each time point, over a period of 6 weeks.

Parasitaemia (*T. borreli*/ml blood) is shown as a line graph. Relative gene expression was normalized to the reference gene and the non-infected controls at time point zero and is shown as bar graphs. White bars represent non-infected control and black bars represent *T. borreli*-infected carp. Values represent mean ± S.D. of n=3-5 fish. Significant differences to the time point control are indicated with an asterisk (***) (P<0.001).

Modulation of *tlr20* gene expression by parasite lysate

Induced gene expression of *tlr20* was high in head kidney, spleen and PBL. Constitutive gene expression on *tlr20* was examined in a tissue and leukocyte cDNA library of naïve carp. A relatively high basal gene expression level of carp *tlr20* was observed in several organs, especially in gut and PBL (Figure 8a). Further, basal gene expression of carp *tlr20* was particularly high in B lymphocytes sorted from PBL (Figure 8b). Although in vitro stimulation of HEK-pNiFty-Luc cells transiently transfected with carp Tlr20 with parasite (*T. borreli*) lysate did not lead to cell activation (data not shown), in vitro stimulation of PBL from naïve fish with *T. borreli* lysate induced a clear (3-fold) upregulation of *tlr20* (Figure 8c). In addition, in vitro re-stimulation of PBL from carp that survived a *T.borreli* infection with *T.borreli* lysate also induced a clear (3-fold) upregulation of *tlr20* (data not shown).

DISCUSSION

The complexity of the Toll-like receptor families still is increasing, owing to the continuous discovery of additional members that do not seem to have clear homologues to mammalian TLRs. Apparently, there are several Tlrs that have been lost during evolution but are present in reptiles, amphibians and/or fish [8, 11, 12, 56]. Tlr20 is a non-mammalian Tlr without clear homology to any of the known mammalian Tlrs, which presence seems unique to the modern bony fish. The function of Tlr20 has remained unknown. We identified full-length cDNA sequences for *tlr20* of both, zebrafish and common carp, two closely-related cyprinid fish species. Previously, full-length cDNA sequences for *tlr20* had only been described for channel catfish [11], a fish species that is among the closest living relatives to the cyprinids. Catfish Tlr20 is found in two copies as a close proximity tandem duplication in the catfish genome. At present it is not clear if the second, shorter gene (*tlr20*-2) encodes a functional protein [11]. Although salmonid fish also appear to express Tlr20; at least in rainbow trout a partial *tlr20* EST has been identified [20] and a *tlr20* sequence has been retrieved from a whole genome shotgun sequencing of Atlantic salmon [11], so far attempts to retrieve *tlr20* from other teleost groups have failed.

In silico analysis of an early version of the zebrafish genome (ZV2) predicted six *tlr20* sequences with some found as close proximity tandem duplications on chromosome 9 [15]. The first expression studies with reverse transcriptase PCR suggested that at least two *tlr20* genes in zebrafish were expressed, whereas also modulation by mycobacterium infection was reported [15]. Indeed, we confirmed the presence of six zebrafish *tlr20* genes in the latest assembly of the zebrafish genome (ZV9), but found that two copies (*tlr20e* and *tlr20f*) in the genome contain mutations in the reading frame that are not leading to the expected products, in one case leading to a premature stop codon. The four full-length zebrafish *tlr20* all have a signal peptide. Zebrafish *tlr20* displayed a low constitutive gene expression level which was not significantly modulated upon infection with *M. marinum* or *S. epidermis*, at least not to an extent detectable by RNAseq.

Surprisingly, in common carp, which is a very close relative of zebrafish, we could detect only a single *tlr20* sequence in the genome. This is surprising because usually in the tetraploid carp genes are found as duplicated copies of those found in diploid zebrafish [22]. Although it cannot

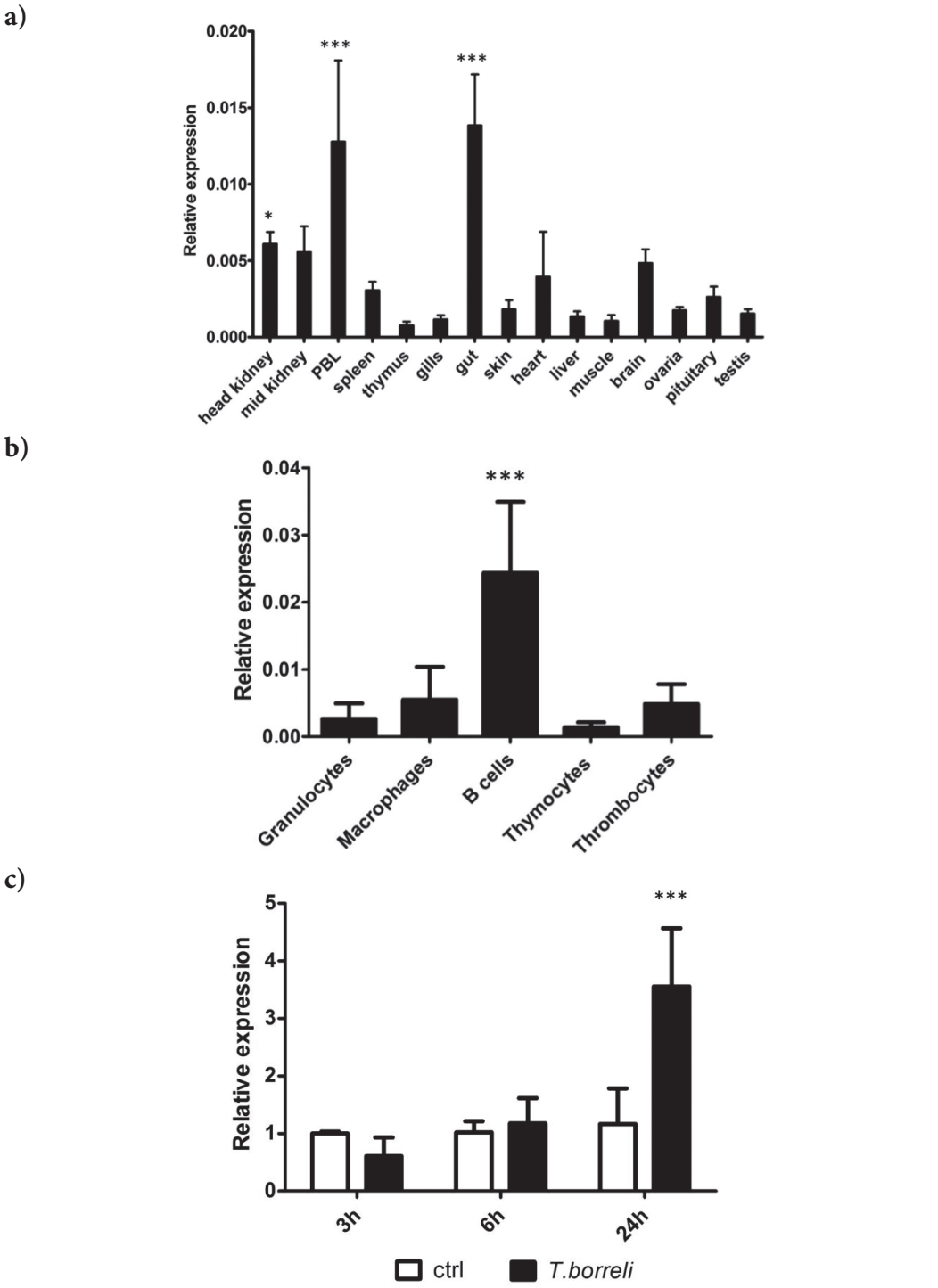


Figure 8. Peripheral blood leukocytes (PBL) of carp express relatively high basal *tlr20* gene expression levels that can be induced by parasites. Gene expression profiles in a) organs from naïve fish, b) leukocyte cell populations from naïve fish and c) PBL stimulated in vitro with parasite lysate. Constitutive mRNA levels of *tlr20* in different organs and different leukocyte cell populations of carp (a and b). Induced mRNA levels of *tlr20* in PBL of

naïve fish stimulated in vitro with *T. borreli* parasite lysate (equivalent of 1:2 parasites:cells) (c). Relative gene expression was normalized to the reference gene and is shown as bar graphs. Significant differences were calculated in comparison with the lowest expressed values in thymus (a) or thymocytes (b). Bars represent mean ± S.D. of *n*=3-5 healthy carp (a and b, respectively) or triplicate wells of one representative experiment out of three independent experiments. Significant differences were calculated using one-way ANOVA and are indicated by asterisks (*) (P <0.01) and (***) (P <0.001).

be excluded that some mistakes are present in the current assembly of the carp genome it is highly unlikely that up to a number of 11 possible copies of the carp *tlr20* genes would have been missed during the assembly.

Sequence analyses place teleost Tlr20 in the TLR11 family which also comprises three Tlrs unique to mouse: Tlr11, Tlr12 and Tlr13, characterized by 25, 26 or 27 LRRs, respectively [3]. Assumed important for the recognition of PAMPs, the extracellular domain of Tlr20 molecules from carp, zebrafish and catfish (Tlr20-1) all have an identical number of 26 LRRs. Assumed important for protection of the hydrophobic core of the first LRR [23], the $Cx_{14}Cx_8C$ LRRNT motif is the same among the teleost Tlr20 molecules, but different from the LRRNT motifs found in mouse Tlr11-13. Assumed important for protection of the hydrophobic core of the last LRR, the $CxCx_{28}Cx_{16\text{-}18}C$ LRRCT motif is similar, although not exactly the same, in the teleost Tlr20 molecules and comparable to the LRRCT motifs found in mouse Tlr11-13 ($CxCx_{24}Cx_{16\text{-}19}C$). These molecular characteristics suggest that Tlr20 fits well the TLR11 family but also point at clear differences between teleost Tlr20 and Tlr11 family members found in the mouse.

Synteny analysis of the zebrafish genome showed that Tlr20 is not orthologous to any of the mouse members of the Tlr11 family because no conservation of synteny was found with genes neighboring *tlr11*, *tlr12* or *tlr13*. However, conserved synteny was observed between the region downstream of the zebrafish *tlr20-f* gene and carp *trl20*. The region upstream of the carp *tlr20* gene could not be analyzed due to the limited length of the relevant contig.

Three-dimensional modelling based on the crystal structure of human TLR8, an intracellular TLR that senses RNA, showed a similar structure for all teleost Tlr20 molecules characterized by a slightly distorted horseshoe shape the effect of which, if any, is unknown. TLRs can be expressed in different compartments of the cell; on the cell surface, in intracellular vesicles such as endosomes, or as part of the endoplasmatic reticulum (ER). TLRs can also translocate from one compartment to another. Our confocal microscopic analysis of Tlr20 suggested a possible sub-cellular localization of Tlr20 in the ER. This could correspond with the best three-dimensional fit to intracellular TLR8.

In vitro reporter assays based on NF-κB activation following overexpression of carp Tlr20 in human cell lines, or fish cell lines (EPC and CLC, data not shown), could not identify a ligand unique to Tlr20. It could be that the cell lines used, represent cell types that could not fully support natural sub-cellular localization, ligand binding and/or Tlr20 signalling. Such an observation has been made for salmon Tlr9 which, when overexpressed in salmonid cell lines, failed to translocate to CpG-containing endosomes. Apparently, only specific immune cell types in salmon have the ability to relocate the Tlr9 receptor to the appropriate cellular compartments where it may become activated by its ligand [57]. UNC93B1 is a transmembrane protein required for TLR3, TLR7, TLR9, TLR11, TLR12, and TLR13 function, which controls trafficking from the ER to endolysosomes. UNC93B1 remains associated with TLRs through post-Golgi sorting steps, but these steps are different among endosomal TLRs. For example, TLR9 requires UNC93B1-mediated recruitment of adaptor protein complex 2 (AP-2) for delivery to endolysosomes whereas TLR7, TLR11, TLR12, and TLR13 utilize alternative trafficking pathways. Thus, endosomal TLRs are differentially sorted

by UNC93B1 [58]. Despite the identification of a sequence encoding for a *unc93b1* homologue in teleosts [59], it cannot be excluded that this molecule, or other accessory molecules [60], crucial to the natural function of Tlr20 could be too different, or absent, in the (human or fish) cell lines used. Indeed, a preliminary investigation of the transcriptome of the EPC fish cell line suggests these cells do not express *unc93b1* (Pietretti, unpublished data). The apparent absence of *unc93b1* from the EPC transcriptome could maybe have affected the functional characterization of Tlr20 overexpressed in this particular cell line.

Phylogenetic analyses place Tlr20 closest to Tlr11 and Tlr12 of the TLR11 family, two TLRs that sense ligands from protozoan parasites (*Toxoplasma gondii*) in the mouse. Only few studies have looked at the expression of Tlr20 *in vivo* after infections. Initial studies in whole zebrafish embryos infected with *M. marinum* [15] suggested increased expression of *tlr20a* 8 weeks after intraperitoneal injection of bacteria. In adult channel catfish, infection with *E. ictaluri* led to increased expression of *tlr20* six hours after injection [61]. We re-examined biological sample collections from both zebrafish and carp for *tlr20* gene expression. Infection of zebrafish with *S.epidermis* [55] and with *M.marinum* [54] did not clearly modulate *tlr20* gene expression. Infection of carp with spring viraemia of carp virus (SVCV) [52] did not significantly modulate *tlr20* gene expression after bath challenge with this virus. In contrast, infection of carp with the blood parasite *Trypanoplasma borreli* [29], induced a clear upregulation of *tlr20* gene expression after the peak of parasitaemia, at 6 weeks after infection. Moreover, we observed a similar upregulation of *tlr20* at 6-8 weeks post infection with another carp blood parasite *Trypanosoma carassii* (data not shown). Subsequent analysis of constitutive gene expression in different organs and leukocyte cell types confirmed a high constitutive expression of Tlr20 in PBL and in B lymphocytes sorted from PBL. We looked in more detail at *tlr20* gene expression induced by *T. borreli*. Stimulation of PBL from naïve fish with *T. borreli* lysate induced a clear upregulation of Tlr20. Also re-stimulation of PBL from fish that had survived a *T. borreli* infection induced a clear upregulation of Tlr20. In humans, immature transitional B cells and naïve B cells exhibit some responses to Tlr ligands, in particular CpG-containing oligodinucleotides, but exhibit strong responses when simultaneously stimulated *via* the B cell receptor and CD40. Also IgM-positive memory B cells exhibit strong responses to Tlr ligands [62]. In future experiments, it would be of interest to study the role of Tlr20 in fish B lymphocytes and putative effect of B cell receptor co-stimulation. Although our data indicate that Tlr20 plays a role in the immune response to trypanosomes, it is difficult to define a clear ligand for Tlr20 based on our *in vivo* studies. In general, the sub-cellular localization of TLRs often corresponds to the place at which recognition of particular PAMPs occurs; TLRs expressed at the cell surface generally recognize outer membrane components of microbes such as lipids and (lipo)proteins, whereas TLRs expressed intracellularly recognize microbial nucleic acids [63]. The intracellular localization of Tlr20 could point at a nucleic acid type of ligand or pathogen-derived protein produced by the host. Our *in vitro* reporter assays could not clearly identify a ligand for Tlr20. Future studies could take into account accessory proteins present or absent in cell lines used for *in vitro* studies. Such accessory molecules can be divided based on their functions as: mediators of ligands delivery and/or recognition, chaperones, trafficking and TLR processing factors [60]. The identification of several, although not all of the accessory molecules in fish [59] will allow for combinations of studies on Tlr molecules and accessory molecules and may shed further light on the function of fish-specific TLRs such as Tlr20.

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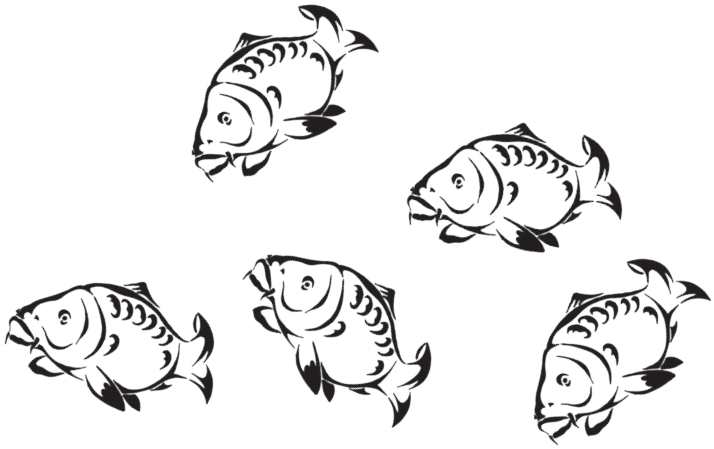

Chapter 5

Functional study of Toll-like receptor 4 of fish

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Manuscript in preparation

ABSTRACT

It has been long established that TLR4 senses Gram-negative bacterial lipopolysaccharide (LPS), at least in mammalian vertebrates. Fish have been shown relatively resistant to the toxic effects of LPS and the receptor remains elusive. In fish the *tlr4* gene seems present particularly in members of the cyprinid and silurid families, which include species such as zebrafish (*Danio rerio*), grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*) and channel catfish (*Ictalurus punctatus*). The presence, however, of *tlr4* genes in these fish species does not necessarily lead to recognition of LPS, possibly due to the apparent absence of essential co-stimulatory molecules such as MD-2 and CD14. To understand the role of *tlr4 in vivo*, zebrafish larvae lacking the *tlr4ba* and *tlr4bb* genes were infected with *Salmonella typhymurium*. Adult carp were infected with Spring Viraemia of Carp Virus (SVCV) and *tlr4* gene expression measured. No difference in fish survival after bacterial infection could be observed between knockout and wild-type zebrafish, suggesting the presence of Tlr4 is not crucial for protection against *S.typhimurium*. Carp infected with SVCV showed an upregulation of *tlr4* gene expression at 4 days post-infection, possibly suggesting a role for carp Tlr4 in the immune response to viruses. Preliminary results from *in vitro* reporter assays indicated that carp Tlr4 alone is not able to directly bind the surface glycoprotein of SVCV and activate NFkB. Further investigations revealed the presence of three novel *tlr4* genes in common carp (preliminary referred to as *tlr4bc*, *tlr4bd* and *tlr4be*), among which *trl4be* leading to the expression of a soluble protein. We discuss, based on three-dimensional modelling of carp Tlr4, the possibility that fish Tlr4 could sense LPS in the presence of the MD-2 co-receptor.

INTRODUCTION

Toll-like receptor 4 (TLR4) is a protein that in humans, together with the co-receptor myeloid differentiation protein-2 (MD-2) senses lipopolysaccharides (LPS), a major component of the outer membrane of Gram-negative bacteria. LPS-binding protein (LBP) and CD14 are two proteins whose coordinate actions assist delivery of LPS to the TLR4-MD-2 complex [1]. In contrast to humans, fish tolerate relatively high concentrations of LPS [2] and the absence of *md-2*, *cd14*, and a prototypical *lbp* from fish genomes [3, 4] could help explain this phenomenon of high tolerance to LPS. Although initial genome studies on pufferfish suggested that fish might not express the *tlr4* gene [5], by now it has become clear that at least Cypriniform and Siluriform fish species do express *tlr4* genes. To date, *tlr4* genes have been cloned and characterized in zebrafish (*Danio rerio*) [6, 7], rare minnow (*Gobiocypris rarus*)[8], common carp (*Cyprinus carpio*) [9], grass carp (*Ctenopharyngodon idella*)[10] and channel catfish (*Ictalurus punctatus*)[11].

Only limited functional studies have been performed and mostly on zebrafish Tlr4. Dual-luciferase reporter assays were used to study NF-κB activation in whole zebrafish embryos, suggesting that LPS signals via a TLR4- and MyD88-independent manner but also indicating that zebrafish Tlr4 negatively regulates the Myd88-dependent signalling pathway [12]. Additional functional studies used chimeric molecules combining zebrafish Tlr4 extracellular leucine rich repeat (LRR) domains with mouse intracellular Tlr4 Toll/interleukin-1 receptor (TIR) domains, demonstrating a lack of responsiveness to LPS [13]. To date, overall consensus seems to be that fish Tlr4 molecules do not play a major role in the sensing of LPS [14]. Most likely fish Tlr4 molecules have evolved to sense ligands alternative to LPS. Human TLR4 does not only sense LPS from Gram

negative bacteria but also, for example, viral envelope proteins. However, these proteins (at least Respiratory Syncytial Virus (RSV) F protein) may also require the presence of CD14 and MD-2 for signalling [15]. Also in mouse, Tlr4 signaling has been shown to play an important role in controlling infection with paramyxovirus [16] or RSV [17], again linking Tlr4 to viral infections. Of interest, in fish, PCR-based gene expression studies showed upregulation of *tlr4* in cyprinid fish after infection with grass carp reovirus [8, 10], whereas the first detection of common carp *tlr4* was in cDNA pools of viral (KHV)-infected carp [9]. Alternative to LPS and viral ligands; human TLR4 can also sense fungal cell wall components such as β-glucans: although of the many TLR genes TLR2 may be the most important TLR for recognition of β-glucans, also TLR4 has been implicated in stimulations by fungal-derived PAMPs [18]. In the present study we provide a molecular and functional characterization of Tlr4 from zebrafish and common carp, two cyprinid fish species. We investigated the role of Tlr4 *in vivo* in zebrafish larvae lacking the previously described *tlr4ba and tlr4bb* genes (knockouts) following infection with Gram negative *Salmonella thyphimurium* bacteria. We also investigated the role of *tlr4* in carp following *in vivo* infection of adults with Spring Viraemia of Carp Virus (SVCV). Analysis of constitutive *tlr4* gene expression was performed in organs and leukocytes from healthy carp. Confocal microscopy was applied to study sub-cellular localization of carp Tlr4 whereas *in vitro* reporter assays were used to study candidate ligands for Tlr4, including viral proteins and ß-glucans. We did not only identify several additional copies of *tlr4* in the genome of common carp but we also further characterized the phylogenetic origin of a third copy of a *tlr4* gene present in the genome of zebrafish and recently reported in the databse but not yet fully characterized. We discuss, based on our three-dimensional modeling of carp Tlr4, the possibility that fish Tlr4 could sense LPS in the presence of the MD-2 co-receptor.

MATERIALS AND METHODS

Animals

European common carp (*Cyprinus carpio carpio*) were reared in the central fish facility of Wageningen University at 23±2°C in recirculating UV-treated water and fed pelleted dry food (Sniff, Soest, Germany) daily. R3xR8 heterozygous carp are the offspring of a cross between fish of Hungarian (R8 strain) and of Polish (R3 strain) origin [19]. Carp were between 9 and 11 months old. All studies on carp were performed with approval from the animal experimental committee of Wageningen University. Wild-type zebrafish (*Danio rerio*) were obtained from the Zebrafish International Resource Center (ZIRC) and maintained as described in the zebrafish handbook [20] and in a 12 h light/dark cycle at 28.5°C. The knock-out zebrafish *tlr4ba-/-* and *tlr4bb-/-* were obtained from the Moens lab TILLING project for unrec_tlr4ba and unrec_tlr4bb. All animal studies on zebrafish were carried out in accordance with the European Union regulations for animal experimentation.

In vivo infection in zebrafish and carp

Zebrafish larvae 72 hours post fertilization were microinjected with *Salmonella typhymurium* strain SL 1027. Bacteria were taken from a -80˚C glycerol stock, plated on fresh LB agar plates and incubated overnight at 37˚C. Individual colonies were resuspended in sterile phosphate-buffered saline (PBS) and directly used as fresh suspension for the injections. Bacterial suspension was mixed in microinjection buffer (0.5× Tango buffer and 0.05% phenol red solution) and 50 bacteria/larvae

microinjected (0.5–1 nl) into the yolk sac of zebrafish embryos using a Narishige IM300 microinjector. Zebrafish larvae were monitored for 8 days after injection. SVCV strain CAPM V 539 [21] was propagated in EPC cells (Epithelioma Papulosum Cyprini, [22]) at 15°C. Cells were grown in Eagle's Minimal Essential Medium (MEM) containing 2% fetal bovine serum (FBS) and standard concentration of antibiotics. Virus titers are given as tissue culture infective dose (TCID50/ml; [23]. Fish were exposed, by immersion, to 10^3 $TCID_{50}$/ml for 2 h [24]. Briefly, ten-month-old carp were raised at 15°C, the temperature optimal for SVCV infectivity [25], to an average weight of 30–40 g. Fish were sampled at 0h, 2, 4 and 7 days-post-infection. At each time point the level of viral N gene expression was determined in samples from mid kidney (primers used: SVCV-N_Fw TGAGGTGAGTGCTGAGGATG and SVCV-N_Rv CCATCAGCAAAGTCCGGTAT).

Molecular cloning of carp *tlr4*

Oligonucleotide primers for carp *tlr4ba* and carp *tlr4bb* were designed based on known partial carp *tlr4* and full length zebrafish *tlr4aa* and *tlr4bb* sequences from GenBank (accession number: carp *tlr4ba* GU321982.2 and *tlr4bb* HQ229652.1; zebrafish tlr4ba NM_001131051.1 and zebrafish tlr4bb NM_212813.1). Gene-specific primers were designed using Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) primers and are listed in **Table 1**. Primers to complete the 3' end of carp tlr4ba and tlr4bb were: tlr4baFW1 and tlr4baRV1 and tlr4baFW2 and tlr4baRV2; tlr4bbFW1 and tlr4bbRV1 and tlr4bbFW2 and tlr4bbRV2. Primers to complete the 5' end of carp tlr4ba and tlr4bb were: tlr4baFW3 and tlr4baRV3 and tlr4baFW4 and tlr4baRV4; tlr4bbFW3 and tlr4bbRV3 and tlr4bbFW4 and tlr4bbRV4. The full length coding sequence was amplified using as template RNA isolated from head kidney tissue of carp three weeks after infection with the parasite *Trypanoplasma carassii* [26] using a LongRange 2Step-RT-PCR kit (Qiagen) and gene specific primers tlr4baFw5 in combination with Ttlr4baRv5 or tlr4bbFw5 in combination with Ttlr4bbRv5 (**Table 1**). A second PCR was performed using the same gene specific primers and Expand High Fidelity Plus PCR System (Roche). The products were cloned in JM109 competent *E. coli* using pGEM-Teasy kit (Promega) and both strands of eight positive clones were sequenced using ABI Prism-Bigdye Terminator Cycle Sequencing Ready Reaction kit and analysed using ABI 3730 sequencer. Nucleotide sequence data were analysed for identity to other sequences using the GenBank database [27].

Table 1. Primers used

Primer	Sequence (5′-3′)	Used
tlr4baFw1	ACTTGATTCCCTTGAGATTC	Cloning
tlr4baRv1	TAGGTGGAACACCGTTCTCTAG	Cloning
tlr4baFw2	CTAGAGAACGGTGTTCCACCTA	Cloning
tlr4baRv2	TTTAATCAGAATACACACAATGA	Cloning
tlr4bbFw1	GCACTTGTTCCGTTGTATG	Cloning
tlr4bbRv1	ATCTGTCTGGGAGCAGGAG	Cloning
tlr4bbFw2	ATTTGTCCTCTAACCCCATC	Cloning
tlr4bbRv2	AAAAGCTGCTATGAAAATGTAA	Cloning
tlr4baFw3	ATGAATGAAGGGAGAGACAT	Cloning
tlr4baRv3	TAGTCTTGATGTAGGAGGATTTGAT	Cloning
tlr4baFw4	GAGTACTCATGTTCTGG	Cloning
tlr4baRv4	ATGTCTCTCCCTTCATTCAT	Cloning
tlr4bbFw3	CTTAGAGTCGGGACAAATAAC	Cloning
tlr4bbRv3	GACTAGTTTTGTATGGTGGAG	Cloning
tlr4bbFw4	TGATATAAATTGAAACAGATTGTAG	Cloning
tlr4bbRv4	ATGAATGAAGGGAGAGACAT	Cloning
tlr4baFw5	CCAGCAGTCTTCCCTTCACTGT	Cloning
tlr4baRv5	TGTAACATGACTGGAAAACCATACTGA	Cloning
tlr4bbFw5	TTGCTGTAGGATGTAGAATCTCCTG	Cloning
tlr4bbRv5	GCTGCTATGAAAATGTAACATGACTGG	Cloning
q40SFw	CCGTGGGTGACATCGTTACA	Cloning
q40SRv	TCAGGACATTGAACCTCACTGTCT	qPCR
qtlr4baFw	ATTGATGAGATGGAGTATGTATTT	qPCR
qtlr4baRv	TAGTTTTTCTAAAGTATGGAGA	qPCR
qtlr4bbFw	AGCCCCCACTTTATTATCTG	qPCR
qtlr4bbRv	GAACAACAGTCCTTCAAAA	qPCR
cyca*tlr4bb*FW1	CTGGACAAGGAGATTACAAGGATGACGATGAC AAGCAGGAATGTACCACGATAATCAAG	Tlr4bb-GFP construct
cyca*tlr4bb*FW2	TGATTTTTCTAGGCTCAGTCTTATTTTTGGCGA GTTCTGGACAAGGAGATTACA	Tlr4bb-GFP construct
cyca*tlr4bb* **BamHIFW3**	ACGTACGGATCCAACATGGTCATGTCATATGGG GAATGGATGATTTTTCTAGGCT	Tlr4bb-GFP construct
cyca*tlr4bb* **XhoIRv1**	ACGTACCTCGAGTTGGTTTGTGGCAAAAATAG CTTTCCTGAG	Tlr4bb-GFP construct

Highlighted sequence indicate Flag tag (GATTACAAGGATGACGATGACAAG); underlined sequence indicated the BamHI restriction site (GGATCC); double underlined sequence indicate XhoI restriction site (CTCGAG).

RNA isolation and cDNA synthesis

For real-time quantitative polymerase chain reaction (RT-qPCR), total RNA was isolated from different different cell types, as previously described: monocytes [28], macrophages [26], granulocytes [29], thrombocytes ([30], thymocytes [31], B lymphocytes [29] and endothelial cells [32].

RNA was isolated from different carp organs using Trizol® (Invitrogen, Breda, The Netherlands) according to the manufacturer's protocol and stored a -80˚C until use. RNA concentration was measured by spectophotometry (GeneQuant, Pharmacia Biotech) at OD_{260} nm and the purity determined as the OD_{260nm}/OD_{280nm} ratio with expected values between 1.8 and 2.0. The integrity of RNA was determined by gel electrophoresis on 1% agarose gel containing 0.1% of SYBR® Safe DNA Gel Stain (Invitrogen™). For cDNA synthesis 1 µg total RNA was used and a DNase treatment was performed using DNase I amplification grade (Invitrogen) according to the manufacturer's instructions. Synthesis of cDNA was performed with Invitrogen's SuperScript™ III First Strand Synthesis Systems for RT-PCR using random primers, according to the manufacturer's instructions. A non-reverse transcriptase control was included for each sample. Before use as template in RT-qPCR analysis, the cDNA was further diluted 25-50 times in nuclease-free water.

Real-time quantitative polymerase chain reaction (RT-qPCR)

To investigate gene expression of *tlr4ba* and *tlr4bb* from carp, RT-qPCR using ABsolute QPCR SYBR Green Mix (no Rox) (Thermoscientific) was performed with a Rotor-Gene™ 6000 (Corbett Reasearch) as previously described [33]. Primers used for RT-qPCR were designed to amplify the S11 protein of the 40S subunit as a reference gene (**Table 1**). Primers were designed using OligoAnalyser 3.1 IDT (Integrated DNA Technologies) (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx). To 5 µl of 50 times-diluted cDNA, 7 µl Master SYBR Green mix, forward and reverse primer (300 nM each) and MilliQ water up to 14 µl was added. Following cycling conditions were used: one holding step of 15 min at 95˚C; followed by 40 cycles of 15 sec at 95˚C for denaturation, 20 sec at 60˚C for annealing and 20 sec at 72˚C for elongation, followed by a final holding step of 1 min at 60˚C. A melting curve was then created with continuous fluorescence acquisition starting at 60˚C with a rate of 0.5˚C/5 sec up to 90˚C to determine the amplification specificity. In all cases, amplification was specific and no amplification was observed in negative control samples (non-template control and non-reverse transcriptase control). Fluorescence data from RT-qPCR experiments were analysed using Rotor-Gene software version 1.7 (built 87) and exported to Microsoft Excel. Relative expression ratios were obtained using the Pfaffl method [34], using average efficiencies per run, per genes. Gene expression of the house keeping gene was highly constant as determined by the BestKeeper software [35] and used to normalize the data. Products were checked at least once by sequencing.

Generation of carp Tlr4bb-GFP construct

The PCR product amplifying the complete carp *tlr4bb* coding sequence was used as template for a PCR using the cyca-FLAG-tlr4bbFW1 in combination with cyca-tlr4bbXhoIRv1 (**Table 1**) followed by a second PCR using the cyca-tlr4bbPFW2 in combination with cyca-tlr4bbXhoIRv1. The PCR products were purified and used as template for a final PCR using cyca-tlr4bbBamHIFW3 and cyca-tlr20-XhoI-Rv1. Primers were designed to add a *BamHI* site at the 5'end upstream of the leader pepetide and a FLAG tag and an *XhoI* site at the 3' end, excluding the *tlr4bb* stop codon.

Subsequently, this product was ligated into the BamHI and XhoI sites of a pcDNA3.1 plasmid (Promega) in frame with the sequence of the Green Fluorescent Protein (GFP) that was already inserted in the vector, to obtain the Tlr4bb-GFP fusion product.

In vitro ligand studies

HEK 293 (human embryonic kidney) cells were transfected with 3.5 µg of pNiFty-Luc, a plasmid encoding for the luciferase reporter gene under the control of the NF-κB-inducible ELAM-1 composite promoter (Invivogen). HEK293 cells were cultured in DMEM F12 (Gibco®) medium supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamin and 1% streptomycin/penicillin at 37˚C, or 27˚C with 5% CO_2. Stably transfected cells (HEK-NFκB-Luc) were selected using 250 µg/ml Zeocin (Life technologies™).

For transient transfection of the Tlr4bb-GFP vector, stably-transfected HEK-NFκB-Luc cells were first plated at 5 x 10^4 cells/well in 96 well plate and incubated for 24 h, followed by transfection with JetPRIME™ (Polyplus) with 0.125 µg of carp Tlr4bb-GFP vector alone or in combination with 0,125 µg of the pcDNA3-SVCV-G plasmid encoding for the Glycoprotein of SVCV (kindly provided by Dr. Niels Lorenzen). Alternatively, cells were transfected with the same amount of a pcDNA3-GFP plasmid as negative control, or with pcDNA3-TLR2-YFP (Addgene plasmid 13016 encoding for human TLR2) as positive control and incubated for 72 h. After this incubation period, cells were stimulated with different ligands for 5 h, medium was replaced with Bright glow luciferase (Promega), the suspension transferred to a white 96 well plate with opaque bottom (Corning®, Cat nr. 3300) and luminescence measured (Filtermax 5, Molecular Devices). In parallel, as additional source of SVCV-G protein EPC cells (epithelioma papulosum cyprini [22]) were used. EPCs were cultured in RPMI 1640 (Cambrex) supplemented with 10% Foetal Bovine Serum (FBS), 1% L-glutamin and 1% streptomycin/penicillin at 27˚C with 5% CO_2. Cells were seeded in 6-well plates (1 x 10^6 cells/well) 24 h prior to transfection and subsequently transfected with 2 µg of pcDNA3-SVCV-G plasmid using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Cells were stimulated with one of the following TLR ligands: ultra-pure LPS from *Escherichia coli* O111:B4 (LPS-EB), ultrapure lipopolysaccharide from *Porphyromonas gingivalis* (LPS-PG), purified lipoteichoic acid from *Staphylococcus aureus* (LTA), ultrapure endotoxin-free single-stranded DNA from *E. coli* (tlrl-ssec), CpG ODNs 1668 (tlrl-1668), polyinosinic-polycytidylic acid poly(I:C) (tlrl-pic), flagellin from *S. typhimurium* (tlrl-stfla), 23S rRNA (ORN Sa19) from *S. aureus* (tlrl-orn19), all purchased from InvivoGen.

Immunofluorescence analysis

Experimental determination of the sub-cellular localization was performed using the carp Tlr4bb-GFP construct. To this end, a human cell line (HEK 293) and a fish cell line (EPC) were used. Cells were cultured as described above, seeded in 6-well plates (0.5 x 10^6 cells/well [HEK 293] and 1 x 10^6 cells/well [EPC]) 24 h prior to transfection and transfected with 2 µg of carp Tlr4bb-GFP plasmid. For detection of intracellular or cell surface localization of carp Tlr4bb-GFP, cells were harvested 72 h post-transfection and fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature (RT), followed by a washing step with PBS containing 1% (w/v) bovine serum albumin (PBS-BSA). Nuclei were stained with VECTASHIELD® Mounting Media containing propidium iodide (Vector Laboratories) after overnight incubation. Sub-cellular localization of Tlr4bb-GFP was determined with the help of a Zeiss LSM-510 laser scanning microscope. Green fluorescent

signal was excited with a 488 nm argon laser and detected using a band-pass filter (505-530 nm).

Three-dimensional modelling

Structural models were obtained using the amino acid sequence alignment of carp Tlr4ba, Tlr4bb and human TLR4, and dimer structure of human TLR4 (PDB-id: 3fxi) as template using the Modeller program (version 9.12) [36]. In addition, the N-acetylglucosamine (NAG) and water molecules present in the crystal structure were included in the modelling procedure. Thirty comparative models were generated, after which the model with lowest corresponding DOPE score [37] was selected for image generation with Pymol (Pymol).

Identification of novel Tlr4 sequences: bioinformatic, phylogenetic and synteny analyses

Novel carp *tlr4* genes were identified in the draft genome of common carp Bioproject PRJNA73579 [38] blasting the known carp *tlr4* sequences (*tlr4ba* and *tlr4bb*) using the program CLC bio Genomics Workbench version 4.9 (CLC bio, Aarhus, Denmark). The putative coding regions within the genomic DNA were identified with FGENESH (http://linux1.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind) and GENSCAN (http://genes.mit.edu/GENSCAN.html), the predicted amino acid sequences were confirmed by using these sequences as template in BLAST [39] and FAST [40] to compare with the most similar hits of previously annotated genes. A number of 5 contigs within the carp genome (scaffold_63298, scaffold_52039, scaffold_140, scaffold_37770 and scaffold_26868) with regions coding for *tlr4* homologs were identified. Nucleotide sequences of carp *tlr4* genes were translated using the ExPASy translate tool program (http://us.expasy.org/tolls/dna.html)[41] and aligned with Multiple Sequence Alignment by CLUSTALW v2.0 (http://www.genome.jp/tools/clustalw/). The predicted amino acid sequences were examined for the presence of a signal peptide using the SignalP program (http://www.cbs.dtu.dk/services/SignalP-2.0/) [42] and the TMHMM2.0 program (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Identification of protein domains was done with SMART [43] (http://smart.embl-heidelberg.de/) and LRRfinder (http://www.lrrfinder.com/), individual LRRs were identified manually annotated according to the definitions from previous studies [44, 45].

A multiple alignment was performed using ClustalW v2.0 and a phylogenetic tree based on the LRR extracellular domains was constructed using the Neighbor-Joining method [46] in MEGA5 software [47]. The tree was built using LRRs domains only, to have possibility to include the Tlr4 soluble form fish. Evolutionary distances were computed using the Poisson correction method [48], all positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were done with 10,000 bootstrap replicates.

Genome synteny of the loci harbouring the *tlr4* gene was undertaken by analysis of human (genome assembly GRCh37, project number GCA_000001405.12), mouse (genome assembly GRCm38, project number GCA_000001635.3), chicken (genome assembly Galgal4, project number GCA_000002315.2), lizard (genome assembly AnoCar2.0 project number GCA_000090745.1) zebrafish (genome assembly Zv9, project number GCA_000002035.2), pufferfish (Genome assembly FUGU4) genomes that were retrieved from the Ensemble Genome Browser [49] (http://www.ensembl.org/index.html) and carp Bioproject PRJNA73579 [38].

Statistical analysis

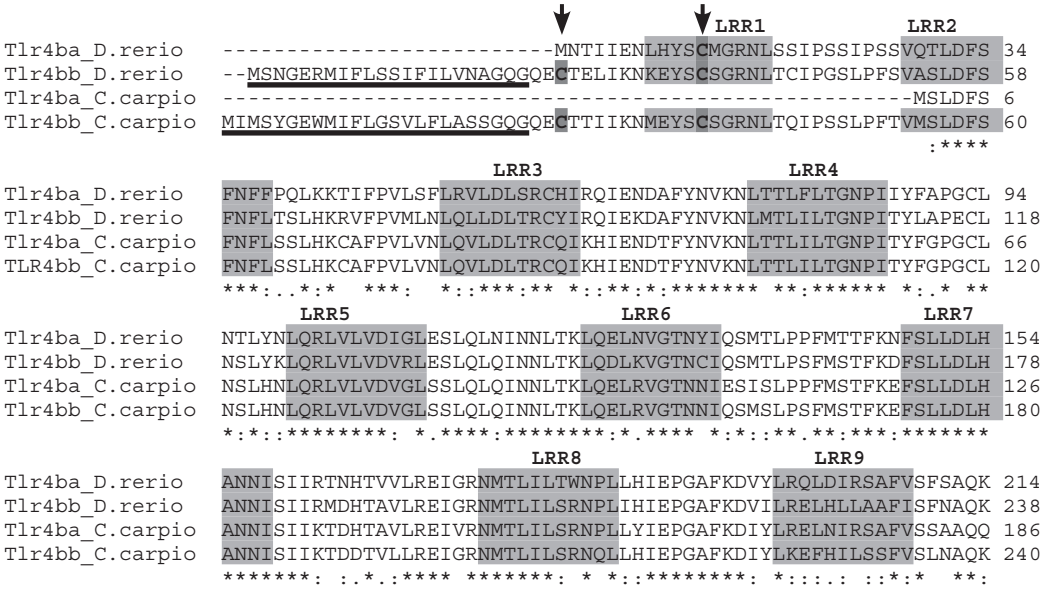
Relative expression ratios (*R*) were calculated as described above. Transformed (LN(*R*)) values were used for statistical analysis in GraphPad prism version 5. For all tests, homogeneity

of variance was assessed using Levene's test. Significant differences (P<0.05) were determined by two-way ANOVA followed by Sidak test. In case of unequal variances between groups, a two-way ANOVA was performed followed by a Games-Howell test.

RESULT

Identification of *Toll-like receptor 4 (tlr4)* in carp

For zebrafish *tlr4*, it has been argued that the origin of the gene(s) raises questions regarding nomenclature. The names *tlr4* and *TLR4* would suggest misleading orthology, which can be avoided by calling the zebrafish genes *tlr4ba* and *tlr4bb.* This has been suggested based on the hypothesis that two ancestral *TLR4* genes (referred as *TLR4a* and *TLR4b*) were present in the common ancestor of teleosts and mammals but that during evolution the *TLR4b* was lost from the human lineage while it is still present in (some) teleosts' genomes. In zebrafish (as in other cyprinids and siluriform fish) the tlr4b gene duplicated, leading to *tlr4ba* and *tlr4bb*. Vice versa, the *TLR4a* gene was lost from the teleosts' lineage and retained in the mammalian one and is currently known as *TLR4* [13]. We adopted this proposal and nomenclature for zebrafish and carp *tlr4* genes. Using the information from zebrafish *tlr4ba* and *tlr4bb* and partial sequences for carp *tlr4a* (accession number GU321982.2) and *tlr4b* (HQ229652.1) we cloned two full-length cDNA sequences for carp *tlr4* (accession numbers: *tlr4ba* KF582561, *tlr4bb* KF582562). The carp *tlr4ba* and *trl4bb* genes have open reading frames of 2325 and 2466 bp, encoding for proteins of 774 and 821 aa and predicted molecular weights of 103.1 and 109.0 kDa, respectively. Alignment of the carp and zebrafish Tlr4 sequences identified carp Tlr4ba as most similar to zebrafish *tlr4ba* (75%) and carp *tlr4bb* similar to zebrafish *tlr4bb* (76%) (**Figure 1**). Carp and zebrafish Tlr4ba sequences do not contain a signal peptide and the N-terminal LRR (LRRNT) whereas both, carp and zebrafish tlr4bb contain a signal peptide (24 aa) and a LRRNT signature ($Cx_{10}C$). Carp Tlr4ba has 21 LRR domains whereas tlr4bb has 23, both have a C-terminal LRRCT ($CxCx_{23}Cx_{18}C$), a transmembrane domain and a TIR domain. The complete carp protein sequences are 90% similarity to the partial sequences in the database.



