Targeting the innate immune system to develop novel prophylactic strategies: lessons from amphioxus (B. lanceolatum) and zebrafish (D. rerio)

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Abstract

Immunization through vaccination is one of the most effective strategies to control infectious diseases. However, effective vaccines and alternative prophylactic tools for many fish diseases are still lacking. More studies on basic and applied immunology are required to improve the prevention and control of diseases in aquaculture. In this context, the thesis presents both basic and applied research. The Toll-like receptors (TLRs) are important for raising innate immune defense and their ligands are used as vaccine adjuvants to improve the immune responses. We studied the TLR system in the amphioxus *B. lanceolatum*. We identified 28 new putative TLR genes which consist in both non-vertebrate- and vertebrate-like TLRs. We cloned one of these genes, Bl_TLRj. The phylogenetic analysis together with functional analysis showed that it clusters with TLR11 family and particularly with subfamily 13. Moreover, Bl_TLRj responded against viral stimuli and showed high sequence identity with fish TLR13 and TLR22. Second, we developed two different infection models in zebrafish and we tested two potential nanoparticle adjuvants, IBs$^{TNF\alpha}$ and NL$_c$. The IBs$^{TNF\alpha}$ are a highly stable, non-toxic, and low-cost protein-based biomaterial formed with nano-structured trout tumor necrosis factor alpha cytokine. Via oral intubation of adult zebrafish, combining flow cytometry, histology, and confocal microscopy, we show that IBs$^{TNF\alpha}$ are able to cross the intestinal mucosal epithelial barriers, pass through the lamina propria, and reach the muscle layer. The expression of innate immune-related genes was significantly up-regulated in zebrafish intestine. Finally, IBs$^{TNF\alpha}$ could protect zebrafish against a *Mycobacterium marinum* lethal infection when i.p. injected. The second particle tested, NL$_{c,}$ was previously developed in our lab and is composed by nanoliposomes encapsulating LPS and Poly I:C. The NL$_c$ was tested in our *M. marinum* bacterial infection model and it could protect zebrafish against a lethal infection when i.p. injected. Next, we explored the infective possibilities of two fish pathogens, *M. marinum* and *Aeromonas hydrophila,* in zebrafish larvae by immersion. The mortality of zebrafish larvae immersed with *M. marinum* showed no significant differences but zebrafish larvae infected with *A. hydrophila* by immersion showed significant differences compared to controls in a dose-dependent manner. NL$_c$ and IBs$^{TNF\alpha}$ localized in the pharynx and intestine of zebrafish larvae at 3 and 5 dpf, respectively. The expression of immune-related genes such as IL-1β and IRF1α was significantly up-regulated after 48 h treatment with NL$_c$ in 2 dpf larvae. The 5 dpf larvae immersion in IBs$^{TNF\alpha}$ could not significantly alter immune-related gene expression and IBs$^{TNF\alpha}$ could not protect zebrafish larvae against *A. hydrophila* lethal infection.

Resum

La vacunació és una de les estratègies més efectives de control de les malalties infeccioses. Tot i així hi ha una clara falta de vacunes eficients o d'eines profilàctiques efectives per moltes especies de peixos d'interès comercial. Es necessiten més estudis de recerca bàsica i aplicada per millora la prevenciIBs$^{TNF\alpha}$) i els liposomes miniaturitzats NL$_c$. Els IBs$^{TNF\alpha}$ són altament estables, no toxics, i són un tipus de biomaterial proteic amb un baix cost de producció. Mitjançant intubació oral de peixos zebra adults i l'ús combinat de citometria, histologia i microscòpia confocal hem demostrat que els IBs$^{TNF\alpha}$ poden atravessar l'epiteli de la mucosa intestinal, passar per la lamina propria i arribar a la capa muscular subjacent. A més l'expressió de gens relacionats amb la resposta innata està significativament regulada a l'alça en intestins de peix zebra. Finalment hem demostrat que els IBs$^{TNF\alpha}$ poden protegir al peix zebra d'una infecció per *Mycobacterium marinum*. D'altra banda el sistema de nanoliposomes encapsulant LPS i Poly(I:C) o NL$_c$, desenvolupats previament al laboratory, també protegeix al peix zebra contra una infecció letal de *M. marinum*. També hem explorat la viabilitat d'utilitzar *M. marinum* i *A. hydrophila* per desenvolupar un model d'infecció en larves de peix zebra. El model d'infecció de *M. marinum* no és viable ja que no podem induir mortalitats per inmersió; però el model amb *A. hydrophila* ha demostrat ser adequat ja que la mortalitat de les larves és depenent de la dosi infective *d'A. hydrophila*. Els NL$_c$ i els IBs$^{TNF\alpha}$administrats per inmersió els localitzem a la faringe i l'intestí de les larves de peix zebra a dia 3 i 5 post fertilització. L'expressió de gens de resposta immune es veu regulada a l'alça després del tractament amb NL$_c$. Encanvi no observem una expression a l'alça de gens inmunes després del tractament amb IBs$^{TNF\alpha}$ i això correlaciona amb el fet que els IBs$^{TNF\alpha}$ no protegeixen d'una infecció letal per *A. hydrophila*.

Contents

Introduction

Aquaculture and disease

Aquaculture is the farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants. Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated (1). Nowadays, aquaculture supplements with the wild capture fisheries, feed more than 7 billion people in the context of climate change, economic and financial uncertainty, and growing competition for natural resources (1) (2) (3). Fishery by the capture of wild fish is the major source of fish in the past and the production is relatively static since the late 1980s. In the other hand, aquaculture has increased significantly in the last few decades. Whereas aquaculture provided only around 7 million tonnes of production for human utilization in 1980, this quantity had increased to 57 million tonnes in 2005 and 106 million tonnes in 2015 (Figure 1) (4). Only in 2015, the total aquaculture production was estimated first-sale value of US$163.0 billion, consisting of US$67.5 billion of freshwater fishes, US$10.2 billion of marine fishes, US$17.9 billion of molluscs, US$4.8 billion of aquatic plants, US$38.5 billion of crustaceans and other aquatic products (US$24.1 billion) (4). Almost all fish produced from aquaculture are destined for human consumption, although by-products may be used for non-food purposes (2). In general, aquaculture has the potential to make a significant contribution to the increasing demand for aquatic food in many regions of the world.

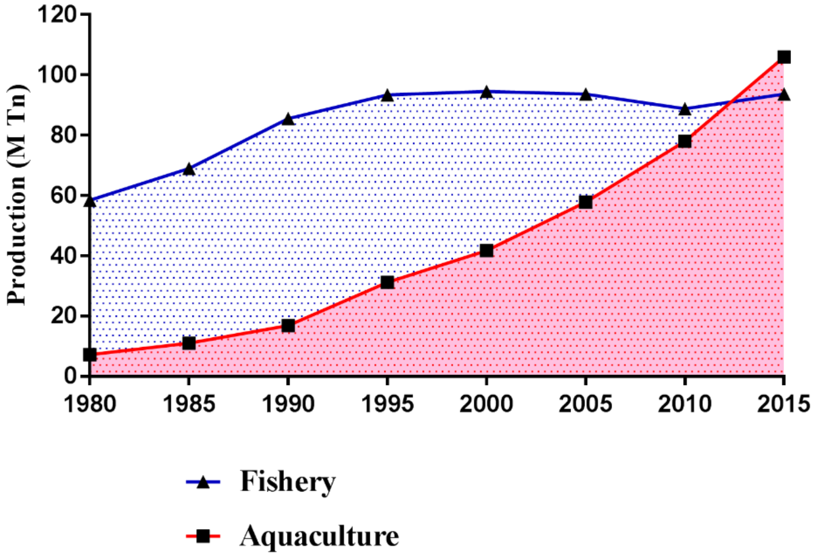


Figure 1. **World aquaculture and fishery production**

By 2014, a total of 580 species and/or species groups were farmed around the world. These species items include 362 finfishes (including hybrids), 104 molluscs, 62 crustaceans, 6 frogs and reptiles, 9 aquatic invertebrates, and 37 aquatic plants. Despite the large number of farmed species, the majority of total aquaculture production output relies on several dozens of species led by the farming of carps, barbels and other members of the cyprinid family. In order to optimize the cost-benefit ratio, high density stocking, artificial feeds and fertilization of the pond water have become common husbandry practices in both fresh water and marine aquaculture. However, modern intensive and semi-intensive aquaculture practices are extremely vulnerable to the pollution and disease outbreaks. Infectious disease is a major problem as water-borne pathogens can spread at very fast rates and transmit diseases across vast geographic regions. When combined with the crowded conditions of the aquaculture facilities and the warmer water temperatures, it provides ideal conditions for disease outbreaks. Disease outbreaks cost the global aquaculture industry some US$6 billion per year and represent the major farm-level risk (2) (5) (6) (7).

Thousands of viral, bacterial, protozoan, fungi, and metazoan parasites in the aquatic environment can cause infectious diseases in aquaculture. The most common bacterial pathogens in aquaculture are *Aeromonas*, *Pseudomonas*, *Vibriosis*, *Edwardsiella*, *Flavobacterium*, *Streptococcus*, and *Mycobacteria* (8). On the other hand, viral diseases are more difficult to control due to the lack of therapeutics. The main fish virus are aquabirnavirus, betanodavirus, infectious salmon anemia virus (ISAV), infectious hematopoietic necrosis virus (IHNV), epizootic hematopoietic necrosis virus (EHNV), and viral hemorrhagic septicemia virus (VHSV) (9). In addition, the most common parasites of fish are protistans and metazoan which infect gills, skin, and internal organs. For instance, *Ichthyophthirius multifiliis*, belonging to ciliates, invades epithelial tissue of gills, skin, or fins, leaving a small wound and visible white spot or nodule (10). *Dactylogyrus intermedius*, belonging to monogeneans, cause gill inflammation, excessive mucous secretions, accelerated respiration, and secondary bacterial infections (11). Several drugs, synthetic chemicals and vaccination have been in practice to prevent and control the diseases, but only partial success has been achieved. Although, antibiotics can overcome bacterial diseases, consumer health and food safety issues prevent their use in aquaculture (12). Moreover, it is not possible to treat the viral diseases once they established. Therefore, there is an urgent need to develop alternative approaches or improve the existing strategies in the prevention and control diseases in aquaculture.

Vaccination is a crucial disease management strategy developed in aquaculture against the lethal infection of bacteria, parasite and virus. Vaccination is a process by which the immune system is induced in aquatic animals by administration of vaccines. Vaccines are various preparations of antigens derived from specific pathogenic organisms that are rendered non-pathogenic. They stimulate the immune system and increase the resistance to disease from subsequent infection by the specific pathogen. There are various vaccine administration methods. The most frequently used are injection, immersion, and oral administration. Oral vaccination with the antigen mixed in the feed is the ideal method of vaccine delivery to fish. It is time and labor-saving and avoids any manipulation stress. Therefore, much effort has been put into the development of such vaccines. However, poor and inconsistent responses have been reported by conventional oral vaccines due to antigen destruction in the gut (13) (14). Some approaches to protect the antigen from degradation, such as nanoparticle encapsulation or entrapment with adjuvants (15) (16), have made some promising results. However, a large quantity of antigen is usually necessary and the protection achieved is generally weak and of short duration. Therefore, the two main methods of vaccine delivery to fish are still, immersion in a diluted suspension of the vaccine or injecting it into the body cavity (typically by intramuscular, intradermal or subcutaneous injection). Immersion vaccines are effective for a number of bacterial pathogens and are cheap, easy to administer to small fish. However, large quantities of vaccines are always needed and the protection is always weak (17). In contrast, injection is labor intensive and requires the fish to be over a certain size, making vaccination of larvae or fry difficult. Nevertheless, many fish vaccines today are multicomponent injection vaccines. The advantages of injection vaccines are that the volume of vaccine needed is relatively low and that every fish is vaccinated with the correct dose (18). The first report of disease prevention using vaccine was reported by Duff in 1942. The vaccine protected immunized trout against *A. salmonicida* infection by parenteral inoculation and oral administration (19). Up to date, fish vaccines are commercially available against many of serious bacterial diseases, a few of viral diseases, and none of parasitic disease (Table 1) (18).

Table 1. **Common fish diseases and vaccines**

	Pathogen	**Major fish species affected**	**Primary region(s)/ country(s)**	**Commercially available?**
Bacteria	Vibriosis (*Listonella anguillarum* and V. spp.)	Salmonids, Cod, Halibut, Seabass, Seabream, Amberjack, Yellowtail	Globally	Yes
	Coldwater vibriosis (*Vibrio salmonicida*)	Salmonids	Northern Europe, Canada, USA	Yes
	Wound disease (*Moritella viscosa*)	Salmonids	Northern Europe	Yes
	Furunculosis including carp Erythrodermatitis/Ulcer disease (*Aeromonas salmonicida subsp.salmonicida*)	Salmonids, carp	Northern Europe, Canada, USA, Asia	Yes
	Atypical *Aeromonas salmonicida*	Salmonids/ Various freshwater and seawater species	Globally	Yes/no
	ERM/Yersiniosis (*Yersinia ruckeri*)	Salmonids freshwater	Northern Europe, Chile,Canada,USA	Yes
	Piscirickettsiosis (*Piscirickettsia salmonis*)	Salmonids	Chile	Yes
	Bacterial gill disease (*Flavobacterium branchiophilum*)	Various species, e.g., salmonids and carp, freshwater	Canada, USA, Europe, Chile, Japan	No
	Flavobacteriosis (*Flavobacterium psychrophilum*)	Salmonids freshwater	Chile, Canada/ USA(West)	Yes
	Columnaris (*Flavobacterium columnare*)	Channel catfish/Salmonids freshwter	USA/Chile	Yes/yes
	Rainbow trout fry syndrome (*Flavobacterium psychrophilum*)	Salmonids freshwater	Europe, Canada, USA, Chile	No
	Enteric septicaemia of catfish (*Edwardsiella ictaluri*)	Catfish species	USA/Asia	Yes/no