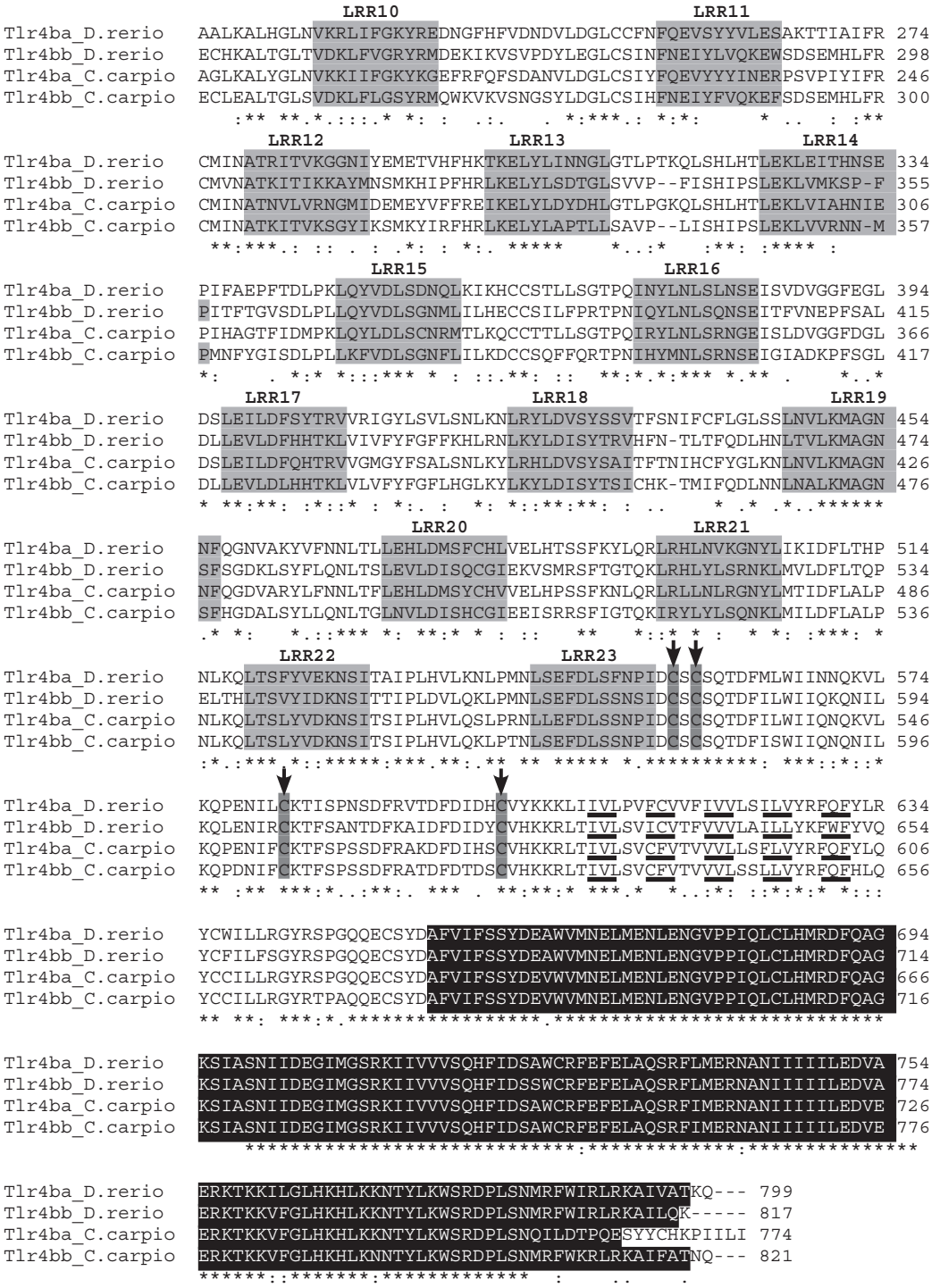


Figure 1. Protein sequence comparison of zebrafish and carp Tlr4. Amino acid alignment of carp Tlr4ba (KF582561) and Tlr4bb (KF582562) with zebrafish Tlr4ba (NP_001124523.1) and Tlr4bb (NP_997978.1). Alignment was performed usin ClustalW v2.0. The putative signal peptide is underlined. Conserved cysteine residues important for the N-terminal domain (LRRNT) and C-terminal domain (LRRCT) are indicated by

arrows above the sequence alignment and highlighted in dark grey. Predicted leucine rich repeat domains are highlighted in grey and numbered LRR1-23. The transmembrane region is underlined with dash line, whereas the TIR domain is highlighted in black. Asterisks (*) indicate identities, (:) double dots indicate conserved substitutions, (.) single dots indicate semi-conserved substitution and (-) dashes gaps used to maximize the alignments.

Constitutive gene expression of *tlr4* in carp

Constitutive expression of carp *tlr4ba* was relatively high in mid kidney, peripheral blood leukocytes (PBL), brain and liver, whereas expression of *tlr4bb* was more equal between organs (**Figure 2a)**. Overall, *tlr4ba* basal gene expression was always higher than *tlr4bb* gene expression. Of interest, *tlr4bb* gene expression was particularly high in macrophages but, in contrast to *tlr4ba* absent in B and T lymphocytes (**Figure 2b**), suggesting cell type-specific expression of *tlr4ba* and *tlr4bb*.

Role of zebrafish and carp Tlr4 during in vivo bacterial and viral infections

To investigate the role of zebrafish Tlr4 *in vivo* we injected larvae from knockout zebrafish (*tlr4ba*-/- and *tlr4bb* -/-) and wild type (wt) controls at 72 h post-fertilization with *Salmonella tyhimurium* or with PBS. Survival rate was approximately 50% in zebrafish injected with bacteria. No difference in survival could be observed between *tlr4ba*-/- and *tlr4bb* -/- and wt zebrafish (**Figure 3a**), indicating that the absence of either of the two *tlr4b* genes does not exacerbate the infection, either suggesting that both Tlr4b might not play a crucial role in the protection against *S.typhimurium* or that there is redundancy in the function of these two molecules. Interestingly, although *tlr4ba*-/- uninfected zebrafish were always less viable than *tlr4bb* -/- animals, they did not show higher susceptibility to the infection.

Carp, which can naturally be infected with Spring Viraemia of Carp Virus (SVCV), were bath challenged with SVCV and *tlr4* gene expression was measured during infection using common primers amplifying carp *tlr4ba* and *tlr4bb*. We observed a clear upregulation of *tlr4* gene expression in mid kidney of carp at 4 days post-infection, coinciding with the peak of vireamia as determined by SVCV N-protein gene expression (**Figure 3b**). These data suggested a role for Tlr4 in the immune response against viral rather than bacterial infection.

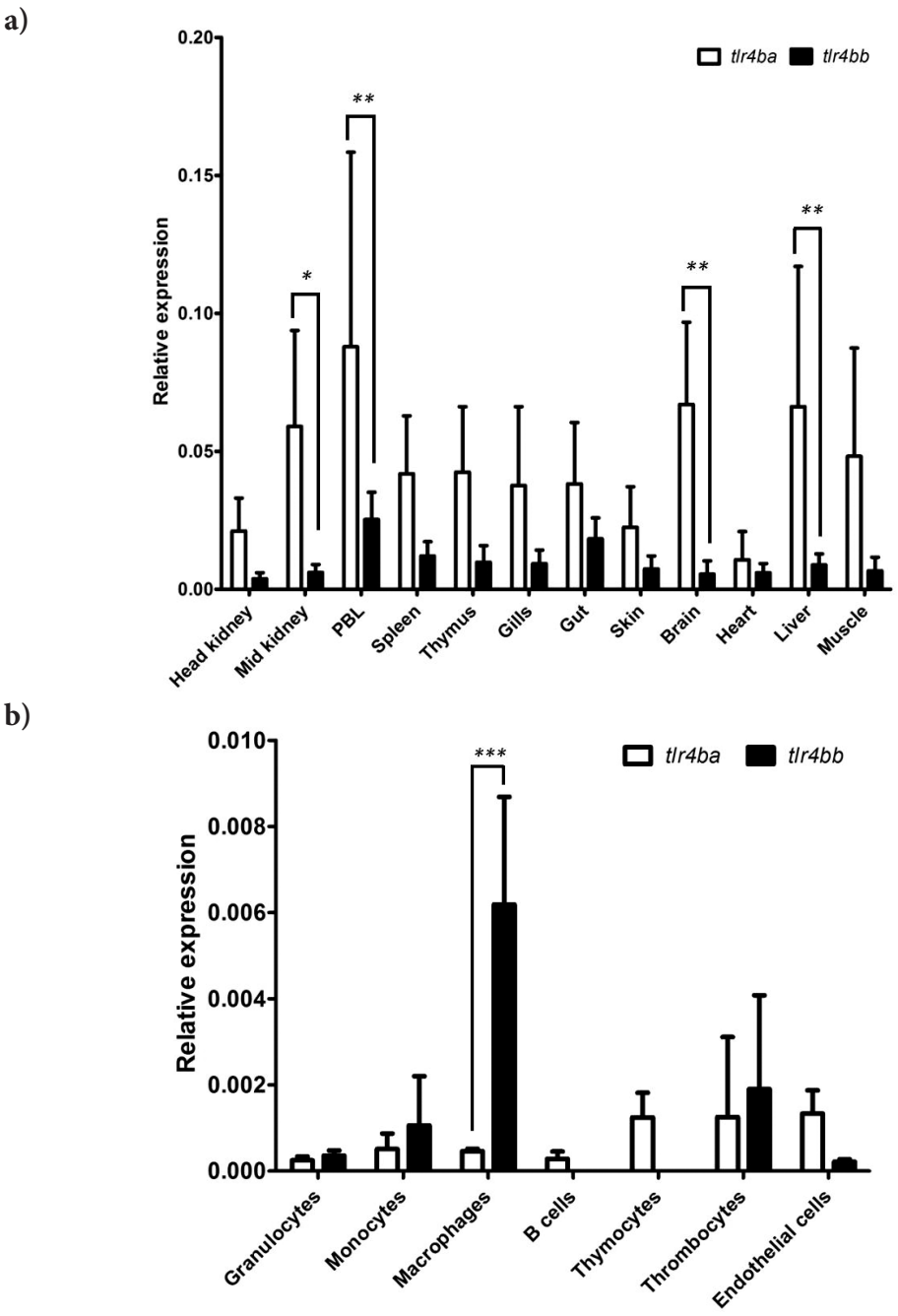


Figure 2. Real-time quantitative PCR analysis of carp *tlr4ba* and *tlr4bb gene expression*. Constitutive gene expression in different organs a) and in different cell types b). mRNA levels are expressed relative to the house keeping gene (S11 protein of the carp 40S subunit). Data are represented as mean ± SD (n=5 healthy carp). Significant differences between expression levels of *tlr4ba* and *tlr4bb* are indicated by * (P<0.05) and ** (P<0.001). Abbreviation: PBL, Peripheral Blood Leukocytes.

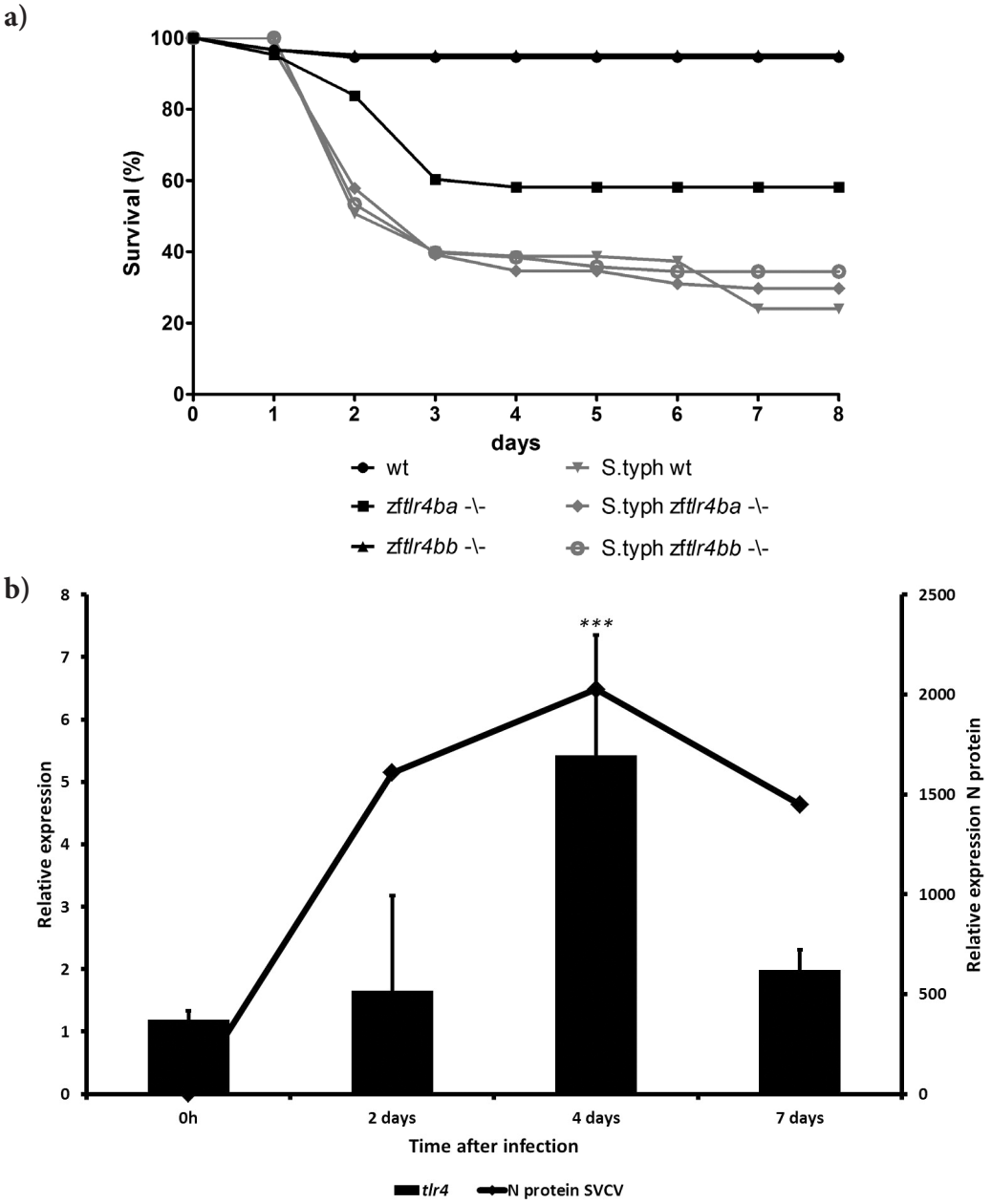


Figure 3. In vivo modulation of zebrafish and carp *tlr4*.

a) Survival rate of zebrafish *tlr4ba* and *tlr4bb* knockout larvae after infection with *Salmonella typhimurium*. Larvae (25 individuals per treatment) 72 hours post-fertilization (dpf) were anaesthetized and injected in the yolk with PBS (negative control) and with *S.typhymurium* (50 bacteria/larvae) and then we counted the number of survival fish during the following eight days. Values represent mean of three independent experiments with similar results.

b) Real-time quantitative PCR analysis of carp *tlr4* gene expression after bath challenge with spring viraemia of carp virus (SVCV).Fish were exposed to SVCV (10^3 $TCID_{50}$/ml) by bath challange for 2 hours. At each time point, fish were sacrificed, mid kidney was collected and the level of Tlr4 and the level of N protein gene expression analysed. Primers were designed to amplify both, *tlr4ba* and *tlr4bb* genes. Gene expression was

normalized relative to the S11 protein of 40S subunit (40S) as reference gene. Data are shown as mean ± SD (n=6 individuals). Significant differences are indicated by *** (P <0.0010).

In vitro ligand binding of Tlr4

To investigate the putative ligands of Tlr4 we developed a reporter assay based on a human (HEK 293) cell line stably transfected with an NFκB luciferase reporter construct (pNiFty-Luc) and transiently transfected with carp Tlr4bb. We chose to perform these experiments with Tlr4bb, rather than Tlr4ba, because we wanted to investigate putative extracellular ligands of Tlr4 and since Tlr4ba misses a signal peptide, it was not predicted to be expressed on the cell surface. As positive control, we used transient transfection with human TLR2. Successful transfection was confirmed by microscopy, evaluating the percentage of fluorescent cells (40-50% approximately), visualizing GFP for carp Tlr4bb or YFP for human TLR2. Responses to ligands were measured as luminescence and expressed as relative light units (RLU). Overexpression of human TLR2 and stimulation with the prototypical TLR2 ligand lipoteichoic acid (LTA) induced a specific response (**Figure 4**). Stimulation of cells transfected either with the Tlr4 or with control constructs with different preparations of LPS always induced very high reporter gene expression. Whereas stimulation with G-protein of SVCV did not lead to activation of the NFκB promotor, stimulation with β-glucans (zymosan > MacroGard® > depleted zymosan (DPZ)) did suggest carp Tlr4bb might play a role in the recognition of β-glucans (**Figure 4**).

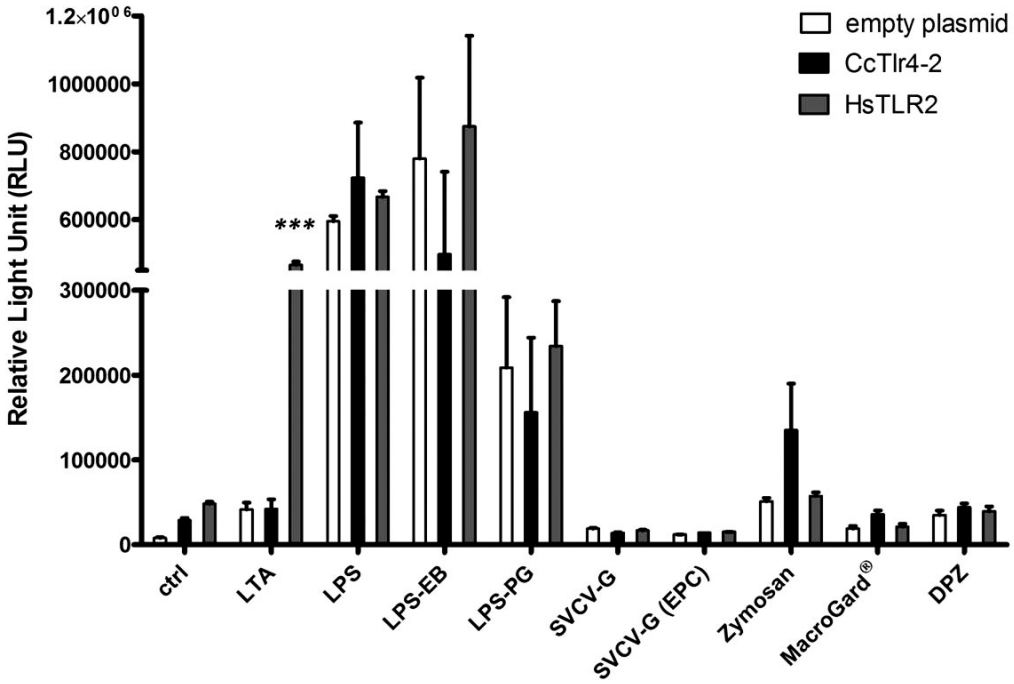


Figure 4. Ligand-binding study of carp Tlr4bb. HEK-NFκB-Luc were transiently transfected with carp Tlr4bb-GFP or human TLR2-YFP constructs. pcDNA3-GFP vector was used as negative control. Cells were cultured as described in the method section. After 72 h cells were stimulated for further 5 h with PBS, lipoteichoic acid (LTA) (50 μg/ml) (positive control), LPS (50 μg/ml), ultra-pure LPS from *E.coli* (LPS-EB, 50 μg/ml), LPS from *P.gingivalis* (LPS-PG, 50 μg/ml) or with different preparations of β-glucans: zymosan, MacroGard®, depleted-zymosan (DPZ) (all 100 μg/ml). For stimulation with the SVCV-G protein, cells were either co-

transfected with the pcDNA3-SVCV-G vector (SVCV-G) or incubated with SVCV-G-expressing EPC cells (SVCV-G(EPC)) at a 1:1 ration for the last 5h of culture. After stimulation, luminescence was measured and es expressed as relative light units (RLU). Values represent mean ± S.D. of triplicate wells. Significant differences are indicated with *** (P<0.001).

Sub-cellular localization of carp Tlr4

To investigate the sub-cellular localization of Tlr4bb, human HEK 293 cells and fish EPC cells were transfected with Tlr4-GFP (**Figure 5**). Detection of proteins expressed on the cell surface of non-permeabilized cells using an antibody against the FLAG-tag did not show clear results (data not shown). However we could detect the presence of GFP-Tlr4 in the cytoplasm suggesting a preferential expression of Tlr4 in intracellular compartments of the two cell lines used.

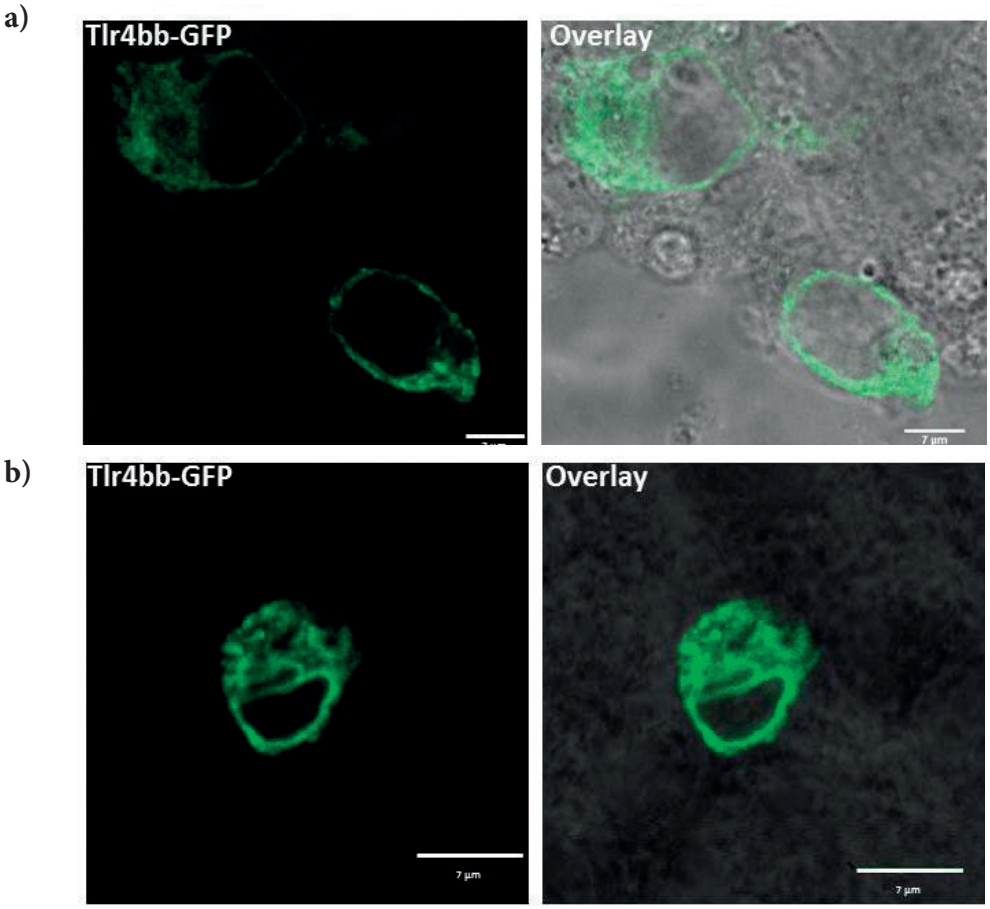


Figure 5. Sub-cellular localization of Tlr4bb in human (HEK 293) and fish (EPC) cell lines. Confocal microscopy of cells (HEK 293, EPC) overexpressing carp Tlr4bb. Cells were seeded 24 h prior to transfection with Tlr4bb-GFP vector. Three days later, cells were examined by confocal microscopy. Left panels: Tlr4bb-GFP expression (green); right panels: overlay with bright field. **a) human cell line HEK 293. b) fish cell lie EPC.** White bar indicate 7 µm.

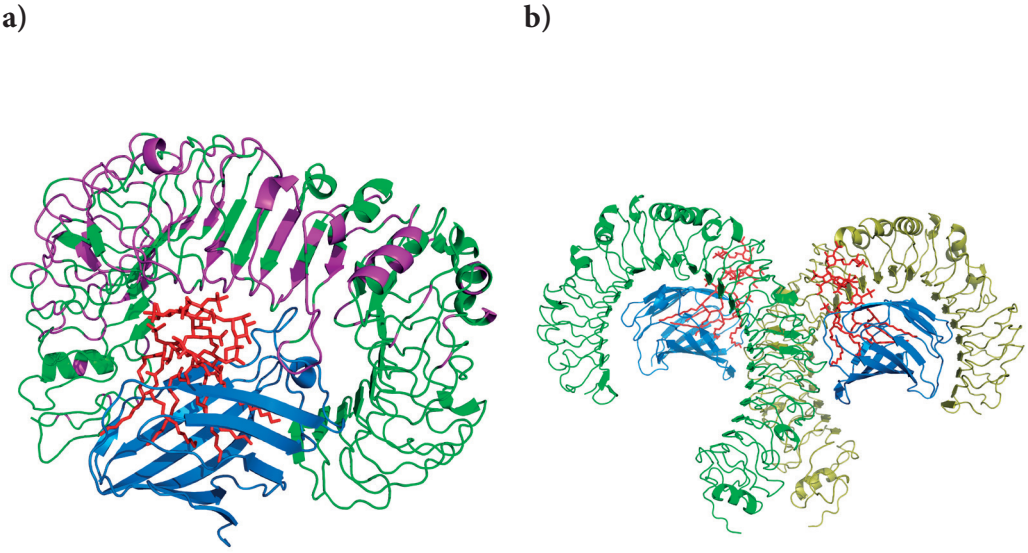
Three-dimensional structure of Tlr4

The three-dimensional structure of carp Tlr4ba and Tlr4bb were modelled using as template the crystal structure of the human TLR4-human MD-2-E.coli LPS Ra complex (PDB-id: 3fxi). In human TLR4 the receptor multimer is composed of two copies of the TLR4–MD-2–LPS complex arranged in a symmetrical fashion. Three-dimensional modelling of the carp Tlr4bb homodimer (**Figure 6a**) shows the horseshoe shape typical of Tlrs. Modelling of carp Tlr4bb (green) in comparison with carp Tlr4ba (purple) provided a three-dimensional approach to the aa differences between the two molecules. Differences between carp Tlr4ba and Tlr4bb were most prominent in the region of LPS (red) binding, rather than MD-2 (blue) binding (**Figure 6b**). This could point at different ligand specificities for carp Tlr4ba and Tlr4bb molecules. The three-dimensional structure of carp Tlr4 does not provide a clear indication that the Tlr4 molecules would be unable to bind LPS. Yet, of the three aa important for hydrophobic interaction between TLR4 and MD-2 (F440, L444, F463), only one aa was conserved (F436, H440, T459) (**Figure 6c**). Of the four aa important for hydrogen bonds between TLR4 and MD-2 (S416, N417, E439, Q436), none were conserved in carp Tlr4. However, of four aa important for charge interaction with phosphates between TLR4 and LPS (K388, R264, K341, K362), three are conserved (K383, R260, K316, M357).

Identification of novel *tlr4b* genes

Upon closer examination, the zebrafish genome assembly Zv9 (GCA_000002035.2) shows the presence of a third *tlr4*-like gene located on chromosome 13 between the previously described *tlr4ba* and *tlr4bb* (region 18.569.711-18.572.351). This novel *tlr4* gene for zebrafish has only recently been deposited in the database (1st August, 2013; Genbank accession number XM_001919664.4). The zebrafish gene, currently referred to as *tlr4a-like* (*tlr4al*), has an open reading frame of 2451 bp encoding for a protein of 816 aa with a predicted molecular weight of 109 kDa. The previously described zebrafish genes are both named *tlr4b* (*tlr4ba* and *tlr4bb*) based on paralogy to the human *TLR4(a)* and, as further discussed below, we believe the novel zebrafish *tlr4al* should have rather been referred to as *tlr4ba-like* or *tlr4bc*. Upon closer examination, the carp genome (PRJNA73579) also contains additional (three) Tlr4-like genes. For carp, a total number of five putative *tlr4* genome sequences could be identified in five different scaffolds. The three new carp Tlr4 sequences (Tlr4bc, Tlr4bd, Tlr4be) have open reading frames of 2463 bp, 2610 bp and 2034 bp encoding for proteins of 820 aa, 869 aa and 677 aa with predicted molecular weights of 108.9 kDa, 115.1 kDa and 89.4 kDa, respectively. Tlr4be is predicted to be a soluble molecule.

Alignment of zebrafish and carp Tlr4 protein sequences with other Tlr4 sequences (**Figure 7**) confirmed conservation of structural features of Tlr4 including (the number of) LRRs (23), except for grass carp Tlr4bb (18) and common carp Tlr4ba (21), presence of LRRNT and LRRCT in most but not all Tlr4 molecules, presence of transmembrane domain in most Tlr4 molecules and presence of an intracellular TIR domain (**Table 2**). The three soluble Tlr4s (Tlr4bb from channel catfish, Tlr4ba from grass carp and Tlr4be from common carp) were not included in the alignment.

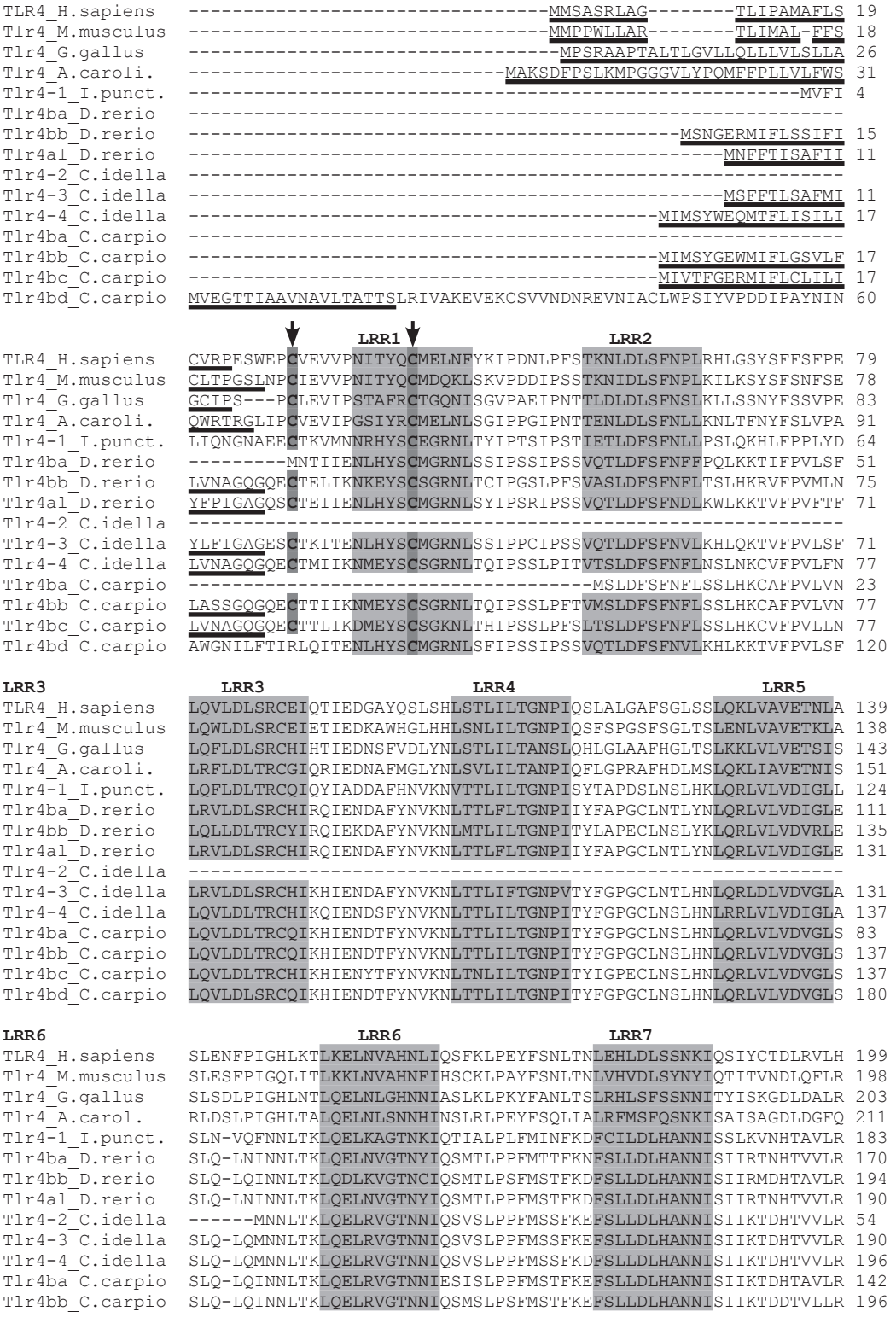


c) Contact points between Tlr4, MD-2 and LPS; comparison between human and carp. Amino acids conserved in common carp are underlined.

Interaction	HsTLR4	CcTlr4bb
hydrophobic interaction TLR4 and MD-2	F440	F436
	L444	H440
	F463	T459
Hydrogen bonds TLR4 and MD-2	S416	D411
	N417	K412
	E439	Y435
	Q436	L432
Phosphate interaction TLR4 and LPS	K388	K383
	R264	R260
	K341	K316
	K362	M357

Abbreviations Hs=*Homo sapienes*; Cc=*Cyprinus carpio*

Figure 6. Three–dimensional modelling of carp Tlr4-MD-2-LPS. a) Homodimer of carp Tlr4bb, MD-2 and *E.coli* LPS complex. b) Interaction of carp Tlr4ba and Tlr4bb monomer with human MD-2 and *E.coli* LPS. c) Contact points between Tlr4, MD-2 and LPS; comparison between human and carp. Structural models were obtained using the amino acid sequence alignment of carp Tlr4ba (purple) and Tlr4bb (green) with human Tlr4 using the dimer structure of human TLR4 (PDB-id: 3fxi) as template. In addition to Tlr4, MD-2 (blue) and the LPS molecule (red) as well as N-acetylglucosamine NAG, water and Mg^{2+} present in the crystal structure were included. This is the best model with lowest corresponding DOPE score out of thirty comparative models generated with Pymol (Pymol). Contact points for hydrophobic interaction between TLR4 and MD-2, for hydrogen bonds between TLR4 and MD-2 and contact points for charge interaction with phosphates between TLR4 and LPS are listed.