	Edwardsiella septicaemia (*Edwardsiella tarda*)	Channel catfish/Eel, Japanese flounder	USA/Asia	No/no
	Bacterial kidney disease (*Renibacterium salmoninarum*)	Salmonids	Chile, Canada, USA/ Europe, Japan	Yes/no
	Lactococciosis (*Lactococcus garvieae*)	Rainbow trout/Amberjack, yellowtail	Italy, France, UK/Japan	Yes/yes
	Pasteurellosis (*Photobacterium damsela subspecies piscicida*)	Sea bream, sea bass/Amberjack, yellowtail	Mediterranean/Japan	Yes/no
	Streptococciosis (*Streptococcus iniae*) (*Streptococcus phocae*) (*Streptococcus agalactiae*)	Tilapia/Asian sea bass/Salmonids	Asia/Asia/Chile	Yes/no/no
	Dropsy (*Aeromonas hydrophila*)	Indian carps	India	Unknown
Virus	Infectious pancreatic necrosis/IPNV, other aquatic birnaviruses	Salmonids/Various marine species	Globally	Yes/no
	Pancreas disease/PDV	Salmon	UK, Ireland, Norway	Yes
	Infectious salmon anemia/ISAV	Salmonids	Canada, USA(East), Norway, UK	Yes
	Infectious hematopoietic necrosis/IHNV	Salmonids	Canada,USA (West)	Yes
	Viral hemorrhagic septicemia/VHSV	Rainbow and brown trout, turbot, Japanese flounder	Europe, Asia	No
	Viral nervous necrosis/SJNNV and several other betanodavirus	Several marine fish species, e.g., sea bass, groupers, barramundi, halibut	Globally	No
	Iridoviral disease/RSIV	Red sea bream, amberjack, yellowtail	Asia	Yes
	Channel catfish virus disease/CCV	Channel catfish	USA	No
	Spring viremia of carp: /SVCV	Mostly carp species	Europe	No
	Grass carp hemorrhage disease/GCHDV	Grass carp	China	Yes
	Koi herpesvirus (CyHV-3)	Common carp and KOI	Asia, South America	Yes
Parasite	Amoebae *Paramoeba* spp. (Amoebic gill disease)	Salmonids	Europe, Asia, America, Australia	No

Flagellates, Cryptobia salmositica/Ichthyobodo spp.	Salmonids/Various fish	North America/Globally	No
Ciliates *Ichthyophthirius multifilis* (White spot disease)/*Cryptocaryon irritans*/*Trichodina* spp.	Freshwater fish/Seawater fish/Various fish	Globally	No
Microsporidia *Tetramicra brevifilum*/*Pleistophora anguillarum*/*Nucleospora salmonis*	Turbot/Japanese eel/Salmonids	Europe/Japan/North America	No
Myxosporeans *Myxobolus cerebralis* (Whirling disease)/*Tetracapsula bryosalmonae* (proliferative kidney disease; PKD)/*Kudoa thyrsites*	Salmonids freshwater/Salmonids/Salmonids	Europe, North America/ Europe/North America (West coast)	No
Monogeneans *Gyrodactylus* spp./*Dactylogyrus* spp./*Benedinia* spp.	Various fish	Globally/Globally/South East Asia	No
Cestodes *Eubothrium* spp.	Salmonids, seawater	Europe	No
Crustaceans *Lepeophtheirus salmonis/Caligus* spp.	Salmonids/Various fish	Europe, North America/ Globally	No

CCV: Channel catfish virus; GCHDV: Grass carp hemorrhage disease virus; IHNV: Infectious hematopoietic necrosis; IPNV: Infectious pancreatic necrosis virus; ISAV: Infectious salmon anemia; PDV: Pancreas disease virus; RSIV: Red sea bream iridovirus; SJNNV: Striped jack nervous necrosis virus; SVCV: Spring viremia of carp virus; VHSV: Viral hemorrhagic septicemia virus. (Updated from (18))

Fish immune system

Vaccinology includes different disciplines and immunology is one of them. There are two types of immunity in the host fighting against infections: innate and adaptive. The innate immune system, which mainly consists of dendritic cells, macrophages, and neutrophils, is genetically programmed to detect invariant features of invading microbes. In contrast, the adaptive immune system, which is composed of T and B lymphocytes, employs antigen receptors that are not encoded in the germ line but are generated de novo in each organism (20). Innate immune system is the first line of defense against infectious disease (21). Immediately after infection, innate response is mediated to kill the pathogens and concurrently to synthesize inflammatory mediators and cytokines. Next, components of the pathogen are presented to T cells, resulting in the activation of the adaptive immune response and the establishment of protective immunity (22). Thus, the innate immune system can fight against a wide range of pathogens and mount a rapid response. In opposite, the adaptive immune system is conferred specificity to pathogen and provides a long-term protection. They are complementary to each other.

From an evolutionary point of view, innate immune system plays a critical role in all metazoans including invertebrate and vertebrate whereas the adaptive immune system is only seen within vertebrates (23). The innate immune system is more crucial in invertebrates and low vertebrates than mammals because the adaptive immunity of low vertebrates is not as diversified. For example, there are three classes of immunoglobulins, M, D, and Z/T in teleost but five categories (M, D, G, A, and E) in mammals, and the major histocompatibility complex (MHC) is not present in all teleost, like Atlantic cod (*Gadus morhua*), but in mammals (24) (25). Most of the mechanisms of innate immunity are conserved between invertebrates and vertebrates. The major biological host defense systems of invertebrates, including hemolymph coagulation system, pro-phenoloxidase (pro-PO) activating system, lectin-complement system, agglutinin-lectin system, anti-bacterial, -fungal, and -viral systems mediated by Toll-like receptors and peptidoglycan binding protein (PGBP), reactive oxygen-producing system, phagocytic system, are also found in mammals (26) (21). Moreover, both invertebrates and vertebrates respond to microbial surface antigens like lipopolysaccharides (LPS), lipoteichoic acids, lipoproteins, peptidoglycan (PGN) and (1,3)-β-D-glucans (26). Adaptive immunity aroses early in vertebrate evolution, 500 million years ago in jawed fish (27), more accurate, 450 million years ago between the divergence of cyclostomes (lampreys) and cartilaginous fish (sharks) (Figure 2) (28). Low vertebrates are on the crossroad between invertebrates and mammals, between innate immune response and adaptive immune response. Therefore, the study of innate and adaptive immunity in low vertebrates could provide new insights in the understanding of the evolutionary history of immune system.

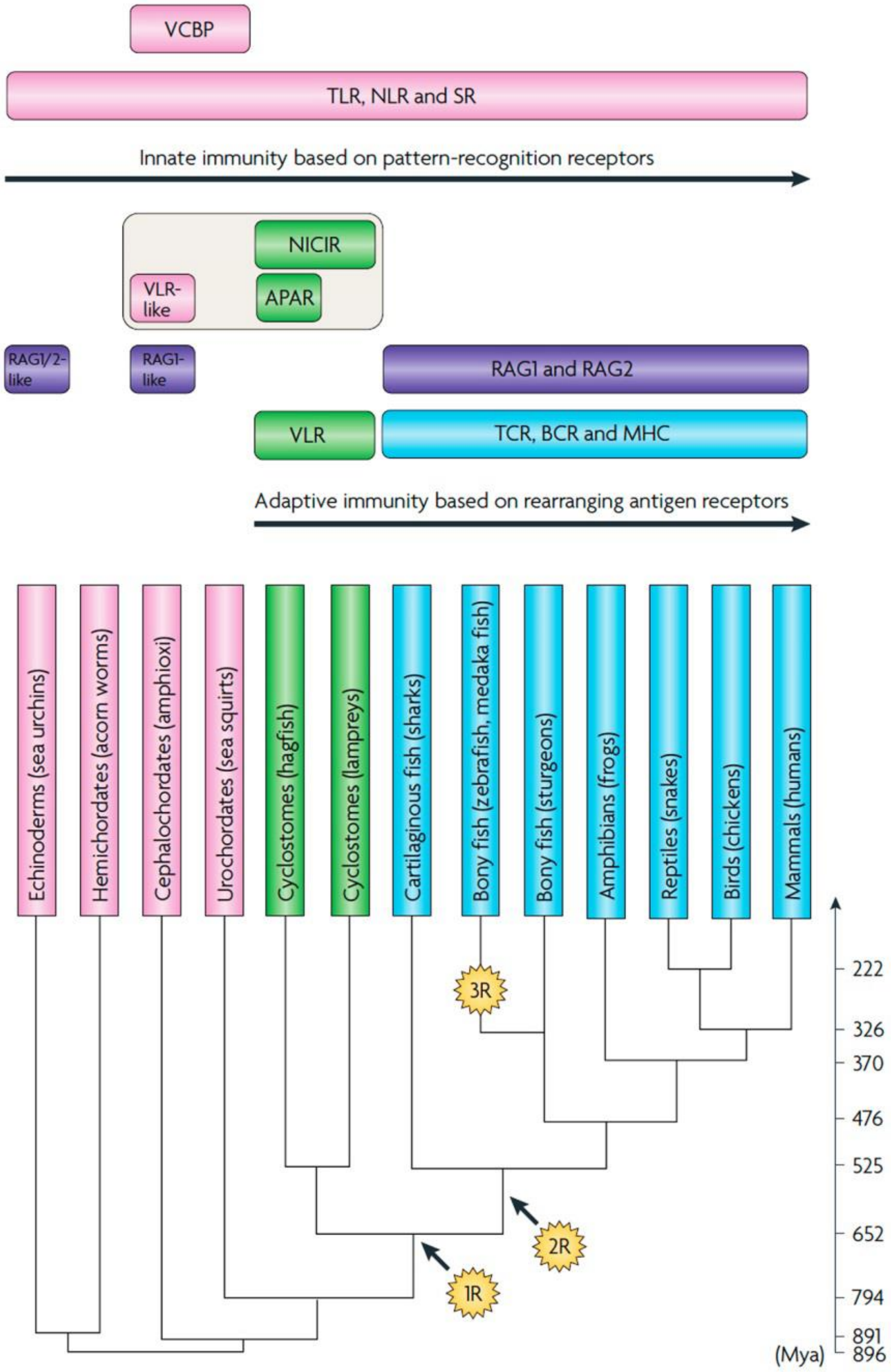


Figure 2. **Overview of the evolution of the immune system in deuterostomes.** Molecules restricted to jawed and jawless vertebrates are indicated in blue and green, respectively. Molecules that emerged at the stage of invertebrates are in pink. Recombination-activating gene (RAG)-like genes (indicated in purple) are of viral or bacterial origin (from the transib transposon family) and are also present in the genomes of sea urchins and amphioxi. Agnathan paired receptors resembling antigen receptors (APAR) and novel immune receptor tyrosine-based activation motif-containing immunoglobulin superfamily receptor (NICIR, also known as T cell receptor (TCR)-like) are agnathan immunoglobulin superfamily (IgSF) molecules that are thought to be related to the precursors of TCRs and B cell receptors (BCRs).

1R and 2R indicate the two rounds of whole-genome duplication (WGD). Whether the 2R, the second round of WGD, occurred before or after the divergence of jawed and jawless vertebrates is controversial. The divergence time of animals (shown in Mya (million years ago)) is based on Blair and Hedges (29). MHC, major histocompatibility complex; NLR, Nod-like receptor; SR, scavenger receptor; TLR, Toll-like receptor; VCBP, V-region containing chitin-binding protein; VLR, variable lymphocyte receptor (27)

Innate immune system

Innate immune system is generally divided into two parts, the cellular and humoral responses. Cellular responses, include the physical barrier such as mucus and epithelial tissues on the surfaces of mucosa-associated lymphoid tissues (MALT), and specialized cells (like monocytes/macrophages, granulocytes and nonspecific cytotoxic cells), play directly role of fighting against invasion microorganisms. Humoral responses, on the other hand, employ a variety of proteins and glycoproteins capable of destroying or inhibiting growth of infectious microorganisms, which include among others anti-bacterial peptides, proteases, complement, transferrins and the antiviral myxovirus resistance-1 protein (Mx1) (30). In teleost, the mucosae are the first barrier against infection because it directly contacts with the microbial-rich water environment. The important of mucus in the innate immune response has been studied in several fish species (31) (32) (33). Beside of being the physical barrier, mucosae are also active immunological sites against microbes and stressors (34). Phagocytosis is an important process in cellular response by which the phagocytes engulf diverse particulate targets. Expert or professional phagocytes in fish are monocytes/macrophages and granulocytes. Nevertheless, in recent years phagocytic properties have also been attributed to teleost dendritic cells, lymphocytes and thrombocytes (35) (36). Macrophages are the first cells that encounter non-self-material, especially bacteria, and engulf and degrade them by using hydrolytic enzymes and oxidative attack (35). Regarding granulocytes, neutrophils have a crucial role in the host tissue protection by engulfing the microbes by phagocytosis and secreting antimicrobials (37). Dendritic cells (DCs) are specialized antigen presenting cells that bridge innate and adaptive immunity in mammals. The mammalian homologous DCs were identified in some fish species (zebrafish, rainbow trout, and Atlantic salmon) (38) (39) (40).