LRR1
LRR2
LRR3
LRR3
LRR4
LRR5
LRR6
LRR6
LRR7

```
Tlr4bc_C.carpio  SLQ-LQMNNLTKLQELRVGTNNIQSVSLPPFMSTFKDFSLLDLHANNISIIKSNDTVVLR 196
Tlr4bd_C.carpio  SLM-LQMNDLTKLQELRVGTNNIESISLPPFMSTFKEFSLLDLHANNISIIKTDHTAVLR 239
                       :..*  *:.*. . * * :  ** ::  :  :  :.:  * *  :   .   ::
                            LRR8                    LRR9
TLR4_H.sapiens   QMPLLNLSLDDLSLNPMNFIQPGAFKEIRLHKLTLRNNFDSLNVMKTCIQGLAGLEVHRLV 259
Tlr4_M.musculus  ENPQVNLSLDMSLNPIDFIQDQAFQGIKLHELTLRGNFNSSNIMKTCLQNLAGLHVHRLI 258
Tlr4_G.gallus    ETNRLNLTLVLSLNNIKYIQSGSFAKIHLGELILRSSFENLNAMHSSLQGLAGLQVNRLI 263
Tlr4_A.carol.    SR---NLTLVLSKNIIKYIELNSFSGVYFQELSLRGCFETSAHMQTSLQNLSGLHANRLV 268
Tlr4-1_I.punct.  EMRG-NITLILSSNPILHIEPGVFKDIYLKELNIHSAFVSFDAMRYGLKALSGLNVGKLA 242
Tlr4ba_D.rerio   EIGR-NMTLILTWNPLLHIEPGAFKDVYLRQLDIRSAFVSFSAQKAALKALHGLNVKRLI 229
Tlr4bb_D.rerio   EIGR-NMTLILSRNPLIHIEPGAFKDVILRELHLLAAFISFNAQKECHKALTGLTVDKLF 253
Tlr4al_D.rerio   EIGK-NMTLILSRNPLLHIEPGSFKGVHLVELDIRSTFVSFASKKQGLNGLHGLNVTRLM 249
Tlr4-2_C.idella  KIGR-NMTLILSRNPLLYIEPGAFKDVYLRQLDIRSAFVSFAAQKFGLKALYGLNVKKLL 113
Tlr4-3_C.idella  KIGR-NMTLILSRNPLLYIEPGAFKDVYLRQLDVRSAFVSFAAHSVGLKALYGLNVKRLM 249
Tlr4-4_C.idella  QIGR-NMTLILSRNPLLHIEPGAFRDIYLRELHIQASFVSTNAQKKCLNALTGLSVDKLF 255
Tlr4ba_C.carpio  EIVR-NMTLILSRNPLLYIEPGAFKDIYLRELNIRSAFVSSAAQQAGLKALYGLNVKKII 201
Tlr4bb_C.carpio  EIGR-NMTLILSRNQLLHIEPGAFKDIYLKEFHILSSFVSLNAQKECLEALTGLSVDKLF 255
Tlr4bc_C.carpio  EIGR-NMTLILSRNPLLHIEPGAFKGMYLKEFHIQSAFVSLNAQKECLEALAGLSVDKLI 255
Tlr4bd_C.carpio  EIVR-NMTLILSRNPLLYIEPGAFKDIYLRELNIRSAFVSSAAQKAGLKALYGLNVKRLM 298
                      *::* :: * : .*:   *  : : :: :   * .        : * ** . ::
                     LRR10                    LRR11                    LRR12
TLR4_H.sapiens   LGEFRNEGNLEKFDKSALEGLYNLTIEEFRLAYLDYYLDDIIDLFNCLTNVSSFSLVSVT 319
Tlr4_M.musculus  LGEFKDERNLEIFEPSIMEGLCDVTIDEFRLTYTNDFSDDIVK-FHCLANVSAMSLAGVS 317
Tlr4_G.gallus    VGEFTNILKITAFQNGLLSGLCQVQMQEFVLMCFREFDNDTDTLFDCIGNVTTIRLVDLN 323
Tlr4_A.carol.    LGAYRNADRLEDFSKDHLDGLCHMCLQEIALVQISSLYR-TDSLSDCLNNIHSVRLVNTD 327
Tlr4-1_I.punct.  IGNYIEHRAIKISDADYLDGLCLINFKEIYFLQR-ECSDSENNVFHCMANATKITLKQGC 301
Tlr4ba_D.rerio   FGKYREDNGFHFVDNDVLDGLCCFNFQEVSYYVL-ESAKTTIAIFRCMINATRITVKGGN 288
Tlr4bb_D.rerio   VGRYRMDEKIKVSVPDYLEGLCSINFNEIYLVQK-EWSDSEMHLFRCMVNATKITIKKAY 312
Tlr4al_D.rerio   FGMYKDDPKLYPSDLDYFDGLCSIHFYEAYYYMK-ERLDWKMNIFRCMINATVVVVKGGV 308
Tlr4-2_C.idella  FGKFRGDFKFKYSDANILDGLCSIFFQEVYYYIS-DRPDELISIFPCMINATIVVVKDGV 172
Tlr4-3_C.idella  FGNNRDDYKIFPPDSDYLDGLCSIYFHEVYYYIK-ERPSGQINIFRCMINATIIAVKGGL 308
Tlr4-4_C.idella  MGSYRMQWKSSVSDSNYLDGLCSVNFNEIYLIQK-EYSHSEMNIFHCMINATKITLKIGY 314
Tlr4ba_C.carpio  FGKYKGEFRFQFSDANVLDGLCSIYFQEVYYYIN-ERPSVPIYIFRCMINATNVLVRNGM 260
Tlr4bb_C.carpio  LGSYRMQWKVKVSNGSYLDGLCSIHFNEIYFVQK-EFSDSEMHLFRCMINATKITVKSGY 314
Tlr4bc_C.carpio  IGSYRMQWKVKVSDASYLDGLCSVNFNEIYFVLK-EWSDSEMHLFRCMINATKITVKKGY 314
Tlr4bd_C.carpio  FGKYRCDYKILLSDANYLDGLCFINFHEVYYYMK-EKHDVPVSIFRCMINATIVAVKGGI 357
                 .*           . :.**  . : *                *: *   . :
                          LRR13                    LRR14
TLR4_H.sapiens   IERVKDFSYNFGWQHLELVNCKFGQFPTLKLKSLKRLTFTSNKGG------NAFSEVDLP 373
Tlr4_M.musculus  IKYLEDVPKHFKWQSLSIIRCQLKQFPTLDLPFLKSLTLTMNKGS------ISFKKVALP 371
Tlr4_G.gallus    LETLSEVPMFSQVKHLEWKRCKFQELPAEKLSLFKELRVLRITKS-KDLNGFEQKFGSLT 382
Tlr4_A.carol.    IEQVSPFPENSSIHHLEINNCRLRGVPAESLSSLKELRVLRITKSGRFLTRFAEDFNGPP 387
Tlr4-1_I.punct.  IGAVQHVSFH-QLKELHIHRTQFPLIL--ELSHLHFLEKLVVVTY---KYTVFRGLSDLP 355
Tlr4ba_D.rerio   IYEMETVHFH-KTKELYLINNGLGTLPTKQLSHLHTLEKLEITHNS--EPIFAEPFTDLP 345
Tlr4bb_D.rerio   MNSMKHIPFH-RLKELYLSDTGLSVVP--FISHIPSLEKLVMKSP---FPITFTGVSDLP 366
Tlr4al_D.rerio   IRVIGYVPFH-KIKELYLINTQLYTVPGKQLSHIRTLEKFVFTHNS---ATQVEKFLDMP 364
Tlr4-2_C.idella  IGDMEYVFFH-ELKELYLISNHLSILPGKQLSHLYTLEKLVITHNS--ELFSAATFIDMP 229
Tlr4-3_C.idella  IRGIPYVTFN-KIKELYLIQNQLDTVPGRLLSHLHTLEKVVFTRN---VATQVETFIDMP 364
Tlr4-4_C.idella  IKRMERVPFC-RLKELYLGHTLLSVVP--SVSHIPSLEKLVVKNT---IPMTFFGIGGLP 368
Tlr4ba_C.carpio  IDEMEYVFFR-EIKELYLDYDHLGTLPGKQLSHLHTLEKLVIAHNI--EPIHAGTFIDMP 317
Tlr4bb_C.carpio  IKSMKYIRFH-RLKELYLGPTLLSAVP--LISHIPSLEKLVVRNN---MPMNFYGISDLP 368
Tlr4bc_C.carpio  FRSMEYIQFR-HLKELYLGPTSLSVLP--FISHIPSLEKLVVKNN---IPMSFNGISDLP 368
Tlr4bd_C.carpio  IREIANVPFY-EIKELYLISNQLDTVPGKKLAHLHTLEKLVFTNNV--ATQIP-DFIDMP 413
                 :  :  .     : *      :  .    :  :  *                      .
                    LRR15                    LRR16                    LRR17
TLR4_H.sapiens   SLEFLDLSRNGLSFKGCCSQSDFGTTSLKYLDLSFNGVITMS-SNFLGLEQLEHLDFQHS 432
Tlr4_M.musculus  SLSYLDLSRNALSFSGCCSYSDLGTNSLRHLDLSFNGAIIMS-ANFMGLEELQHLDFQHS 430
Tlr4_G.gallus    YLEVVDLSENRLSFLTCCSPKFPRSPNLKHLNLSFNSDISLT-GEFANLRNLLYLDLQHT 441
Tlr4_A.carol.    NLEILDLSENSLVVSFAWSSLMEGLPNLKHLNLSFNSKITFP-PECSGVSKLEYLDLRHT 446
Tlr4-1_I.punct.  KLQHVDLSENQLNLFDCCSKYFTGMPNIHTLNLSFNAIIYLSKNPFSGLDSMRILDIQST 415
Tlr4ba_D.rerio   KLQYVDLSDNQLKIKHCCSTLLSGTPQINYLNLSLNSEISVDVGGFEGLDSLEILDFSYT 405
Tlr4bb_D.rerio   LLQYVDLSGNMLILHECCSILFPRTPNIQYLNLSQNSEITFVNEPFSALDLLEVLDFHHT 426
Tlr4al_D.rerio   KLQYVDLNSNQITLQSCCIDVLSGTPQIRYLNLSLNPQISLDKGGFEGLESLEILDFHHT 424
Tlr4-2_C.idella  KLQYVDLTSNRITLKRCCSTLLSGTPQIRYLNLSLNAEIDLDVGAFDGLDSLEILDFHHT 289
Tlr4-3_C.idella  KLQYLDLSSNQITLSSCCTEVLLNTPQIRYLNLSLNPQICLSFEAFVGLDSLEILDFHHT 424
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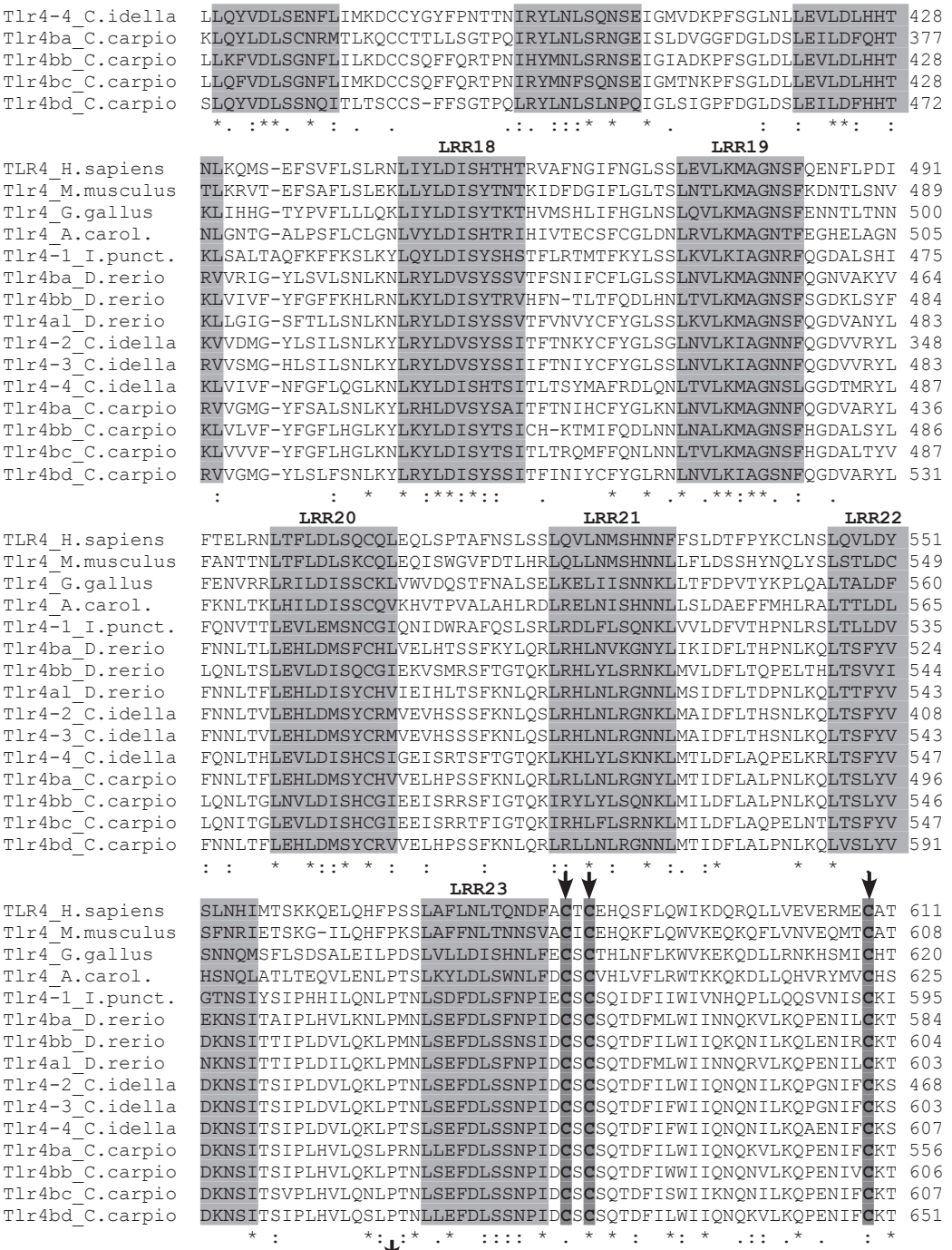
LRR18
LRR19
LRR20
LRR21
LRR22
LRR23

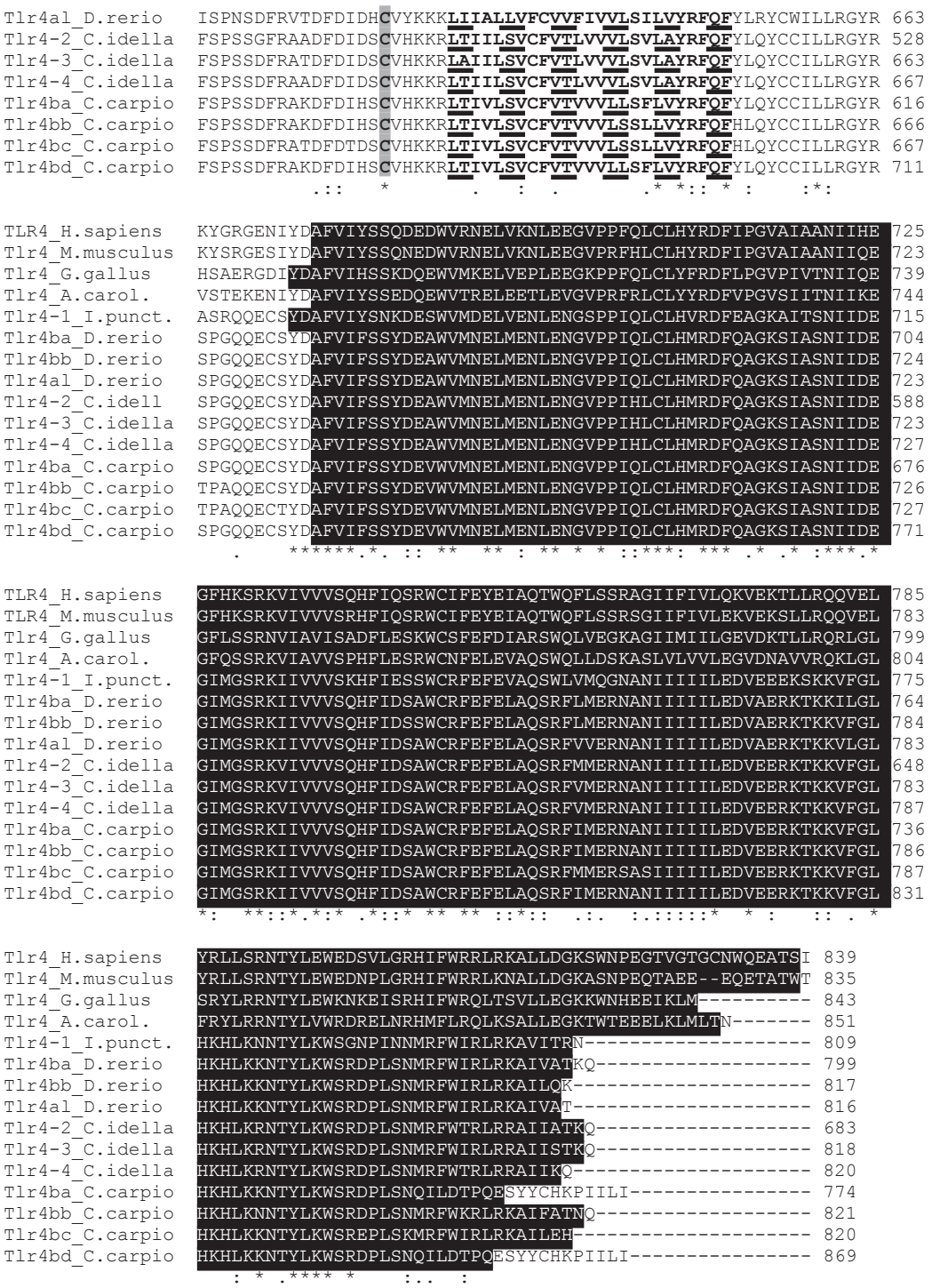

Figure 7. Multiple amino acid alignment of vertebrate TLR4. Multiple alignment of amino acid sequences between human (*Homo sapiens*, accession number: NP_612564.1); mouse (*Mus musculus*: NP_067272.1); chicken (*Gallus gallus*: ACR26315.1), lizard (*Anolis carolinensis*: XP_003227123.1); channel catfish (*Ictalurus punctatus*: Tlr4-1 AEI59665); zebrafish (*Danio rerio*: Tlr4ba NP_001124523.1, Tlr4bb NP_997978.1 and

Tlr4al LOC795671); grass carp (*Ctenopharyngodon idella*: Tlr4-2 AEQ64878.1, Tlr4-3 AEQ64879.1, Tlr4-4 AEQ64880.1) and common carp (*Cyprinus carpio*, Tlr4ba KF582561, Tlr4bb KF582562, Tlrbc scaffold-140 and Tlr4bd 37770). Alignment was performed using ClustalW v2.0. The putative signal peptide is underlined. Conserved cysteine residues important for the N-terminal domain (LRRNT) and C-terminal domain (LRRCT) are indicated by arrows above the sequence alignment and highlighted in dark grey. Predicted leucine rich repeat domains are highlighted in grey and numbered LRR1-23. The transmembrane region is underlined with dash line, whereas the TIR domain is highlighted in black. Asterisks (*) indicate identities, (:) double dots indicate conserved substitutions, (.) single dots indicate semi-conserved substitutions and (-) dashes denote gaps used to maximize the alignments. Abbreviations: A.carol. = *Anolis carolinensis;* I.punct.= *Ictalurus punctatus*.

Phylogenetic and synteny analysis of tlr4

Phylogenetic analysis was conducted based on the amino acid sequences of the extracellular domains of known Tlrs as to also include the soluble Tlr4 molecules (Tlr4-2 from channel catfish, Tlr4-1 from grass carp and Tlr4be from common carp) (**Figure 8**). Phylogenetic analysis supported our previous observations that carp tlr4ba is most similar to zebrafish Tlr4ba and carp Tlr4bb most similar to zebrafish Tlr4-bb (see also **Figure 1**). The novel carp Tlr4bc is highly similar to carp Tlr4bb and could be a duplicated copy of the same gene (paralogs). The novel carp Tlr4bd and Tlr4be genes are very similar to each other, cluster together with carp Tlr4bb and could be paralogs as well. Interestingly the zebrafish Tlr4al clusters close to the Tlr4ba clade (and not the mammalian TLR4 clade), indicating that the currently assigned name is misleading and the gene should have rather been referred to as *tlr4ba-like* or simply Tlr4bc as it is not more similar to the zebrafish Tlr4ba than the carp or grass carp Tlr4ba proteins are. A similar phylogenetic tree was obtained also when including the full-length protein sequences for all knowns Tlr4 (data not shown). We conducted synteny analysis to investigate conservation in the TLR4 region on the chromosomes of zebrafish, pufferfish (that have no *tlr4*), carp (only scaffold containing *tlr4bc*) and other vertebrate species (**Figure 9**). We observed a conserved synteny between human, mouse, and chicken. The three *tlr4* genes in zebrafish are found as a close proximity tandem duplication on chromosome 13. The genes flanking human, mouse, chicken and even lizard *trl4*, however, are located in different regions of the zebrafish genome. For example *trim32* (scaffold Zv9_NA154:29810-32054) and *dbc1* (chromosome 5), and thus show no conserved synteny. Likewise, the genes flanking zebrafish *tlr4* (*stox1*, *ccar*, *tet1*, and *slc25a16*) showed synteny with regions on human chromosome 10 and mouse chromosome 10, but not with regions on chromosome 9 (human) or chromosome 4 (mouse) that contain *tlr4*. Conserved synteny was observed between the genes flanking the zebrafish *tlr4* genes and those flanking carp *tlr4bc* gene. An improved version of the carp genome is underway that will help revealing the location of carp *tlr4ba* and *tlr4bb* genes as well as of the newly identified carp *tlr4bc-e* genes to better understand the syntenyc relationship of the genomic region and the phylogenetic origins of the genes. Synteny between the cyprinid (zebrafish and carp) and the pufferfish genomes was highly conserved for all genes flanking the *tlr4* genes but no *tlr4* gene could be identified in the pufferfish genome as previously reported [13].

Table 2. Comparison of structural features of vertebrate Tlr4.
Comparison of length, presence of signal peptide, number of LRR and signature of LRRNT and LRRCT from Tlr4 of human (Hs, *Homo sapiens*); mouse (Mm, *Mus musculus*); chicken (Gg, *Gallus gallus*); lizard (Ac, *Anolis carolinensis*); channel catfish (Ip, *Ictalurus punctatus*); zebrafish (Dr, *Danio rerio*), grass carp (Ci, *Ctenopharyngodon idella*) and common carp (Cc, *Cyprinus carpio*). '-' means 'not present'

Name	aa lenght	Signal peptide	LRR	LRRNT	LRRCT
Hs Tlr4	839	23	23	$Cx_{10}C$	$CxCx_{23}Cx_{17}C$
Mm Tlr4	835	25	23	$Cx_{10}C$	$CxCx_{23}Cx_{18}C$
Gg Tlr4	384	30	23	$Cx_{10}C$	$CxCx_{23}Cx_{18}C$
Ac Tlr4	851	37	23	$Cx_{10}C$	$CxCx_{23}Cx_{18}C$
Ip Tlr4-1	809	-	23	$Cx_{10}C$	$CxCx_{23}Cx_{18}C$
Ip Tlr4-2	263	26	9	-	-
Dr Tlr4ba	799	-	23	-	$CxCx_{23}Cx_{18}C$
Dr Tlr4bb	819	22	23	$Cx_{10}C$	$CxCx_{23}Cx_{18}C$
Dr Tlr4al	816	18	23	$Cx_{10}C$	$CxCx_{23}Cx_{18}C$
Ci Tlr4-1	585	18	22	-	$CxCx_{23}Cx_{18}C$
Ci Tlr4-2	683	-	18	-	$CxCx_{23}Cx_{18}C$
Ci Tlr4-3	818	18	23	$Cx_{10}C$	$CxCx_{23}Cx_{18}C$
Ci Tlr4-4	820	24	23	$Cx_{10}C$	$CxCx_{23}Cx_{18}C$
Cc Tlr4ab	774	-	21	-	$CxCx_{23}Cx_{18}C$
Cc Tlr4bb	818	24	23	$Cx_{10}C$	$CxCx_{23}Cx_{18}C$
Cc Tlr4bc	820	24	23	$Cx_{10}C$	$CxCx_{23}Cx_{18}C$
Cc Tlr44bd	869	19	23	-	$CxCx_{23}Cx_{18}C$
Cc Tlr4be	677	21	22	-	-

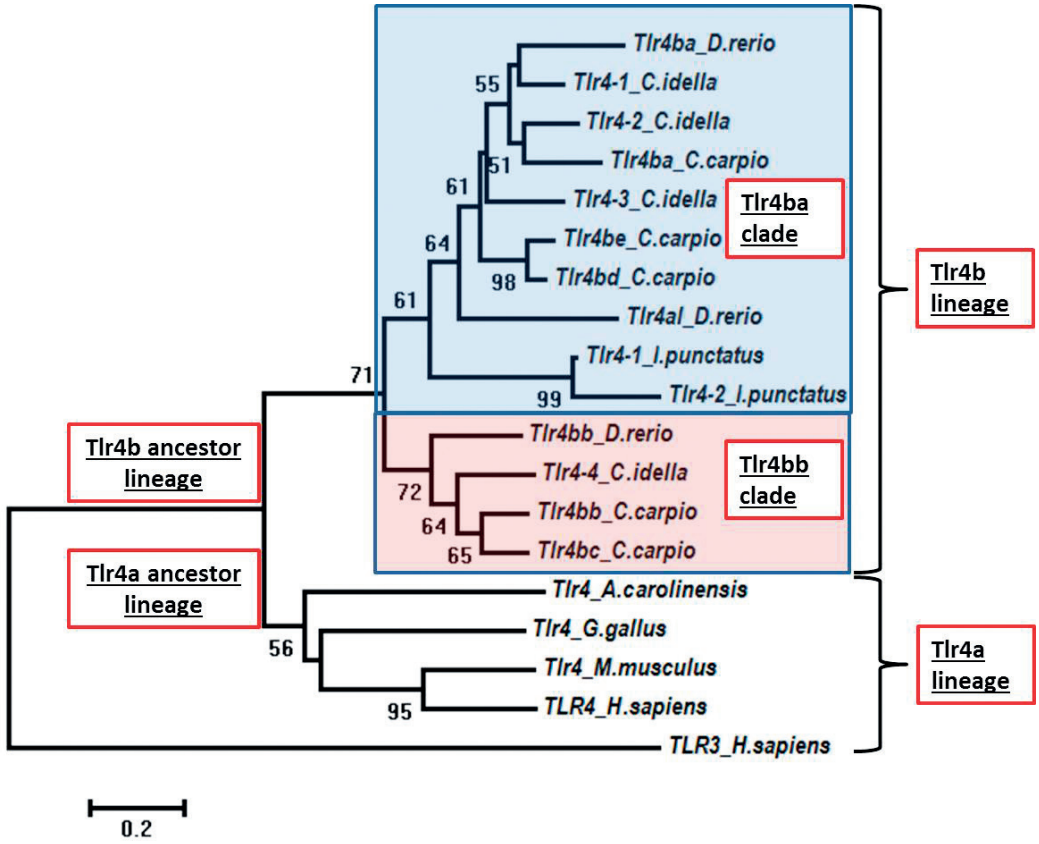

Figure 8. Molecular phylogenetic tree of TLR4. Neighbor-Joining tree based on the extracellular domains of vertebrate's Tlr4 sequences, with evolutionary distances computed using the Poisson correction method using the complete deletion option. Phylogenetic analysis was conducted with 10,000 bootstrap replicates. Bootstrap values at major branching points are shown as percentage. The sequences were derived from *Anolis carolinensis* (Tlr4 XP_003227123.1) *Cyprinus carpio* (Tlr4ba KF582561, Tlr4bb KF582562, Tlr4bc scaffold-140, Tlr4bd scaffold-37770 and Tlr4be scaffold-26868); *Ctenopharyngodon idella* (Tlr4-1 AEQ64877.1, Tlr4-2 AEQ64878.1 , Tlr4-3 AEQ64879.1, Tlr4-4 AEQ64880.1); *Danio rerio* (Tlr4ba NP_001124523.1, Tlr4bb NP_997978.1 and Tlr4al LOC795671); *Gallus gallus* (Tlr4 ACR26315.1), *Ictalurus punctatus* (Tlr4-1 AEI59665); *Homo sapiens* (TLR4 NP_612564.1); *Mus musculus* (Tlr4 NP_067272.1).

DISCUSSION

TLR4 is a protein that in humans, together with the co-receptors myeloid differentiation protein-2 (MD-2) and CD14, recognizes LPS, the major component of the outer membrane of Gram-negative bacteria. In fish, the tlr4 gene is present especially, although not exclusively, in members of the cyprinid and silurid families, including zebrafish [6, 7], grass carp [10], rare minnow [8], common carp [9] and channel catfish [11]. Most recently, in silico analysis predicted a Tlr4 TIR domain in a whole genome shotgun dataset of Atlantic salmon [11]. However, the function of the Tlr4 receptor in fish remains elusive. To better understand the role of Tlr4 in vivo, zebrafish larvae knockout for tlr4ba and tlr4bb genes were infected with S. typhimurium. No difference in

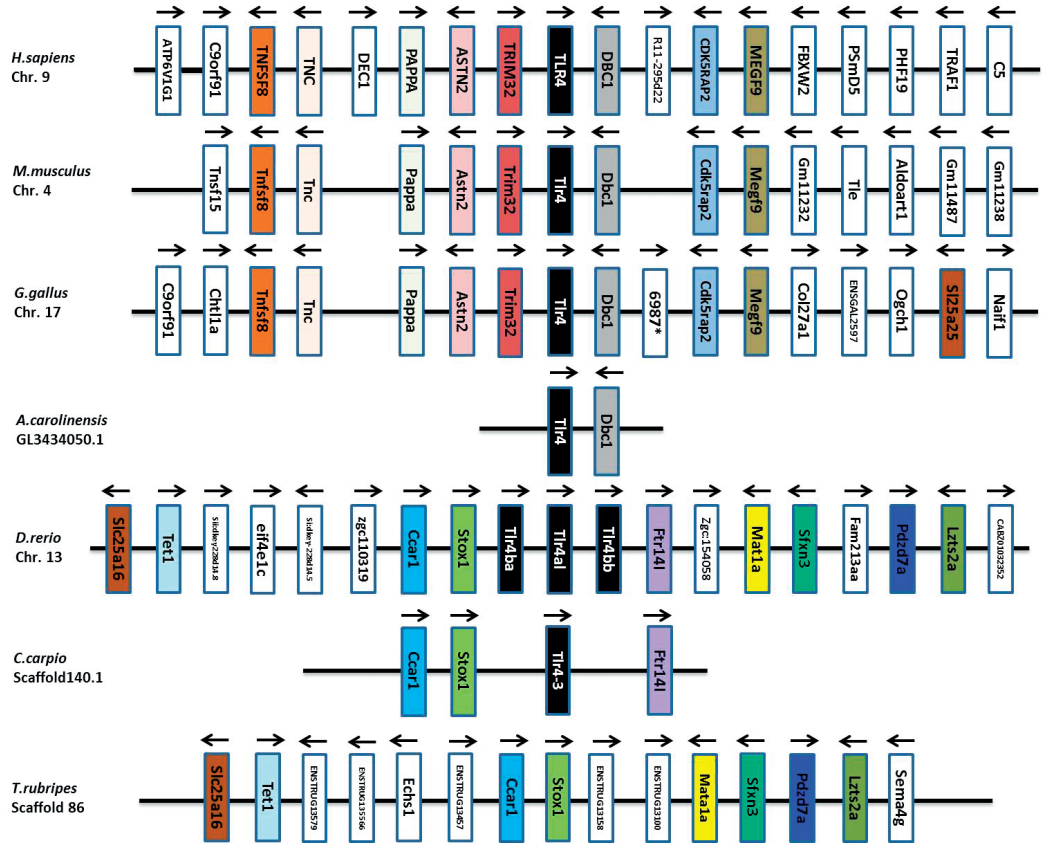

Figure 9. Synteny analysis of the TLR4 region in vertebrates. Comparative gene location map of the regions where TLR4 genes are located in human (*H. sapiens*), mouse (*M. musculus*), chicken (*G. gallus*), lizard (*A. carolinensis*), zebrafish (*D. rerio*), pufferfish (*T. rubripes*) and carp (*C. carpio*) genomes. For this analysis, the human genome assembly (GRCh37), mouse genome assembly (GRCm38), chicken genome assembly (Galgal4), lizard genome assembly (AnoCar2.0), zebrafish genome assembly version 9 (Zv9) pufferfish genome assembly version 4 (FUGU 4.0) and the carp genome Bioproject PRJNA73579 [38] were used.

survival after bacterial infection could be observed between the knockout and wild-type zebrafish, suggesting the presence of Tlr4 would not be crucial for the protection against this Gram-negative bacterium or that zebrafish Tlr4 proteins have redundant functions and can compensate for the absence of one of the two genes. As further discussed later, the discovery of a novel tlr4 gene in zebrafish, for which knockout lines are not yet available, and the possibility that more tlr4 genes

might be present in the zebrafish genome, further complicates the interpretation of the results. In mammals, Tlr4 has also been shown to play an important role in viral infections, including paramyxovirus [16] and respiratory syncytial virus [17]. Zebrafish larvae express an inability to mount a protective antiviral response to waterborne spring viraemia of carp virus [50], and thus the approach using the knockout fish lines could not be taken. Instead, adult carp were infected with SVCV by bath and up-regulation of tlr4 gene expression at 4 days post-infection was observed,

possibly suggesting a role for teleost Tlr4 in the immune response to viruses. In contrast, carp infected with the blood parasite Trypanoplasma borreli [29] or skin parasite Argulus japonicus [51] did not show an up-regulation of tlr4 gene expression (Pietretti, unpublished data) although other tlr genes such as tlr20 (Pietretti Chapter 4, this thesis) were upregulated. Of interest, viral infections of other cyprinid fish species, including rare minnow [8] and grass carp [10] with grass carp reovirus also induced tlr4 gene expression. These findings suggest that teleost Tlr4 could possibly play a role in sensing viral proteins.

Although our in vivo results indicated a putative role for Tlr4 in the immune response to viruses, in vitro NFκB-luciferase reporter assays with a human cell line overexpressing carp Tlr4, did not provide clear evidence for viral ligand recognition by carp Tlr4. One reason for these results could be that human HEK 293 cells used for our in vitro studies would not express all accessory proteins necessary for optimal functioning of carp Tlr4. For example, in vitro studies on human and mouse cell lines revealed that TLR4 interactor with leucine-rich repeats (TRIL) interacts with TLR4 to enhance recognition of LPS [52]. This may also be true for fish, at least fish genomes, including those of zebrafish and carp, do contain the tril gene [4]. In our case, HEK 293 might express accessory proteins, such as TRIL, that are too different to enhance ligand recognition by carp Tlr4. We also developed a NFκB-luciferase reporter assays based on a fish cell line (epithelioma papulosum cyprini, EPC) overexpressing carp Tlr4. However, also the use of this fish cell line did not lead to clear evidence for viral ligand recognition by carp Tlr4. Of interest, deep sequencing of the EPC revealed that this cell line does not express all accessory molecules of potential importance to the functioning of carp Tlr4 [4], among which tril (Pietretti, unpublished data). The exact implications of these findings are as yet unknown.

Our analysis of sub-cellular localization of carp Tlr4 by confocal microscopy pointed at an intracellular rather than cell surface localization of carp Tlr4 in transfected cells. At this moment it is difficult to conclude whether this sub-cellular localization would be natural to the carp Tlr4 molecule or a result of aberrant expression in a human cell line. Alternatively to recognition of LPS and viral ligands, TLR4 has also been implicated in recognition of fungal-derived PAMPs such as β-glucans [18]. Indeed, our in vitro reporter assay did provide indications that carp Tlr4 could maybe play a role in sensing β-glucans. The intracellular localization of carp Tlr4 would suggest that β-glucans would first need to be phagocytosed or internalised by the cell before binding to Tlr4. Nevertheless, this preliminary finding would need to be confirmed in further experiments.

Only recently, the sequence for a novel zebrafish *tlr4* gene, currently referred to as *tlr4a-like* gene, was deposited in the database. As already discussed by Sullivan et al. [13] the previously described zebrafish genes (tlr4ba and tlr4bb) and other teleost's *tlr4* genes might not to be true orthologues of human TLR4 (here also referred to as TLR4a). The teleost *tlr4* described thus far might in fact be phylogenetically related to an ancestral TLR4b gene and duplication events within the teleost's lineages have led to an expansion of *tlr4b* paralogs. Based on this hypothesis and also based on our extensive phylogenetic and synteny analysis of the currently known teleost *tlr4* genes, we propose to name the novel zebrafish gene *tlr4ba-like* or simply *tlr4bc*. Only further functional analysis will determine whether these paralogs have similar or divergent functions and their phylogenetic relationship to the other teleost's *tlr4b* genes.

Detailed investigations of the carp genome revealed the presence of three novel *tlr4* genes (*tlr4bc*, *tlr4bd* and *tlr4be*), among which *tlr4be* is predicted to be expressed as soluble protein. Phylogenetic analysis supports the suggestion that carp *tlr4ba* is orthologous to zebrafish *tlr4ba* and that carp *tlr4bb* and *tlr4bc* are duplicated copies of a gene orthologous to zebrafish *tlr4bb*. At

this moment, phylogenetic analysis does not clearly identify the novel carp *tlr4bd* and *tlr4be* genes as orthologous to the zebrafish *tlr4al* although they cluster close to each other in the Tlrba clade. Functional research on the newly discovered zebrafish and carp *tlr4* genes is required to determine their potential to sense bacterial LPS and other ligands. At this moment, the three-dimensional structure of carp Tlr4 does not provide a clear indication that teleost Tlr4 molecules would not be able to bind LPS. Of interest, of all contact points between Tlr4, MD-2 and LPS, the aa important for charge interaction with phosphates between TLR4 and LPS were almost completely conserved. This could imply that the absence of MD-2 from fish genomes [3, 4] is a factor most crucial to the relative insensitivity of fish to low concentrations of LPS.

ACKNOWLEDGEMENTS

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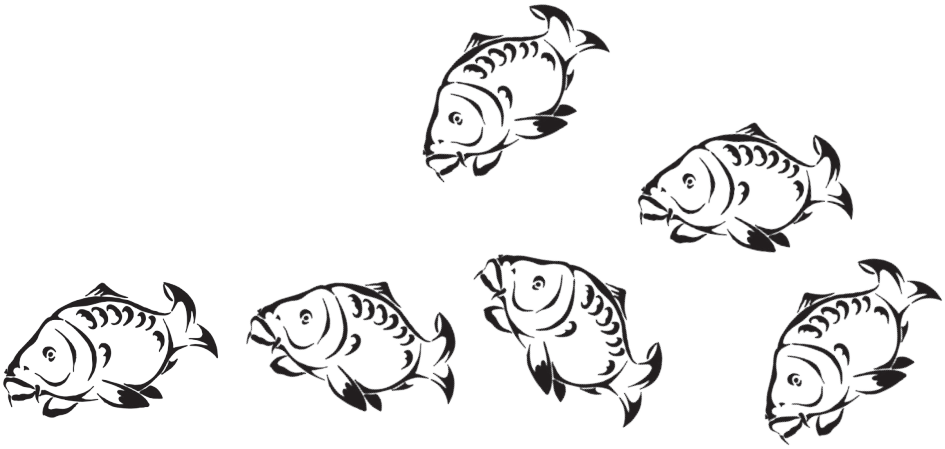

Chapter 6

Ligand specificities of Toll-like receptors in Teleost fish: indications from infection studies

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ABSTRACT

Toll like receptors (TLRs) are present in many different fish families from several different orders, including cyprinid, salmonid, perciform, pleuronectiform and gadiform representatives, with at least some conserved properties among these species. However, low conservation of the leucine-rich repeat ectodomain hinders predictions of ligand specificities of fish TLRs based on sequence information only. We review the presence of specific TLR genes, and changes in their gene expression profiles as result of infection, in the context of different fish orders and fish families. The application of RT-qPCR and availability of increasing numbers of fish genomes has led to numerous gene expression studies, including studies on TLR gene expression, providing the most complete dataset to date. Induced changes of gene expression may provide (in)direct evidence for the involvement of a particular TLR in the reaction to a pathogen. Especially when findings are consistent across different studies on the same fish species or consistent across different fish species, up regulation of TLR gene expression could be a first indication of functional relevance. We discuss TLR1, TLR2, TLR4, TLR5 and TLR9 as presumed sensors of bacterial ligands and discuss as presumed sensors of viral ligands TLR3 and TLR22, TLR7 and TLR8. More functional studies are needed before conclusions on ligands specific to (groups of) fish TLRs can be drawn, certainly true for studies on non-mammalian TLRs. Future studies on the conservation of function of accessory molecules, in conjunction with TLR molecules, may bring new insight into the function of fish TLRs.

Introduction to Toll-like receptors

Once a pathogen has breached physical barriers such as the skin or mucosal tissue, recognition by receptors on dendritic cells, phagocytes, B cells, endothelial cells and other cell types can trigger a series of reactions aimed at the final removal of the pathogen. Macrophages and dendritic cells, generally assigned to the innate immune system, not only have an important regulatory role in the early recognition of pathogens but also are crucial instructors of adaptive immunity. Activation of these cell types can be triggered by the recognition of pathogens by germ line-encoded receptors that recognize conserved patterns of pathogens [1]. These Pattern Recognition Receptors, or PRRs, come in distinct classes that together are able to recognize a large array of ligands, also named Pathogen Associated Molecular Patterns (PAMPs). Toll-Like Receptors (TLRs) are one group of well-known PRRs, each TLR binding to its own set of preferred ligands [2-4]. Thereby TLRs trigger a rapid inflammatory response *and* prime adaptive immunity [5-7]. The Toll receptor itself was first described in the fruit fly and was initially characterized for its developmental function; only later recognition of fungal pathogens was ascribed to the same receptor [8, 9]. To date, Toll-*like* receptors have been described in virtually every class of the animal kingdom including fish from several different orders, among which cyprinid, salmonid, perciform, pleuronectiform and gadiform representatives.

Toll-like receptors are type-I transmembrane proteins with numerous extracellular leucine-rich repeat (LRR) motifs which, together, form a horseshoe-like shaped solenoid directly involved in the interaction with a ligand. TLR specificity is determined by variation in the sequence and number of LRR motifs that can interact with ligands as diverse as lipids, carbohydrates, proteins and nucleic acids. When a ligand binds to the concave side of the extracellular domain of a TLR, conformational changes initiate receptor homo- or heterodimerization. Owing to receptor dimerization two

intracellular Toll/interleukin-1 receptor homology (TIR) signaling domains are brought close together initiating the recruitment of adaptor molecules. In contrast to the extracellular LRR motifs, the cytoplasmic TIR domain is highly conserved; not only between different TLRs of one species but also between different animal species, and has a central role in recruiting adaptor molecules [10]. The TIR domain-containing adaptor proteins MyD88, MAL, TRIF, TRAM and SARM can trigger one of two main signaling pathways [11]. One pathway leads to the activation of the transcription factor nuclear factor-κB (NF-κB) whereas the other pathway leads to the activation of activator protein-1 (AP-1). But both pathways trigger transcription of pro-inflammatory cytokines such as interleukin-1 and interleukin-6, or tumor necrosis factor alpha. Several reviews with detailed descriptions of the intracellular routes of activation following receptor-ligand interaction in fish have been published [12-14] and these routes of activation will not be subject of the present review. Since the LRR-rich ectodomains of TLRs are not very well conserved, predictions of ligand specificities of TLRs in fish may be unreliable when based only on sequence information, requiring additional, functional studies.

Evolution of Toll-like receptor families and genes

Some of the building blocks of TLRs go far back in evolution, for example, LRRs have been identified as important motifs in disease resistance proteins in plants [15]. The first combination between vertebrate-type TIR and LRR domain may have occurred after the divergence of Cnidaria and Bilateria. Subsequently, a recombination of both domains possibly occurred before or during the evolution of primitive vertebrates, leading to the generation of vertebrate TLR molecules [16]. The ascidian sea squirt *Ciona intestinalis* and the nematode *Caenorhabditis elegans* seem to have only one or two TLR genes [17, 18]. In contrast, sea urchin (*Strongylocentrotus purpuratus*) and amphioxus (*Branchiostoma lanceolatum*) possess a (very) large number of TLRs of more than two-hundred in the case of sea urchin [19-24]. Japanese lampreys (*Lethenteron japonicum*), representing a very ancient lineage of jawless vertebrates, have not many more than 16 TLR genes [25], close to the number of TLR genes found in higher vertebrates. Overall, since most vertebrate genomes are recognized to have at least one gene representing each of the six major TLR1, TLR3, TLR4, TLR5, TLR7 and TLR11 families [26] this suggests, but does not demonstrate, conservation of vertebrate TLRs.

Although within the modern bony fish (Teleostei) the number of TLR families generally is consistent with what is found for most vertebrates, it is not unusual to find duplicated TLR genes in fish. First postulated by Ohno, two rounds of whole genome duplication (WGD) have occurred during early vertebrate evolution [27], whereas in teleosts a third, fish-specific genome duplication (FSGD) occurred later in a basal teleost [28-30]. To complicate matters, some 25-100 million years ago (MYA) in salmonids and more recently (11-21 MYA), also in (some) cyprinids, a fourth WGD event took place [27, 31]; [32-34]. Fish-specific gene duplications as a result of WGDs may lead to the appearance of paralogues with partitioned functions of the ancestral gene (subfunctionalization) [35, 36], or may lead to the development of new functions (neofunctionalization) [37]. Evolution of sub- or neofunctionalization of TLRs can be particularly well studied in tetraploid species such as common carp in comparison with a related diploid species such as zebrafish. A comparative study into the additional genome duplication event that occurred in the common carp (*Cyprinus carpio*) lineage but not in zebrafish (*Danio rerio*) showed an almost complete synteny of genes [38].

With respect to partitioning of functions of duplicated genes it is of interest to mention the TLR repertoire of Atlantic cod (*Gadus morhua*), a cold-adapted teleost. Besides a highly

expanded number of MHC class I genes, Atlantic cod expresses a unique composition of TLR families; most TLR genes seem absent from the genome but instead, a single *tlr21*, two *tlr23* and 12 *tlr22*-related genes have been found [39]. The large number of *tlr22* genes seems a result of positive selection pressure, supporting the hypothesis that the *tlr22* genes in cod are undergoing neofunctionalization [40]. In general, positive selection pressure is often taken as an indication of a history of host-pathogen interactions, which would confirm a role for TLR(22) genes in the recognition of pathogens.

Conservation of Toll-like receptors

Molecular analyses can provide information on the molecular structure of TLRs *per se* thereby providing the most 'clean' indication of TLR conservation, which is often displayed in a phylogenetic tree. Sometimes, phylogenetic trees may be good predictors of function. A good example is TLR7, one of the TLR molecules with a remarkably high sequence conservation among vertebrates and with one of the lowest evolutionary rates of the LRR domains of all TLRs [41, 42]. Although the word conservation suggests a black or white situation in which a protein is either conserved or not, these situations do not exist and percentage amino acid identity is used to indicate a *degree* of conservation. But also these percentages do not describe perfectly the evolutionary process because not all domains that make up a (TLR) protein contribute equally to its function. For example, LRR and TIR domains have very different functions. In general, studying sequences only, may lead to false presumptions that sequence homology equates to functional conservation [43]. For example, conclusions on TLR function based on TLR sequences only may be obscured because of the requirement for co-receptors necessary for ligand recognition by the different TLRs [44, 45].

According to the phylogenetic definition, two homologous genes are orthologs if they diverged through a speciation event. In general, the topology of phylogenetic trees of TLRs are based on the more conserved cytoplasmic TIR domain [42, 46]. Their outlines, however, are similar to the topology of phylogenetic trees based on the more variable LRR domains [47] (see **Figure 1**). Both, phylogenetic trees based on TIR domains and phylogenetic trees based on LRR domains, show a strong identity to the topology of the species tree, suggesting the TLRs of fish are orthologs of TLRs found in other animals.

Some Toll-like receptors are more equal than others

TLRs can be referred to as 'highly conserved' or even 'archetypical' PRRs, but are they? The definition of archetype would refer to unchanging forms of receptors. But TLRs *did* change through evolution so this definition would not apply. Others might refer to TLRs as prototypical, which would define them as primordial, usually unrefined versions. However, prototypical would maybe best describe the Toll receptor but not the Toll-*like* receptors. Maybe *all* TLRs should be considered true reproductions of the archetypical Toll receptor and thus *all* TLRs should be referred to as archetypes. No matter what, the functional relevance of the degree of conservation (or better: degree of *change*) of homologous TLRs is primarily determined by their ability to recognize the same, similar (or different) ligands and subsequently trigger (or not) a rapid inflammatory response and prime acquired immunity. Conservation of function may be best determined by the (few) ligand binding studies on fish TLRs, to which we will come back later in this review. Maybe less informative, but certainly more frequent are studies on changes in gene expression.

At present, a large part of our knowledge on the fish immune system comes from studying fish species important for aquaculture. Traditionally, the most studied species include common

and crucian carp (both Cypriniformes), channel catfish (Siluriformes), rainbow trout and Atlantic salmon (both Salmoniformes), tilapia, sea bass and seabream (all Perciformes), Japanese flounder (Pleuronectiformes)

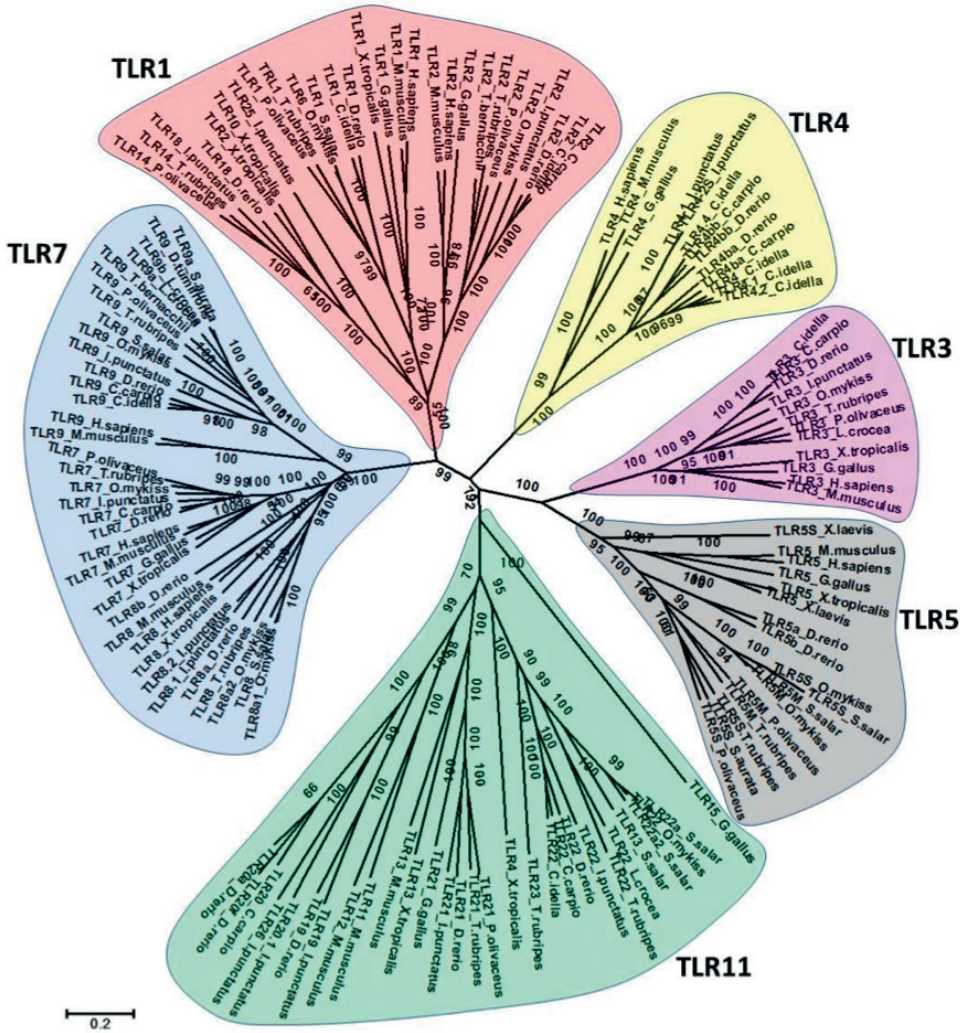

Figure 1 Phylogeny of Toll-like receptors. Phylogenetic relationships of fish TLRs based on amino acid alignments of the LRR domains. Bootstrap values based on 10,000 replicates are indicated on each branch. The evolutionary history was inferred using the neighbor-joining method [163]. All positions containing gaps and missing data were eliminated from the dataset (pairwise deletion) [164]. Phylogenetic analyses were conducted in MEGA5 [165] with 10,000 bootstrap replicates. Bootstrap values of major branching points are show as percentages. Accession numbers of sequences used to build the tree are as follows: *Ctenopharyngodon idella* (TLR1: ACT68332.1, TLR2: ACT68333.1, TLR3: ABI64155.1, TLR4: ACT68334.1, TLR4.1: AEQ64873.1, TLR4.2: AEQ64874.1, TLR4.4: AEQ64876.1, TLR9: ADB96920.1, TLR22: ADX97523.2); *Cyprinus carpio* (TLR2: ACP20793.1, TLR3: ABL11473.1, TLR4ba: own data, TLR4bb: own data, TLR7: BAJ19518.1, TLR9: ADC45018.2, TLR20: own data, TLR22: ADR66025.1); *Danio rerio* (TLR1: AAI63271.1, TLR2: AAQ90474.1, TLR3: NP_001013287.2, TLR4ba: NP_001124523.1, TLR4bb: NP_997978.1, TLR5a: XP_001919052.2, TLR5b: NP_001124067.1, TLR7: XP_003199309.1, TLR8a: XP_001920594.2, TLR8b: XP_001340186.2, TLR9: NP_001124066.1, TLR18: AAI63840.1, TLR19: XP_002664892.2, TLR20a: AAQ91318.1, TLR20f:

XP_003199280.1, TLR21: NP_001186264.1, TLR22: NP_001122147.1); *Dentex tumiforms* (TLR9: ABY79218.1), *Gallus gallus* (TLR1: BAD67422.1, TLR2: BAB16113.2, TLR3: ACR26371.1, TLR4: CAF31361.1, TLR5: AFV92631.1, TLR7: NP_001011688.1, TLR15: NP_001032924.1, TLR21: NP_001025729.1); *Homo sapiens* (TLR1: AAC34137.1, TLR2: AAY85647.1, TLR3: ABC86909.1, TLR4: AAI17423.1, TLR5: AAZ17473.1, TLR7: AAF78035.1, TLR8: AAI01076.1, TLR9: BAB19259.1); *Ictalurus punctatus* (TLR2: ABD17347.1, TLR3: AEI59664.1, TLR4.1: AEI59665.1, TLR4.2S: AEI59666.1, TLR5-1: AEI59668.1, TLR5.2: AEI59669.1, TLR5S: AEI59667.1, TLR7: AEI59670, TLR8.1: AEI59671.1, TLR8.2: AEI59672.1, TLR9: AEI59673.1, TLR18: AEI59674, TLR19: AEI59675, TLR20.1: AEI59676, TLR21: AEI59678.1, TLR22: AEI59679, TLR25: AEI59680, TLR26: AEI59681); *Larimichthys crocea* (TLR3: ADZ52858.1, TLR9a: ACF60624.1, TLR9b: ACF60625.1, TLR22: ADK77870.1); *Mus musculus* (TLR1: AAG37302.1, TLR2: AAD46481.1, TLR3: NP_569054.2, TLR4: AAD29272.1, TLR5: NP_058624.2, TLR7: AAL73191.1, TLR8: AAK62677.1, TLR9: AAK28488.1, TLR11: AAS37672.1, TLR12: AAS37673.1, TLR13: AAS37674.1); *Oncorhynchus mykiss* (TLR2: CCK73195.1, TLR3: NP_001118050.1, TLR5M: NP_001118216.1, TLR5S: NP_001117680.1, TLR6: NP_001159573.1, TLR7: ACV41797.1, TLR8a1: ACV41799.1, TLR8a2: ACV41798.1, TLR9: ACC93939.1, TLR22: NP_001117884.1); *Paralichthys olivaceus* (TLR1: AFW04264.1, TLR2: BAD01046.1, TLR3: BAM11215.1, TLR5M: AEN71825.1, TLR5S: AEN71823.1, TLR7: ADX32854.1, TLR9: BAE80691.1, TLR14: BAJ78226.1, TLR21: AFW04263.1); *Salmo salar* (TLR1: AEE38252.1, TLR5M: AEE38253.1, TLR5S: AEE38254.1, TLR8: NP_001155165.1, TLR9; ABV59002.1, TLR13: NP_001133860.1, TLR22a: CAJ80696.1, TLR22a2: CAR62394.1); *Sparus aurata* (TLR5S: CCP37739.1, TLR9a: AAW81697.1); *Takifugu rubripes* (TLR1: AAW69368.1, TLR2: AAW69370.1, TLR3: AAW69373.1, TLR5M: AAW69374.1, TLR5S: AAW69378.1, TLR7: AAW69375.1, TLR8: AAW69376.1, TLR9: AAW69377.1, TLR14: AAW69369.1, TLR21: NP_001027751.1, TLR22: BAF91187.1, TLR23: AAW70378.1); *Trematomus bernacchii* (TLR1: ACT64128.1, TLR9: ACT64130.1); *Xenopus tropicalis* (TLR1:XP_002938702.1, TLR2: XP_002942485.1, TLR3: XP_002934448.1, TLR4: XP_002942581.1, TLR5: XP_002940742.1; TLR7: NP_001120883.1, TLR8: XP_002933859.1, TLR10: XP_002943096.1, TLR13: XP_002935047.1); *Xenopus laevis* (TLR5; NP_001088449.1, TLR5S: AAY67754.1).

and Atlantic cod (Gadiformes). Increasingly, the knowledge on the fish immune system is substantiated by information from fish species of economic importance to Asia, including grass carp and Indian carp (Cypriniformes) and pufferfish (Tetraodontiformes), but also the miiuy and the yellow croaker as well as the orange-spotted grouper (all Perciformes). Further, not to be underestimated, there are a number of freshwater fish species that have become important experimental models in immunology, including medaka (*Oryzias latipes*, Beloniformes/ Cyprinodontiformes), stickleback (*Gasterosteus aculeatus*, Gasterosteiformes) and zebrafish (Cypriniformes). Although the list of fish species studied for their immune system still is increasing, it may also be clear that our knowledge of fish immunology is built on only a minor fraction of the approximate 27,000 fish species known. We therefore should not be surprised if the organization of the immune system is not exactly the same across fish species. However, in general, the closer the phylogenetic relationship between fish species, the closer the similarities. That is why, in this manuscript, we have reviewed the information on TLRs from different fish species phylogenetically, i.e. per (super)order (**Figure 2**).

The superorder Ostariophysi comprises the order Cypriniformes (cyprinids), with the order Siluriformes (catfishes) as their closest living relatives. In the review, we will discuss the cyprinids and catfishes as one related group. The cyprinids are a diverse group comprising goldfish, common carp and zebrafish but also various Indian major carp species including rohu (*Labeo rohita*), catla (*Catla catla*) and mrigal (*Cirrhinus mrigala*). Where the zebrafish has become maybe the best-studied fish in the laboratory, common carp is worldwide the most cultured fish species for food consumption.

The superorder Acanthomorpha, also known as the spiny-rayed fishes, comprises over 60% of existing teleosts. This superorder includes the orders Salmoniformes (salmonids), Pleuronectiformes

(flatfishes), Tetraodontiformes (pufferfish) and Perciformes (perciforms). The order Salmoniformes has the Salmonidae as the only living family currently placed in the order and includes salmon and trout. Rainbow trout is not only an important fish species for aquaculture but also one of the most studied fish species with respect to immunology.

The Acanthomorpha superorder, next to the Salmoniformes, also comprises the orders Pleuronectiformes (flatfishes), Tetraodontiformes (pufferfish) and Perciformes (bass). The flatfishes include, among others, the half-smooth tongue sole (*Cynoglossus semilaevis*), a genus of tonguefish indigenous to the Indo-West Pacific region and the olive flounder (*Paralichthys olivaceus*) native to the north-western Pacific Ocean (also referred to as bastard halibut, Japanese or Korean flounder or flatfish). *Takifugu rubripes* is one of 25 species belonging to the genus Takifugu, a salt water fish well-known for its lethal amounts of the poison tetrodotoxin in internal organs and for being the first fish species for which the genome was sequenced. The perciforms underwent an explosive radiation 60 million years ago and taxonomically contain a large number of teleost taxa among which gilthead sea bream and seabass. The Perciformes are the most 'young' fish order and also comprise medaka and stickleback. In this review, we will discuss the flatfish, pufferfish and perciform seabass as a more closely related group.

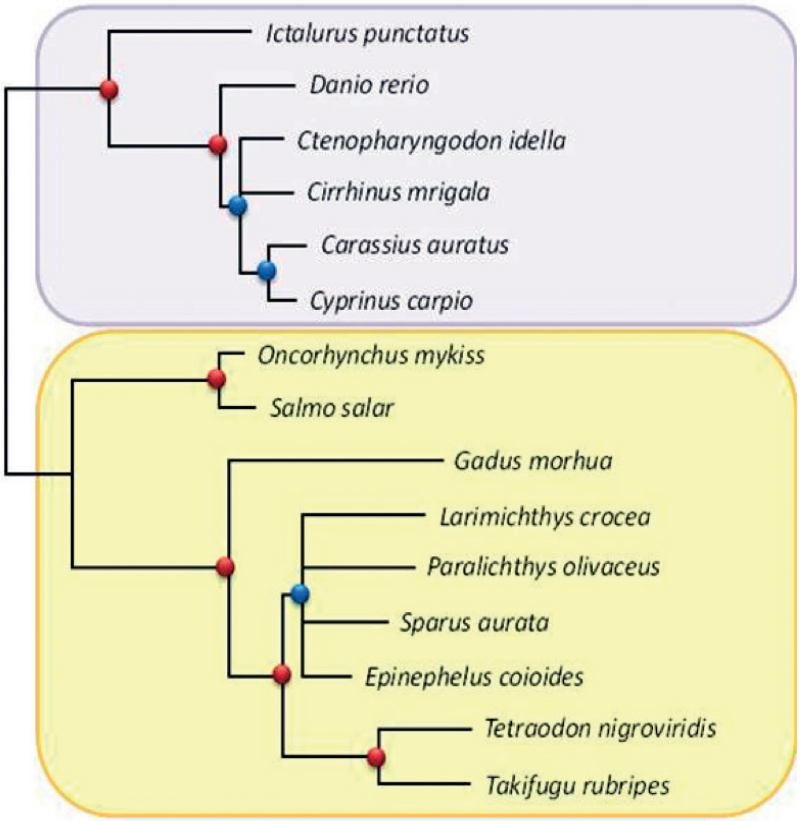

Figure 2. Rectangular phylogram illustrating the phylogenetic relationship between teleosts, with Ostariophysi and Acanthomorpha superorders indicated by blue and yellow boxes, respectively. The phylogram was built on teleost-specific TLR22 proteins. Bayesian analysis was performed using a mixed fixed-rate amino acid model and the consensus tree was built after burning 1,000 trees from the 105 generations. Only bayesian probability values above 0.8 are indicated as tree nodes: 0.81 to 0.89 (blue circles) and 0.9 to 1 (red circles). Accession numbers of TLR22 sequences are as follows: crucian carp (*Carassius auratus*) AY162178; common

carp (*Cyprinus carpio*) HQ452813; grass carp (*Ctenopharyngodon idella*) HQ676542; Indian carp (*Cirrhinus mrigala*) ADQ74638; zebrafish (*Danio rerio*) BC163527; orange-spotted grouper (*Epinephelus coioides*) AER11138; Atlantic cod (*Gadus morhua*) JX074773; channel catfish (*Ictaluris punctatus*) HQ677725; yellow croaker (*Larimichthys crocea*) GU324977; rainbow trout (*Oncorhynchus mykiss*) AJ628348; Japanese flounder (*Paralichthys olivaceus*) BAD01045; seabream (*Sparus aurata*) CAP47202; Atlantic salmon (*Salmo salar*) AM233509; green spotted pufferfish (*Takifugu nigroviridis*) ENSTNIT00000016840; pufferfish (*Takifugu rubripes*) AB197916.

Studying basal gene expression of Toll-like receptors

A eukaryotic cell contains thousands of genes, several of which are constitutively expressed at high levels and do not respond to external stimuli (housekeeping genes). Most genes, however, have a low basal expression level that may increase when a cell enters a particular pathway or differentiation stage. In these cases, the level of gene expression can provide an indication for the presence of activated or differentiated cells. Gene expression is often used to imply a function of that particular gene in a particular organ; if TLRs function as sensors of PRRs also in fish, indeed one would expect basal TLR gene expression to be high in immune organs. Up-regulation of TLR gene expression could indicate an increase in numbers of (differentiated) cells bearing the same TLRs. Expression studies on genes conserved between mouse and human indicate an enrichment of these genes in the same organs in zebrafish, defining organ-specifics with functionally-related genes as conserved between species [48]. This indicates that studying basal gene expression levels of particular (groups of) genes can indeed be informative.

Although mouse and human diverged from a common ancestor more than 60 million years ago, it should not be surprising that besides commonalities also differences exist between TLRs of these species. For example, they do not display the exact same TLR repertoire; the human genome contains 10 functional TLRs whereas the mouse genome contains 12 TLRs, with *tlr10* being a pseudogene and *tlr11*, *tlr12* and *tlr13* being mouse-specific and represented by pseudogenes in humans [49]. Not only do differences in repertoire exist, also differences in ligand specificity for true human:mouse TLR orthologs are common. For example, human TLR8 but not mouse TLR8 recognizes the imidazoquinoline compound R848 [3]. Furthermore, differences in cell-specific expression of human:mouse TLR orthologs are present; often a specific human immune cell population will respond differently to a distinct set of TLR ligands compared to its mouse counterpart. The same is true for TLR expression; for example, lipopolysaccharide (LPS) u-pregulates *tlr3* and *tlr6* in mouse but not human macrophages. In general, TLRs seem to have a narrower range of protective functions against infectious diseases in humans as compared to mice [49]. Bearing in mind the above-described differences in function between TLRs of humans and mice, fish immunologists run the risk of underappreciating fish-specific subtleties when comparing outcomes to the 'mammalian' immune system.

In research areas such as fish immunology, where investigators are forced to rely heavily on genetic information rather than on protein expression, basal gene expression is often taken as an indicative measure of function. Over the last 5-10 years, real time-quantitative PCR (RT-qPCR) has become the method of choice for accurate measurement of mRNA expression levels [50]. The application of RT-qPCR and availability of the first fish genomes has led to numerous gene expression studies. Several of these publications describe a single TLR gene and its (basal) expression in different organs, frequently followed by the conclusion that the TLR of interest indeed is expressed higher in immune organs than in non-immune organs. However, comparison of outcomes from these different studies, even when dealing with the same fish species, is difficult when analyses were

not performed on the same cDNA samples. For this reason, in our overview of basal TLR gene expression (**Figure 3**), we included selective information only, taken from scrutinized databases on genes from human and mouse (BioGPS www.biogps.org). For fish, we only included studies that provided an overview of basal TLR gene expression using a single, common source of cDNA. We included three fish species; zebrafish and common carp (Cypriniformes) and channel catfish (Siluriformes); all Ostariophysi. The information from zebrafish was taken from an early publication [51]. The information on common carp TLR gene expression was taken from our own (unpublished) observations, whereas the information on channel catfish TLRs was summarized from two recently published studies [42, 52]. We used the information refered to above to produce an overview (**Figure 3**) with arbitrary values in an attempt to compare TLR basal gene expression in different organs from both mammalian and fish species.

Overall, as can be seen in **figure 3**, TLR genes are more abundantly expressed in peripheral blood leukocytes (PBL) than in spleen, kidney and liver, and lowest expressed in skin and gut. This seems to be a common finding for human and mouse but also for fish. This finding may be skewed, however, by the fact that PBL preparations consist of leukocytes only, whereas organ tissues are 'diluted' with non-leukocyte cell types. Differences in composition of PBL preparations further complicate the comparison between human, mouse and fish. Comparison of TLR gene expression profiles over different organs show a relatively good conservation of tissue distribution per gene. The exceptions are *tlr4*, with a basal expression in fish kidney that is very low compared to *Tlr4* gene expression in bone marrow of mouse and human and *Tlr9*, with a high expression in fish kidney compared to that in bone marrow of mouse and human. These exceptions may be, of course, the result of our attempt to compare TLR gene expression profiles in hematopoietic (head) kidneys of fish with hematopoietic bone marrow of mammalian species. TLR gene expression profiles were not always fully consistent between human and mouse and not always fully consistent across the different fish species either. For example, *tlr1* is higher expressed in skin of zebrafish than in skin of carp, but *tlr2* is lower expressed in gut of zebrafish than in gut of carp. Also, *tlr7-tlr8-tlr9* genes are highly expressed in the spleen of catfish but low expressed in the spleen of zebrafish. Of course, the level of gene expression is difficult to compare between studies but our use of arbitrary colour gradients is an attempt to reduce interpretation errors. In a number of cases, the expression between two isoforms (double circles) is quite different, especially true for *tlr4* isoforms in zebrafish (gut), catfish (liver) and carp (gut, liver), but also true for *tlr*8 in zebrafish (gut). Summarizing, it appears difficult to use basal gene expression of TLRs as an indicative measure of function.

Studying changes in TLR gene expression induced by pathogens or PAMPs

In homeostatic situations gene expression is more or less constant whereas infections can bring about an up-regulation of gene expression due to the migration or proliferation of cells or because of altered rates of transcription per cell. Down-regulation of TLR gene expression can indicate an altered rate of transcription but also a migration of the relevant cell type away from the organ analysed. Overall, although no common rule, induced changes in gene expression provide (in)direct evidence for the involvement of a particular gene in the reaction to the pathogen. For example, injection of a TLR ligand such as polyinosinic polycytidilic acid (poly I:C) may lead to the up-regulation of gene expression of the TLR gene suspected to be important for ligand recognition, in this case TLR3. For the same reason, up-regulation of *Tlr3* following infection with a double-stranded (ds)RNA virus could be taken as evidence for the involvement of this particular TLR in the recognition of viral dsRNA.

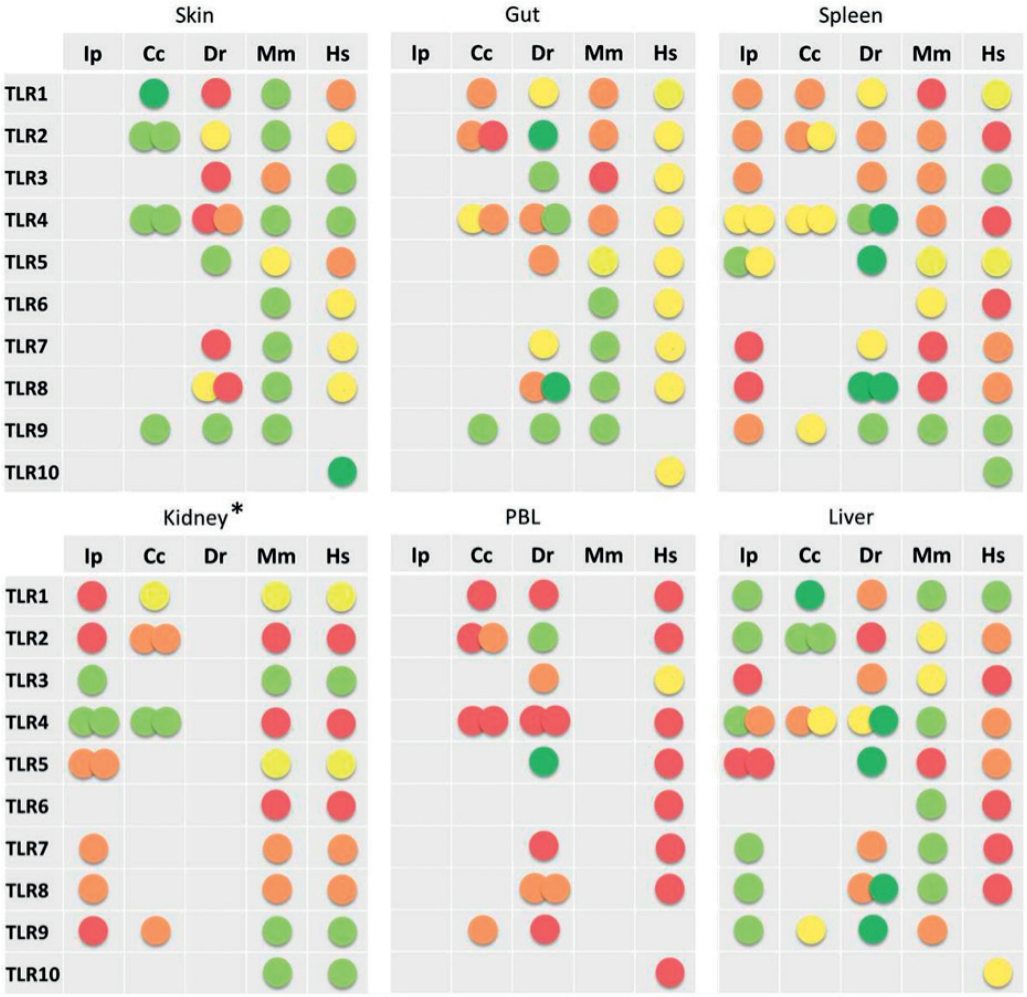

Figure 3 Overview of basal TLR gene expression in different organs of channel catfish, common carp, zebrafish, mouse and human. The arbitrary colour gradient indicates the expression level of each TLR; red indicates highest gene expression followed by decreasing levels of expression indicated by orange, yellow and green. Dark green indicates the lowest TLR gene expression. Double circles indicate gene expression of duplicated TLR genes. TLR gene expression is displayed for skin, gut, spleen, kidney, peripheral blood leukocytes (PBL) and liver. We restricted ourselves to the analysis of TLR1-TLR10. Abbreviations: Ip (*Ictalurus punctatus*), Cc (*Cyprinus carpio)*, Dr (*Danio rerio*), Mm (*Mus musculus*) and Hs (*Homo sapiens*).

- Asterisk (*) indicates comparison of bone marrow (mouse and human), head kidney (carp) and kidney from (catfish).
- Double circles: Ip TLR4 (left: membrane mTLR4 and soluble sTLR4; right: mTLR4), Ip TLR5 (left: mTLR5 and sTLR5; right: mTLR5). Cc TLR2 (left: TLR2a; right: TLR2b), Cc TLR4 (left: TLR4ba; right: TLR4bb). Dr TLR4 (left: TLR4ba; right: TLR4bb), Dr TLR8 (left: TLR8a; right: TLR8b).
- Channel catfish (Ip) TLR gene expression was taken from two recently published studies using catfish tissues from n=3 1-year-old fish [42, 52]
- Common carp (Cc) TLR gene expression was collected from n=5 healthy animals of 9 to 12 months of age (unpublished data).
- Zebrafish (Dr) TLR gene expression was taken from a single source of cDNA [51].
- Human (Hs) and mouse (Mm) TLR gene expression was collected from www.biogps.org.

There are a number of bacteria more commonly studied, and most often the decision to study these particular bacteria is based on their impact on aquaculture. Among the Gram-negative bacteria more commonly mentioned in studies on TLR gene expression are several Vibrio species, including *Vibrio anguillarum*, *Vibrio parahaemolyticus* and *Vibrio alginolyticus*, but also *Aeromonas hydrophila, Edwardsiella tarda* and *Edwardsiella ictaluri*. Gram-negative bacteria typically have an outer LPS layer that is sensed, at least in mammals, by TLR4. Both *E. tarda* and *E. ictaluri* are motile bacteria and express flagellin, generally accepted as ligand for TLR5. There are two additional bacteria commonly used in zebrafish, more frequently as models for infections of humans rather than for their relevance to aquacultured fish species: *Mycobacterium marinum*, a fish equivalent of Gram-positive *Mycobacterium tuberculosis* and *Salmonella thyphimurium* (Gram negative). Gram positive bacteria lack LPS but express high amounts of peptidoglycan (PGN) and lipoteichoic acid (LTA) in the cell wall, both considered ligands for TLR1/TLR2. As an alternative to infections with live bacteria, fish often are injected in the laboratory with 'pure' ligands such as LPS from *Escherichia coli* or PGN and LTA from *Staphylococcus aureus*.

As it is important to differentiate between Gram-negative and Gram-positive bacteria when considering recognition of bacterial ligands by TLRs, it is equally important to recognize the nature of a virus when considering viral infections: is it a DNA or an RNA virus? Does it carry single-stranded (ss) or double-stranded (ds) genomes? These features will influence the subsequent TLR recognition because, at least in mammals, DNA is sensed by TLR9, dsRNA by TLR3 and ssRNA by TLR7 and TLR8. Viruses studied in the context of TLR gene expression in fish are viral haemorrhagic septicaemia virus (VHSV; ssRNA) and grass carp reovirus (GCRV, dsRDNA). As an alternative to viral infections, fish are frequently injected with poly I:C as a double-stranded polyribonucleotide mimicking viral infection with a dsRNA virus.

Under the assumption that TLR1 and/or TLR2 sense molecular patterns from Gram-positive bacteria, TLR4 senses molecular patterns from Gram-negative bacteria, TLR5 senses flagellin and TLR9 senses CpG motifs in bacterial (and viral) DNA, it is logic to study these TLRs in the context of bacterial infections. Likewise, under the assumption that TLR3 senses dsRNA and TLR7/TLR8 sense ssRNA, it is logic to study these TLRs in the context of viral infections. In this review we first discuss TLR1, TLR2, TLR4, TLR5 and TLR9 as presumed sensors of bacterial ligands and then TLR3, TLR7 and TLR8 as presumed sensors of viral ligands. We will try to detect commonalities in the responses of fish TLRs to bacterial or viral pathogens, mostly based on changes in gene expression. Of course, infections of whole animals with live pathogens can induce changes in gene expression that can vary due to the presence of multiple PAMPs, time, dose etcetera. This, for example, makes it difficult to claim direct correlations between up-regulation of expression of a TLR gene and recognition of specific pathogen-derived ligands by that particular TLR. In other words, infection with a dsRNA virus and subsequent up-regulation of TLR3 gene expression does not necessarily prove TLR3-dsRNA recognition. However, if such findings are consistent over different studies on the same species and maybe even across different fish species, up-regulation of TLR gene expression could at least be considered an indication of functional relevance and stimulate further investigations on the proposed relationship.

Do fish TLR1 and TLR2 sense bacterial lipopeptide ligands?

In humans, TLR2 recognizes a great diversity of ligands and by the ability to form heterodimers, not only with TLR1, but also with TLR6 (all members of the TLR1 family), the spectrum of ligand-recognition is even broader [53]. Formation of a heterodimer of TLR1 and TLR2, or TLR6 and

TLR2, is dependent on the fatty acid pattern as well as the assembly of the polypeptide tail of the lipopeptide ligands [54, 55]. Triacylated lipoproteins and mycobacterial products are sensed by TLR2 molecules that form a heterodimer with TLR1, whereas TLR2 senses bacterial lipopeptides and lipoteichoic acids as well as glycosylphosphatidylinositol anchors from parasites as heterodimer with TLR6 [56].

Initial screening of the zebrafish genome for the presence of TLR and TLR-associated genes predicted the presence of *tlr1* and *tlr2*, but not *tlr6* and *tlr10* [13, 51, 57], leaving TLR1 as the most likely partner for heterodimerization with TLR2. In support of a conserved lipopeptide recognition by a putative TLR1-TLR2 heterodimer are conserved amino acids at TLR1 and TLR2 loops previously shown to interact with each other during dimerization [58]. Also in support is the fact that fish TLR1 molecules do not have a LRR at the N-terminal domain (LRRNT), a characteristic believed to be important for the dimerization process with TLR2 [42]. Functional studies will have to provide more conclusive evidence for TLR1-TLR2 heterodimerization in fish.

Further studies on TLR2 within the order Cypriniformes were performed on (Indian rohu and common) carp, whereas TLR(2) studies in the model zebrafish species mostly turned to large-scale transcriptomic approaches. Gene expression of *tlr2* in rohu could be increased following *in vitro* stimulation of primary cell cultures obtained from heart tissue using two well-known ligands, PGN and LTA [59]. Infections of rohu with Gram-positive *Staphylococcus uberis*, with *E. tarda* [59] or with *A. hydrophila* [60] all induced *tlr2* gene expression. Apparently, both Gram-positive (*Staphylococcus*) and Gram-negative (*Edwardsiella, Aeromonas*) bacteria can induce *tlr2*2 gene expression in rohu. Alternatively, these studies are good examples of how difficult it is to correlate up-regulation of expression of a particular TLR gene to the recognition of specific pathogen-derived ligands.

The fold change in *tlr2* gene expression is generally low, both in rohu and common carp. This would suggest gene expression per se not to be the best read-out for TLR2 activation. We investigated in more detail the function of TLR2 in common carp [61, 62]. Activation of carp macrophages by PGN resulted in (moderate to low) up-regulation of *tlr2* gene expression and enhanced *tlr2* mRNA stability [61]. The effects were always greater using PGN than LTA. Overexpression of carp *tlr2* in a human cell line (HEK 293) confirmed recognition by carp TLR2 by both, PGN and LTA, using phosphorylation of MAPK-p38 as read-out. However, the synthetic triacylated lipopeptide Pam_3CSK_4 required high concentrations to activate cells overexpressing carp TLR2, whereas the diacylated lipopeptide MALP-2 did not activate TLR2-transfected human cells at all. In mammals, triacylated lipoproteins are sensed by TLR2 forming a heterodimer with TLR1, whereas triacylated lipoproteins are sensed by TLR2 as heterodimer with TLR6. The complete absence of response to diacylated MALP-2 could be related to the absence of a TLR6 homologue in fish genomes and thus the inability of TLR2 to heterodimerize with TLR6 in fish. With regard to the possibility of TLR2-TLR1 heterodimer formation; the presence of *tlr1* in fish geneomes has at least been confirmed for zebrafish [51, 57], common carp (unpublished data) and channel catfish [42]. Functional studies on TLR2-TLR1 heterodimerization, however, have not yet been reported.

Early studies on infection of blue catfish and channel catfish with *E. ictaluri* indicated that *tlr2* gene expression was not up- but modestly down-regulated in head kidney, whereas in the spleen a modest increase in *tlr2* gene expression was observed later during infection [63]. Another study showed minor changes in *tlr2* gene expression in the head kidney of channel catfish infected with *E. ictaluri* [64]. Most recently [52], in a comparison of three organs, nine TLR genes and in particular *tlr20* were again found down-regulated in head kidney 6 days after bath challenge with *E. ictaluri*. In contrast, TLR genes and in particular *tlr18*, *tlr25* and *tlr26* were found up-regulated in spleen and

liver, indicating clear differences in induced gene expression per organ.

Salmonid TLR2 has not been studied extensively, although recently a rainbow trout *tlr2* sequence has been submitted to the database. Trout TLR1 was identified using a bacterial artificial chromosome (BAC) library, but putative ligands have not been extensively studied. Gene expression levels of *tlr1* in head kidney leukocytes were neither affected by diacylated lipoprotein PAM_2CSK_4, nor by triacylated lipoprotein PAM_3CSK_4 [65].

Early genomic sequence screens in pufferfish predicted the presence of several TLRs, with one (partial) sequence showing equally high amino acid identity to human TLR1, TLR6 and TLR10, which was named TLR1 [66]. The full molecular cloning and characterization of pufferfish (*T. nigroviridis*) TLR1, took until 2008 however. *tlr1* transcripts in spleen were found increased (3-fold) after stimulation with LPS [67]. Regretfully, prototypical TLR1 ligands were not tested. Recently, TLR2 of not only pufferfish (Tetraodontiformes) both also miiuy croaker (Perciformes) was cloned and proven to share high sequence identities [68]. Both *tlr1* and *tlr2* were cloned for orange-spotted grouper, another perciform species, and *tlr1* and *tlr2* gene expression found increased in spleen upon stimulation with LPS and poly I:C, or upon injection with *V. alginolyticus* [69]. Gene expression of *tlr2* was also up-regulated in grouper infected with the ectoparasite *Cryptocaryon irritans*, with significant changes in skin, gill but also head kidney and spleen [70]. True for most fish species, changes in TLR gene expression profiles after infection with fish parasites have hardly been studied.

Of interest are early studies in flounder (Pleuronectiformes) that established a link between induction of *tlr2* gene expression in peripheral blood leukocytes (PBLs) and stimulation with PGN but also with poly I:C [71]. Of further interest are the studies where olive flounder *tlr2* gene expression was linked to infection with the VHS virus [72], and a mapping study where *tlr2* was found linked to a microsatellite marker tightly associated with resistance to lymphocystis, another viral disease of flounder [73]. These findings could suggest a putative link between fish TLR2 and recognition of viral rather than bacterial ligands [74].

Fish TLR4 does not sense bacterial LPS

TLR4 is a protein that in humans, together with the co-receptors myeloid differentiation protein-2 (MD-2) and CD14, recognizes LPS, the major component of the outer membrane of Gram-negative bacteria and an important endotoxin. LPS is a highly amphipathic molecule that naturally exists in solution as large aggregates. LPS-binding protein (LBP) and CD14 are two proteins whose coordinate actions result in the disaggregation and delivery of LPS monomers to the TLR4 MD-2 complex. Fish, in contrast, are known to tolerate relatively high concentrations of LPS and thus, initially, the high tolerance of fish to LPS seemed easily explained by the absence of *tlr4* from the pufferfish genome [66]. However, the subsequent identification of *tlr4* in the zebrafish genome [51, 57] challenged the idea that *tlr4* would be absent from *all* fish genomes. Nevertheless, also zebrafish tolerate high concentrations of LPS. Subsequently, the absence of *md-2*, *cd14*, and of a prototypical *lbp* [45, 75] from all fish genomes examined to date, was put forward as an explanation for the high tolerance of fish to LPS [76]. Interestingly, mammalian LBP and CD14 have been shown to sensitize cells not only to LPS but also to lipopeptides and lipoproteins, disaggregating these molecules for delivery to CD14. Cell-based assays showed that the sensitivity of cells to minute amounts of Pam_3CSK_4 agonist was enhanced by the addition of either LBP, or soluble CD14 [77]. In other words, absence of MD2, CD14 and LBP from fish genomes could have an effect on not only the sensitivity of fish to Gram-negative bacterial ligands but also Gram-positive bacterial ligands.

To date, *tlr4* has been cloned and characterized in zebrafish (*D. rerio*) [51, 57], rare minnow (*Gobiocypris rarus*) [78], common carp (*C. carpio*) [79], grass carp (*C. idella*) [80] and channel catfish (*I. punctatus*) [42]. The most detailed studies on the function of TLR4 have been performed in zebrafish. Using a dual-luciferase reporter assay to study NF-κB activation in whole zebrafish embryos, it became clear that LPS signaled via a TLR4- and MyD88-independent manner but also that zebrafish TLR4 negatively regulated the *myd88*-dependent signaling pathway [81]. In support, the use of chimeric molecules in which the extracellular LRR domains of zebrafish TLR4 proteins were fused to intracellular TIR domains of the mouse TLR4 protein, conclusively demonstrated a lack of responsiveness of these Tlr4 proteins to LPS [43]. Catfish TLR4 was reported to lack the important structural features required for TLR4 functions, since only one of the four critical residues for LPS/TLR4 interaction [82] is conserved in catfish [42]. By now it has become clear that the Cypriniformes and Siluriformes do express *tlr4* genes, of which the ligand most likely would not be LPS but remains uncharacterized. This does not need to be a full surprise. Human TLR4 does not only sense LPS from Gram negative bacteria but also, for example, viral envelope proteins. However, similar to LPS, these proteins (at least Respiratory Syncytial Virus (RSV) F protein) also require the presence of CD14 and MD-2 for signaling [83], leaving the function of (cyprinid and silurid) fish TLR4 uncharacterized.

Despite the high tolerance of fish to LPS [66], LPS does have multiple biological effects on fish [76, 84] and perhaps remains the most commonly used stimulator of fish cells. This would suggest LPS *can* be sensed by fish cells, but maybe via a receptor other than TLR4. LPS is best described as consisting of three structural components, of which the most important is lipid A. Lipid A, acting alone or as a component of LPS, is a potent modulator of the mammalian immune response and primarily responsible for the effects of the endotoxin. Linked to Lipid A is a short chain of sugars named the core antigen, or R polysaccharide. The third structural feature is attached to the core polysaccharide and contains up to 40 repeating subunits of 3-5 sugars, named the O antigen. One problem analyzing immune responses of fish to LPS is that the majority of studies use preparations of LPS with various degrees of purity, making it difficult to ascribe the effects to one of the three structural components and to recognition by TLR4. Indeed, using gene expression of the cytokines *il-1β* and *il-6* as read out, crude phenol-extracted LPS, but not ultrapure preparations of LPS, could activate rainbow trout macrophages. In these studies, the activity of the phenol-extracted LPS preparations could be ascribed to PGN rather than LPS. Interestingly, the stimulatory effect of PGN was only true for PGN from Gram-negative but not from Gram-positive bacteria [85]. In future attempts to identify the LPS receptor in fish, it would be of great interest to use ultrapure LPS preparations from fish-specific bacteria rather than LPS from primarily *E. coli*, although this would still leave the identification of the ligand(s) of fish TLR4 unexplored.

Human TLR4 activates the MyD88-dependent pathway, which gives rise to strong and early activation of NF-κB, but also activates the TRIF-dependent and MyD88-independent pathway that primarily drives strong activation of IRF-3 [86]. As mentioned above, TLR4 does not only sense LPS but is also well-known for binding to other ligands. For example, mouse TLR4 signaling has been shown to play an important role in controlling infection with paramyxovirus [87] or RSV [88]. There are a number of reports that could link fish TLR4 to viral infections. PCR-based gene expression studies showed up-regulation of *tlr4* in cyprinid fish after infection with grass carp reovirus [78] [80]. The first detection of common carp *tlr4* was in cDNA pools of viral (KHV)-infected carp [79]. We have studied *tlr4* gene expression during infection of common carp with SVCV and preliminary results indicate that *tlr4* gene expression can be induced by this virus as well

(unpublished data). These findings argue for more studies into viral PAMPs as ligands of fish TLR4.

Interestingly, multiplication of the *tlr4* gene seems a factor common to all fish species for which TLR4 sequences have been described so far. In zebrafish *tlr4* genes are duplicated, where one copy lacks a secretory signal [43]. In common carp, although initially only a single *tlr4* was described [79], we could detect at least five *tlr4* genes in the genome [38] (Pietretti, unpublished data). Grass carp has five, copies of *tlr4* [80]. Channel catfish has two *tlr4* genes, of which one is a membrane and the other a soluble form [42]. Channel catfish *mtlr4* had the highest basal expression in gills, whereas *stlr4* is highest expressed in the gonads. Thus, the expression profile of the soluble form of *tlr4* is very different from the membrane-bound counterpart indicating that soluble TLR4 may play a different role rather than simply act as an agonist or antagonist of the membrane form [42]. Clearly, further research into the different isoforms of *tlr4* is required, while keeping an open mind for the possibility that neofunctionalization of TLR4, membrane-bound or soluble, may have led to novel, unknown functions.

Fish have both a membrane and a soluble form of TLR5 that senses bacterial flagellin

Human TLR5 recognizes flagellin, a protein monomer that makes up the filament of bacterial flagella and is found on nearly all motile bacteria. Indeed, there are a number of regions in the flagellin protein that are highly conserved among all bacteria. Structural and biochemical studies on the TLR5-flagellin interaction in mammals have been challenging due to the technical difficulty in obtaining bioactive recombinant protein of the ectodomain of TLR5. The successful production of the zebrafish ectodomain of TLR5 as a hybrid with the variable lymphocyte receptor (VLR) of hagfish, allowed for the determination of the crystal structure of its complex with Salmonella flagellin (FliC) D1-D2-D3 domains [89]. Although the reason to use zebrafish rather than human TLR5 was technical, this does provide the ultimate proof that flagellin is the best ligand for studies on fish TLR5. Conservation of flagellin binding by TLR5 had already been suggested by a morpholino *tlr5* knockdown, reducing flagellin-induced inflammation in zebrafish [90], and by a modulation of *tlr5* gene expression induced by flagellin and infection of mrigal Indian carp with *A. hydrophila* or with *E. tarda* [91].

Similar to human and mouse TLR5, cyprinid TLR5 seems to be present in membrane form only. In contrast, many other fish species also express a soluble form of the TLR5 ortholog (sTLR5). In channel catfish, the expression profiles of m*tlr5* (two isoforms) and *stlr5* (single isoform) are very different; *mtlr5* isoforms have their highest expression in liver and gonads whereas *stlr5* is more expressed in the kidney, indicating that *stlr5* may play a different role than *mtlr5* [42]. With this new information at hand, it can be important to re-interpret some previous data sets. For example, with the present knowledge on catfish *tlr5* it is likely that primers used previously [92] amplified *stlr5* rather than *mtlr5*. The primers used in another study on catfish TLR5, in retrospect, amplified all rather than membrane or soluble forms of *tlr5* genes specifically [93]. With the past knowledge, of course, this could not be prevented. But with the present knowledge on multiple isoforms, if indeed sTLR5 and mTLR5 would play clearly different roles, future studies will require the design of primers for specific detection of either membrane or soluble forms.

Rainbow trout also express a membrane and soluble TLR5. Whereas *mtlr5* is ubiquitously expressed, *stlr5* was found predominantly in the liver [94]. Similar to the situation in channel catfish, this again indicates clear differences in basal expression between membrane and soluble forms of *tlr5*. The rainbow trout extracellular LRR region of sTLR5 or mTLR5 was combined with

the TIR domain of human TLR5 and expressed in mammalian cell lines. This study suggested that both sTLR5 and mTLR5 could sense flagellin, as *V. anguillarum*-derived flagellin stimulated NF-κB activation for both membrane and soluble forms of TLR5 [94]. Using a rainbow trout hepatoma cell line (RTH-149) the same research group showed that stimulation of mTLR5 with *V. anguillarum* or its flagellin, up-regulated the gene expression of *stlr5*. Flagellin-mediated NF-κB activation was more significant in the presence or simultaneous expression of *stlr5*. These observations led the authors to propose a two-step mechanism: (a) flagellin first induces basal activation of NF-κB through mTLR5, facilitating the production of sTLR5, and (b) the inducible sTLR5 amplifies mTLR5-mediated cellular responses in a positive feedback fashion. Further, up-regulation of *stlr5* in RTH-149 cells in response to *V. anguillarum* or its purified flagellin amplified the NF-κB response of *human* TLR5 to flagellin [95]. Physical binding of flagellin to sTLR5 was detected under the conditions where NF-κB activation by human TLR5 was further amplified by sTLR5. Signal amplification by sTLR5 was specific to human TLR5: no other human TLRs tested responded to sTLR5. These results suggested that sTLR5 serves as an adjuvant amplifying flagellin-TLR5-mediated NF-κB activation, even in human cells [96]. Most recently, in salmon, flagellin was shown to have the ability to stimulate both *stlr5* and *mtlr5*. Recombinant flagellin (FlaD from *V. anguillarum*) was produced in a full-length (FDL) and a truncated form (FDS). In cell cultures using COS-7 cells, the full-length FDL stimulated the NF-κB pathway more effectively than truncated FDS. *In vivo*, both FDL and FDS induced gene expression of *tlr5*, with *stlr5* being more highly up-regulated than *mtlr5*, as well as pro-inflammatory cytokines such as *tnfα*, *il-6*, *il-8*, and *il-1β*. Both forms of flagellin were used as an adjuvant together with hemocyanin antigen (*Limulus polyphemus*) but neither form of flagellin helped induce antibody production [97]. In conclusion, flagellin clearly has stimulating capacities in several fish species but its potential to act as an adjuvant requires more investigation.

Also flounder has a soluble and a membrane form of TLR5 with high but not always similar basal gene expression levels in head kidney and gills (*mtlr5*>*stlr5*) but also liver (*mtlr5*>*stlr5*) and brain (*stlr5*>*mtlr5*). Stimulation with flagellin induced an up-regulation of gene expression of *stlr5* but a down-regulation of *mtlr5* in PBL and liver. Infection with *E. tarda* showed a similar strong up-regulation of *mtlr5* gene expression in liver but down-regulation of *stlr5* in intestine. These data were supported by *in situ* hybridization studies that showed mTLR5>sTLR5 transcripts in the lamina propria of the intestine but sTLR5>mTLR5 transcripts in the liver after *E. tarda* infection [98]. Building on the ligand studies, fragments of the flagellin protein from *E. tarda* (FliC) were expressed *in vivo* upon intramuscular injection of DNA plasmids in combination with *E. tarda* antigen, showing the conserved N-terminal 163 residues of FliC were the best adjuvant, with respect to improvement of the relative percent survival of vaccinated fish, comparable to the adjuvant effect of full-length FliC [99]. Most recently, over-expression of the promotor of *stlr5* in a fish cell line (hirame natural embryo or HINAE cells), containing sequence elements for two *ap-1* binding sites, two C/EBP sites and one *nf-κb* site showed induced luciferase reporter activity upon stimulation with flagellin, but also showed luciferase activity after stimulation with LPS. Deletion clones and a site-directed mutant of *nf-κb* were generated. Co-transfection of the *p65* sub-unit of with the wild-type *stlr5* promoter increased luciferase activity by more than nine-fold compared with the *nf-κb* mutant. Further, wild-type *stlr5* promoter activity was increased synergistically by more than 159.5-fold in the presence of both, flagellin and *p65*. Furthermore, *stlr5* gene expression could be up-regulated by *p65* and flagellin. Translocation of sTLR5 in the HINAE-sTLR5 stable cell line after flagellin stimulation was observed by confocal microscopy. Altogether, these results suggest that NF-κB and flagellin are essential components for a maximal induction of the *stlr*5 promoter [100].

Interestingly, comparative genomic and phylogenetic analyses not only reveal a proximal location in the fish genomes but a pattern of co-evolution for *mtlr5* and *stlr5* genes across fish species [101]. Co-evolution of mTLR5 and sTLR5 would support evidence of a functional interaction between soluble and membrane forms of TLR5 in fish.

Fish TLR9 may sense DNA from bacteria and from viruses

Human TLR9 recognizes foreign DNA molecules from bacteria and/or viruses that typically contain short sequences of unmethylated CpG dinucleotides in higher frequency than eukaryotic DNA, where CpG motifs are mostly methylated. The extracellular domain of human TLR9 is composed of 25 LRR contributing to the binding to CpG motifs, with LRR11 having the highest affinity for CpG motifs. Single- or multiple-site mutants at five positively charged residues, but in particular Arg-337 and Lys-367, were shown to contribute to TLR9 binding of CpG oligodeoxynucleotides (ODN) [102]. Activation of the cell by CpG motifs requires trafficking of TLR9 from the endoplasmic reticulum through the Golgi to the endolysosomes that contain the foreign DNA, which seems to be a safeguard against inappropriate activation by self DNA [103, 104]. Full-length TLR9 is then cleaved by resident proteases [105, 106] and it is the truncated form of TLR9 that binds to the CpG ODN and initiates signalling cascades via the recruitment of adaptor molecules. CpG ODNs can be classified into three different types (A, B, C) based on their structural organization and CpG content [107]. Many studies into CpG recognition by TLR9 use synthetic, nuclease-resistant and therefore more stable phosphorothioate-modified forms of CpG ODN (PS-ODN). Most recently, TLR9 has been claimed to preferentially recognize curved DNA backbones rather than short sequences of unmethylated CpG dinucleotides [108], which could mean the importance of CpG motifs as ligands for TLR9 might need re-assessment in the future.

In fish, immunostimulatory activities for CpG ODNs of the A, B or C class have been reported using cytokine gene expression, cell proliferation and radical production as read-out. For example, initial in vitro studies in grass carp using head kidney macrophages suggested that several CpG-ODNs, including ODN-1670 (AACGTT), could activate macrophages as shown by increased levels of superoxide anion, hydrogen peroxide, acid phosphatase and increased bactericidal activity [109]. Or, CpG-ODNs could induce the expression of pro-inflammatory cytokines in common carp head kidney leukocytes [110]. Common carp full-length *tlr9* is contained in a single large exon with conserved predicted protein domains [111]. Our preliminary data suggest that CpG ODNs of the A, B or C class do not readily stimulate common carp leukocytes, however (unpublished data). Overall, studies on the function of TLR9 in cyprinids have been few. Yet, a study into purifying selection for TLR9 of teleosts, among which zebrafish, identified eleven sites subjected to positive selection of which 10 sites were associated with LRRs [112]. Positive selection of LRR in TLR9 may indicate an functional flexibility of TLR9 and an adaptation of Teleost to different CpG ODNs motifs present in different bacteria.

Rainbow trout *tlr9* gene expression could be strongly induced in muscle by DNA vaccination with a plasmid bearing the gene of the VHSV glycoprotein and unmethylated CpG motifs in the plasmid backbone itself [113, 114]. In Atlantic salmon, plasmid DNA and synthetic ODNs containing unmethylated CpG induced production of antiviral cytokines in leukocytes, whereas ODNs with an inverted motif (GpC) or with methylated cytosines had practically no effect. Macrophages seemed to be the cell types directly activated by CpG-ODN [115]. A series of investigations using stimulation with CpG were undertaken in salmon. Salmon *tlr9* gene expression could be up-regulated in head kidney leukocytes after *in vitro* treatment with CpG ODNs or by recombinant (trout) interferon-γ

[116]. *In vivo* treatment with CpG ODNs, however, induced only a minor fold-change in *tlr9* gene expression in Atlantic salmon spleen and head kidney [117]. In fact, the most recent data suggest that Atlantic salmon TLR9 interacts with synthetic ODN via a CpG-independent but pH-dependent mechanism [118]. The authors suggest that TLR9, expressed by primary mononuclear phagocytes, should colocalize with CpG ODNs in endosomes. When overexpressed in salmonid cell lines, TLR9 spontaneously activates interferon-stimulated response element (ISRE)-containing promoters of genes involved in the IFN response; however, transgenic TLR9 fails to translocate to the CpG-containing endosomes. These data may indicate that only specific immune cell types have the ability to relocate the receptor to the appropriate cellular compartments where TLR9 may become activated by its ligand [118]. As yet, additional research is required to unequivocally confirm TLR9 is the receptor sensing CpG ODN in salmonids.

For both gilthead seabream and large yellow croaker two alternative splicing variants of *tlr9* have been described [119, 120], suggesting a mechanism of post-transcriptional processing to produce different forms of structurally related proteins. Injection of large yellow croaker with formalin-inactivated *V. parahaemolyticus* confirmed high expression in spleen and down-regulation in liver for both variants of *tlr9* [120]. Gilthead seabream *tlr9* splice variants were broadly expressed in spleen, head kidney, gills, skin and gut [119]e, but their expression pattern was not investigated upon infection. Human *Tlr9* is also expressed in at least two splice forms, of which one is monoexonic and the other is biexonic, the latter encoding a protein with 57 additional amino acids at the N-terminus [121]. Alternatively spliced forms can have a different function or be expressed in different cell types or tissues. Alternative splicing may also result in differential sub-cellular localization, stability and translational efficiency. In general, alternative splicing in teleost fish genomes is lowest in large genomes with a high number of duplicated genes (17% of which are alternatively spliced), e.g. zebrafish, and highest in those species with compact genomes, e.g. pufferfish (43% of genes are alternatively spliced) [122]. Fish species with smaller genomes may rely more heavily on alternative splicing to generate necessary protein diversity.

Half-smooth tongue sole *tlr9* gene expression could be induced by immunization with inactivated *V. anguillarum*, especially in spleen and in head kidney [123, 124]. Japanese flounder challenged with *E. tarda* showed up-regulation of *tlr9* gene expression in blood, gill, kidney and spleen. Interestingly, this is one of few studies where an antibody against a TLR protein was used to study the immune response. Three days after infection with *E. tarda*, immunostaining with anti-MyD88 polyclonal antibody revealed an increased population of MyD88-positive cells in the kidney and spleen [125]. Furthermore, MyD88 immunostaining in combination with an anti-TLR9 antibody revealed that *tlr9* and *myd88* were expressed in the same kidney cells. Only few *tlr9*-expressing cells were found in gill, kidney and spleen of healthy fish, but many after *E. tarda* challenge, coinciding with lesions that had been colonized by the bacteria [126]. Furthermore, overexpression of TLR9 in HINAE cells showed activation upon stimulation with synthetic CpG ODN [126]. Overall, studies on TLR9 in several fish species seem to point at the importance of studying TLR expression in cell types with the inherent ability to relocate TLR9 to the appropriate sub-cellular compartments where TLR9 may become activated by its ligand.

Fish TLR3 may not be the only receptor that senses viral double-stranded RNA

Human Toll-like receptor 3 (TLR3) is localized in the endosomal membrane or cell surface and signals the presence of extracellular double-stranded (ds)RNA or, for example, the synthetic

analog of dsRNA, poly I:C. Grass carp reovirus (GCRV) is a dsRNA virus that can cause disease in grass carp (*C. idella*) and rare minnow (*G. rarus*), and can infect but does not cause disease in common carp (all are cyprinids). Infection of grass carp [127] and rare minnow [128] up-regulated *tlr3* gene expression. In contrast, infection of common carp with GCRV lead to a down-regulation of *tlr3* gene expression during the first 1-3 days [129]. Rare minnow not only expresses a full-length *tlr3* but also a splice variant [128] and the expression of both *tlr3* variants was significantly increased in liver following GCRV infection, although the full-length variant was expressed highest. Interestingly, *in vivo* knock-down (by siRNA) and overexpression of full-length *tlr3* in rare minnow zygotic embryos indicated that the full-length form is mainly responsible for the induction of an anti-viral state upon poly I:C stimulation, as measured by the induction of *mx* gene expression. These data indicate a role for TLR3 in the recognition of dsRNA molecules and in the following anti-viral response. This is supported by a recent study identifying putative markers for selective breeding of GCRV-resistant grass carp based on eight single nucleotide polymorphisms (SNPs) for *tlr3* [130]. Full-length *tlr3* has now also been identified for rohu (*L. rohita*) [131]; [132] and common carp [79, 129]. Zebrafish *tlr3* transcripts were up-regulated in response to infection with snakehead rhabdovirus (SHRV), a ssRNA virus [133]. The authors used a mutant *tlr3* expression construct that lacked the LRRs and most of the extracellular domain, but retained the TIR domain, and could show a dramatic increase in luciferase expression, indicating TLR3 can activate the NF-κB signal transduction pathway in zebrafish.

Whereas TLR3 is assumed to be mainly localized in the endosomal membrane sensing dsRNA in endosomes, a number of studies suggest TLR22 would be a cell surface analog sensing the presence of dsRNA outside the cell. The first studies on this proposed function of TLR22 were performed in pufferfish. Grass carp *tlr22* is expressed in many tissues, most highly in the gills. Infection of grass carp with GCRV rapidly induces up-regulation of *tlr22* gene expression in spleen. Infection of a grass carp kidney cell line with GCRV also induced rapid changes in gene expression of *tlr2* [134]. Recently, a comparison of gene expression profiles was made, including *tlr3* and *tlr22*, following GCRV infection of grass carp [135]. Phylogenetic analysis of, among others, *tlr22* genes from nine different fish species showed evidence of positive selection at three sites within the leucine-rich repeat regions of Tlr22. Tlr22, in particular, is evolving under positive selection[136], suggesting an important role for TLR22 with regard to sensing ligands from pathogens.

Rainbow trout *tlr3* was identified based on information from bacterial artificial chromosome (BAC) and expressed sequence tags (ESTs) [137]. Injection of rainbow trout with poly I:C up-regulated *tlr3* gene expression (1 day) and challenge with infectious hematopoietic necrosis virus (IHNV; ssRNA) also induced gene expression of *tlr3*, with a peak at day 3. In contrast, bath challenge with bacterial *Y. ruckeri* did not lead to an up-regulation of *tlr3* gene expression. *In vitro* stimulation of rainbow trout anterior kidney leukocytes with poly I:C also enhanced *tlr3* gene expression [137]. In Atlantic salmon, poly I:C and imidazoquinoline R848 were used to study induction of different type I interferons (IFNs). In this study, poly I:C was assumed to induce *ifns* via MDA5 and TLR3/TLR22 signaling, whereas R848 was assumed to induce *ifns* via TLR7 signaling. In cell lines, poly I:C strongly induced *ifna*, whereas R848 mainly triggered *ifnb* and *ifnc* up-regulation [138].