The innate immune recognition is based on non-specific recognition of conserved motifs on pathogens. This recognition relies on a variety of germline-encoded pattern recognition receptors (PRRs) and the conserved motifs were proposed calling pathogen-associated molecular patterns (PAMPs), which are essential for microbial survival and are structurally conserved among many microorganisms (41) (42) (43). To date, most of PRRs can be classified into one of five families based on protein domain homology, they are Toll- like receptors (TLRs), C-type lectin receptors (CLR), Nucleotide-binding domain, leucine-rich repeat (LRR)-containing (or NOD-like) receptors (NLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and the AIM2-like receptors (ALRs) (44). These families can be separated into two main classes: membrane-bound receptors and unbound intracellular receptors. The TLRs and CLRs, which are found at the cell surface or on endocytic compartments, belong to the former group. They survey for the presence of microbial ligands in the extracellular space and within endosomes. The NLRs, RLRs, and ALRs form the latter group, they are located in the cytoplasm and survey for the presence of intracellular pathogens (45). Various PAMPs, including lipoprotein, lipopolysaccharide (LPS), Flagellin, peptidoglycan, cyclic dinucleotides (CDNs) from bacteria and double-stranded RNA (dsRNA), uncapped single-stranded RNA (ssRNA) and DNA from virus can be recognized by these PRRs. The recognition of PAMPs by PRRs enhances phagocytosis, activates complement cascades, triggers inflammatory cytokine production, and induces dendritic cell maturation (46).

Cytokines

Cytokines are a family of low molecular weight proteins that are often glycosylated and are secreted by activated immune-related cells upon induction by various pathogens such as parasitic, bacterial, or viral components (47). They can modulate immune responses through an autocrine or paracrine manner upon binding to their corresponding receptors. Cytokines are derived from macrophages, lymphocytes, granulocytes, DCs, mast cells, and epithelial cells, and can be divided into interferons (IFNs), interleukins (ILs), tumor necrosis factors (TNFs), colony stimulating factors, and chemokines (48). Cytokines act as modulators of the immune responses in related to both innate and adaptive responses. In particular, pro-inflammatory cytokines, including interleukin-1β, TNF-α, and IL-6 are commonly used immune-regulatory genes in fish (49) (50) (51) (52).

Interleukin-1β is a member of the β-trefoil family of cytokines. In mammals it is produced as an inactive precursor molecule that is processed by interleukin converting enzyme (ICE) to give a biologically active 'mature' peptide. IL-1β could be secreted by monocytes, activated macrophages, granulocytes, endothelial cells, activated T lymphocytes, and many other cell types after activation of host pattern recognition receptors (PRRs) by pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs) (53) (54) (55). IL-1β genes have been identified in various teleost fish species, including rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*), sea bass (*Dicentrarchus labrax*), channel catfish (*Ictalurus punctatus*), and yellowfin sea bream (*Acanthopagrus latus*) (56) (57) (58) (59) (49). IL-1β has diverse physiological functions and its roles in regulating the inflammatory process are conserved in fish (60). To date, most in vivo studies have been focused on the transient and local effects of IL-1β on the immune system. For example, Two IL-1β genes were identified in catfish (*I. punctatus*) and both genes may play different roles in anti-bacterial response (59).

TNFα is a member of the β-jellyroll family of cytokines which including the well-known TNF, Fas ligand, CD27 ligand, CD40 ligand, and TNF related apoptosis inducing ligand (TRAIL) (61) (62). Most of them are produced as a type II transmembrane protein or glycoprotein which share a conserved extracellular C-terminal domain called the TNF homology domain (THD). The THD binds to the cysteine-rich domain (CRD) of their corresponding receptors to initiate appropriate biological responses (63). Evidence gathered to date demonstrates that multiple TNF homologues/paralogues exist, which can be categorized into three phylogenetic groups, the type I and II TNF-α group and the TNF-N group (64) (65). TNF fulfills its functions by interacting with its specific receptors. The TNF receptor (TNFR) family members are classified into three groups: TNF receptor-associated factor (TRAF) binding receptors, death domain (DD)-containing receptors, and decoy receptors (66) (67) (68). These receptors binding to their own adaptors result in the activation of NF-κB and activator protein-1, which in turn leads to apoptosis, inflammation, or cell survival. As a pro-inflammatory cytokine, TNF-α is one of the early immune genes expressed at an early stage of infection in fish and has a key role in regulating inflammation. Like its mammalian counterparts, fish TNF-α displays overlapping functions with IL-1β. Many fish TNF-αs have been produced in bacteria as monomers, dimers and trimers and are able to activate macrophages/phagocytes and enhance their microbial killing activity (69) (70) (71). The TNF-α protein enhances the phagocytic activity of fish leucocytes (72). In *M. marinum* infected zebrafish, TNF-α has been shown to promote macrophage survival and also restrict bacterial growth in infected macrophages (73). Fish TNF-αs are suggested to be involved in the regulation of leucocyte homing, proliferation, migration (70). TNF-α activates chemokine expression in local tissue cells such as endothelial cells in zebrafish and gilthead seabream (74). In addition, TNF-α is associated with pathogenesis of several chronic diseases in fish (75) (70) (76).

Interferons (IFN) are cytokines that play a major role in the defense against virus infection of vertebrates. They are classified into three groups with different structures and functions (e.g. type I IFNs, type II IFNs, and type III IFNs) and interact with different cell-surface receptors. Type I IFNs consist of about 20 members, including IFN-α, IFN-β, IFN-ω, IFN-κ, IFN-ε, and limitin, while both type II and type III IFNs only have one member called IFN-γ and IFN-λ, respectively (77) (78). Type I IFNs have been characterized from many fish, as well as putative IFN receptor genes and Mx protein (79) (80). Both Type I and type II IFNs are exist in fish and play important roles in immune responses especially participate in anti-viral defense (81) (82). In addition, type II fish IFN may also be involved in bacterial immune responses (83). In the classical IFN pathways, the type I IFN receptor can trigger rapid phosphorylation and activation of receptor-associated JAKs upon binding to type I IFNs, which in turn activate STAT complexes and allow them to form into homodimers or heterodimers. Activated STATs can translocate into the nucleus and associate with IRFs, followed by attachment of the complexes to ISREs in the promoters of interferon-stimulated genes (ISGs) to induce ISG expression, which ultimately exert host antiviral effects (84). In addition to the classical pathway, there is emerging evidence showing that non-STAT pathways also play important roles in the signaling of IFN-responses (85).