As mentioned before, the most extensive studies assigning a functional role to Tlr22 were performed in pufferfish, linking both TLR3 and TLR22 to the (type I) IFN-inducing pathway via TICAM-1, or TRIF adaptor [139]. The authors show TLR3 to reside intracellularly in the endoplasmic reticulum and recognize relatively short-sized dsRNA and TLR22 to be on the surface of the cell and recognize long-sized dsRNA. Fish cells stimulated with poly I:C recruit TICAM-1,

which acts as a shuttling platform for IFN signalling. When pufferfish cells expressing TLR22 are exposed to dsRNA or aquatic dsRNA viruses, an IFN response is induced to acquire resistance to virus infection [139]. Therefore, at least in pufferfish, both endoplasmic TLR3 and cell surface TLR22 participate in type I IFN production. TLR22 is distinct from mammalian TLR3 in terms of sub-cellular localization, ligand selection and tissue distribution [140, 141] and, at least in pufferfish, TLR22 could be a functional substitute of human cell surface TLR3 and serve as surveillant for infection with dsRNA viruses.

Japanese flounder *tlr22* is mainly expressed in peripheral blood leukocytes (PBL) and could be induced by both PGN and poly I:C [71], whereas *tlr3* gene expression in PBL increased upon stimulation with poly I:C and CpG ODN 1668 [142]. Challenge of flounder with VHSV (ssRNA virus) increased *tlr3* gene expression in blood, liver, head kidney and spleen. Overexpression of TLR3 in a kidney cell line showed that stimulation with intracellular poly I:C induced NF-κB activity whereas stimulation with extracellular poly I:C induced expression of *ifn*-inducible genes [142]. Both TLR3 and TLR22 have also been studied in large yellow croaker, but studies have been mostly limited to gene expression analysis. Basal gene expression was high in several immune organs and could be up-regulated after injection with poly I:C in anterior kidney (*tlr22*), spleen (*tlr3*, *tlr22*), liver (*tlr3*) and blood (*tlr3*), thus not always in the same organs. Challenge with *V. parahaemolyticus* induced a moderate up-regulation of *tlr3* gene expression in blood and moderate down-regulation in liver [142], whereas poly I:C induced up-regulation of *tlr2* primary anterior kidney cells [143]. In orange-spotted grouper, *tlr2* basal gene expression was highest in head- and trunk-kidney, spleen, heart and PBL, which is more or less comparable with the findings in large yellow croaker. Challenge with *V. alginolyticus* induced up-regulation of *tlr22* in the spleen [144]. Overall, most if not all fish species seem to express both *tlr3* and *tlr22*, but not always in the same organs. The allocation of TLR3 as a sensor for short-sized dsRNA and TLR22 as a surface sensor of long-sized dsRNA, as proposed for pufferfish, has to be confirmed in more detail for other fish species.

TLR7 and TLR8 could likely sense viral ligands

Although TLR7 and TLR8 are phylogenetically very close, their natural ligand, ssRNA of viral origin, stimulates human TLR7 and TLR8 and mouse TLR7, but not mouse TLR8 [145, 146]. Although mouse TLR8 is functional, it displays differences with respect to ligand specificity to human TLR8 [49]. For example, human TLR8, but not mouse TLR8, recognizes the imiquimod compound R848 [147]. Anti-viral RNA-like resiquimod R848 and S28463 and imiquimod compounds (R837, S26308) are members of a group of low molecular weight compounds, the imidazoquinolinamines, that have proven to induce antiviral activity via endogenous cytokine production and are often used to study TLR7 and TLR8.

TLR7 has a remarkable conservation across vertebrates [41, 42] with a relatively low evolutionary rate of the LRR domains, suggesting that ligand specificity could be well conserved. Both *tlr7* and *tlr8* were first identified in pufferfish [66] and zebrafish [51, 57]. Common carp *tlr7* gene expression can be up-regulated in head kidney cells stimulated with imiquimod, after which these cells produce elevated levels of pro-inflammatory and type I *ifn* cytokines mRNA [148]. This could indeed point at a conservation of ligand specificity for TLR7. Grass carp *tlr7* gene expression was rapidly up-regulated in spleen, but down-regulated in hepatopancreas after infection with GCRV, a dsRNA virus. Furthermore, *tlr7* gene expression in *C. idella* kidney (CIK) cells was up-regulated following stimulation with poly I:C as TLR3 ligand [149]. Grass carp *tlr8*

gene expression in spleen and head kidney was up-regulated at 24 h post-infection with GCRV. In contrast to *tlr7*, which gene expression was up-regulated in CIK cells following stimulation with poly I:C [149], *tlr8* transcription was rapidly down-regulated by poly I:C in a dose and time-dependent manner [150]. Short hairpin-based inhibition of *tlr8* gene expression in CIK cells slightly increased *tlr7* basal gene expression. TLR8 knock-down induced a strong resistance against GCRV [150], suggesting TLR8 might play a negative role in the antiviral immune response of grass carp. Overall, it remains a challenge to dissect clearly, the existing differences in response of TLR7 and TLR8 to poly I:C and other ligands. The high conservation of TLR7 could indicate a conservation of ligand specificity for ssRNA.

Single copy genes for *tlr7* and *tlr9* have been found in channel catfish, whereas *tlr8* has two representatives. Channel catfish TLR7 is 58.1% identical to human TLR7 [42], in support of the remarkable conservation across vertebrates. In comparison, TLR8 sequences have a lower percentage of identity to human TLR8 (45% approximately). The two catfish *tlr8* genes are not located on the same chromosome and probably did not result from a recent duplication [151]. Both catfish *tlr8* sequences seem to be closer to the *tlr8a*, rather than *tlr8b* of zebrafish [42]. Ligand specificity of TLR7 and TLR8 of catfish has not been resolved yet.

Two *tlr7/8* loci were identified from a rainbow trout BAC library using DNA fingerprinting and genetic linkage analyses [152]. Trout *tlr7* and *tlr8* were found in duplicate copies, but one of the TLR7 genes is present as putative pseudogene. Stimulation with R848 and poly I:C produced elevated levels of pro-inflammatory and type I IFN cytokines mRNA in rainbow trout anterior kidney leukocytes. Gene expression of the *tlr7* and *tlr8a1* genes themselves were not affected by these treatments, but *tlr8a2* expression was moderately down-regulated by R848. Inhibition of acidification of the endosome did not clearly modulate R848-induced cytokine expression, however, so it remains questionable whether recognition of R848 in rainbow trout requires endosomal maturation [152].

Early studies in Atlantic salmon described clear effects of typical ligands for endosomal TLRs, including poly I:C (*tlr3*), imiquimod R837 (*tlr7*) and CpG-ODN (*tlr9*) on gene expression of, among others, *ifn* and *mx* genes in liver and head kidney. One major difference between gene induction by S27609 (an analog of imiquimod R837) and poly I:C was that S27609 induced much lower levels of type I *ifn*, possibly because the two ligands were not always sensed by the same cells [153]. *tlr8* gene expression of Atlantic salmon was tissue-restricted with a high level of gene expression in the spleen. Although *tlr8* gene expression could be up-regulated in TO cells treated with recombinant type I and type II IFN, TLR8, but not MyD88, gene expression in spleen of infected fish was not affected by challenge with salmon alphavirus subtype 3 (ssRNA). In vitro stimulation of salmon head kidney leukocytes with CpG ODNs and type II *ifn* gamma also up-regulated *myd88* gene expression, but not gene expression of *tlr8* [154]. Recently, as already discussed above in the context of TLR3, R848 was shown to induce a typical type I IFN response in Atlantic salmon [138]. Further, fluorescence *in situ* hybridization showed that poly I:C induced IFNa and IFNc in a variety of cells in several organs, whereas R848 induced coexpression of IFNb and IFNc in distinct cells in head kidney and spleen. The latter could be specialized high IFN producers. Most recently, studies have identified two *tlr7* genes in Atlantic salmon (of which one is possibly a pseudogene) and three *tlr8* (*tlr8a1*, *tlr8b1* and *tlr8b2*) genes. Promoter analysis predicted the presence of several transcription factor binding sites and cytokine regulation of these TLRs. Indeed, *tlr7* and *tlr8a1* gene expression was influenced by treatment with type I IFN and IFNγ, whereas *tlr8a1* and *tlr8b1* were most sensitive to treatment with IL-1β [155]. Not all *tlr7* and *tlr8* genes reacted the same to cytokine treatment and

future studies may want to address putative differences between the duplicated genes. Overall, it is clear that compounds such as RNA-like resiquimod R848 induce type I *ifn* responses in salmonids, but it remains difficult to unequivocally ascribe this response to sensing by TLR7 and/or TLR8. Certainly, studies on TLR7 and TLR8 in fish species other than cyprinids or salmonids are urgently needed to create a more complete overview.

Large-scale transcriptome studies can provide unbiased views

The first teleost homologue of the TLR family was found in goldfish [156]; this homologue is now believed to be *tlr22* [71]. In this study, an initial EST sequence detected in a subtractive library of macrophage activation factor- and LPS-stimulated macrophages, was completed by full-length cDNA sequencing. Indeed, gene expression for this TLR could be induced in macrophages following treatments with LPS, heat-killed *A. salmonicida*, and live *Mycobacterium chelonei* [156]. This may be the only study in fish where function was the leading factor for characterization of a TLR sequence, rather than first identifying a TLR sequence and subsequently studying gene expression and function of the molecule. In general, most studies of TLRs are based on the attractive but simplified assumption that ligand-binding properties would be conserved. For example, in many studies changes in TLR gene expression are examined following injection of whole fish with a prototypical ligand such as LPS or poly I:C. Or, changes in TLR gene expression are examined following infections with (economically important) pathogens that do not necessarily express the PAMPs most relevant to the TLR studied.

Given the evolutionary distance between fish and mammals it would be good to approach fish TLR function in a more unbiased manner. For example, it would be more objective to always study, for any given fish TLR, the complete range of ligands used for mammalian TLR studies or, study ligands from fish pathogens. This may be even more required in studies on non-mammalian TLRs for which no prototypical ligand can be indicated. Also, larger scale transcriptome studies maybe can provide us with unbiased views on ligands able to modulate gene expression of particular fish TLRs. Zebrafish have become a widely used model for in vivo studies of host-pathogen interactions, and novel high-throughput deep sequencing technologies are changing dramatically our approaches to study functional complexity of transcriptomes. For example, the Solexa/Illumina's digital gene expression (DGE) system, a tag-based transcriptome sequencing method, was used to investigate mycobacterium-induced transcriptome changes in zebrafish. Comparison of data with a previous multi-platform microarray analysis showed that both types of technologies identified regulation of similar functional groups of genes, but with an unbiased nature of DGE analysis that provided insights that microarray analysis could not have achieved [157]. It is exactly this unbiased approach that could make novel high-throughput deep sequencing technologies highly valuable.

There are several larger-scale transcriptomic studies that have taken unbiased approaches to infections of fish. For example, embryonic zebrafish were used for a deep sequencing analysis of the host response to *S. typhimurium* [158]. Using both tag-based (Tag-Seq) and whole transcript (RNA-Seq) sequencing approaches they extended and validated previous microarray data of this infection model. Combining sequencing-based and microarray-based transcriptome data resulted in an annotated reference set of Salmonella-responsive genes in zebrafish embryos, not only including genes homologous to immune-related genes found in human but also many known or novel genes not previously linked to the immune response. Furthermore, comparison of deep sequencing data of Salmonella infection in zebrafish embryos with previous deep sequencing data of Mycobacterium infection in adult zebrafish [157], defined a set of innate host defense genes

common to both infections. We have summarized the outcomes of a number of similar larger-scale transcriptomic studies where clear changes in TLR gene expression were detected after bacterial or viral infections (**Figure 4**).

Overall, the expression of a large number of TLR genes (*tlr1*, *tlr2*, *tlr3*, *tlr4*, *tlr5*, *tlr8*, *tlr9*, *tlr21*), but not *tlr7* or *tlr22*, have been reported up-regulated after infection with either bacterium, or virus, in at least one study. In addition, the expression of several TLR genes (*tlr1*, *tlr2*, *tlr3*, *tlr8* and non-mammalian *tlr18* and *tlr22*), but not *tlr4*, *tlr5*, *tlr7*, *tlr9* nor non-mammalian *tlr21* have been reported down-regulated after infection with either bacterium, or virus. This suggests that regulation of TLR gene expression per se, can indeed be detected after *in vivo* challenge and possibly be taken as (in)direct evidence of the involvement of a particular TLR molecule in the reaction to the pathogen. With the exception of *tlr3*, up-regulation of gene expression after bacterial infection seems particularly evident for *tlr1*, *tlr2*, *tlr4* and *tlr5*, presumed important for the recognition of bacterial ligands. In contrast, with the exception of *tlr1* and *tlr2*, down-regulation of gene expression after bacterial infection is particularly evident for *tlr3*, *tlr8*, *tlr18* and *tlr22*, presumed less important for the recognition of bacterial ligands. Similarly, with the exception of *tlr2* and *tlr5*, up-regulation of gene expression after viral infection is particularly evident for *tlr8*, *tlr9* and *tlr22*, presumed important for the recognition of viral ligands. Although the brief summary in **figure 4** indicates it remains important to verify the effects of both bacterial and viral pathogens, it also suggests that (up)regulation of TLR gene expression may provide first indications for the involvement of a particular TLR molecule in the reaction to a group of pathogens.

Fish-specific TLRs and accessory proteins: two future subjects of research

Several interesting differences exist between the TLR repertoires of teleost fish with respect to the mammalian TLR repertoire. The most obvious are TLRs not present in mammals that often are, but not always, specific for fish. Examples already discussed above include soluble forms of known TLR molecules such as sTLR4 and sTLR5 and 'fish-specific' TLR22. Initially, the existence of six 'non-mammalian' TLR types were reported [46], but recently two new TLR types were discovered in channel catfish [42]. Non-mammalian TLRs generally are grouped within the TLR11 family. Of these, TLR20 has the lowest conservation rate of all TLRs among fish [42] and its ligand remains uncharacterized, although zebrafish *tlr20* gene expression was induced after infection with *M. marinum* [57] and catfish *tlr20* gene expression was induced after infection with *E. ictaluri* [93]. Fish express *tlr21* but also express *tlr9* whereas, intriguingly, the chicken genome lacks a TLR9 homolog and has instead the TLR21 as the intracellular nucleotide receptor that senses synthetic CpG DNA and bacterial genomic DNA [159]. Chicken TLR21 shares many functional characteristics but displays minimal sequence similarity with mammalian TLR9. Grass carp *tlr21* gene expression is down-regulated after infection with aquareovirus (dsRNA virus), but up-regulated after infection with Gram-negative *A. hydrophila* [160]. In orange spotted grouper, both *tlr9* and *tlr21* gene expression was induced at local infection sites (skin and gill), but suppressed in systemic immune organs (spleen and head kidney) after infection with the parasite *Cryptocaryon irritans* [161]. Also in channel catfish, the combination of *tlr9* and *tlr21* gene expression was studied after infection with a parasite, but this time induced gene expression did not coincide; whereas *tlr9* was induced in the skin and gills by *Ichthyophthirius multifiliis*, *tlr21* gene expression was induced in head kidney and spleen [162]. Clearly, fish-specific TLRs will require further studies with respect to their function and, since no obvious mammalian references exist, determination of ligand binding and signaling

			Up	Down	No change
Bacteria	*A.hydrophila*	*L.crocea*	TLR1, 3[1]	TLR2, 22[1]	
		S.aurata	TLR2[2]		
	A.salmonicida	*S.salar*	TLR5[3]		TLR1, 3, 5, 20[4]
	E.tarda	*D.rerio*	TLR2, 4, 5[5]		
	F.psychrophilum	*O.mykiss*			TLR2, 3, 5, 9, 22[6]
	S.typhimurium	*D.rerio*	TLR5[7]	TLR8, 18[8]	
	V.harveyi	*L.japonicus*		TLR1, 3, 18[8]	
	M.marinum	*D.rerio*	TLR5, 21[9]		

			Up	Down	No change
Virus	HRV (dsRNA)	*P.olivaceus*	TLR2[10]		
	IPNV (dsRNA)	*S.salar*	TLR8, 9, 22[12]		
	SGIV (dsDNA)	*E.coioides*	TLR2, 5[11]		

Figure 4. Detection of regulation of TLR gene expression in transcriptome studies following bacterial or viral infection. Summary of data from large-scale transcriptome studies indicating the detection of clear changes in TLR gene expression following bacterial or viral challenge. The colour code indicates red for up-regulated TLR gene expression; green indicates down-regulated TLR gene expression; grey indicates no change in TLR gene expression. Abbreviations: HRV = hirame rhabdovirus; IPNV = infectious pancreatic necrosis virus; SGIV = singapore grouper irido virus.References: [1][166], [2][167], [3][168], [4][169], [5][170], [6][171],[7][90], [8][172] , [9][173], [10][174], [11][175], [12][176] , [13][177], [14][178].

functions of non-mammalian TLRs will remain a challenging task. Most likely, the total number of eight 'non-mammalian' TLRs will even further increase with future genomic research.

A second subject of interest regards molecules accessory to TLR function. In mammals, several accessory proteins have been characterized as required for the biosynthesis and activation of Toll-like receptors [44]. Already mentioned examples of mediators of ligand-delivery and/or ligand-recognition are LBP, MD2, CD14 and CD36. With the exception of CD36, a scavenger receptor of the class B family, these accessory proteins could not be identified in fish genomes analysed thus

far. Thus, CD36 is one of the few members of this particular group of accessory molecules present in fish. However, other groups of accessory proteins have been defined to include TLR chaperones, TLR trafficking factors and TLR-processing enzymes [44]. In contrast to the above-mentioned mediators of ligand-delivery and/or ligand-recognition, studies indicate an almost ubiquitous presence and conservation of these groups of accessory proteins important for TLR function in fish [45]. Combining studies on TLR molecules with studies on accessory molecules present in fish may bring new insights into the function of fish TLRs.

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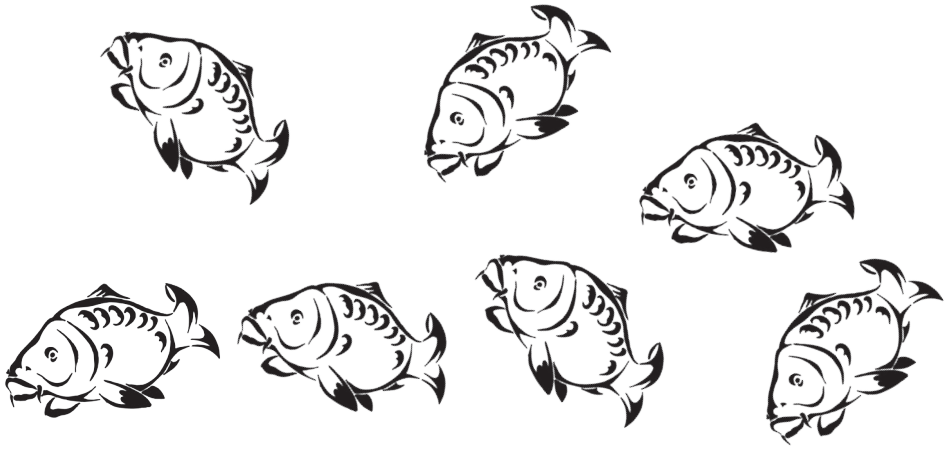

Chapter 7

Accessory molecules for Toll-like receptors in Teleost fish. Identification of TLR4 interactor with leucine rich repeats (TRIL)

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ABSTRACT

The biosynthesis and activation of Toll-like receptors (TLRs) requires accessory proteins. In mammals, a number of accessory proteins have been characterized, that can be classified based on their function as ligand-recognition and delivery cofactors, chaperones and trafficking proteins. We identified the homologs in teleost fish genomes of mammalian accessory molecules and show their expression in transcriptome data sets. Further, we annotate in detail tlr4 interactor with leucine-rich repeats (*tril*) in zebrafish (*Danio rerio*) and in common carp (*Cyprinus carpio*). In mammals, TRIL is a functional component of the TLR4 complex and is important for TLR3 signaling, and is mainly expressed in the brain. In fish, the Tril molecule has many conserved features of mouse and human TRIL, containing 13 leucine-rich repeat domains, a fibronectin and a transmembrane domain. Zebrafish *tril* could not be detected in the latest assembly of the zebrafish genome (Zv9) and required manual annotation based on genome and transcriptome shotgun sequencing data sets. Carp *tril* was found in two copies in the draft genome. Both copies of carp *tril* are constitutively expressed in several organs, with the highest gene expression in muscle, skin and brain. In carp, the *tril* gene is expressed at high levels in endothelial cells and thrombocytes. We discuss the implication of the presence of most, but not all, accessory molecules for the biosynthesis and activation of tlr molecules in fish.

INTRODUCTION

Toll-like receptors (TLRs) constitute an important class of pattern-recognition receptors (PRRs) that recognize a multitude of pathogen-associated molecular patterns, or (PAMPs) [1]. TLRs are type I transmembrane proteins consisting of three domains: an extracellular ectodomain containing tandem arrays of leucine-rich repeats (LRR) that bind to PAMPs and define the specificity of the TLR, a transmembrane region and an intracellular Toll/IL-1 receptor (TIR) domain, involved in downstream signaling cascades [2]. In general and probably true for most animal species, TLR receptors recognize and respond to a wide range of exogenous and endogenous ligands [3], either at the plasma membrane (e.g. human TLR1, TLR2, TLR4, TLR5, TLR6, TLR10) or intracellularly (e.g. human TLR3, TLR7, TLR8, TLR9). The number of TLR genes can vary among organisms. For example, ten functional TLRs are expressed in human, whereas the murine genome shows the presence of three additional TLRs, i.e. TLR11, TLR12 and TLR13, but not TLR10 [4, 5]. Thus far, homologs of TLR6 and TLR10 have not been identified in any of the teleost genomes [6-8] but several TLRs additional to the ones found in mammalian vertebrates have been described [6-15]. In mammals, several accessory proteins have been characterized that are required for the biosynthesis and activation of the different Toll-like receptors or required for proper TLR folding in the endoplasmatic reticulum [16]. To our knowledge, the presence and conservation of TLR accessory proteins have not been studied extensively in fish. In general, accessory molecules can be defined as required for TLR function whereby they facilitate interaction with other TLRs or with TLR ligands. Accessory molecules can be broadly divided into i) mediators of ligand delivery and/or recognition, ii) TLR chaperones, iii) trafficking factors and iv) TLR processing factors [16].

i) Well-known examples of mediators of ligand delivery and/or recognition include LBP, CD14,

MD2 and CD36. LBP (lipopolysaccharide (LPS)-binding protein) is an acute phase protein that mediates innate immune responses to PAMPs from both Gram-positive and Gram-negative bacteria by facilitating their presentation to CD14 [17, 18]. CD14, a GPI-linked protein found on the surface of many TLR4 expressing cells [19], binds directly to LPS [19] and is known to lead LPS molecules to the TLR4-MD2 signaling complex [20-22]. MD2 (Myeloid Differentiation factor-2) and TLR4 bind to LPS and initiate downstream signaling [23]. Neither TLR4−/− nor MD2−/− knockout mice respond to LPS, indicating that both members of the TLR4/MD2 complex are essential for LPS responses [24] [25]. CD36 is a scavenger receptor of the class B family and fine-tunes TLR assembly and responses to ligands, especially some TLR2-TLR6 ligands [26].

Other examples of mediators of ligand delivery and/or recognition include (pro)granulin, HMGB1, LL37 and TRIL. Granulin is produced as a result of the proteolytic processing of its percursor progranulin by serine proteases, binds to oligonucleotides and facilitates the delivery of CpG DNA to TLR9 [27]. HMGB1 (high-mobility group box 1) is a nuclear protein that binds to DNA and displays pro-inflammatory functions once released by the cell. HMGB1 binds to both DNA (through TLR9) and RNA (through TLR7 and TLR8) [28]. LL37 is a 37 amino acid amphipathic peptide that is activated through the cleavage of its precursor, the antimicrobial peptide cathelicidin, by a serine protease. LL37 may serve mostly as a DNA-delivery molecule in situations of cell injury [29]. TRIL (TLR4 interactor with leucine-rich repeats) is a recently described mediator of ligand delivery which is highly expressed in brain and facilitates recognition of LPS and poly(I:C) [30, 31]. Knockdown experiments demonstrated that TRIL mediates TLR4 and TLR3, but not TLR2 and TLR9 signaling [30, 31]

ii) Examples of TLR chaperones include Gp96 and PRAT4. Gp96 (also known as GRP94, HSP90b1) is a member of the heat shock protein 90 family and functions as a chaperone for TLR1, TLR2, TLR4, TLR5, TLR7 and TLR9 [32]. Macrophages deficient for Gp96 show a defective cytokine production in response to signaling via most TLRs [33]. PRAT4 (protein associated with TLR4) associates with TLR4 and TLR9 and is required for the trafficking of these TLRs to the plasma membrane and endolysosome, respectively [34].

iii) Examples of TLR trafficking factors include UNC93B1 and AP3. UNC93B1 (uncoordinated 93 homolog B1) is responsible for the translocation of TLR7 and TLR9 from the ER in unstimulated cells to lysosomes after ligand stimulation [35]. UNC93B1 -/- knockout mice show defects in cytokine production and upregulation of costimulatory molecules in response to ligands of TLR7, TLR9 as well as TLR3 and are more susceptible to viral and bacterial infection [36]. UNC93B1 specifically binds to the transmembrane region of TLR3, TLR7 and TLR9 in the ER [33]. AP3 (adaptor protein 3) is a tetrameric complex involved in protein trafficking from the endosomes to the lysosomes [37] and is a required component of the trafficking machinery of TLR9 [38].

iv) TLR-processing enzymes include cathepsins and AEP. Cathepsins are important for the cleavage of TLR9, an event required for optimal signaling [39]E. This proteolytic process has also been reported for TLR3 and TLR7 and may be a general event for endosomal TLR activation [40]. AEP (asparagine endopeptidase) is a lysosomal protein that cleaves asparagine residues; AEP has been shown to cleave TLR9 and mediate its activation in dendritic cells [41].

In this manuscript we identify the presence in teleost fish genomes of the above-described accessory molecules defined as required for TLR function and analyze their expression in

transcriptome data sets. We characterize in detail, TLR4 interactor with leucine-rich repeats (*tril*) in zebrafish (*Danio rerio*) and common carp (*Cyprinus carpio*). In mammals, only recently, TRIL has been identified as functional component of TLR4 and TLR3 signaling. Fish Tril has many of the conserved features of mammalian TRIL containing a 13 leucine-rich repeat domain, a fibronectin domain and a short transmembrane domain. Common carp *tril* is constitutively expressed in a large number of organs, with highest gene expression in muscle, skin and brain tissue. The screening of a cDNA library made from different cell types of common carp showed that carp *tril* is expressed at high levels in endothelial cells and thrombocytes.

Studies on teleost Tlrs, aimed at the characterization of their biological activity, are frequently hampered by the lack of suitable cell lines that could act as expression systems. One of the reasons that, for example, mammalian cell lines may not always support biological activity of fish Tlrs could be that not all accessory molecules required for function of fish Tlrs would be present. The identification of TLR accessory molecules may help refine studies on the biological activity of Tlrs in fish and will be further discussed.

MATERIALS AND METHODS

Identification of TLR accessory molecules in fish

To investigate the presence of TLR accessory molecules in different fish species we used the protein database (http://www.ncbi.nlm.nih.gov/protein/) or nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore/) from the National Center of Biotechnology Information (NCBI), as a representative source of information from several other sources, including GenBank, RefSeq, Third-Party Annotation (TPA) and Protein Data Bank (PDB). Accession numbers of previously annotated accessory proteins, as retrieved from the nucleotide or protein databases and accession numbers of newly annotated proteins, based on their presence in expressed sequence tag (EST) databases, are provided in **Table 1**. Genome databases used in this study were: zebrafish (*Danio rerio*): genome assembly Zv9 (GCA_000002035.2); Atlantic cod (*Gadus morhua*): genome assembly gadMor1 (GCA_000231765.1); stickleback (*Gasterosteus aculeatus*): genome assembly BROADS1; human (*Homo sapiens*): GRCh37 (GCA_000001405.11); Coelacanth (*Latimeria chalumnae*): genome assembly LatCha1 (GCA_000225785.1); mouse (*Mus musculus*) GRCm38 (GCA_000001635.3); pufferfish (*Takifugu rubripes*) FUGU4; xenopus (*Xenopus tropicalis*) JGI_4.2 (GCA_000004195.1) and common carp (*Cyprinus carpio*): draft genome Bioproject (PRJNA73579; [42]. Genomic information was then used to predict nucleotide and protein sequences using FGENESH version 2.5 (www.softberry.com).

Sequence retrieval and bioinformatic analysis of carp and zebrafish *tril*

Carp *tril* was first identified in the draft genome of common carp based on conservation of synteny between the genomes of human, mouse, pufferfish, xenopus and common carp. The putative coding regions within the genomic DNA were identified using FGENESH and the predicted amino acid sequences were confirmed by using these sequences as template in BLAST [43] and FAST [44] to compare with the most similar hits of previously annotated genes. We identified two contigs within the carp genome (scaffolds 13327 and 23328) with regions coding for TRIL homologs. Initially, zebrafish *tril* could not be detected in the NCBI database or in the most recent assembly of the zebrafish genome Zv9 from ENSEMBL. Therefore, a blast search using the carp *tril* sequences was performed against the EST database and the whole genome shotgun

assembly from Tübingen (http://blast.ncbi.nlm.nih.gov/Blast.cgi). We confirmed the gene structure using zebrafish transcriptome data as previously published [45]. The sequence of the zebrafish *tril* gene based on these results has been submitted to the NCBI database.

Nucleotide sequences were translated using the ExPASy translate tool program (http://us.expasy.org/tolls/dna.html) and aligned with Multiple Sequence Alignment by CLUSTALW (http://www.genome.jp/tools/clustalw/). The predicted amino acid sequences were examined for the presence of a signal peptide using the SignalP program (http://www.cbs.dtu.dk/services/SignalP-2.0/) [46] and the TMHMM2.0 program (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Identification of protein domains was done with SMART (http://smart.embl-heidelberg.de/). A multiple sequence alignment was made with CLUSTALW and a phylogenetic tree was constructed using the Neighbor-Joining method [47] in MEGA5 software [48]. Evolutionary distances were computed using the Poisson correction method [49], all positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were done with 10000 bootstrap replicates. Three-dimensional protein structures were modeled using 3D-JIGSAW (http://bmm.cancerresearchuk.org/~3djigsaw/) the figures were generated using the PyMOL (http://www.pymol.org) (see Supplementary Figures 1a and 1b).

Animals

European common carp (*Cyprinus carpio carpio*) were reared in the central fish facility of Wageningen University at 23±2°C in recirculating UV-treated water and fed pelleted dry food (Sniff, Soest, Germany) daily. R3xR8 heterozygous carp are the offspring of a cross between fish of Hungarian (R8 strain) and of Polish (R3 strain) origin [50]. Carp were between 9 and 11 months old. All studies were performed with approval from the animal experimental committee of Wageningen University.

RNA isolation

For real-time quantitative polymerase chain reaction (RT-qPCR), total RNA was isolated from different carp organs (gills, gut, head kidney, mid kidney, liver, muscle, peripheral blood leukocytes (PBL), skin, spleen, thymus, brain, pituitary, heart, ovary and testis) and different cell types (head kidney-derived macrophages [51], thrombocytes [52], endothelial cells [53], granulocytes and B-cells [54]).

RNA was isolated using Trizol® (Invitrogen, Breda, The Netherlands) according to the manufacturer's protocol and stored a -80˚C until use. RNA concentration was measured by spectophotometry (GeneQuant, Pharmacia Biotech) at OD_{260nm} and the purity determined as the OD_{260nm}/OD_{280nm} ratio with expected values between 1.8 and 2.0. The integrity of RNA was determined by gel electrophoresis on 1% agarose gel containing 0.1% of SYBR® Safe DNA Gel Stain (Invitrogen™).

cDNA synthesis

Prior to cDNA synthesis, a second DNase treatment was performed using DNase I amplification grade (Invitrogen). Briefly, 1 µg of total RNA from each sample was combined with 1 µl 10x DNase reaction buffer and 1 U DNase I up to a final volume of 10 µl, mixed and incubated at room temperature for 15 minutes, followed by inactivation of DNAase I by addition of 1 µl of 25 mM EDTA. Synthesis of cDNA was performed with Invitrogen's SuperScript™ III First Strand

Synthesis Systems for RT-PCR, according to the manufacturer's instructions. Briefly, DNase I treated RNA samples (11 µl) were mixed with 5 times first strand buffer, 300 ng random primers, 10 mM dNTPs, 0.1 M DTT, 10 U RNase inhibitor and 200 U SuperScript™ III Reverse Transcriptase (Invitrogen) up to a final volume of 20 µl. The mixture was incubated at 50˚C for 60 min followed by an inactivation step of 70˚C for 15 min. A non-reverse transcriptase control was included for each sample. Before use as template in RT-qPCR experiments, the cDNA was further diluted 25 times in nuclease-free water.

Real-time quantitative polymerase chain reaction (RT-qPCR)

To investigate gene expression of *tril*, RT-qPCR using ABsolute QPCR SYBR Green Mix (no Rox) (Thermoscientific) was performed with a Rotor-Gene™ 6000 (Corbett Reasearch) as previously described [55]. Primers used for RT-qPCR were designed to amplify the S11 protein of the 40S subunit as a reference gene (forward 5'-3' CCGTGGGTGACATCGTTACA, reverse 5'-3' TCAGGACATTGAACCTCACTGTCT), carp *trila* (forward 5'-3' GACAACGAGGCTCTCAAT, reverse 5'-3' GTCACTGAAGTCCAGGTTC) and carp *trilb* (forward 5'-3' AAAGAAGGAGAGGAAACTGG, reverse 5'-3' TTAAACTGCTCCTGTGGG), all designed with Primer Express software. To 5 µl of 10 times-diluted cDNA, 7 µl Master SYBR Green mix, forward and reverse primer (300 nM each) and MilliQ water up to 14 µl was added. Following cycling conditions were used: one holding step of 15 min at 95˚C; followed by 40 cycles of 15 sec at 95˚C for denaturation, 20 sec at 60˚C for annealing and 20 sec at 72˚C for elongation, followed by a final holding step of 1 min at 60˚C. A melting curve was then created with continuous fluorescence acquisition starting at 60˚C with a rate of 0.5˚C/5 sec up to 90˚C to determine the amplification specificity. In all cases, amplification was specific and no amplification was observed in negative control samples (non-template control and non-reverse transcriptase control). Fluorescence data from RT-qPCR experiments were analyzed using Rotor-Gene software version 1.7 (built 87) and exported to Microsoft Excel. Data were further analyzed using the Pfaffl method [55, 56], using average efficiencies per run, per gene. Gene expression of the house keeping gene was highly constant and used to normalize the data. Each product was checked once by sequencing, both strands of at least six samples were sequenced using the ABI Prism-Bigdye Terminator Cycle Sequencing Ready Reaction kit, and analyzed using an ABI 3730 sequencer. Nucleotide sequence data were analyzed for identity to other sequences using the GenBank database [57].

Statistical analysis

Gene expression data were statistically analyzed by using two-way ANOVA and Bonferroni's multiple comparisons to determine significant differences between the different treatments and their respective control groups. GraphPad Prism v5 software was used for creating the graphs and statistical analysis.

RESULTS

Accessory molecules of teleost fish

We investigated the presence of homologues of mammalian TLR accessory molecules in different teleost fish species, including fish of different orders among which Cypriniformes (zebrafish and common carp), Salmoniformes (Atlantic salmon), Gadiformes (Atlantic cod), Gasterosteiformes (stickleback) and Tetraodontiformes (pufferfish). We first investigated the

presence of already annotated and published accessory molecules in the NCBI database. In case of absence of an annotated sequence, molecules were identified based on their presence in EST or genomic databases. The presence or absence of accessory molecules in teleost fish, based on previous and new annotations, is summarized in **Table 1**. As also previously reported [58, 59], MD2 and CD14 could not be detected in databases of any fish species. Also, prototypical LBP could not be found in fish, which instead express an ancestral bactericidal/permeability-increasing protein (BPI)/LBP gene, at least in rainbow trout [60], common carp [61], Atlantic cod [62], catfish [63], gilthead seabream [64], Atlantic salmon [65], Japanese flounder [66] and rock bream [67]. All other accessory molecules were present in the NCBI, EST or genomic databases, except for LL37 which we could not be retrieved from zebrafish, carp or pufferfish databases. In contrast to all other fish species, including the closely-related carp, zebrafish *tril* could not be detected in the assembly of the zebrafish genome (Zv9). Amino acids alignments of the accessory molecules, present in databases or predicted from genomes, are shown in **Supplementary Figure 2**. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2013.07.012.

Identification of carp *tlr4 interactor with leucine-rich repeats (tril)*

Using a predicted sequence for *tril* from pufferfish (XP_003969101) we identified two putative *tril* molecules (*trila, trilb*) in the draft genome of common carp. We obtained two full-length sequences from scaffold 13327 and scaffold 23328, with open reading frames of 2277 bp for both carp *trila* and *trilb*, encoding for proteins of 758 aa with a predicted molecular weight of 82.25 kDa or 82.23 kDa for *trila* and *trilb*, respectively. Carp Trila and carp Trilb share 91% similarity between each other. Protein alignment of the carp sequences with TRIL from pufferfish, human and mouse (**Figure 1a**) revealed that TRIL proteins are highly conserved between species: they all contain a signal peptide of 24 aa (except for pufferfish which has a signal peptide of 29 aa), 13 leucine-rich repeats , a fibronectin domain and a short transmembrane domain. The percentage of sequence similarity between TRIL proteins of carp, pufferfish, mouse and human is approximately 50% indicating a remarkable degree of conservation (**Figure 1b**).

Genomic organization of TRIL

Using the carp *tril* sequences and carp genome information from scaffold 13327 and scaffold 23328, we predicted the genome organization of carp *tril*. In addition, we investigated the genome organization of TRIL from pufferfish (scaffold 346), human (chromosome 7), mouse (chromosome 6), and xenopus (scaffold GL172692) (**Figure 2**). In all species, TRIL is encoded by a single exon, except for xenopus where the *tril* gene is divided over 3 exons and 2 introns. The intron splicing consensus of the xenopus (GT/AG) was conserved at the 5' and 3' ends of the introns.

Conservation of synteny

Using synteny analysis we detected the genes flanking the *tril* gene in different species (human, mouse, xenopus, pufferfish, zebrafish and carp). We observed a relatively high degree of synteny, not only between human and mouse but also with the fish species (**Figure 3**). Despite the relatively short scaffolds of the carp genome we were still able to identify at least one flanking gene, *creb5*, directly flanking the *tril* gene in all species. In carp, similar to *tril,* the *creb5* gene was also found in duplicate, suggesting a recent duplication of the entire gene locus. The zebrafish assembly Zv9 suggested the absence of *tril* from the zebrafish genome, which seemed unlikely given the high

degree of conservation of this gene.

Table 1 Accessory molecules present in teleost fish species. Accessory molecules were divided into i) mediators of ligand delivery and/or recognition, ii) TLR chaperones, iii) trafficking factors and iv) TLR processing factors based on a previously published classification [16].

		Cypriniformes		Salmoniformes	Gadiformes	Gasterosteiformes	Tetraodontiformes
		D.rerio	*C. carpio*	*S.salar*	*G. morhua*	*G.aculeatus*	*T.rubripes*
	LBP[a]	ENSDARG00000088486	AU279378	NP_001135199	AAM52336	Nd	XP_003973736
	MD2	Nd	Nd	Nd	Nd	Nd	Nd
	CD14	Nd	Nd	Nd	Nd	Nd	Nd
	CD36	AAH76048	D	CB502085	ENSGMOG00000011706	ENSGACG00000019957	XP_003972763
	TRIL	EV756356; EH605673; EH588231	KF241720; KF241721	Nd	ENSGMOG00000014043	DT987621; DN696904	XP_003969101
	progran.	NP_001001949	D	NP_001133519	ENSGMOG00000000761	DN711800	XP_003969441
	HMGB1[c]	NP_955849	CA964438	ACN12566	FJ007668	BT028552	XP_003979516
i	LL37	Nd	Nd	ADN34602	ACF21013	Nd	Nd
	GP96	AAP47138	EX826012; DY655865	DY737961; GO062123; GE781136	GO393826; ES781184; FF410258	CD508321; DT967242; DW597352	XP_003967565
ii	PRAT4	NP_001034602	D	NP_001167214	ENSGMOG00000005480	ENSGACG00000019316	XP_003966977
	UNC93B[1]d	XP_002660582	D	NP_001167066	GW858817	DW597638; CD499027	XP_003972059
iii	AP3	NP_001038480	EC394060	DY715934; DY729337; CA057704	EG638362	DN717984	XP_003974179
	cathep.[b]	NP_001017778	EX883770	NP_0011338711	AEI61876	EX725539; ES476079	XP_003965855
iv	AEP	NP_999924	DC997151; AU052102	NP_001158867	EX725474	BT026620	XP_003962395

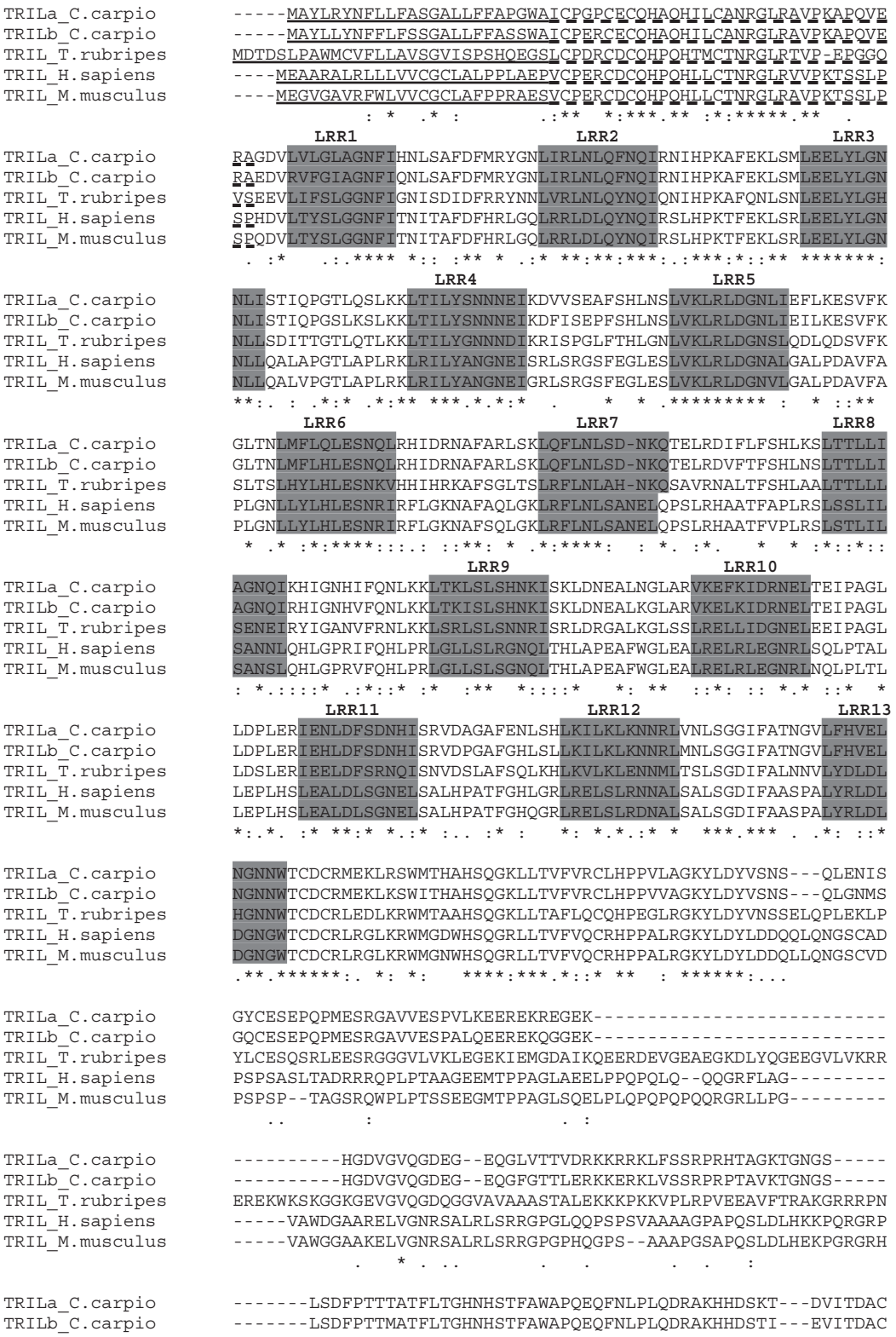
[a]BPI/LBP ancestral gene of LBP; [b]Cathepsins F; [c]Hich mobility group-T protein; dUNC93B1-like protein MFSD11.

Nd = Not detected after blasting against the database and the genomes available ;

D = detected in the carp genome.

Genome databases used were: zebrafish (*Danio rerio*): genome assembly Zv9 (GCA_000002035.2); Atlantic cod (*Gadus morhua*): genome assembly gadMor1 (GCA_000231765.1); stickleback (*Gasterosteus aculeatus*): genome assembly BROADS1; pufferfish (*Takifugu rubripes*) FUGU4; common carp (*Cyprinus carpio*): draft genome Bioproject (PRJNA73579).

a)


TRILa_C.carpio
TRILb_C.carpio
TRIL_T.rubripes
TRIL_H.sapiens
TRIL_M.musculus
LRR1
LRR2
LRR3
LRR4
LRR5
LRR6
LRR7
LRR8
LRR9
LRR10
LRR11
LRR12
LRR13

```
TRIL_T.rubripes      VLPRTDPPPIFPTSHAGERHENATETLSATVLVQTGEKYDLLMLNHEESL---PVITDPC
TRIL_H.sapiens       TRADPALAEPTPTASPGSAPSPAGDPWQRATKHRLGTEHQERAAQSDGGAGLPPLVSDPC
TRIL_M.musculus      TRANLSQTEPTPTSEPASGTPSARDSWQRAAKQRLASEQQESAVQSVSGVGLPPLVSDPC
                               **: .         ..    :    :      :     :          :::*.*
                        <           Fibronectin Domain type III
TRILa_C.carpio       QFNHHSILNVSVEDVTSSTATVRWSTIPDVGLVHG-----KELIFRVLFDRFGHAFRFPR
TRILb_C.carpio       QFNRHSILNVSVEDVTSSTATVRWSTTLDAGLVLG-----KELLFRVLFDRFGHAFRFPR
TRIL_T.rubripes      MFNHYHLTNVSVDHVTSNTVTVYWTTKEHRRYTPAPGPGLDEVHYRIMYDRFGTPERFPR
TRIL_H.sapiens       DFNKFILCNLTVEAVGADSASVRWAVREHRSPRPL-----GGARFRLLFDRFGQQPKFHR
TRIL_M.musculus      DFNKFILCNLTVEAVSANSASVRWAVREHRSPRPQ-----GGARFRLLFDRFGQQPKFQR
                      **:. : *::*: * :.:.:* *:.  .               :*:::****   :* *
                                  Fibronectin Domain type III         >
TRILa_C.carpio       YVYTDGSDRAVTLQELRPDSTYITCVESVVGGALCQVAPRDHCTGFVTLLPSVTS----E
TRILb_C.carpio       YVYTDGSDRAVTLQELRPDSTYITCVESVVGGALCQVAPRDHCTGFVTPLPSVTS----E
TRIL_T.rubripes      YVYTOGTARSVTLRELSSEVTYMICVEGVVGRSVCQVAPRDHCAGLVTLPAGLSPGWTLT
TRIL_H.sapiens       FVYLPESSDSATLRELRGDTPYLVCVEGVLGGRVCPVAPRDHCAGLVTLPEAGSRG---G
TRIL_M.musculus      FVYLPERSDSATLHELRGDTPYLVCVEGVLGGRVCPVAPRDHCAGLVTLPEAGGRG---G
                     :**       :.**:**  : .*: ***.*:*  :* *******:*:**   .
                               Transmembrane
TRILa_C.carpio       VNLQLVTIAALAANALLLLLVGGVWLGRVLKRRIRS-RKSSTHAHVRHMYSTRHPFRSTV
TRILb_C.carpio       VNLQLVTVAALVANALLLLLVGGVWLGRVLKRRIRS-RKSSTHAHVRHMYSTRHPFRSTV
TRIL_T.rubripes      SDLQLLTLATLAANIMLLVVIGGIWLGRRLKRRLQR-RKS--AVHVRHMYSTRRPFRSAL
TRIL_H.sapiens       VDYQLLTLALLTVNALLVLLALAAWASRWLRRKLRARRKGGAPVHVRHMYSTRRPLR--S
TRIL_M.musculus      VDYQLLTLVLLAVNALLVLLALAAWGSRWLRRKLRARRKGGAPVHVRHMYSTRRPLR--S
                      : **:*:. *..* :*:::  . * .* *:*:::  **.   .*********:*:*