TLRs

Toll-like receptors (TLRs) are type-I transmembrane proteins consist of extracellular leucine-rich repeat (LRR) motifs, transmembrane (TM) domains, and intracellular Toll/interleukin-1 receptor (TIR) domains (Figure 3) (86). The ectodomain, which mediating the recognition of PAMPs, is arranged by tandem LRRs (87). The TIR domain is present in the cytoplasmic region and required for downstream signal transduction (88). Upon PAMPs recognition, TLRs recruit TIR domain-containing adaptor proteins such as MyD88 and TRIF, which initiate signal transduction pathways that culminate in the activation of NF-κB, IRFs, or MAP kinases to regulate the expression of cytokines, chemokines, and type I interferons (IFNs) (Figure 4) (89). To date, ~10 TLRs were identified in mammals (10 in human and 12 in mouse). These TLRs localize to the cell surface or to intracellular compartments such as the ER, endosome, lysosome, or endolysosome and recognize distinct or overlapping PAMPs. For instance, TLR2 along with TLR1 or TLR6 recognizes a variety of PAMPs including lipopeptides from bacteria, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, zymosan from fungi, tGPI-mucin from *Trypanosoma cruzi* and the hemagglutinin protein from measles virus (88). TLR3 homodimer recognizes double-stranded RNA (dsRNA) on the cell surface (90). TLR4 recognizes bacterial lipopolysaccharide (LPS) (91). TLR5 recognizes bacterial flagellin (92). TLR7 and TLR8 mediate the recognition of single-stranded RNA (ssRNA) (93); TLR9 homodimers recognize agonistic unmethylated CpG-containing DNA in the bacteria (94). TLR10 is pseudogene in mouse due to an insertion of a stop codon, but human TLR10 collaborates with TLR2 to recognize ligands from listeria (95). Mouse TLR11 and TLR12 were reported that can recognize profilin from *Toxoplasma gondii* by forming homo- and hetero-dimers on endosomal membrane (96) (97); TLR13 in mice recognizes a conserved 23S ribosomal RNA (rRNA) in bacteria (98). Therefore, vertebrate TLRs can be divided into six families according to the protein structure and the ligands of human and mouse TLRs (99) (Table 2).