TRILa_C.carpio       AATCVSSEFSGYQSGRQLAEE-----GDLIQFPGDRFFDNS------HARRDDDVIMLRY
TRILb_C.carpio       ATTCVSSEFSGYQSGRQLAEE-----GDLIQFPGDHFFDNS------HARRKDDAIMLRY
TRIL_T.rubripes      ATATASTDFASYQSSRAARLVPHA-DGDLIEFPCDRFFDSN------SVRRDSEMQRFVD
TRIL_H.sapiens       MGTGVSADFSGFQSHRPRTTVCALSEADLIEFPCDRFMDSAGGGAGGSLRREDRLLQRFA
TRIL_M.musculus      MGTGVSADFSGFQSHRPRTTVCALSEADLIEFPCDRFMDSTGGGTSGSLRREDHLLQRFA
                       : .*::*:.:** *          .***:** *:*:*.         **..
TRILa_C.carpio       SD-
TRILb_C.carpio       SD-
TRIL_T.rubripes      ---
TRIL_H.sapiens       D--
TRIL_M.musculus      D--
```

b)

	Trila	Trilb
***C.carpio* Trila**	**100**	**91**
***C.carpio* Trilb**	**91**	**100**
T.rubripes	**59**	**59**
H.sapiens	**50**	**50**
M.musculus	**49**	**50**

Figure 1. Sequence comparison of carp TLR4 Interactor with Leucine-rich Repeats (TRIL) with fish and mammalian vertebrate.

a) Amino acid alignment of carp Trila and Trilb with other TRIL molecules. The predicted signal peptide is underlined, the leucine-rich repeat N-terminal domain (LRRNT) is underlined by a dashed line, leucine-rich repeats (LRRs) are indicated by a grey shadow, the fibronectin domain type III (FN3) is boxed and the transmembrane domain is indicated by white letters on a dark background. Asterisks (*) indicate identities, (:) double dots indicate conserved substitutions, (.) single dots indicate semi-conserved substitutions and (-) dashes denote gaps used to maximize the alignments.

b) Percentage sequence identity of TRIL determined by Clustal W alignment. GeneBank accession numbers: *Cyprinus carpio* (Trila KF241720 Trilb KF241721), *Homo sapiens* (NP_055632.2), *Mus musculus* (NP_080093) and *Takifugu rubripes* (XP_003969101).

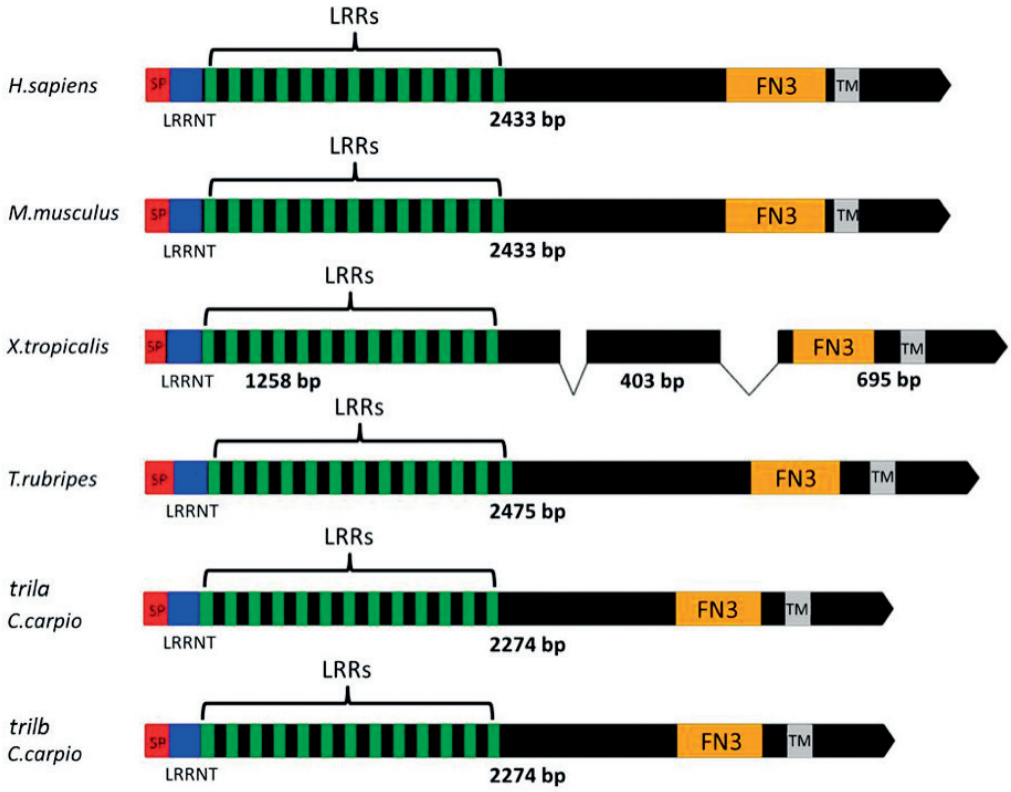


Figure 2. Predicted genomic organization of TRIL genes. Comparison of the intron-exon organization of carp *trila* and *trilb* with TRIL from *Homo sapiens* (NP_055632), *Mus musculus* (NP_080093), *Takifugu rubripes* (XP_003969101) and *Xenopus tropicalis* (ENSXETP00000057398).

Danio rerio tril

To detect zebrafish *tril*, we investigated in detail EST databases and re-analyzed whole genome shotgun sequences. We found three ESTs (EV756356, EH605673 and EH588231) for zebrafish *tril* and from the whole genome shotgun assembly Tübingen (CABZ01002620). Furthermore, we identified a *tril* transcript based on a *de novo* assembly of a recently published transcriptome data set of zebrafish [45]. This allowed us to retrieve the full nucleotide sequence of zebrafish *tril*, predicting an open reading frame of 2277 bp, encoding for a protein of 758 aa with a predicted molecular weight of 99.15 KDa. Zebrafish Tril contains a signal peptide of 24 amino acids, 13 LRRs, a fibronectin domain and transmembrane domain, similar to other Tril molecules (**Figure 4**). The similarity between both carp molecules (Trila and Trilb) and zebrafish Tril is 83% and 84%, respectively. Based on this information the Genome Reference Consortium (GRC, Wellcome Trust Sanger Institute, UK) is currently re-sequencing the area (present in clone DKEY-18N21) were the *tril* gene is supposed to be located (issue identifier 5422) and that is absent in the present zv9 assembly.

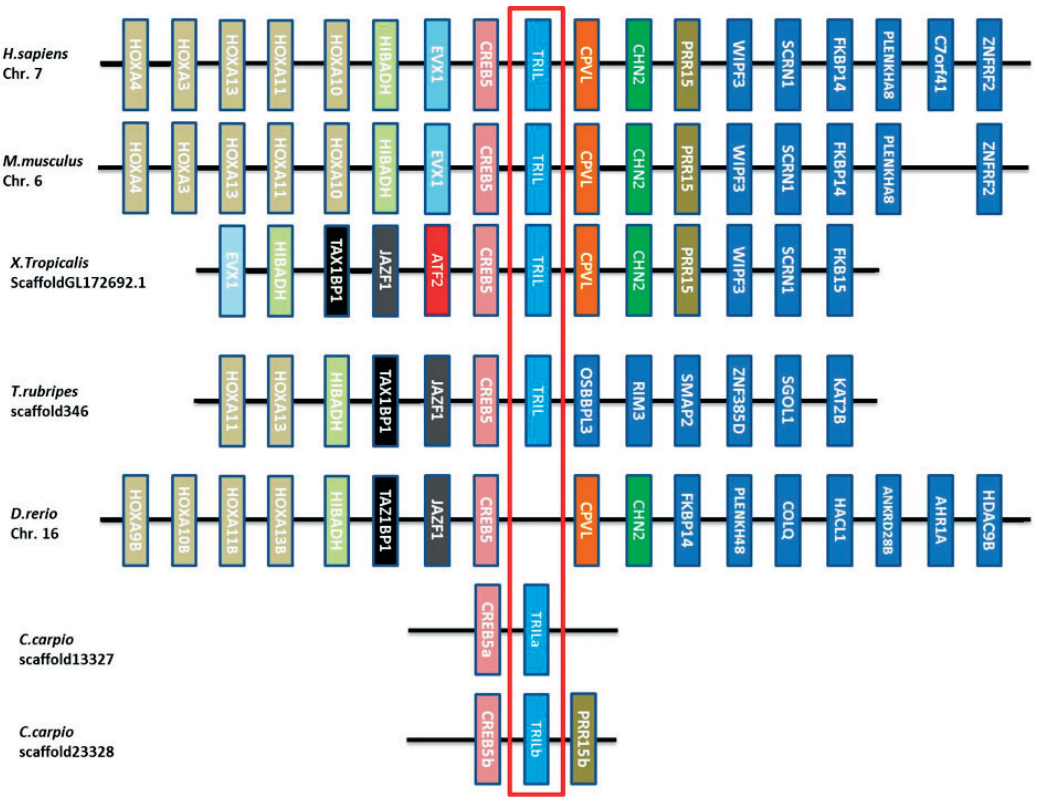


Figure 3. Synteny of the TRIL gene. Comparative gene location map of the regions where TRIL is located in human, mouse, xenopus, pufferfish, zebrafish and carp genomes. For this analysis, the human genome assembly (GRCh37), mouse genome assembly (GRCm38), pufferfish genome assembly version 4 (FUGU 4.0), xenopus genome assembly (JGI_4.2) and the zebrafish genome assembly version 9 (Zv9) were used.

```
1     atggcatatttgctctcaaacttatttttgttcggcagtggattgcttttattcttcgcc 60
        M  A  Y  L  L  S  N  L  F  L  F  G  S  G  L  L  L  F  F  A
61    cctgtttgcgccatttgtccggagcgatgtgattgccagcacgcgcagcatatcctgtgc 120
        P  V  C  A  I  C  P  E  R  C  D  C  Q  H  A  Q  H  I  L  C
121   gcaaaccgcggtctccgcgcggtgcccaaagcgccgcaggtggagcacgcagaggatgtg 180
        A  N  R  G  L  R  A  V  P  K  A  P  Q  V  E  H  A  E  D  V
181   cgggttctcagtctcgcgggaaacttcattcacaacatcagtgctttgacttcatgcgc 240
        R  V  L  S  L  A  G  N  F  I  H  N  I  S  A  F  D  F  M  R
241   tatggcgacctaatgagacttaatctccagtttaatcaaataaggagtatacatcctgaa 300
        Y  G  D  L  M  R  L  N  L  Q  F  N  Q  I  R  S  I  H  P  E
301   tcattcaagaaactatccaaactagaggaactatttctaggaaacaacctaatatcgacg 360
        S  F  K  K  L  S  K  L  E  E  L  F  L  G  N  N  L  I  S  T
361   atacaacctgggactctacaatcgctgaaaaaactcacaatattatatagcaataacaac 420
        I  Q  P  G  T  L  Q  S  L  K  K  L  T  I  L  Y  S  N  N  N
421   gaaatcaacgagtgcatccctaaatcatttacccatttaaacagtttagtgaaactacga 480
        E  I  N  E  C  I  P  K  S  F  T  H  L  N  S  L  V  K  L  R
481   cttgatggaaattcgatcgaagtgttaaaggagtccgtcttcgaaggtttgccaaactta 540
        L  D  G  N  S  I  E  V  L  K  E  S  V  F  E  G  L  P  N  L
541   atgtttctccacttagaatcgaaccaccttcggcgtatcgaccggaatgcattcttgcgg 600
        M  F  L  H  L  E  S  N  H  L  R  R  I  D  R  N  A  F  L  R
601   ctcagcaaactgcagtttttaaacctgtcggacaataaacaaacagagctgcaagatgtt 660
```

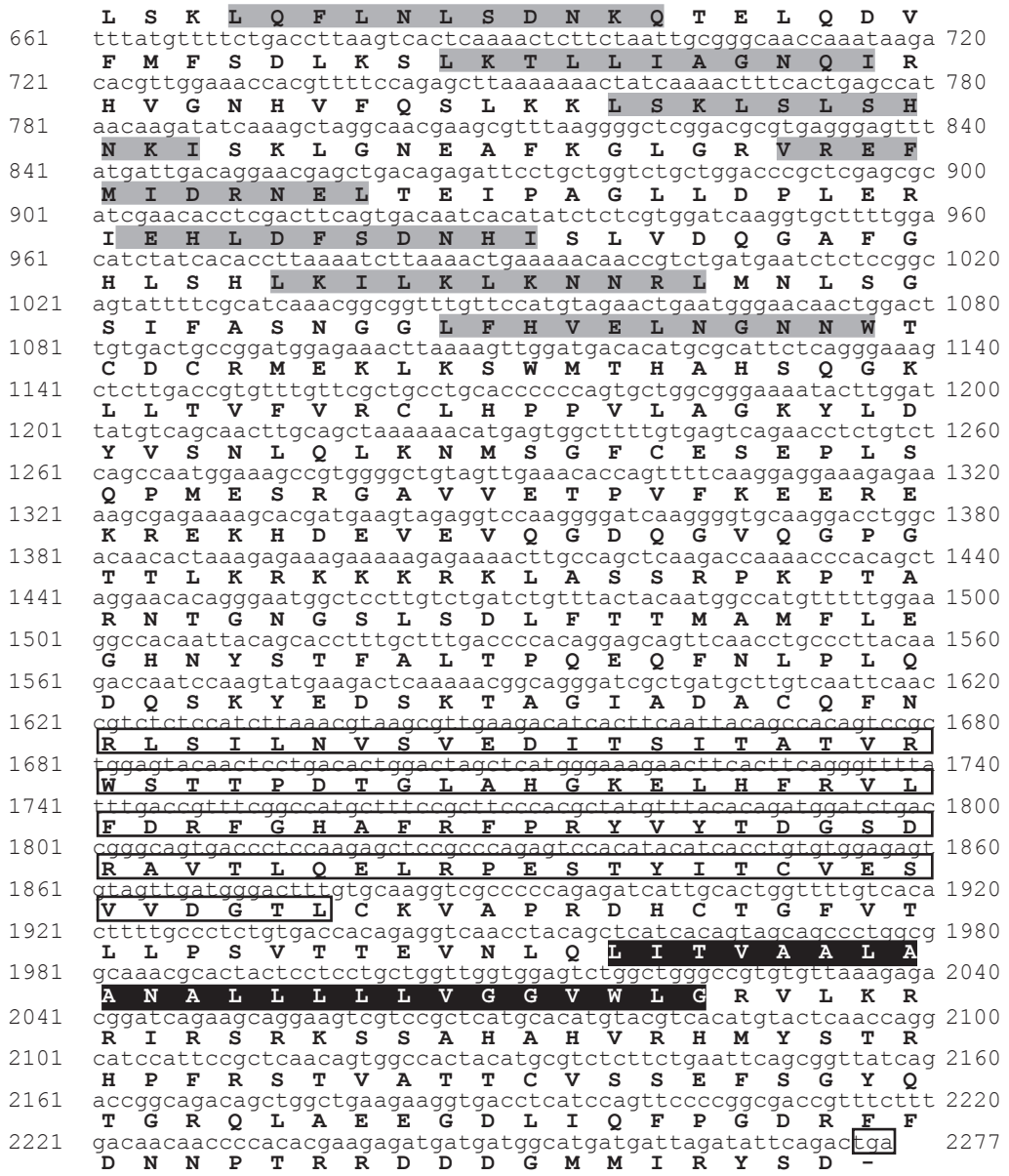


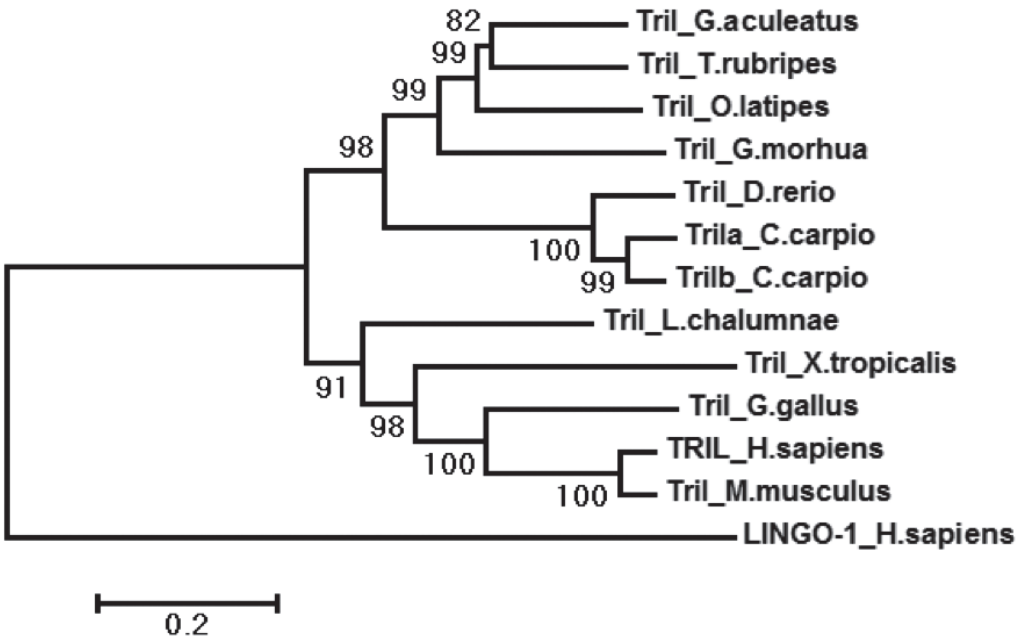
Figure 4. Nucleotide and predicted amino acid sequence for Tril of zebrafish. Small letters indicate nucleotide sequence and capital letters indicate amino acid sequence. Start codon (atg) and stop codon (tga) are boxed. The predicted signal peptide is underlined, the leucine-rich repeat N-terminal region (LRRNT) is underlined by a dashed line, leucine-rich repeats (LRRs) are indicated by a grey shadow, the fibronectin domain type III (FN3) is boxed and the transmembrane domain is indicated by white letters on a dark background.

Phylogeny of TRIL

Phylogenetic analysis shows that the Tril orthologues follow the species tree (**Figure 5**), and that Tril of fish have an ancestor in common with the mammalian vertebrates. Clearly, Tril of stickleback, pufferfish and japanese rice fish (medaka) cluster together, whereas also carp and

zebrafish Tril form a separate branch. Interestingly, Tril of the coelacant (*Latimeria chalumnae*), a lobe-finned fish species related to lungfishes and considered ancestral to land animals [68], is more related to amphibians (xenopus), birds (chicken) and mammals (human and mouse) than to the teleost fish species.

Figure 5. Phylogenetic tree of TRIL. Neighbor-Joining tree with evolutionary distances computed using the



Poisson correction method using the complete deletion option. Phylogenetic analysis was conducted with 10000 bootstrap replicates. Bootstrap values at major branching points are shown as percentages. LINGO-1 from *Homo sapiens* (Q96FE5.2) was used as an outgroup. The sequences were derived from *Cyprinus carpio* (Trila KF241720 and Trilb KF241721), *Danio rerio* (CABZ01002620), *Gadus morhua* (ENSGMOG00000014043), *Gallus gallus* (XP_425996), *Gasterosteus aculeatus* (ENSGACG00000007203), *Homo sapiens* (NP_055632), *Latimeria chalumnae* (predicted from genome assembly LatCha1, ENSLACP00000017776), *Mus musculus* (NP_080093), *Oryzias latipes* (XP_004074067), *Takifugu rubripes* (XP_003969101) and *Xenopus tropicalis* (ENSXETP00000057398).

Constitutive gene expression of *trila* and *trilb* in carp

We observed a relatively high level of basal gene expression of carp *trila* and *trilb* in many organs, with highest gene expression in muscle, skin and brain, and moderate-to-high gene expression in heart, ovary and testis but also in immune organs such as mid kidney and gills (**Figure 6a**). Constitutive expression of the *tril* gene in other immune organs such as head kidney, spleen and thymus was much lower whereas in liver, *trilb* was not expressed at all. In general, *trilb* was more highly expressed than *trila*, with the exception of gills, where expression levels were equal. We observed a relatively high level of *trila* and *trilb* basal gene expression in endothelial cells and thrombocytes (*trilb* only) (**Figure 6b**). Granulocytes did not express *tril*, whereas macrophages and B-cell express *trilb* only and at relatively low levels.

Modulation of *tril* gene expression in carp and zebrafish

We investigated the modulation of *tril* gene expression in carp and zebrafish tissues using different collections of cDNA samples, including transcriptome datasets or cDNA samples from stimulated leukocytes. In zebrafish larvae *tril* is constitutively expressed but not induced by infection with *Mycobacterium marinum*, *Salmonella typhymurium*, or *Staphylococcus epidermidis* as based on reads frompublished transcriptome data[45, 69]. The gene is also not induced in adult zebrafish chronically infected with *M. marinum,* as analyzed by serial analysis of gene expression (SAGE) [70](**Supplementary Table 1**).

For carp, cDNA from skin tissue collected from adult fish infected with the skin parasite *Argulus japonicus* [71] did not provide evidence for a clear modulation of *tril* gene expression (data not shown), despite the high basal gene expression of *tril* in carp skin. We could detect constitutive gene expression of carp *tril* in a cell line from common carp brain (CCB; [72]) and freshly isolated head kidney leukocytes, but we could not observe any modulation of gene expression upon stimulation with LPS or poly(I:C) (data not shown). Transcriptome data collected from head kidneys isolated from carp infected with the parasite *Trypanoplasma borreli* [54] did not show a significant modulation of *tril* gene expression (data not shown). In conclusion, our preliminary data indicate fish *tril* gene expression is not readily modulated.

DISCUSSION

TLRs may be only one group of PRRs that activate the immune system, but comprise a particularly well-studied group with each TLR molecule binding with its own set of prefered ligands [73-75], thereby activating a rapid inflammatory innate response and priming specific immunity [76-78]. To date, TLRs have been described in virtually every class of the animal kingdom and studied in many different fish species including representative species of cyprinid and salmonid families, but also cod, stickleback and pufferfish. However, additional to the TLR molecules *per se* other molecules contribute to the function of TLRs, for example because they can act as signaling adaptors, cofactors or regulators of TLRs [79-81]. The function of some accessory proteins has been described in more detail, for example UNC93B1 [33] and CD14 [82] appear to be important for the functioning of several TLRs. However, the exact role for many accessory molecules is still unknown and many questions remain unanswered with respect to TLR biosynthesis, trafficking, ligand recognition and activation. In fish, the presence and conservation of accessory proteins important for TLR function had not been studied.

Accessory molecules can be divided based on their function into mediators of ligands delivery and/or recognition (LBP, MD2, CD14, CD36, TRIL, Progranulin, HMGB1 and LL37), chaperones (GP96 and PRAT4), trafficking molecules (UNC93B1 and AP3) and TLR processing factors (cathepsin and AEP) [16]. It has been recognized that fish do not express a true *lbp* gene but rather express bpi/lbp, a gene ancestral to the LPS-binding protein gene found in mammals [60-67]. We investigated the presence of MD2 and CD14 homologs in all fish genomes and EST databases but could not identify such sequences, in accordance with previous studies [58, 59] whereas, for example, C36 a scavenger receptor of the class B family, is present in fish genomes (see **Table 1**). Also, the chaperone protein Gp96, although initially described in zebrafish to contribute to the formation of otoliths [83]@ was later identified a master chaperone for TLRs and important for innate function of macrophages [32]. Prat4, also known as canopy 4 precursor, also has been

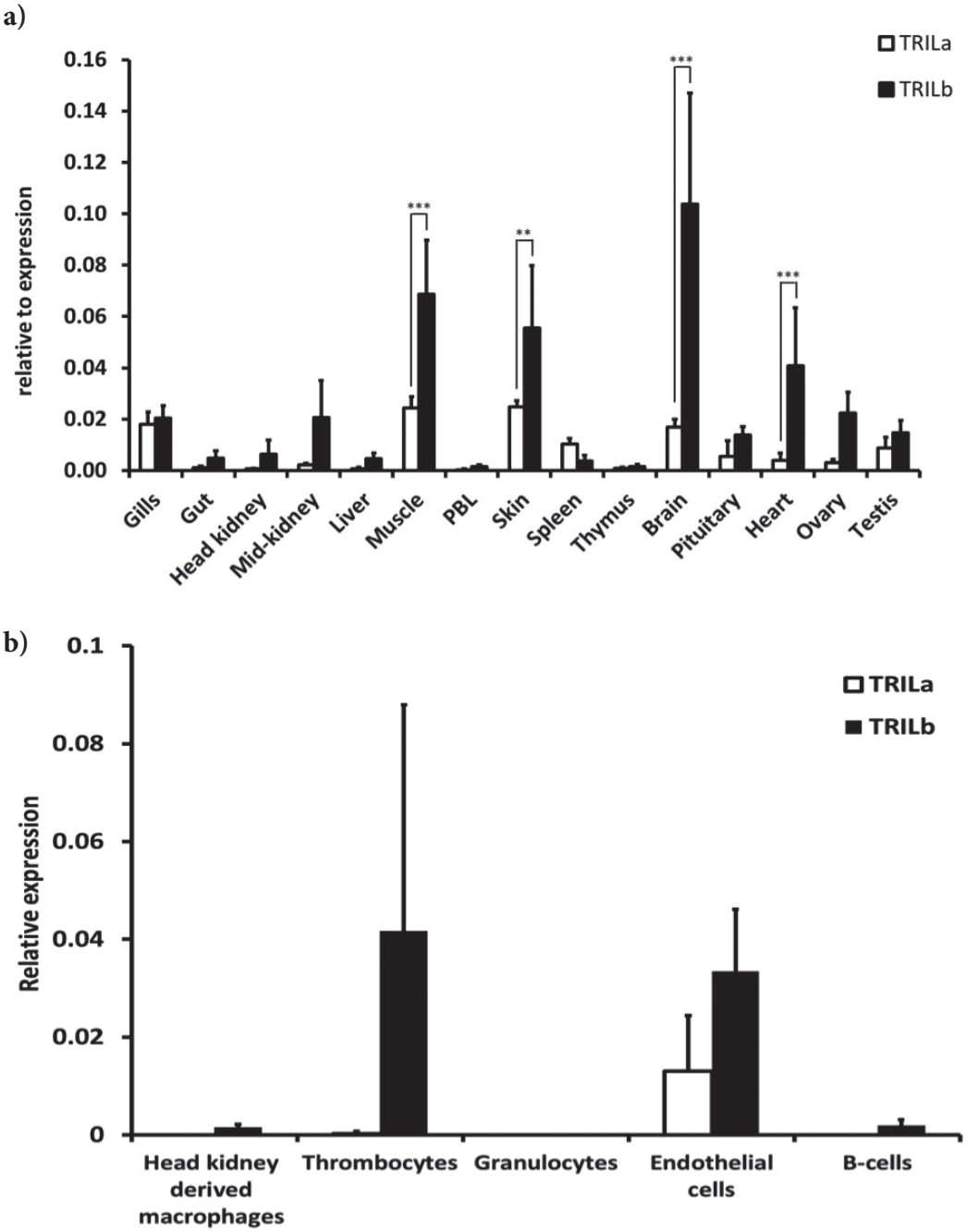


Figure 6. Real-time quantitative PCR analysis of constitutive gene expression of carp *tril*. a) Constitutive gene expression of carp *trila* and *trilb* in different organs. Constitutive mRNA levels are expressed relative to the house keeping gene (S11 protein of the carp 40S subunit). Data are represented as mean ± SD (n=5 healthy carp). Significant differences are indicated by asterisks ** (P<0.01) and *** (P<0.001). Abbreviation: PBL Peripheral Blood Leukocytes. **b) Constitutive gene expression of carp *trila* and *trilb* in different cell types.** Constitutive mRNA levels are expressed relative to the house keeping gene (S11 protein of the carp 40S subunit). Data are represented as mean ± SD (n= 5 healthy carp). No significant differences were detected.

identified in zebrafish, reported to regulate the fibroblast growth factor FGF in the brain [84]. Most accessory molecules, however, had not been previously identified in teleost fish. Our study of fish genomic and/or transcriptomic databases allowed for the identification of most accessory molecules important for the functioning of TLRs in mammals, supporting a broad conservation of function for these regulatory molecules.

We described for the first time in teleost fish in more detail the accessory molecule TLR4 interactor with leucine-rich repeats (Tril) of both carp and zebrafish. *Tril* could be identified in the genomes of most fish species including common carp but surprisingly, at least initially, not in zebrafish. Given the close genetic relationship of zebrafish and common carp [42], we re-examined zebrafish genomic and transcriptomic (EST) data and subsequently were able to also identify *tril* in zebrafish. Apparently, automatic annotation failed because of the high number of repeats in the Tril gene sequence. This shows that Zv9 sometimes requires manual annotation.

The gene sequences of carp and zebrafish *tril*, but also the *tril* sequences from other teleost species, confirm that *tril* is highly conserved. TRIL was previously described in mammals as a protein containing a domain with 13 leucine-rich repeats, a fibronectin domain and a putative transmembrane domain [30]. A similar domain structure was found for fish Tril. The carp genome contains two *tril* genes, located in two different scaffolds, which we named *trila* and *trilb*. The two carp Tril proteins share a high percentage of sequence identity between each other (91%), but also with other vertebrate TRILs (50% identity). Synteny analysis revealed that the genes flanking TRIL are conserved between mammals, amphibians and fish, probably sharing similar functions.

In mammals, TRIL was initially identified as a novel modulator of TLR4 signaling showing high expression in the brain. TRIL expression could be induced upon LPS stimulation *in vitro* in the astrocytoma cell line U373s, murine bone marrow-derived macrophages (BMDM) and human peripheral blood mononuclear cells (PBMC) and expression of the TRIL protein could also induced by LPS *in vivo* in mouse brain [30]. Later, TRIL was shown to also play a role in TLR3 signaling as an important component in endosomal signaling. Interaction of TRIL with TLR3 could be enhanced by stimulation with poly(I:C) [31]W(F. Carp *tril*, in line with finding in mammals, was highly expressed in brain. Further, carp *tril* was highly expressed in thrombocytes and endothelial cells but could not easily be up- nor downregulated, neither following infection with parasites or bacteria nor following stimulation with LPS or poly(I:C) (this study; data not shown).

As mentioned above, in mammals, TRIL expression could be induced upon LPS stimulation and by stimulation with poly(I:C). Zebrafish Tlr4 fails to recognize LPS [85], leaving unknown the receptor most important for recognition and leaving undefined the exact ligand for fish Tlr4. Pufferfish Tlr3 is expressed in the endoplasmatic reticulum and recognizes relatively short-sized dsRNA, whereas Tlr22 recognizes long-sized dsRNA on the cell surface of the cell [86]. This leaves the exact mechanism for recognition of poly(I:C) in fish open for discussion. For these reasons, it may not be surprising that we did not find a clear modulatory role of carp or zebrafish Tril with respect to TLR4, or TLR3 signaling. Differences between mammals and fish in the recognition of important ligands such as LPS and poly(I:C) could point at different regulatory roles for Tril in fish. Our observations support the need for studying the putative roles of accessory molecules, among which Tril, in future studies on the function of TLRs in fish.

Studies on teleost Tlrs, aimed at the characterization of their biological activity, can be hampered by the lack of suitable cell lines that could act as expression systems [87]. One of the explanations that, for example, mammalian cell lines may not always support biological activity of fish Tlrs could be that not all accessory molecules would be present or functional in combination

with fish Tlrs. This could also be true for fish cell lines used as expression systems because, most likely, not all cell types express the whole set of accessory molecules to the same extent. Our study supports a broad conservation of most accessory molecules important for the functioning of TLRs. However, potentially crucial molecules such as CD14 and MD-2 are not present in fish genomes. In addition, the exact roles of most of the accessory molecules that are present in fish genomes have not been examined, and certainly not in combination with TLRs. The identification of TLR accessory molecules may help refine studies on the biological activity of Tlrs in fish.

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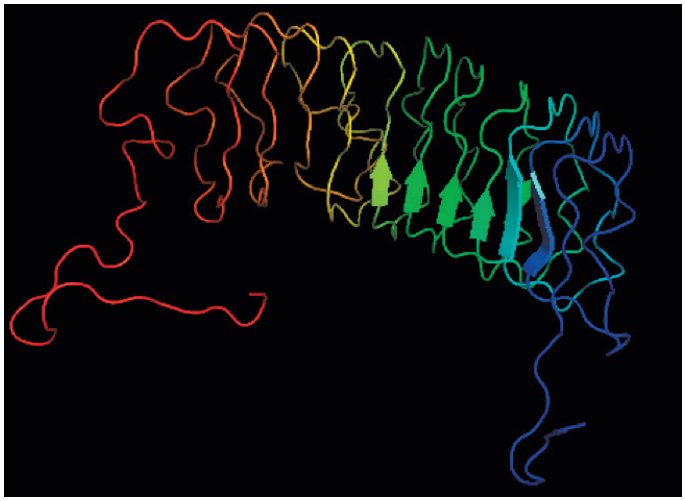
Supplementary Figures

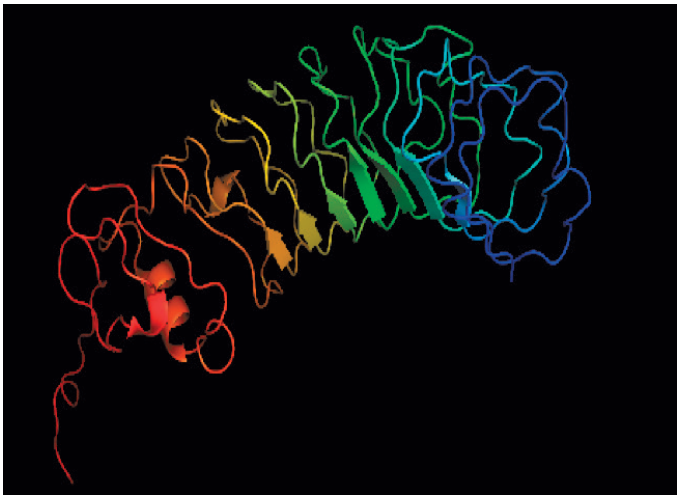
Supplementary Table 1. Gene expression patterns of zebrafish tril after infection of zebrafish with different bacteria.

Infection	Reference	Reads control	Reads infection	Ratio
M.marinum	(Veneman et al., 2013)[45]	83	72	0,86747
S.typhimurium	(Stockhammer et al., 2009)[69]	82	49	0,597561
M. marinum	(Hegedűs et al., 2009)¤[70]	5	9	1,8

a)

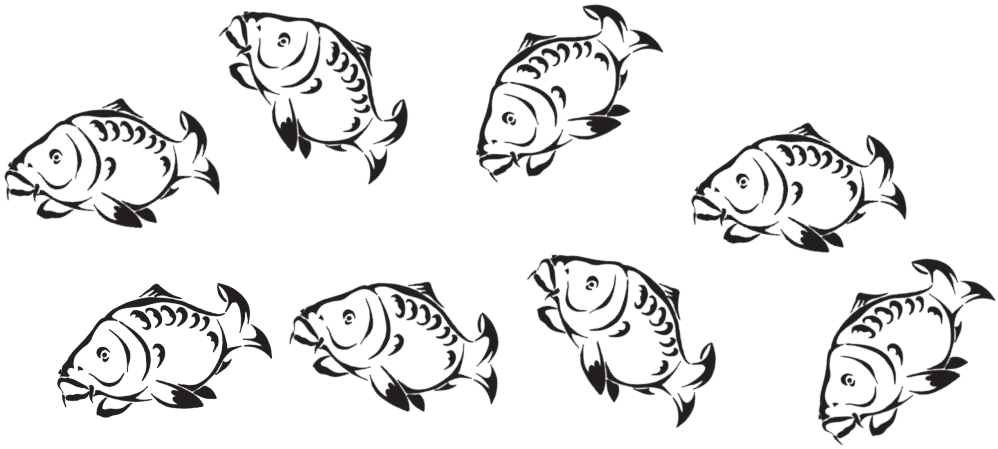


b)



Supplementary Figure 1. Predicted tertiary structure of carp Trila (A) and carp Trilb (B) proteins. Alignments were conducted using Pymol (http://www.pymol.org/). Proteins were modeled with 3D-JIGSAW (http://bmm.cancerresearchuk.org/~3djigsaw/) selecting the model with the best C-score. The three-dimensional model for carp tril was based on the crystal structure of human LINGO-1 (Q96FE5.2).

Supplementary Figure 2 data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/ j.molimm.2013.07.012.



Chapter 8

General discussion

Danilo Pietretti

GENERAL DISCUSSION

The innate immune system is the first line of defence against pathogens and rapidly responds to pathogen-associated molecular patterns (PAMPs). Teleosts possess an innate immune system that shares the same features and elements of the innate immune system of warm-blooded vertebrates. In the Initial Training Network NEMO, we took an integrated approach to the optimisation of the use of β-glucans, components of the cell wall of baker's yeast, as feed ingredient aiming at a direct effect on the innate immune system. These studies contributed to the valorisation and use of β-glucans as immunostimulants for sustainable aquaculture, achieving a strategic improvement of fish health. The overall aim of this thesis was to study the immune-modulating effect of β-glucans, including MacroGard®, on the carp innate immune system, macrophages in particular. A molecular and functional characterization was made of candidate receptors on carp leukocytes sensing β-glucans. In this chapter we discuss the modulation of innate immune responses of carp by different forms of β-glucans including MacroGard® (key objective 1). We also discuss candidate pattern recognition receptors on carp leukocytes that could sense and initiate innate immune responses (key objective 2). Finally, we discuss lessons learnt and directions of future research.

Do β-glucans compose immune symphonies?

To elucidate the innate immune responses induced by β-glucans a suite of immune parameters was measured by the different partners in the NEMO Network. Serum, immune organs and leukocytes were collected to measure both, humoral and cellular reactions that make up innate immunity. Cellular studies targeted macrophage activation assessing oxygen or nitrogen radical production and induced (cytokines) gene expression. Humoral studies included the measurement of serum immunoglobulin levels, but also included the measurement of complement levels. Our own studies focused in particular on candidate receptors on carp leukocytes that could sense β-glucans. All these measurements were part of the scientific program of NEMO to help ascertain how β-glucans modulate innate immune responses in carp. Thereby, the Network established optimal protocols for the use of β-glucans in the improvement of fish health.

The effects of β-glucans on the innate immune system of fish have been extensively reviewed [1, 2]. In fact, one of the most downloaded reviews in fish immunology is on β-glucans, referring to these compounds as "conductors of immune symphonies" [3]. In this paragraph we discuss a number of the effects of β-glucans on the immune system of common carp as found within the NEMO Network. This short paragraph does not aim to provide a complete review but rather highlights a number of findings that could contribute to the immune symphony directed by β-glucans.

Carp that were fed ß glucan-enriched diets responded with increased serum levels of C-reactive protein (CRP), but not with increased values of complement activation during infection with the bacterium *Aeromonas salmonicida* [4]. At the same time, carp that were fed ß glucan-enriched diets showed a reduction in their inflammatory response to *A.salmonicida* as measured by gene expression of a number of inflammation-related cytokines [5]. These apparent positive effects of β-glucan could be substantiated by the absence of β-glucan-induced apoptosis in immune organs [6]. Positive effects of β-glucans on resistance to disease induced by *A. salmonicida* might be

explained, in part, by the ability of β-glucans to stabilize neutrophil extracellular traps (NETs) and protect these NETs from degradation by bacterial nuclease, at least from A. hydrophila [7]. Also of interest are the findings that β-glucans provided to carp not via diet but via bath, can modulate wound healing [8] [9]. Similar to what is seen in mammals, most of the data collected by the Network support immune-stimulating effects of β-glucans on the innate immune system of carp both *in vitro* and *in vivo*.

A particularly interesting feature of β-glucans relates to their ability to increase the concentration of cholesterol in lipid rafts. Lipid rafts are found in cell membranes as liquid-ordered domains enriched in for example cholesterol and sphingomyelin and are thought to play important roles in the immune responses against pathogens [10-13]. Lipid rafts have not been extensively characterized in teleost fish. Only recently (2012), evidence in goldfish (C*arassius auratus*) showed the presence of lipid rafts biochemically and functionally similar to lipid rafts found in warm-blooded vertebrates, in goldfish leukocytes [14]. Of interest, carp fed with β-glucan-enriched diets showed an increase in the concentration of cholesterol located in lipid rafts of leukocytes from the head kidney (G. Brogden, unpublished observations).

Accumulation of cholesterol in macrophage induces formation of so-called foam cells, contributing to artherosclerosis [15]. Removal of excess cholesterol from foam cells is facilitated by high-density lipoprotein (HDL) that releases apolipoprotein (Apo A-1). Thereby, Apo A-1 is considered one of the key factors for artheroprotection [16, 17]. It has already been known for some time that HDL has the ability to directly bind and sequester LPS, thereby suppressing TLR4 signalling [18]. Of interest is that cholesterol accumulation in the plasma membrane of macrophages from Abca1$^{-/-}$ and Abcg1$^{-/-}$ mice, that have silenced genes of either one of these two important transporters of cholesterol, increased signalling of TLRs via MyD88-NF-κB and which enhanced the inflammatory response to LPS and other ligands [19-21]. These studies point at a clear link between levels of cholesterol, controlled by HDL and ApoA-1, the composition of lipid rafts and innate immune responses modulated by TLRs in lipid rafts (**Figure 1**).

As mentioned above, lipid rafts in immune cells of goldfish are biochemically and functionally similar to lipid rafts found in warm-blooded vertebrates and cholesterol levels are modulated by high-density lipoprotein that releases apolipoprotein. Of interest, HDL is the most abundant protein in carp plasma and has been reported to have bactericidal activity [23]. Possibly, this activity could be explained, at least in part, by a modulation of cholesterol in lipid rafts by the high levels of HDL, affecting bactericidal activity via modulation of inflammatory responses induced via TLR signalling of TLR receptors present in these lipid rafts. If, indeed, β-glucans can increase the concentration of cholesterol in lipid rafts of fish leukocytes, this could explain effects on the immune responses directed by TLRs present in these lipid rafts.

Recently, in vivo studies on carp fed β-glucan-enriched diets for a period of 25 days and injected with the double-stranded RNA analog poly (I:C) provided promising results. In these studies, both tlr3 and mx gene expression were upregulated, but only in groups fed β-glucan-enriched diets and not in carp that received control feed (A. Falco, unpublished data). These data could point at a β-glucan-mediated protection against viral infection, moderated via *tlr3*. Whether this mechanism, as discussed above, could be based on a β-glucan-induced increase of cholesterol concentration in lipid rafts and subsequent effect on the functioning of innate immune responses directed by TLRs such as Tlr3 in these lipid rafts, remains to be investigated.

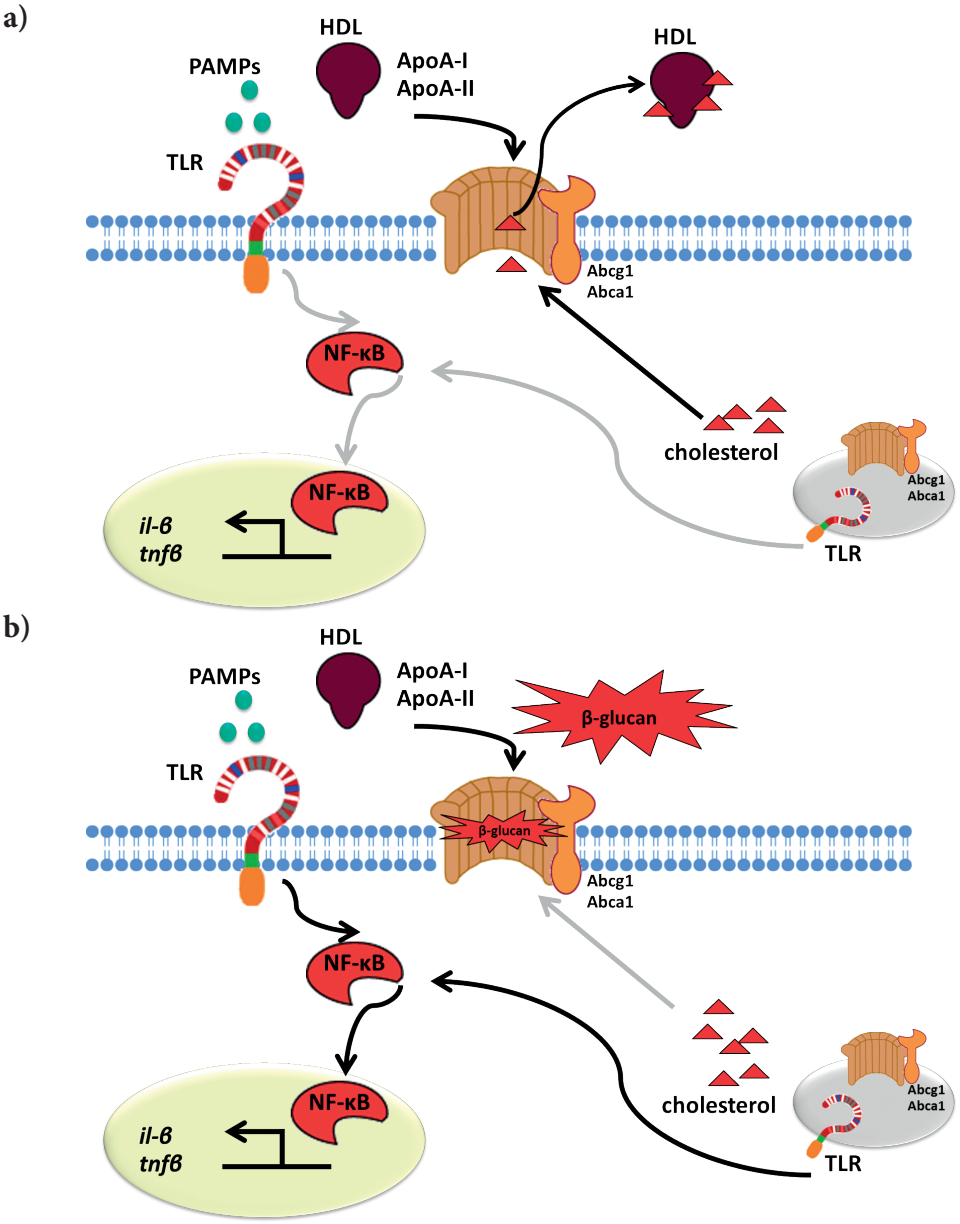


Figure 1. Possible role of β-glucansin blocking apolipoproteins in lipid rafts.a) HDL through ApoA-I and ApoA-II proteins sequesters PAMPs (such LPS) and soak up cholesterol transport from (Abcg1 and Abca1) and leads to neutralization Toll-like receptor pathway. **b)** β-glucans increase the levels of cholesterol in the lipid rafts, probably by blocking ApoA-I and ApoA-II. The high levels of cholesterol increase signalling by cell surface or endosomal Toll-like receptors (TLRs) thereby enhancing the inflammatory response. Grey arrows indicate less activity of the pathway and black arrows indicate enhanced activity of pathway in the presence of b-glucan. **Abbreviations: HDL**= high-density lipoprotein; **apoA-I and apoA-II**= apolipoprotein; **TLR**= Toll-like receptor; **PAMPs**=pathogens-associated molecular patterns; **abcg1**= transporter of cholesterol; **abca1**=transporter of cholesterol. Figure was re-adapted from [22].

The never-ending search for β-glucan receptors in fish

We observed activation of innate immune responses in carp leukocytes by measuring increased production of oxygen radicals in macrophages and neutrophilic granulocytes (**chapter 2**). Levels of these oxygen radicals, when measured in total leukocyte populations, could be taken as indicators of fish health [24]. In addition, we measured induction of oxygen and nitrogen radicals in carp macrophages by particulate β-glucan (such as MacroGard® and zymosan) and by Dectin-1 agonists (depleted-zymosan and curdlan) (**chapter 3**)[25]. β-glucans induced the production of pro-inflammatory cytokines such *il-1β*, *il-6* and *il-11*. Thus, despite the presumed absence of Dectin-1 receptors from fish genomes [26, 27], we found that Dectin-1 ligands could induce both oxygen and nitrogen radicals, although to a lower extent than particulate β-glucans [25]. In conclusion, our studies suggest recognition of β-glucans by multiple pattern recognition receptors that could include Toll-like receptors, but also other receptors that are not TLRs. In this thesis we characterized two Toll-like receptors (Tlr4 and Tlr20) as candidate pattern recognition receptors on carp leukocytes that could sense β-glucans and initiate innate immune responses. In mammals, Dectin-1 can synergize with TLR2 but also with TLR4 to enhance the production of cytokines in response to stimulation with β-glucans [28]. Preliminary data could point at a role for Tlr3 in sensing β-glucans (A. Falco, see above), but this suggestion requires additional experimental confirmation. No convincing evidence was found that would suggest for Tlr4 (**chapter 5**), nor the non-mammalian receptor Tlr20 (**chapter 4**), to act as β-glucan receptor. These results have been discussed as part of the relevant chapters in this thesis and will not be further discussed here. Additional candidate receptors for recognition of β-glucans in fish are discussed below.

The NEMO network

The NEMO network formed a nucleus of young scientists, of which one more PhD student (Inge Rosenbek Fink) and one post-doc fellow (Anders Østergaard) were appointed at Wageningen University, Cell Biology and Immunology group. Next to the work described in this thesis, they undertook a molecular and functional characterization of carp Tlr1 and Tlr2, in combination with the scavanger receptor Cd36 (Inge R. Fink). And, carp scavanger receptor 'Scarf-1' or C-type lectin receptors 'Illrs' (Anders Østergaard) as candidate receptors for recognition of β-glucans. The preliminary results of these studies in progress are relevant to the research described in this thesis and shortly discussed below.

Full-length cDNA sequences of carp Tlr1 and Tlr2 were obtained and synteny analyses support that these molecules are orthologs of mammalian TLR1 and TLR2. Real-time quantitative PCR analysis of basal gene expression revealed expression in immune relevant organs. Of interest, Tlr1 was found to be expressed mainly by B cells and neutrophils, and to a lower extent by macrophages, whereas Tlr2 was expressed by B cells and macrophages. The full-length sequences were used to create fluorescent protein-tagged receptors to visualize their sub-cellular localization. At this moment, transfection experiments are being carried out to determine binding of ligands, among which β-glucan but also previously identified ligands for TLR2 [29], in both human and fish cell lines. Based on conservation of amino acids known in mammals to be important for heterodimer formation, it can be hypothesized that heterodimer formation between Tlr1 and Tlr2 can also occur in common carp [30].

Two full-length *scarf-1* genes have been obtained from the common carp genome, confirming

the hypothesis that carp has undergone an additional genome duplication event compared to zebrafish [31], where only one *scarf-1* gene is found. Sequence analysis of Scarf-11 revealed a high level of amino acid conservation compared to the human orthologue, whereas synteny studies show conserved linkage with several neighbouring genes when comparing genomic regions from different species. Gene expression analysis of *scarf-1* showed highest expression in endothelial cells, but macrophages, granulocytes, thrombocytes and thymocytes also showed *scarf-1* gene expression. Carp *cd36*, in contrast to *scarf-1*, was expressed at low levels only in carp leukocytes. Sub-cellular localization of Scarf-1 and Cd36 was studied by confocal microscopy of cell lines transfected with a fluorescently tagged receptor. These studies showed these receptors to be expressed at the cell surface. Experiments are being carried out to study the possible roles of Scarf-1 and Cd36 as internalizing receptors that could facilitate TLR activation upon phagocytosis of ligands, such as β-glucan [32].

There are more receptors that could be candidates for recognition of β-glucans in fish. In mammals, myeloid cells express several receptors capable of recognizing β-glucan, with the C-type lectin receptor (CLR) Dectin-1 in conjunction with TLR2, considered key receptors for recognition of β-glucan. In our studies we could clearly show that carp macrophages are less, but not unresponsive to selective Dectin-1 agonists, suggesting recognition of β-glucan by multiple pattern recognition receptors that could include TLR but also non-TLR receptors of the CLR superfamily (**chapter 3**). Within the CLR superfamily not only Dectin-1, but also Dectin-2 and Dectin-3 may have an active role in the recognition of β-glucan. Dectin-3 forms a heterodimer with Dectin-2 for recognizing infection with fungi and it is suggested that CLRs may in fact form a scala of different hetero- and homodimers providing host cells with receptors to detect a scala of microbial infections [33]. Although (all) dectin receptors may be absent from fish genomes [26], other CLRs may well play roles in the recognition of β-glucans in fish. Several CLRs of fish have been characterized that show characteristics of both group II and group V receptors. The family of immune-related, lectin-like receptors (Illrs), that are part of the group II CLRs and possess inhibiting and/or activating signalling motifs typical of group V receptors, could be candidate receptors for recognition of β-glucan. The *illr* genes are differentially expressed in the myeloid and lymphoid lineages in zebrafish [34] supporting the idea that *illrs* may function in natural killer (NK) as well as in myeloid cells. In mammals, the first line of defence is represented by macrophages and NK cells, both cell types well developed in teleosts. Two types of NK cell homologues have been described in fish: non-specific cytotoxic cells and NK-like cells [35]. In carp, *illr* genes have been identified, and multiple alignment have shown high identity between *illr* genes in zebrafish and carp suggesting that carp *illrs* are homolog of zebrafish *illrs*. The basal gene expression level of carp *illrs* show a similar expression pattern as observed in zebrafish. (Østergaard, unpublished data).

Besides Dectin-1-like receptors that can detect β-glucan and trigger antimicrobial activity such as phagocytosis and production of reactive oxygen species, the immunoglobulin (Ig) Fc receptor (FcγR) can also play a functional role in the activation of the phagocytosis process [36]. Likewise, the complement receptor 3 (CR3) is involved in the uptake of particulate β-glucan in mice; C3 enhances the uptake of particulate β-glucan and C3 "knockout" mice show reduced capacity to phagocytose [37]. Autophagy is an intracellular degradation process with a number of roles, one of which can be the protection of eukaryotic cells from invading microbes. Microtubule-associated protein light-chain 3 (LC3) is a key autophagy-related protein that is recruited to the double-membrane autophagosome responsible for sequestering material intended for delivery to lysosomes. LC3 can also be recruited to other membranes including single-membrane phagosomes,

in a process termed LC3-associated phagocytosis (LAP). Dectin-1 mediates a phagocytic response to β-glucan via this mechanism [38]. Dectin-1 may be absent from fish genomes, but also TLRs can engage in autophagy through the activation of LC3, facilitating rapid recruitment to the phagosome [39].

Based on existing data in the literature, we hypothesize that recognition of β-glucan in fish could be by an interplay of phagocytic receptors and TLRs (**Figure 2**). Phagocytic receptors such as scavenger receptors, C-type lectin receptors (CLR) and/or FcγR receptors could bind β-glucan and activate phagocytosis, leading to a higher expression of TLRs in the phagosome that could sense β-glucan. The TIR domain of the TLRs could then activate the signal cascade that would lead to NF-κB activation and an upregulation of expression of cytokines such *il-1β*, *il-6* and *il-11*, as well as production of reactive oxygen and nitrogen species that would help clear phagocytosed β-glucan.

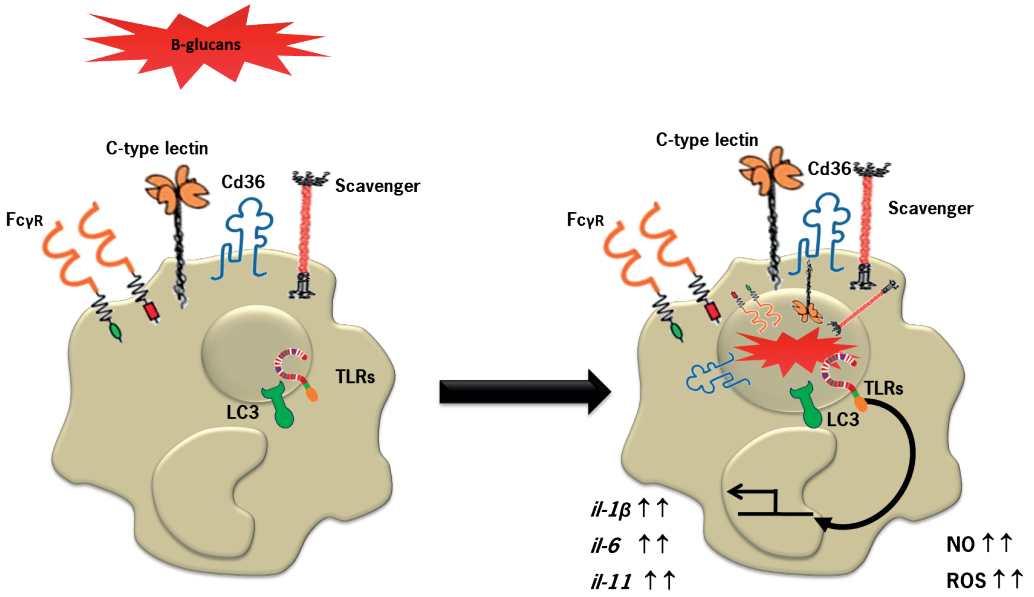


Figure 2. Possible mechanism for recognition of particulate β-glucans in fish. Particulate β-glucans are recognizes by phagocytic receptors such C-type lectin (CLR), the immunoglobulin (Ig) Fc receptor (FCγR) or scavenger receptors (such Scarf-1 and Cd36) which will activate the phagocytosis process. Activation leads to stimulation of TLRs that sense β-glucan and leads to possible recruitment of (microtubule-associated protein light-chain 3) LC3. The activation of the TIR domain and the following intracellular signal cascade leads to the production of oxygen radicals (ROS) and nitrogen radicals (NO) and upregulation of cytokines such *il-1β*, *il-6* and *il-11*.

Of interest also, are recent *in vivo* studies on whole fungal pathogens including *Candida albicans*. The innate immune system controls *Candida* infection in part through Dectin-1. *C. albicans* masks the presence of β-glucan early during infection, but it becomes exposed later, allowing Dectin-1 to recognize *Candida* and mediate immunity [40]. In a recent (2013) paper,

microarray data from zebrafish infected with *Candida albicans* were used to describe an intercellular protein-protein interaction (PPI) network between host and pathogen. As part of this extensive work, important defense-related proteins in zebrafish were predicted [41] (see Figure 3). This dataset may be of particular interest for the identification of β-glucan receptors in fish, identifying in an unbiased manner candidate receptors for β-glucans for future research. This dataset may be of particular interest for the identification of β-glucan receptors in fish, identifying in an unbiased manner candidate receptors for β-glucans for future research. Important to understand which receptors sense the β-glucan it will be investigation of the The figure was taken and adapted from [41].

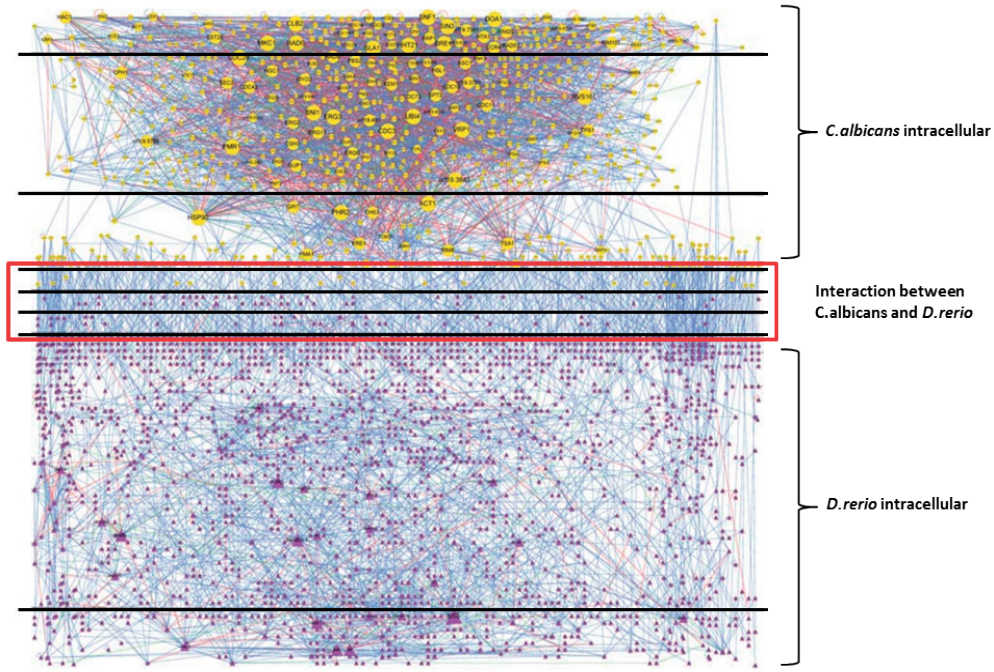


Figure 3. Protein-protein interaction (PPI) network during C. albicans infection of zebrafish. The figure shows protein-protein interaction (PPI) between the *Candida albicans* and *Danio rerio*. Microarray data from *C. albicans* and *D.rerio* were combined to show the proteins interaction between host and pathogen. The red box indicated the region were may be possible to identify the β-glucan receptors in fish.

Twinning: The act of winning

"The combination of the genetically tractable but small-sized zebrafish with the larger-sized common carp creates a very strong combination of animal models that can address disease-related questions relevant to aquaculture based on an increased understanding of fundamental immune mechanisms in comparative immunology" (from: 'zebrafish and carp: non-identical twins', PhD thesis [42]). This statement is supported by the fact that common carp and zebrafish are two phylogenetically closely related fish species, as shown by a high degree of synteny between the

common carp and zebrafish genome [31]. Indeed, access to both the zebrafish and carp genome greatly facilitated the molecular characterization of receptors and other molecules important for the carp immune system (*this thesis*). Where the detailed annotation of the zebrafish genome allows for analysis of synteny, facilitating investigations into orthology of the gene of interest, common carp cDNA libraries allow for detailed analysis of organ- or cell type-specific gene expression.

One factor complicating the 'winning act of twinning', however, is that zebrafish may be diploid but common carp are (semi-)tetraploid after an additional genome duplication event. Gene duplication has played an important role in evolution, providing the supply of new genes to allow organisms to adapt to environmentally-induced stress changes [43]. Two rounds of whole genome duplication (WGD) have occurred during early vertebrate evolution [44] whereas in teleosts, including zebrafish, a third genome duplication occurred [45-47]. Later during evolution, some 11-21 million years ago in the case of cyprinids, a fourth WGD event took place [44, 48, 49]. Indeed, most of the genes found in the carp genome are duplicated with respect to zebrafish [31]. At present, common carp are undergoing a process of re-diploidization of their genome that shows effects of neo-functionalization (development of genes with new functions) and sub-functionalization (appearance of paralogues with partitioned functions of the ancestral gene) [50-52]. Although many of the duplicated genes may degenerate to pseudogenes [53], often the two gene copies usually found in the carp genome may each express (slightly) different functions. Of course, exceptions to this rule do also exist. No matter what, twinning studies on both common carp and zebrafish have already proven their use as exemplified below.

As a first example, during our studies on the characterization of *il-6* we could retrieve back, in the carp genome, two *il-6* genes (**chapter 3**). As a query, we used the zebrafish *il-6* gene annotated the year before [54]. Our identification of carp *il-6* was important in that, until recent, the molecule M-17 was often mistaken for Il-6. This differentiation is relevant because, although M-17 is one of several cytokines that belong to the interleukin-6 family, both cytokines may have very different functions [55, 56]. As predicted, we could find duplicated copies of the single *il-6* gene found in zebrafish. The high similarity between the two copies (more than 91%) required the design of specific primers that can differentiate between the two carp *il-6* genes. Only then studies on neo-functionalization and sub-functionalization can be performed in a reliable manner.

A second, almost opposite example where twinning studies on both common carp and zebrafish have already shown clear benefit is where we identified in the carp genome two copies of a gene that could be assigned as *tril* (**chapter 7**). Assignment could be made with help of the pufferfish, but not the zebrafish genome. Initially, synteny analysis of ZV9 suggested the absence of this gene from the zebrafish genome, an unlikely outcome given the fact that common carp did have two copies of *tril*. Indeed, based on the genome information from carp we could detect in the expressed sequence tag (EST) database partial sequences for zebrafish *tril* and annotate the zebrafish *tril* gene using information from the whole zebrafish genome shotgun assembly from Tübingen.

A last example is where we used genome information from zebrafish on the existence of mutiple copies of non-mammalian *tlr20 genes* to detect carp *tlr20* (**chapter 4**). In the draft genome of carp, despite the short scaffold, it was possible to detect synteny and describe (some of) the genes flanking *tlr20*. Although from the zebrafish genome we could retrieve six copies of *tlr20*, in carp only a single copy could be detected. Given the high number in zebrafish, an even higher number of *tlr20* genes would be expected in carp. The high quality of the carp genome, however, refutes the suggestion that most of these copies would indeed be present but were missed in the carp

genome. Although future improvement of the carp genome, in particular the combination of the high number of short scaffolds into fewer, larger scaffolds will certainly help to shed further light on these intriguing questions, it seems clear not all genes are found in duplicate copies in the carp genome.

Go beyond RT-qPCR down the functional lane

To better understand the function of a particular Tlr molecule, identification of its ligand(s) is crucial. Studies on mammalian Tlrs have identified a range of ligands for the different Tlrs, shedding light on their role in innate immunity. Although these studies may be helpful to direct studies on TLR orthologs in fish, true ligands of fish Tlrs have to be identified with functional studies. Of course, when studying non-mammalian Tlrs such as, for example, Tlr20 (**chapter 4**) there are no studies in mammalian vertebrates than can be referred to. Many conclusions on the putative functions of fish Tlrs and their potential ligands have been based on studies reporting upregulation of *tlr* gene expression by RT-qPCR. Of course, studying up-regulation of *tlr* gene expression can provide indirect evidence of their function (**chapters 4, 6**). However, many of these studies show a bias in their choice of ligands that are selected based on the assumption that fish equal mouse and/or human with respect to ligand recognition by their TLR reportoire. More objective it may be to screen microarray or deep-sequencing datasets for changes in TLR gene expression after infection with whole pathogens.

One of the most common routes taken, and proven successful in the determination of ligands for several TLR [57, 58], is to overexpress the TLR of interest in a cell line that does not naturally express this receptor, for example a human cell line such as the HEK 293. Under the assumption that HEK 293 signalling pathway is conserved between human and fish. Following overexpression, activation by a ligand can be determined by reading, for example, induced levels of luciferase activity using a reporter construct (**chapters 4, 5**). The reporter activity is most often based on activation of NF-κB, a transcription factor highly conserved [59]. In our studies we did not manage to exploit NF-κB-based reporter assays in HEK 293 cells to unequivocally determine ligands for Tlr20 nor Tlr4. Hypothesizing that human cell lines such as HEK 293 may be too different from fish cells in crucial aspects to support ligand-determining studies, we also tried fish cell lines. The CLC (carp leukocyte culture) cell line originates from blood leukocytes of common carp [60], but may have become contaminated in many laboratories by cells from other, non-carp, cell lines (unpublished observations). The EPC (epithelioma papulosum cyprini) cell line originated from carp epidermal herpes virus-induced hyperplastic lesions with a reduced chromosome number (n = 94) compared with common carp [61]. Indeed, it was later discovered to be contaminated with cyprinid fathead minnow [62], but these cells are still considered close relatives of common carp. Thus, in theory, the EPC should support functional studies on carp molecules. Yet, NF-κB-based reporter assays using EPC cells did not unequivocally determine ligands for Tlr20 nor Tlr4. Most recently, we tried to refine our approach as to include a reporter construct based on activation of AP-1, another transcription factor highly conserved [63]. Again, we did not manage to unequivocally determine ligands for Tlr20 nor Tlr4. Future studies should focus on the use of other cell lines derived from carp.

Sub-cellular localization studies on Tlr20 and Tlr4 pointed at intracellular expression of these receptors in human (HEK 293) and fish cell lines. To date, the exact function of the non-mammali-

an Tlr20 is unknown (**chapter 4**) and the same is true for Tlr4 (**chapter 5**). Given these uncertainties, it may well be possible that these Tlrs are expressed intracellulary and not on the cell surface. It may also be possible that overexpression of these molecules will lead to an aberrant expression inside the cell, for example, in the early secretory pathway, endoplasmic reticulum-associated degradation and autophagic pathways specialize in seeking misfolded polypeptides and mediate their degradation [64]. Where aberrant expression patterns of foreign molecules in human cell lines such as HEK 293 could be a true possibility, overexpression of carp Tlrs in EPC cells should lead to correct expression patterns, however.

In this thesis we report the identification, in fish genomes, of a large number of molecules accessory to the function of mammalian Tlrs (**chapter 7**). It could be a realistic option that human cell lines such as HEK 293 express accessory molecules that are (too) different from the fish orthologs to function optimally in conjunction with fish Tlrs, such as carp Tlr4 or Tlr20. At the same time, we can expect these accessory molecules in the fathead minnow cell line (EPC), to be most similar to those in carp. Yet, despite the genetic relation of the two fish species, the fact that these species have different number of chromosomes may result in their inability tot express all the molecules that other (leukocyte) cell types would express. In addition, it is not uncommon that cell lines grown for many years *in vitro* built up mistakes in their genomes [65]. Therefore, we examined the transcriptome of the EPC cell line (courtesy of H.P. Spaink and ZF Screens, Leiden, the Netherlands) for the presence of known accessory molecules (**Table 1**).

As can be concluded from the overview in Figure 4, not all accessory proteins previously thought to be involved in Tlr function were actually present in the transcriptome of the EPC cell line. For example, EPC cells do not express *bpi/lbp*, *cd36*, *tril* and *unc93b1* that can be found in the carp genome. The apparent absence of these molecules in EPC could have affected the functional characterization of Tlrs overexpressed in this cell line. Of the missing accessory molecules, three (*bpi/lbp*, *cd36* and *tril*) are mediators of ligand delivery and or recognition and one (*unc93b1*) is a trafficking factor. Trafficking factors are thought to help localize the Tlr into the correct sub-cellular compartment whereas mediators of ligand delivery and/or recognition may help sense and/or deliver ligands. Future studies should focus on expressing one or several of the missing accessory molecules in EPC to study their role in the functioning of fish Tlrs, among which are Tlr20 and Tlr4.

Overall, the molecular and functional characterization of a number of Tlrs and associated molecules considered important for the carp innate immune system, as described in this thesis, has allowed for much progress on the knowledge of fish Tlrs. Although the search for unique receptors on carp leukocytes sensing β-glucans may still be ongoing, the research described in this thesis has already contributed to the valorization and use of β-glucans as immunostimulants for sustainable aquaculture, potentially achieving a strategic improvement of fish health.

Table 1 Comparison of accessory molecules between carp genome and the EPC transcriptome. [a] *bpi/lbp* ancestral gene of LBP; [b] High mobility group-T protein; [c] *unc93b1*-like protein MFSD11; [d] *cathepsins f.*

	Accessory molecules	Carp genome	EPC transcriptome
Mediators of ligand delivery and/or recognition	***lbp***[a]	+	-
	md2	-	-
	cd14	-	-
	cd36	+	-
	tril	+	-
	progranulin	+	+
	hmgb1[b]	+	+
	ll37	-	-
TLR chaperones	***gp96***	+	+
	prat4	+	+
Trafficking factors	***unc93b1***[c]	+	-
	ap3	+	+
TLR processing factors	***cathepsin***[d]	+	+
	aep	+	+

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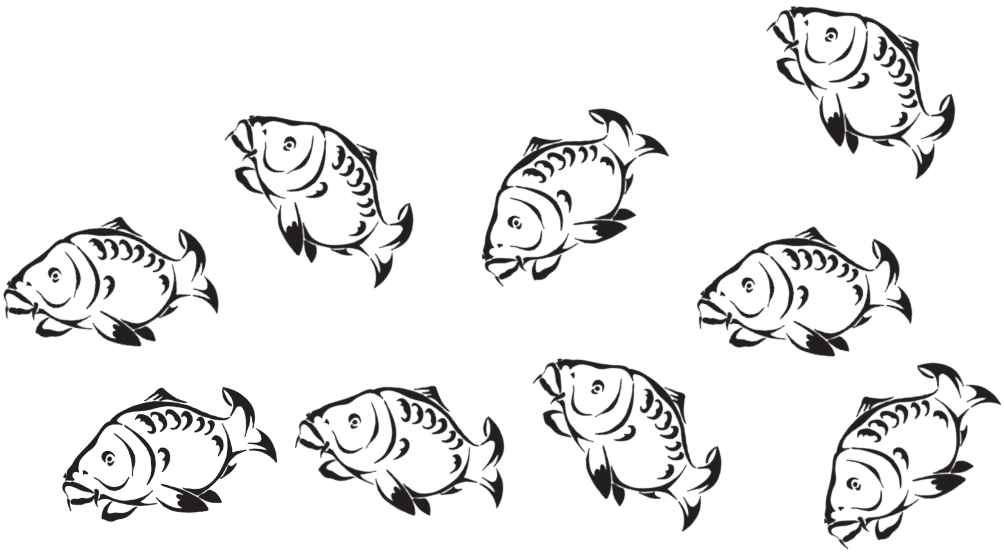

Chapter 9

Miscelleneous

English summary
Nederlandse samenvatting (Dutch summary)
Sommario (Italian summary)
(Serbian Summary)
Acknowledgments
Curriculum Vitae
Publications
Education certificate

SUMMARY

Intensification of aquaculture introduced the risk of increased and widespread infectious with several pathogenic organisms. The main issues in aquaculture are the rapid and uncontrolled growth of pathogens in aquatic organisms and the resulting indiscriminate use of antibiotics that has lead to several resistant pathogens. Thus, alternative strategies and suitable tools are required to control disease outbreaks in this sector. Increased use of vaccine OR use/vaccination rate, dietary supplement of probiotics, prebiotics, and immunostimulants may help to reduce the susceptibility of fish to infections. Feed-compatible immunostimulants such as β-glucans are the modern and primary tools in aquaculture that help in enhancing resistance against infectious diseases by enhancing innate humoral and cellular defense mechanisms. In this context we introduced in **CHAPTER 1** β-glucans as modulators of innate immunity in fish summarizing the effects of what effect of research itself, or reduction in infection rate due to research results?? and the research that has been carried-out in fish over the last decades. However most of this research has not been focused on the immunomodulatory effects of β-glucans in common carp, which is the most cultured fish for food consumption. In this respect, an intra-European training network called NEMO, was formed to develop a sustainable and cost-effective method for using?use of β-glucans as immunostimulant in common carp. Our aim within this project was to measure the modulation of innate immune response by β-glucans and the characterization of pattern recognition receptors (PRR) on carp leukocytes that sense and activate the immune response. In our first study, described in **CHAPTER 2**, we analysed the effect of β-glucans on carp leukocytes in the activation of the innate immune response through the respiratory burst activity on macrophages and neutrophilic granulocytes. The respiratory burst will lead to production of oxygen radicals (ROS), which have a distinct role in the clearance of pathogens and the tissue regeneration process. We compared two methods used for quantitation of ROS produced during the respiratory burst: (1) reduction of nitroblue tetrazolium (NBT) and (2) a real-time luminol enhanced assay based on chemiluminescence. We demonstrated that (1) NBT assay detects only the production of superoxide anions, and (2) the real-time luminol-enhanced assay could detect the production of both superoxide anions and hydrogen peroxide. Moreover, the real time luminol-enhanced assay can record production of ROS in real-time thereby providing more detailed information on the respiratory burst response. However, using both methods (reduction of nitroblue tetrazolium and real-time luminol enhanced assay) we detected activation of the carp immune response through the production of ROS in carp leukocytes. We further investigated in **CHAPTER 3,** the possible receptors involved in activation? expressed on carp macrophages using different sources of β-glucans. We used particulate β-glucan preparations of baker's yeast such as zymosan and MacroGard® (commonly used as feed ingredient for farmed animals including fish), which is known to be sensed through TLR2 and Dectin-1. Next to the particulate β-glucans we also used dectin-1-specific ligands such as depleted-zymosan (treated to deplete the TLR-stimulating properties) and curdlan both known to be sensed by macrophages through dectin-1. We analysed their effects on carp macrophages by measuring the production of reactive oxygen and nitrogen radicals, in addition to induced transcription of cytokine genes. Our results suggest that carp macrophages strongly react to particulate β-glucans with an increase in the production of reactive oxygen, nitrogen radicals and cytokines, such as IL-1β, IL-11 and IL-6. In this study we identified IL-6 for the first time in carp. Moreover, compared to ß-glucans we

observed a decreased response by carp macrophages to selective dectin-1 agonists, suggesting that recognition of β-glucans by macrophages is likely to include TLR but also non-TLR receptors.

To understand which macrophage receptors can actually sense the presence of β-glucans, we investigated and characterized different receptors such as Toll-like receptors (Tlr-1, Tlr-2, Tlr-4 and a non-mammalian Tlr-20) and scavenger receptors (CD36 and Scarf-1) C-type lectin (Illrs) within the NEMO consortium. In our studies described in this thesis we focused mostly on Toll-like receptors (TLRs) that mediate pathogen recognition through detection of conserved microbial ligands. TLRs can be divided in six major families, each of which can recognize a general class of molecular patterns. However, in modern bony fish a number of TLRs with unknown function are present. In **CHAPTER 4** we described Tlr-20 as one of these non-mammalian TLR, which among teleost has so far only been detected in cypriniformes, suluriformes and salmoniformes. We identified full-length cDNA sequences for six *tlr20* genes in zebrafish and one in common carp. To better understand the function of Tlr-20 we made a three-dimensional predictive model that indicates a best fit to the crystal of human TLR-8. However, performing phylogenetic analysis revealed a relationship with a TLR-11 family closest to murine Tlr-11 and Tlr-12, both known to be sensing presence of protozoan parasites (*Toxoplasma gondii*). Synteny analysis of the genes flanking zebrafish *tlr20* did not indicate a conserved synteny between TLR-20 and murine members of the TLR-11 family. To better understand the role of teleost Tlr-20 we performed confocal microscopic analysis of carp Tlr-20 expressing cells, suggesting its sub-cellular localization in the endoplasmatic reticulum. Overexpression of carp Tlr-20 in a human reporter cell line containing a reporter construct detecting NF-κB activation *in vitro* could not identify a unique ligand for Tlr-20. *In vivo* infection experiments using protozoan parasites including *Trypanoplasma borreli* indicated a role of carp Tlr-20 in the induction of a parasite-specific immune response. Analysis of an organ library from carp showed a significant expression level of *tlr-20* mainly in peripheral blood leukocytes (PBL) with B lymphocytes as a primary source. *In vitro* stimulation of PBL with *T. borreli* induced an upregulation of *tlr20*, suggesting a role for Tlr-20 in sensing selective ligands from protozoan parasites.

Another Toll-like receptor which role appears restricted to the teleost orders of siluriformes and cypriniformes is the Toll-like receptor 4 as discussed in **CHAPTER 5.** In mammals TLR-4 senses Gram-negative bacterial lipopolysaccharide (LPS) and it is the most important receptor during induction of septic shock. Teleost Tlr-4 appears not to recognize LPS probably because of a lack of the critical co-stimulatory molecules MD-2 and CD14 in their genomes. To further understand the role of *tlr4* in teleost, we injected in zebrafish larvae with a selective knockdown of *tlr4ba* and *tlr4bb* with *Salmonella typhymurium* but did not observe any detectable response confirming that cyprinid fish Tlr-4 does not sense Gram-negative bacteria. We characterized two putative *tlr4* genes and both genes were expressed in many organs with macrophages as the primary source. Three-dimensional modeling of carp Tlr-4 endorsed the structural possibility of an interaction between carp Tlr-4 with human MD2 and LPS. Unexpectedly, i*n vivo* studies of fish infected with spring viraemia carp virus (SVCV) showed an upregulation of the *tlr4* gene using RT-qPCR analysis. Overexpression of carp tlr-4 in human cell lines using reporter assays based on NF-κB activation, could not identify a ligand unique to Tlr-4. We localized Tlr-4 in the cytoplasm, but not on the cellular membrane, which could explain in part the fact that in vitro we did not observe activation of NF-κB due to lack of exposure of TLR-4 to the extra-cellular stimulus. Further investigation on the genome of zebrafish and carp revealed the presence of several other *tlr4* gene sequences. In **CHAPTER 6**, we summarized the studies that have been performed so far in fish Toll-like receptors (TLRs). The

presence of specific TLR genes, and changes in their gene expression profiles as result of infection, in the context of different fish-orders and fish-families is reviewed. We discussed that studying gene expression may provide (in)direct evidence for the involvement of a particular TLR in the reaction to a pathogen and these findings can be relevant when proven consistent among species but also among families.

Future studies on the conservation of function of accessory molecules between species?, in conjunction with the structure or expression patterns of?TLR molecules, may bring new insight into the actual function of fish TLRs. We investigated the presence of accessory molecules in fish genomes as described in **CHAPTER 7** and our observations suggested that the fish genomes contain most of accessory proteins required for TLR function. In detail, we characterized in zebrafish and common carp a novel accessory protein tlr4-interactor with leucine-rich repeats (Tril) that was recently described in mammals as component of the TLR4 complex and important for the TLR3 signaling. In addition, we discuss the implication of the presence of most, but not all, accessory molecules for the biosynthesis and potential of activation of tlr molecules in fish.

In **CHAPTER 8** we discussed the effects of β-glucans on the innate immune system in common carp highlighting findings within the NEMO consortium. Moreover we discussed which receptors in fish could potentially sense β-glucans by integrating our findings with recent literature. We hypothesized that recognition of β-glucans in fish could require an interplay of phagocytic receptors expressed on macrophages, such as C-type lectin, scavenger receptors and FcγR. In conclusion, we discussed the advantages of using both animal models such as common carp and zebrafish to compensate the missing information in one or other model and bring further the knowledge of the innate immune system.

(NEDERLANDSE) SAMEVATTING

Intensivering van de aquacultuur heeft gezorgd voor een toegenomen risico op het oplopen en verspreiden van infecties met verschillende ziekteverwekkers (pathogenen). De grootste problemen in aquacultuur zijn de snelle en ongeremde groei van pathogenen en het daaruit voortkomende gebruik van generieke antibiotica wat geleid heeft tot resistente pathogenen. Daarom zijn er alternatieve strategiën and methoden nodig om uitbraken van ziekten in de sector te voorkomen. Meer vaccinatie, toevoeging van probiotica en immuno-stimulants kunnen mogelijk de gevoeligheid van vissen tegen ziekten verminderen. Immuno-stimulanten, zoals beta-glucanen (β-glucanen), die geschikt zijn om in het voedsel te gebruiken is de primaire moderne methode in aquacultuur die de weerstand tegen infectieziekten kan verbeteren door het verhogen van de afweerreactie van cellen en door middel van uitgescheiden moleculen. In dit verband introduceren we in **HOOFDSTUK 1** β-glucanen als modulatoren van het innate immuunsysteem van vissen en vatten de effecten en het onderzoek wat in de afgelopen decennia is uitgevoerd in vissen samen. Het meeste onderzoek is echter niet gedaan aan de immuno-modulaire rol van β-glucanen in karpers, wat de meest gekweekte vis is voor consumptie. Om die reden was er een Europees netwerk, NEMO, gevormd om duurzaam en kosten-effectief gebruik van β-glucanen als immuno-stimulant in karpers te onderzoeken. Het doel van het project was om modulatie van de innate immuunreactie tegen β-glucanen te meten en in karpers witte bloedcellen de patroon-herkennings-receptoren (PRR) die het immuunsysteem activeren te karakteriseren. In ons eerste onderzoek, beschreven in **HOOFDSTUK 2**, hebben we het effect van ß-glucanen op de activatie van de innate immuunreactie van witte bloedcellen van de karper door de uitstorting (degranulatie) van pathogeen-dodende moleculen door neutrofielen en macrofagen geanalyseerd. De initiatie van degranulatie lijdt tot de productie van zuurstofradikalen (ROS), die een specifieke rol hebben in het opruimen van pathogenen en in het herstel van weefsels. We hebben twee methoden die gebruikt worden om ROS-productie gedurende degranulatie te meten vergeleken: (1) reductie van *nitroblue tetrazolium* (NBT) en (2) een *real-time* versterkte *luminol* labtest gebaseerd op chemi-luminescentie. We hebben aangetoond dat (1) de NBT-test alleen de productie van superoxide anionen, en dat (2) de *real-time* versterkte luminol-test de productie van zowel superopxide anionen als waterstofperoxide kan detecteren. De *real-time* luminol-test kan *real-time* de productie van ROS meten en geeft daardoor meer gedetaileerde informatie over de degranulatie-reactie. We hebben echter door gebruik van beide methoden de activatie van het immuunsysteem van karpers door de productie van ROS kunnen meten in witte bloedcellen. In **HOOFDSTUK 3** hebben we verder onderzocht wat de mogelijke receptoren zijn die tot expressive komen op macrofagen van karpers door het gebruik van verschillende bronnen van β-glucanen. We gebruikten β-glucaan partikels van bakkers-gist zoals *zymosan* en *MacroGard®* (algemeen gebruikt in voedsel voor vissen), wat herkent wordt door TLR2 en dectin-1. Naast β-glucaan partikels hebben we gebruik gemaakt van liganden specifiek voor dectin-1 zoals aangepast *zymosan* (behandeld zodat het de eigenschap om TLR te stimuleren verliest) en *curdlan* die beiden herkend worden door dectin-1 op macrofagen. We hebben het effect van *zymosan* en *curdlan* op karper-macrofagen geanalyseerd door het meten van zuurstof- en stikstofradikalen, naast de inductie van expressie van cytokienen-genen. Onze resultaten suggeren dat karper-macrofagen sterk reageren op ß-glucaan partikels met een toename van de productie van zuurstofradikalen, stikstofradiklen en cytokienen zoals IL-1β, IL-11 en IL-

6. In deze studie hebben we IL-6 voor het eerst geindentificeerd in karpers. Verder hebben we een verminderde reactie van karper-macrofagen gemeten op een specifieke dectin stimulant, wat suggereert dat ß-glucaan ook door andere TLR en niet-TLR receptoren van macrofagen herkent wordt.

Om te begrijpen welke receptoren op macrofagen de aanwezigheid van ß-glucaan kunnen detecteren, hebben we binnen het NEMO concortium verschillende toll-achtige receptoren (TLR-1, TLR-2, TLR-4 en TLR-20) en *scavenger*-receptoren (CD36 en Scarf-1), C-type lectin (Illrs) onderzocht. In de studies in dit proefschrift hebben we vooral gekeken naar Toll-like receptoren (TLRs) die bijdragen en de indentificatie van pathogenen door het binden aan geconserveerde microbiële moleculen. De TLRs kunnen opgedeeld worden in zes grote families, die elk een groep van moleculaire patronnen kunnen herkennen. Echter, in moderne benige vissen zijn een aantal TLRs aanwezig met onbekende functie. In **HOOFDSTUK 4** beschrijven we TLR-20 als een van deze TLRs die onbekend is in zoogdieren, en tot nu toe alleen gevonden is in cypriniformes, suluriformes en salmoniformes. We hebben de volledige cDNA sequenties van zes *tlr20* genen in zebravissen en één in karper gevonden. Om de functie van TLR-20 beter te begrijpen hebben we een voorspellend drie-dimensionaal model gemaakt dat het beste past op het kristal van humaan TLR-8. Echter, fylogenetische analyse legde een verband tussen muis-TLR-11 en TLR-12, die beiden bekend zijn voor het herkennen protozoe parasieten (*Toxoplasma gondii*). *Syntenie*-analyse van de genen aangrenzend aan zebravis *tlr20* resulteerde niet in geconserveerde *syntenie* tussen zebravis *tlr20* en leden van de TLR-11 familie in de muis. Om de rol van TLR-20 van teleosten beter te begrijpen hebben we een *confocal* microscopische analyse uitgevoerd in karper-cellen die TLR-20 tot expressie brengen, wat suggereerde dat TLR-20 aanwezig is in het endoplasmatsiche reticulum. Over-expressie van karper TLR-20 in menselijke cellen die signaleren bij activatie van NF- κ B, heeft niet geresulteerd in een specifieke ligand voor TLR-20. *In vivo* experimenten met infectie met protozoe parasieten, inclusief *Trypanoplasma borreli*, hebben een rol voor karper TLR-20 geïndentificeerd in de inductie van een parasiet-specifieke afweerreactie. Analyse van een orgaanbank van karpers toonde significante expressie-niveaus van *tlr20* aan, vooral in perifere witte bloedcellen (PBL) met B cellen als belangrijkste bron. *In vitro* stimulatie van PBL met *T. borreli* induceerde een toename in expressive van *tlr20*, wat suggereert dat TLR-20 een rol speelt in het detecteren van specifieke liganden van protozoë parasieten.

Een andere Toll-achtige recepter die beperkt is tot de siluriforme- and cypriniforme- ordes van de teleosten is TLR-4, zoals besproken in **HOOFDSTUK 5.** In zoogdieren herkent TLR-4 Gram-negatief bacterieel lipopolysaccharide (LPS) en is het de belangrijkste receptor voor de inductie van septische shock. Teleosten TLR-4 lijkt geen LPS te herkennen, mogelijk omdat de essentiële *co-stimmulatoire* (mee-stimmulerende) molekulen MD-2 en CD14 ontbreken in het genoom. Om de rol van TLR-4 in teleosten beter te begrijpen hebben we larven van zebravissen geïnjecteerd met een selectieve *knockdown* van *tlr4ba* en *tlr4bb* samen met *Salmonella typhymurium*, maar geen detecteerbaar verschil werd gemeten, wat bevestigt dat TLR-4 van cyprinide vissen geen Gram-negatieve bacteriën herkent. We hebben twee mogelijke genen voor TLR-4 gekarakteriseerd en beide genen werden tot expressie gebracht in veel organen, met macrofagen als primaire bron. Drie-dimensionaal modelleren van karper TLR-4 bevestigde de dat interactie tussen karper TLR-4 met LPS en menselijk MD2 mogelijk is. Onverwacht vonden we in *in vivo* studies van vissen geïnfecteerd met spring viraemia karper virus (SVCV) een toegenomen expressie van het *tlr4* gen zoals gemeten met RT-qPCR analyse. Overexpressie van karper TLR-4 in menselijke cel-kweken gebruikmakend van rapporteer testen gebaseerd op NF- κ B activation, konden geen ligand

identificeren dat uniek was voor TLR-4. We hebben TLR-4 gelokaliseerd in het cytoplasma, wat deels kan verklaren waarom we geen activatie van NF- κ B zagen *in vitro*. Verder onderzoek in het genoom van zebravis en karper leverde verschillende *tlr4* gen sequenties op. In **HOOFDSTUK 6**, vatten we de gedane onderzoeken aan Toll-like receptors (TLRs) in vissen samen. We bespreken de aanwezigheid van verschillende specifieke TLR genen, en veranderingen in hun expressieprofielen ten gevolge van infectie, in de context van verschillende vissen orden en families. We bediscussieren dat het onderzoeken van gen-expressieprofielen (in)direct bewijs voor betrokkenheid van een specifieke TLR in de reactie tegen een pathogeen oplevert en dat deze bevindingen relevant kunnen zijn als ze consistent voorkomen tussen verschillende soorten en families.

Vervolgonderzoek naar de conservering van de functie van *co-stimmulatoire* molekulen van het TLR-complex tesamen met TLR molekulen kan nieuw inzicht geven in de werkelijke functie van vissen TLRs. We onderzochten de aanwezigheid van bijbehorende molekulen in het genoom van vissen zoals beschreven in **HOOFDSTUK 7**. Onze bevindingen suggereren dat het genoom van vissen de meeste benodigde *co-stimmulatoire* eiwitten voor de functie van TLRs. We hebben een nieuw *co-stimmulerend* eiwit dat interacteert met TRL-4 met leucine-rich repeats (Tril) geïndentificeerd in zebravris en karper, dat recent beschreven is in zoogdieren als component van het TLR4 complex en belangrijk is voor activatie via TLR-3. Verder bespreken we de implicaties van de aanwezigheid van de meeste, maar niet alle, *co-stimmulatoire* eiwtitten van TLR-complexen voor de biosynthese en potentiële activatie van TLR-molekulen in vissen.

In **HOOFDSTUK 8** bediscussiëren we de effecten van β-glucanen op ons innate immuunsysteem in karpers en belichten de bevindingen van het NEMO consortium. Verder bespreken we welke receptoren in vissen mogelijk ß-glucanen kunnen detecteren door onze observaties te integreren met de bestaande recente literatuur. We stellen de hypothese voor dat de herkenning van β-glucanen in vissen mogelijk een samenspel van *phagocytic receptoren* zoals C-type lectin, *scavenger*-receptors and FcyR op het membraan van macrofagen nodig hebben om herkent te worden. Tot slot bediscussieren we de voordelen van het gebruik van twee diermodellen zoals karper en zebravis in het ophelderen van de rol van receptoren en hun rol in de herkenning van ß-glucanen en de start van activatie van het immuunsysteem door ß-glucanen als een voedings-ingrediënt.

SOMMARIO (Italian summary)

L' aumento degli allevamenti ittici e l' uso estensivo delle pratiche di acquacultura ha portato ad un incremento delle infezioni e delle diffusioni di malattie dovute alla presenza di diversi organismi patogeni negli allevamenti. Un dei problemi principali in acquacultura e' la rapida e incontrollata crescita dei patogeni all'intero delle vasche contenenti le specie ittiche. Per ridurre la presenza di questi organismi patogeni si e' ricorso ad un uso indiscriminato di antibiotici, l' abuso di antibiotici ha fatto si che gli stessi organismi patogeni diventassero resistenti agli antibiotici.

Percio', si e' reso necessario l' uso di strategie alternative e sostenibili per il controllo e la prevenzione di malattie ed epidemie in questo importante settore. L' aumento dell' uso dei vaccini, dei probiotici (inseriti nella dieta), o dei prebiotici e degli immunostimolanti ha ridotto la suscettibilita' delle specie ittiche alle infezioni. La produzione di mangimi-compatibili con la somministrazione di immunostimolanti, come per esempio i β-glucani, sono una delle possibili strategie utilizzate nella moderna acquacultura. Queste nuove strategie hanno ridotto notevolmente l' uso degli antibiotici ed hanno aumentato i meccanismi difensivi cellulari innati e umorali dei pesci; con conseguente riduzione delle infezioni epidemiche negli allevamenti. In questo contesto abbiamo introdotto nel **CAPITOLO 1** i β-glucani come modulatori dell' immunita' innata dei pesci riassumendo gli effetti e le ricerche portate avanti nel contesto ittico negli ultimi decenni. Molti di questi studi e ricerche nel campo ittico non sono state focalizzate agli effetti benifici dei β-glucani nella CARPA (nome scientifico *Cyprius carpio*); la carpa anche se non molto conosciuta in Europa occidentale e' utilizzata come risorsa alimentare ed e' la specie ittica piu' allevata nel mondo. In questo contesto, un progetto finanziato dalla comunita' EUROPEA chiamato "NEMO" (acronimo del nome NEtwork on immune MOdulation) e' stato formato per conoscere, sviluppare e sostenere l' uso dei β-glucani come immunostimolanti nella carpa. All' interno di questo consorzio il nostro obiettivo era individuare e misurare la modulazione (effetti) della risposta immunitaria innata dovuta all' uso dei β-glucani e la caratterizzazione dei recettori Pattern Recognition Receptors (PRR) presenti nei leucociti di carpa che potrebbero riconoscere e legare i β-glucani, attivando la risposta immunitaria. Il nostro primo studio riguardo l' uso dei β-glucani e' descritto nel **CAPITOLO 2**, in questo capitolo abbiamo analizzato gli effetti dei β-glucani nei leucociti di carpa e la conseguente attivazione della risposta immunitaria atttraverso la produzione ossigeni radicali "Combustione respiratoria" nei macrofagi e neutrofili granulocitari. L' attivazione del "Combustione respiratoria" porta alla produzione degli ossigeni radicali (ROS), i quali hanno il ruolo di eliminare i patogeni ed hanno anche la capacita' di rigenerare i tessuti. Abbiamo confrontato due metodi per quantificare la produzione di ROS durante la combustione respiratoria: (1) il primo metodo quello della riduzione del nitroblue tetrazolium (NBT) e (2) il secondo metodo quello del real-time luminol-enhanced assay basato su un processo chemioluminescente. Abbiamo dimostrato che il (1) NBT assay puo' rivelare solo la produzione di anioni superossidi, mentre il (2) real-time luminol-enhanced assay puo' rivelare la presenza sia degli anioni superossidi ma anche dell' idrossido d' ossigeno. In piu', il secodo metodo real-time luminol-enhanced assay puo' rivelare la produzione di ROS in tempo reale cosi' da fornire informazioni piu' dettagliate durante la combustione respiratoria. Al di la' di queste differenze, entrambi i metodi rendono possibile rilevare l' attivazione della risposta immunitaria della carpa attraverso la produzione di ROS all' interno dei leucociti di carpa. Nel **CAPITOLO 3,** abbiamo approfondito la produzione di ossigeni radicali ma anche la produzione di ossigeno nitrico (un importante radicale utilizzato come meccanismo difesivo) e studiato quali possibili recettori sono coinvolti nel riconoscimento dei β-glucani espressi nei macrofagi di carpa usando differenti tipi di β-glucani derivanti da diverse risorse. Per la nostra ricerca abbiamo utilizzato preparazioni di β-glucani particolati

derivati dal lievito di birra (comunemente utilizzato in cucina *Saccharomyces cerevisiae*) come ad esempio i composti **zymosan** e **MacroGard®** (questo ultimo utilizzato come principale ingrediente dei mangimi per animali d' allevamento agricolo ma anche ittico), questi due composti sono conosciuti nei mammiferi come attivatori dei recettori Toll-like-receptor 2 (TLR2) e del recettore Dectin-1. Oltre all' uso dei β-glucani particolati abbiamo utilizzato dei β-glucani conosciuti nei mammiferi per la capacita' di attivare solo il recettore Dectin-1, questi composti sono il **depleted-zymosan** (cioe' un composto derivato dallo zymosan ma trattato chimicamente per rimuovere la proprieta' stimolante dei recettori TLR) and altri β-glucani come **curdlan**, estratto da un altro lievito chiamato *Candida Albicans* e il **laminarin** estratto da una alga bruna la *Laminaria digitata* anche loro conosciuti come attivatori del recettore Dectin-1 ma non dei recettori TLR. Abbiamo misurato gli effetti di questi diversi β-glucani nei macrofagi di carpa quantificando la produzione di ossigeni radicali, ossigeno nitrico ma anche la variazione d' espressione genica delle citochine. I nostri risultati hanno suggerito che i macrofagi di carpa reagiscono fortemente ai β-glucani particolati con un incremento della produzione sia degli ossigeni radicali ma anche dell' ossigeno nitrico ed abbiamo osservato un incremento dell' espressione genica delle citochine come *il-1*β*, il-11* and *il-6.* Inoltre, abbiamo identificato due geni per *il-6* per la prima volta nella carpa. Per quanto riguarda i β-glucani selettivi per il recettore Dectin-1 abbiamo osservato un decremento della risposta immunitaria dei macrofagi di carpa, suggerendo che il riconoscimento dei β-glucani da parte dei macrofagi e' probabilmente dovuta dai recettori TLRs ma anche da diversi tipi di recettori non TLRs specifici.

Per comprendere quali recettori presenti nei macrofagi di carpa che potrebbero riconoscere i β-glucani, abbiamo ricercato e caratterizzato differenti tipi recettori: recettori TLRs (Tlr1, Tlr2, Tlr4 ma anche recettori che non sono presenti in mammifero come ad esempio Tlr20) alcuni recettori scavenger (come: Cd36 and Scarf-1) e i recettori C-type lectin (come ad esempio Illrs) all'interno del consorzio NEMO. In questa tesi abbiamo descritto con maggior enfasi i recettori Toll-like receptors (Tlrs) che riconoscono i patogeni attraverso il riconoscimento di particolari strutture molecolari conservate negli stessi patogeni (una sorta d' impronta digitale) chiamati Pathogen-associated molecular patterns PAMPs. I recettori TLRs possono essere divisi in 6 grandi famiglie, ognuna delle quali puo' riconoscere una particolare classe di microrganismi ad esempio batteri, virus, lieviti e parassiti. Tuttavia, nei pesci ossei moderni (Teleostei) il numero dei recettori TLRs e' maggiore di quello dei mammiferi. Le funzioni di molti di questi recettori sono ancora sconusciute e molti di questi recettori non sono ancora stati identificati. Nel **CAPITOLO 4** abbiamo descritto uno di questi recettori non presenti nei mammiferi, il recettore Tlr20. Sorprendentemente all'interno dei Teleostei il recettore Tlr20 e' stato solo scoperto, fino ad ora, in pochi ordini dei Teleostei nei carpiformi, siluriformi e salmoniformi. Abbiamo identificato l' intera sequenze nucleotidica (DNA) per sei geni del *tlr20* in zebrafish (pesce zebra) ed abbiamo identificato un unica sequeza nucleotidica nel genoma di carpa. Per meglio comprendere la funzione di questo recettore Tlr20 abbiamo predetto la struttura tridimensionale della proteina che ci ha rivelato un somiglianza a livello strutturale con il recettore TLR8 umano. Tuttavia, analizzando l'origine filogenetica abbiamo riscontrato un omologia con la famiglia del TLR11 che e' solo presente in topo (ma non negli altri mammiferi) questa omologia e' stata riscontrata nei recettori TLR11 e TLR12 che fanno parte della famiglia dei TLR11. Entrambi questi recettori nel topo, riconoscono un parassita protozoico chiamato *Toxoplasma gondii.* Un analisi dettagliata della conservazione dei geni adiacenti i geni del *tlr20* nel pesce zebra, non ci ha rivelato nessuna conservazione con i geni adiacenti i geni della famiglia TLR11 nei cromosomi di topo. Questo tipo di analisi dei geni adiacenti e' chiamata SYNTENY, cioe' la conservazione dei geni adiacenti o regioni fiancheggianti un gene d'interesse nei

cromosomi. Per meglio comprendere il ruolo del TLR20 in Teleostei abbiamo fatto un analisi con il microscopio confocale per localizzare in quale compartimento cellulare il recettore Tlr20 e' espresso. Questa analisi ci ha rilevato che il recettore Tlr20 e' espresso nel reticolo endoplasmatico e non nella membrana cellulare. Un sovraespressione del recettore Tlr20 di carpa *in vitro* nella linea cellulare umana opportunatamente ingegnierizzata per rilevare l' attivazione di un fattore di transcrizione chiamato NF-κB, non ci ha permesso di scoprire la funzione del recettore Tlr20 di carpa. Altri esperimenti condotti *in vivo* utilizzando carpe infettate con un parassita del sistema circolatorio chiamato *Trypanoplasma borreli* (questo parassita infetta naturalmente le carpe), e' stato utilizzato per dimostrare se il recettore Tlr20 di carpa fosse coinvolto nel riconoscimento o nella risposta immunitaria di questo parassita. Questi esperimenti hanno mostrato un aumento dell' espressione del gene *tlr20* di carpa. Ulreriormente abbiamo analizzato l' espressione del gene *tlr20* in diversi tessuti ed abbiamo osservato una maggiore espressione nei leucociti periferali del sangue PBL (peripheral blood leukocytes) e una maggiore espressione nelle cellule lifocitarie B. Stimolazione *in vitro* dei PBL con *T. borreli* induce un aumento dell' espressione del gene *tlr20* suggerendoci un ruolo nel riconoscimento del parassita da parte del recettore Tlr20 di carpa. Un altro recettore ristretto solo agli ordini dei carpiformi, siluriformi e salmoniformi, e' il Toll-like receptor 4 (TLR4) discusso nel **CAPITOLO 5.** Nei mammiferi, il TLR4 riconosce i batteri gram-negativi e piu' precisamente un composto presente nella membrana di questi batteri, che e' il lipopolisaccaride (LPS). LPS e' molto importante e studiato nei mammiferi, specialmente nell' uomo, perche' causa lo shock septico (che puo' portare anche alla morte). Il TLR4 di Teleostei, sembra non riconoscere LPS molto probabilemente perche' i Teleostei non hanno nel loro genoma importanti co-recettori CD14 e MD-2 necessari per il riconoscimento del LPS. Per meglio compredere il ruolo del Tlr4 nei Teleostei, abbiamo iniettato nelle larve dei pesci zebra mutanti (mutanti perche' privati dei geni *tlr4ba e tlr4bb*) un pericoloso batterio gram-negativo la *Salmonella thyphimurium,* sfortunatamente non abbiamo osservato nessuna risposta immunitaria che potesse confermare un riconoscimento o un ruolo attivo del Tlr4 durante l' infezione provocata da questo batterio. Quindi, abbiamo caratterizzato e clonato due geni putativi del *tlr4* nella carpa, entrambi questi geni sono espressi principalmente negli organi immunitari e espressi nelle cellule macrofagiche. La struttura tridimensionale del Tlr4 di carpa, ha confermato la possibilita' che il recettore possa interaggire con i co-recettori MD-2 e CD14 umani e con l' LPS. Inaspettatamente, studi *in vivo* condotti nelle carpe infettate con un virus chiamato spring viraemia carp virus (SVCV)(virus che naturalmente infetta le carpe) ci hanno mostrato un aumento dell' espressione genica del *tlr4*. Un sovraespressione del recettore *tlr4* di carpa nelle cellule umane ingenierizzate con il fattore di transcrizione NF-κB non ci ha rilevato alcuna attivazione del suddetto fattore di transrcizione. Inoltre, abbiamo localizzato il Tlr4 di carpa nella regione citoplasmatica e non nella mebrana cellulare, questo in parte potrebbe spiegare perche' non abbiamo osservato nessuna attivazione del fattore di trascrizione NF-κB. Ulteriori studi che abbiamo effettuato nel genoma di carpa e nel pesce zebra hanno rivelato la presenza di altri geni del *tlr4.* Nel **CAPITOLO 6,** abbiamo riassunto i risultati e le ricerche effettuate negli ultimi anni sui pesci, riguardo i recettori Toll-like receptors (TLRs). Abbiamo riscontrato variazioni dell' espressione genica dei recettori Tlrs come conseguenza di infezioni, queste variazioni di espressione sono conservate in un ampia gamma di specie e famiglie acquatiche. Abbiamo inoltre discusso la validita' delle variazioni di espressione geniche dei Tlrs in questi studi, ed abbiamo discussoche le variazioni di espressioni geniche possono essere prove (in)dirette o evidenze per un coinvolgimento dei recettori Tlrs; ma per comprendere e scoprire la funzione di un recettore occore effetuare studi funzionali diretti. Ulteriori studi nella conservazione e funzione dei co-recettori, in coniugazione

con recettori TLR, potrebbe portare alla scoperta di queste funzioni nei pesci. Per questo motivo abbiamo analizzato la presenza di differenti co-recettori nei genomi di diversi pesci Teleostei nel **CAPITOLO 7.** Il nostro lavoro ha dimostrato la presenza di molti dei co-recettori necessari per il funzionamento dei TLR, all' interno genomi dei dei Teleostei. In specifico, abbiamo caratterizzato nel pesce zebra e nella carpa un corecettore chiamato "Tlr4 interactor with leucine rich repeats (Tril)", che solo recentemente e' stato caratterizato nei mammiferi come componente necessario per il corretto funzionamento del complesso del TLR4 ma anche per l' attivazione del TLR3. In piu', abbiamo discusso il probabile ruolo, nei pesci, di questi corecettori necessari per la biosintesi e la potenziale attivazione dei recettori Tlrs. Nel **CAPITOLO 8,** abbiamo riassunto e discusso tutti i risultati ottenuti nei capitoli precedenti, integrando i nostri risultati con quelli presenti in letteratura. Abbiamo discusso gli effetti dei β-glucani sul sistema immunitario della carpa riassumendo tutti i risultati ottenuti dal consorzio NEMO. In piu', abbiamo proposto e discusso quali recettori protrebbero essere potenzialmente importanti o necessari per il riconoscimeto dei β-glucani. Abbiamo ipotizzato che il riconoscimento dei β-glucani nei pesci potrebbe richiedere l'interazione di diversi recettori come recettori fagocitici, i recettori C-type lectin, i recettori scavenger e i recettori FcγR. In conclusione , abbiamo discusso i vantaggi dell' uso dei due modelli animali il pesce zebra e la carpa per complementare ed ampliare le nostre conoscenze sui meccanismi del sistema immunitario dei pesci ma anche dei mammiferi, senza escludere l' importanza ne dell'uno e ne dell'altro modello animale. Infine, abbiamo discusso nella parte finale l' importanza degli studi funzionali utilizzando come esempio le linee cellulari opportunamente ingegnierizzate o costruzioni di chimere, questo potra' aiutarci in futuro a comprendere la funzione di un determinato Tlr.

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Curriculum Vitae

Danilo Pietretti was born on July 25th 1985 in Tarquinia (VT), Italy. He received his high school diploma at the "Instituto Magistrale Santa Rosa di Viterbo" in 2000 in Viterbo, and started his Bachelor course of Biology at Tuscia University in Viterbo, Italy in 2001. In his BSc thesis he studied auto-regeneration of stem cells and cancer cells, under supervision of Prof. Luigi Bosco and he graduated on Janury 2007. He started his Master course of Biotechonology at the same University and he graduated on Jauary 2009. His Master thesis was on a new system of PCR-array for analysis of transcriptome in a fish model Sea bass (*Dicentrarchus Labrax*), under the superviosion of Prof. Giuseppe Scapigliati, where his passion for fish immunology has started.
In March 2009, he started with his PhD project at the Cell Biology and Immunology group of Wageningen University, under supervision of Prof. Geert F. Wiegertjes and Prof. Huub F. J. Savelkoul. His PhD project was part of intra-European traning network (ITN) on "protective immune modulation in warm water fish by feeding glucans", the scientific aim focused on the development of a sustainable and cost-effective use of β-glucan (MacroGard®) as immune-stimulant in aquaculture. His task was on the effects of β-glucan on carp leukocytes and characterization of possible receptors candidates for recognition of the β-glucan. During his PhD he had a great opportunity to collaborate and visit for four months Prof. Victor Mulero at the Murcia University (Universidad de Murcia) where he conducted his studies on Toll-like receptors using as animal model zebrafish. Within the European network he collaborated tightly with Dr. Michael Engelbrecht Nielsen and Dr. Natalia IvonneVera-Jimenez on production of oxygen radicals and nitrogen radicals. The results of the PhD research are described in this thesis.

Publications

Published

- Accessory molecules for Toll-like receptors in Teleostfish. Identification of TLR4 Interactor with Leucine-rich Repeats (TRIL). **Pietretti D.**, Spaink H., Falco A., Forlenza M. and Wiegertjes G.F. Mol Immunol.2013 Dec 31;56(4):745-56.

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Accepted

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In preparation

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- β-glucan supplemented diets increase TLR3 gene expression in common carp (*Cyprinus carpio*). Falco A. , Miest J. , Pionnier N., **Pietretti D.** , Forlenza M. , Wiegertjes G.F. and Hoole D.

EDUCATION CERTIFICATE

Issue by WIAS

EDUCATION and TRAINING	year	credits
WIAS Introduction Course	2009	1.5
Ethics and Philosophy in Life Sciences	2011	1.5
SCIENTIFIC EXPOSURE		
New Frontiers in Pattern Recognition, (NCMLS):	2009	0.6
European Organisation of Fish Immunology EOFFI	2010	1.4
Dutch Society for Immunology NVVI	2010	0.6
European Association of Fish Pathologists (EAFP)	2011	1.2
European Macrophage and Dendritic Cell Society (EMDS)	2011	0.9
International Society for Development & Comparative Immunology (ISDCI)	2012	1.5
International Society Fish and Shellfish Immunology (ISFSI)	2013	1.2
PRESENTATIONS		
European Organisation of Fish Immunology EOFFI, (poster)	2010	1.0
Dutch Society for Immunology NVVI, (poster)	2010	1.0
Fish Immunology Workshop, (poster)	2010	1.0
European Association of Fish Pathologists (EAFP), (oral)	2011	1.0
European Association of Fish Pathologists (EAFP), (poster)	2011	1.0
European Macrophage and Dendritic Cell Society (EMDS), (poster)	2011	1.0
International Society for Developmental & Comparative Immunology (ISDCI), (oral)	2012	1.0
Fish Immunology Workshop, (oral)	2013	1.0
WIAS Science day (oral)	2013	1.0
International Society Fish and Shellfish Immunology (ISFSI), (oral)	2013	1.0
SEMINARS and WORKSHOPS		
WIAS seminar: Of fish and men: curiosities of immune system	2009	0.2
WIAS seminar: It makes sens to know your enemy	2010	0.2
WIAS seminar: Allergeniticy in food allergy	2011	0.2
WIAS seminar: Mucosal factor regulating allergy	2013	0.2
WIAS science DAY	2013	0.3

IN-DEPTH STUDIES		
Advanced visualisation, integration and biological interpretation of omics data	2009	1.0
Advanced Immunology Training Course,	2010	1.5
Fish Immunology Workshop,	2010	1.5
Three-dimensional protein structure and its determination,	2010	1.0
Elisa course	2010	0.15
Flow Cytometry, BD Biosciences,	2012	0.6
Generic and scientific traiing received on the use of immunomodulators in carp aquaculture	2012	0.3
APC's revisited - NVVI	2012	0.3

PROFESSIONAL SKILLS SUPPORT COURSES		
Project management I NEMO,	2009	0.6
Project management II NEMO	2010	0.3
Intercultural awareness NEMO	2010	0.3
Industrial R&D strategies	2010	0.3
Writing grant proposals NEMO	2011	0.6
Training in Research Presentations at NEMO meetings	2009-2012	1.5
Time management, assertion skills and effective decision making, NEMO	2011	0.3

RESEARCH SKILLS TRAINING		
External training period Murcia, Spain Sep 2011-Feb 2012	2011-2012	2.0

DIDATIC SKILLS TRAINING		
Supervising practicals Fish Workshop, Wageningen, April	2011	0.6
Supervising practicals Fish Workshop, Wageningen, April	2012	0.6
MSc major thesis - Thu Nguyen Thi Anh	2010	2.0
BSc thesis - Mikolaj Babiak	2012	1.0
NEMO Young Scientists' board	2010	1.0

EDUCATIONAL AND TRAINING TOTAL		**39.0**

Design and layout by Danilo Pietretti

The front shows a three-dimensional modelling of carp Toll-like receptor 4, structural model was obtained using the amino acid sequence alignment of carp Tlr4bb with human TLR4 and dimer structure of human TLR4 (PDB-id: 3fxi) as template using the Modeller program.(Chapter 5 of this thesis)

The back shows the expression of the carp Toll-like receptor 20 in human and fish cell lines, the pictures ware obtained in a confocal microscope (Chapter 4 of this thesis).

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