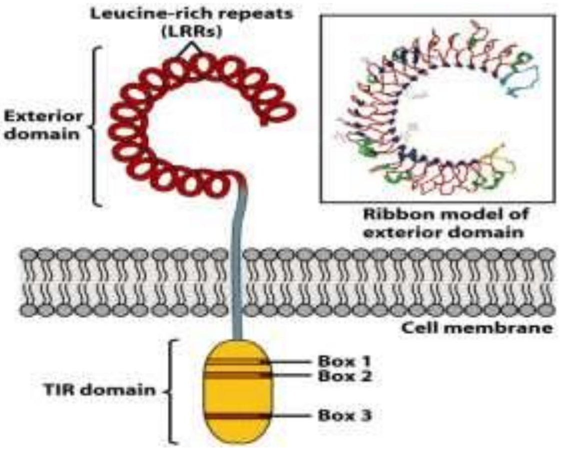


Figure 3. **Basic structure of TLR molecule** (100)**.**

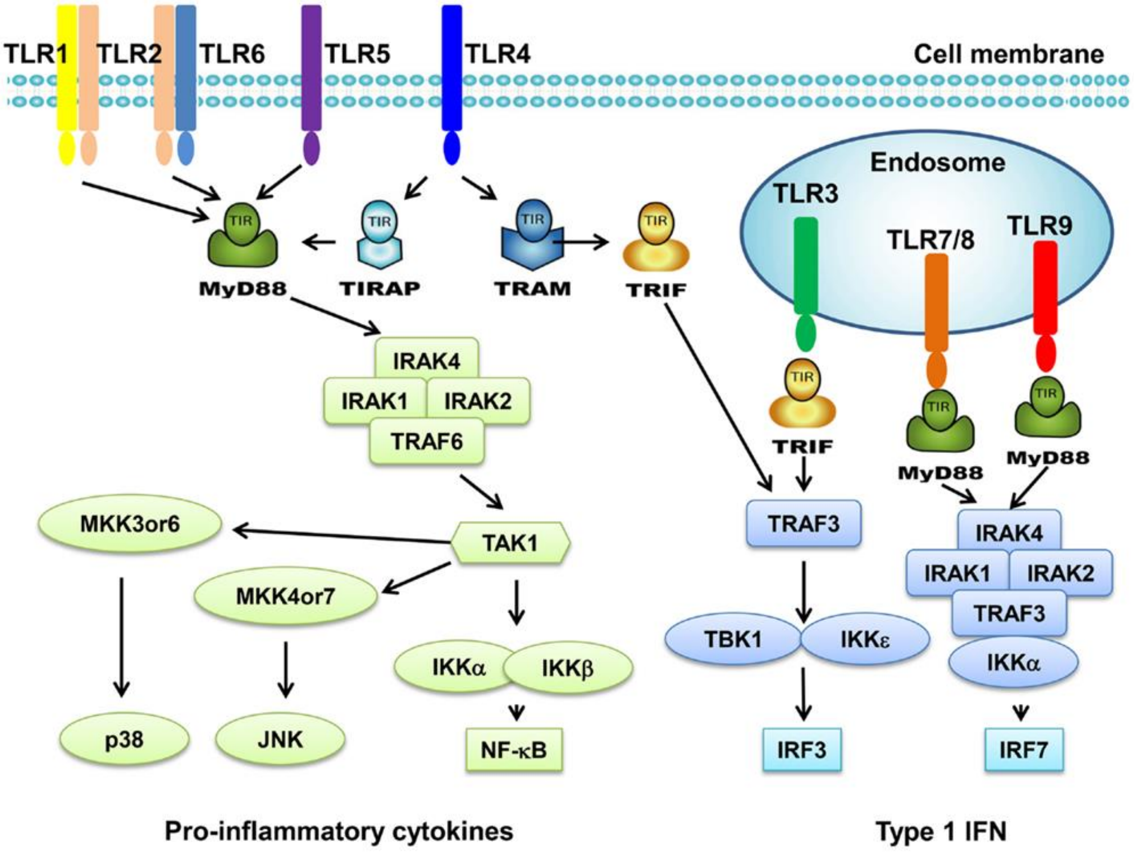


Figure 4. **Toll-like receptors and TLR-mediated signaling pathways.**

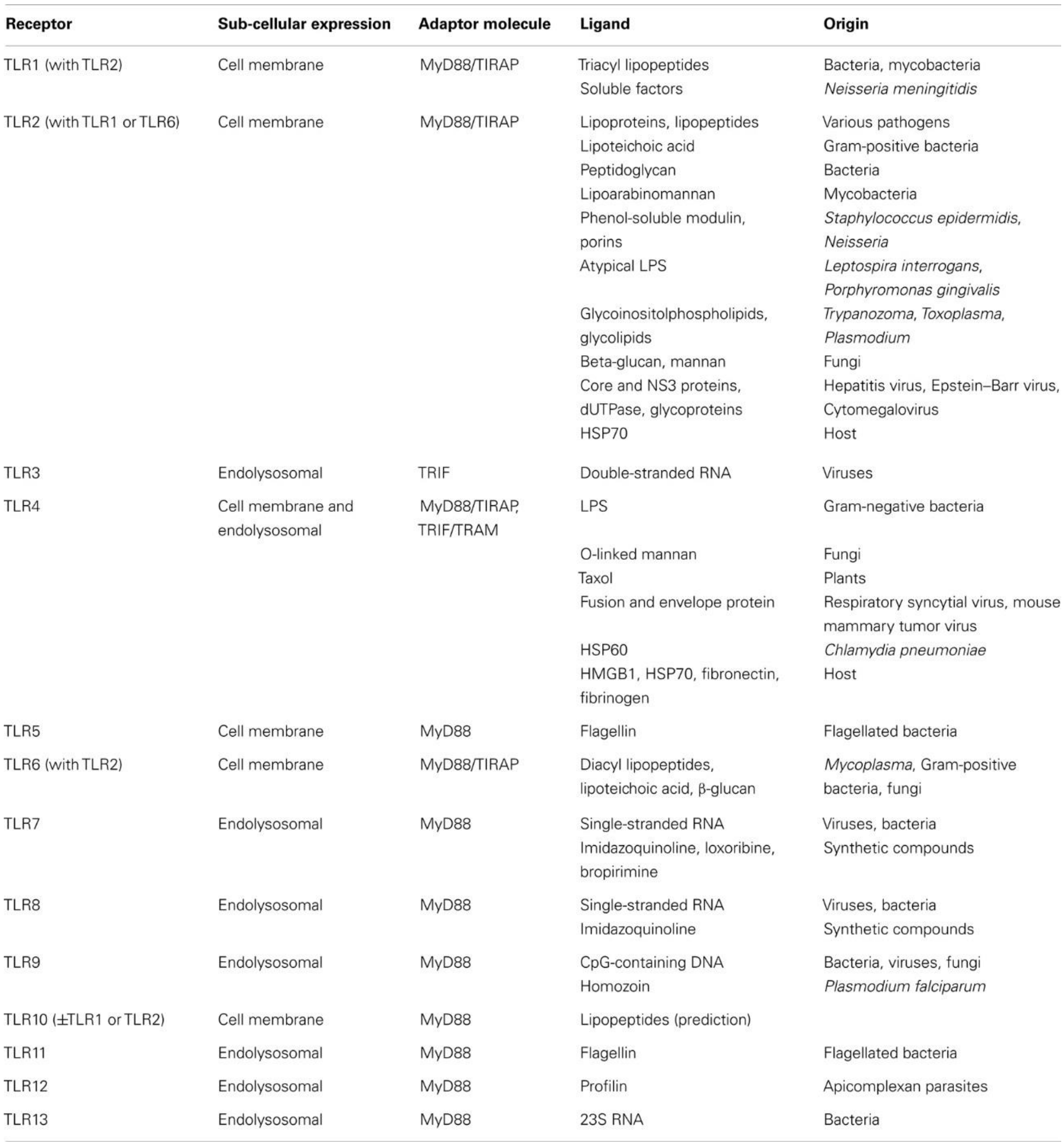
TLR1 and TLR6 recognize their ligands as heterodimers with TLR2. For TLR4, MD2, and CD14 are required for LPS recognition and signaling. TLR3, TLR4, TLR5, TLR7, and TLR9 are currently thought to deliver their signal by forming homodimers after interacting with their ligands. TLR3, TLR7/8, and TLR9 are intracellular TLRs and are involved in the recognition of nucleic acids. Most TLRs, except for TLR3, signal through MyD88 pathway to activate NF-κB and AP1. TLR3 and TLR4 can signal through MyD88-independent pathway (TRIF pathway) to activate INF-β (101).

At least 16 TLR types were discovered in teleost by draft genome sequences and genomic research of five bony fish species (102). The higher number of TLRs are probably due to the whole genome duplication, especially the fish-specific genome duplication (FSGD) occurred later in a basal teleost (103) (104). At least one gene in teleost representing each of six major families hypothesized (but does not demonstrate) the high conservation of TLRs in vertebrate (105), although there are changes through evolution. For instance, Human TLR4 recognizes LPS, the major component of the outer membrane of Gram-negative bacteria, together with myeloid differentiation protein-2 (MD-2) and CD14 (106) (91). In contrast, fish can tolerate relatively high concentrations of LPS and it is explained by the absence of TLR4 (107). However, TLR4 is not absent from all fish genomes. To date, TLR4 has been cloned and characterized in zebrafish (*D. rerio*), rare minnow (*Gobiocypris rarus*), common carp (*C. carpio*), grass carp (*Ctenopharyngodon idella*) and channel catfish (*I. punctatus*) (105). Nevertheless, the ligand of these fish TLR4 most likely would not be LPS and the recognition pathway remains uncharacterized. For example, zebrafish TLR4 lacking the responsiveness to LPS was most likely due to the inability of the extracellular portions. This was proved by using chimeric molecules of zebrafish TLR4 proteins fused with the mouse TLR4 (108). Catfish TLR4 was reported to lack the important structural features that are critical for the LPS recognition of TLR4 in mammals (103). In human, TLR2 recognizes bacterial lipopeptides and lipoteichoic acids as well as glycosylphosphatidylinositol anchors from parasites when forms heterodimers with TLR1 or TLR6 (109). However, the functional studies of TLR2 in different fish species have provided evidence that TLR2 recognition in fish are different and more complex than mammals. For example, activation of common carp macrophages by peptidoglycan (PGN), a bacterial ligand, resulted in (moderate to low) up-regulation of tlr2 gene expression (110). Whereas, the study of blue catfish and channel catfish infected with *E. ictaluri* showed that tlr2 gene expression was down-regulated in head kidney, but a modest up-regulation in spleen (111). In orange-spotted grouper, tlr2 gene expression found increased in spleen upon stimulation with LPS and poly I:C, or upon injection with *V. alginolyticus* (112). Gene expression of TLR2 was also up-regulated in grouper infected with the parasite *C. irritans*, with significant changes in skin, gill but also head kidney and spleen (113). The studies in flounder established a link between fish TLR2 and recognition of viral rather than bacterial ligands (114) (115). Rainbow trout, channel catfish, and flounder have both a membrane and soluble form of TLR5 that senses bacterial flagellin but human only has a membrane form (105). More number of TLRs were found in most of fish species than mammals so far, the existence of six ‘non-mammalian’ TLR types were reported (116) and two new TLR types were discovered in channel catfish recently (117). TLR19 was first identified in zebrafish (118) and in channel catfish which was involved in anti-*I. multifiliis* immune defence (119). The expression of grass carp toll-like receptor 20.2 fluctuated in head kidney cells stimulated by LPS, FLA-ST and poly(I:C) (120). The expressions of TLR20a and TLR22 were up-regulated when zebrafish *in vivo* infected with *M. marinum* (118). The large yellow croaker TLR21 could specifically recognize three CpG-oligodeoxynucleotides but not poly(I:C), LPS, and LTA-SA (121). The expressions of the TLR21 gene was induced to up-regulate in isolated peripheral blood lymphocytes of yellow catfish after stimulation with LPS, PGN, and Poly I:C (122). Peptidoglycan and Poly(I:C) were found to induce the expression of the TLR22 gene in Japanese flounder (123). The expression of rainbow trout TLR22 was induced by the Gram-negative bacterium *A. salmonicida in vitro* (124). The pufferfish TLR22 seems to be located on the cell surface recognizing long dsRNA sequences (125). TLR22 of common carp was highly expressed when fish challenged with poly(I:C) or *A. hydrophila* (126). The Fugu TLR23 was reported that it may participate in LPS recognition (127). TLR24a was identified in lamprey (*Lethenteron japonicum*) and appeared to be transiently down-regulated after Poly I:C stimulation (128). It is reported that TLR25 of grass carp is located in the endosome and could response to virus and bacteria stimulation (129). TLR26 was only found in channel catfish and the ligand of this TLR is currently unknown (117).

Adaptive immune system

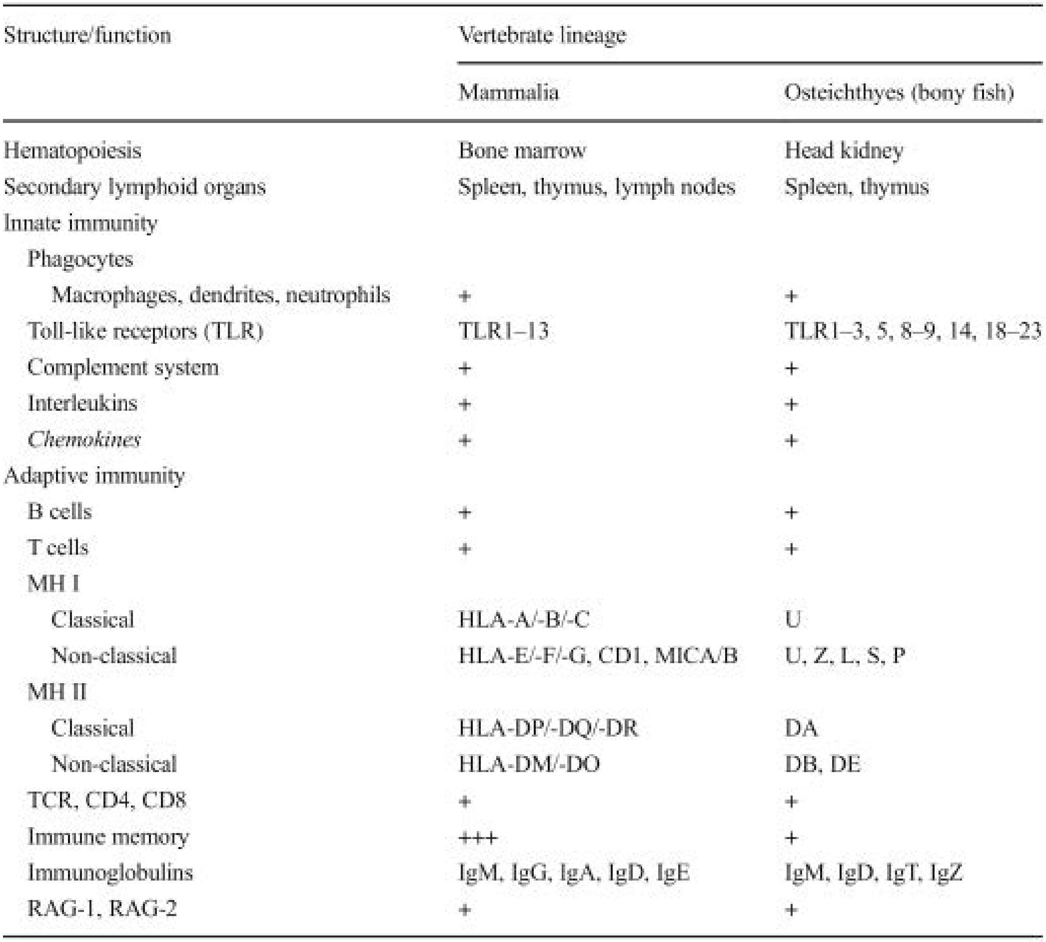
The innate and adaptive immune responses are closely related, as the innate immune responses play an instructive role in the development of acquired immune response. If pathogens successfully evade through the innate immune system, the adaptive immune response is triggered. Adaptive immune system is activated by the innate response and more adapted by its response during an infection to improve its recognition of the pathogen and retainability of response in the form of an immunological memory. While innate immunity is common to all animals, the adaptive immune system has long been considered a key innovation associated with the origin of vertebrates (131). The adaptive immune systems of the earliest gnathostomes (jawed fishes) exhibit a remarkable similarity to those of higher mammals including humans, and contain a full complement of immunoglobulins (Ig), T cell receptors (TCR), and major histocompatibility complex (MHC) molecules associated with a highly specific immune response and immune memory (Table 3) (132). All jawed vertebrates possess the genetic elements essential for the functioning of the adaptive immune response (133). The adaptive immune system (AIS) consists of Ag-recognizing lymphocytes, immunoglobulins (Abs and Ig-family TCR), MHC products, and recombination-activating (RAG) 1 and 2 genes. Adaptive immunity plays a vital role in protection against recurrent infections by generating memory cells (cell-mediated immunity) and specific soluble and membrane-bound receptors (humoral immunity) such as T-cell receptors and immunoglobulin (Ig) (28).

Table 2. **Mammalian Toll like receptors and their ligands** (130).



Receptor	**Sub-cellular expression**	**Adaptor molecule**	**Ligand**	**Origin**
TLR1 (with TLR2)	Cell membrane	MyD88/TIRAP	Triacyl lipopeptides Soluble factors	Bacteria, mycobacteria *Neisseria meningitidis*
TLR2 (with TLR1 or TLR6)	Cell membrane	MyD88/TIRAP	Lipoproteins, lipopeptides Lipoteichoic acid Peptidoglycan Lipoarabinomannan Phenol-soluble modulin, porins Atypical LPS Glycoinositolphospholipids, glycolipids Beta-glucan, mannan Core and NS3 proteins, dUTPase, glycoproteins HSP70	Various pathogens Gram-positive bacteria Bacteria Mycobacteria *Staphylococcus epidermidis*, *Neisseria* *Leptospira interrogans*, *Porphyromonas gingivalis* *Trypanozoma*, *Toxoplasma*, *Plasmodium* Fungi Hepatitis virus, Epstein–Barr virus, Cytomegalovirus Host
TLR3	Endolysosomal	TRIF	Double-stranded RNA	Viruses
TLR4	Cell membrane and endolysosomal	MyD88/TIRAP, TRIF/TRAM	LPS O-linked mannan Taxol Fusion and envelope protein HSP60 HMGB1, HSP70, fibronectin, fibrinogen	Gram-negative bacteria Fungi Plants Respiratory syncytial virus, mouse mammary tumor virus *Chlamydia pneumoniae* Host
TLR5	Cell membrane	MyD88	Flagellin	Flagellated bacteria
TLR6 (with TLR2)	Cell membrane	MyD88/TIRAP	Diacyl lipopeptides, lipoteichoic acid, β-glucan	*Mycoplasma*, Gram-positive bacteria, fungi
TLR7	Endolysosomal	MyD88	Single-stranded RNA Imidazoquinoline, loxoribine, bropirimine	Viruses, bacteria Synthetic compounds
TLR8	Endolysosomal	MyD88	Single-stranded RNA Imidazoquinoline	Viruses, bacteria Synthetic compounds
TLR9	Endolysosomal	MyD88	CpG-containing DNA Homozoin	Bacteria, viruses, fungi *Plasmodium falciparum*
TLR10 (±TLR1 or TLR2)	Cell membrane	MyD88	Lipopeptides (prediction)	
TLR11	Endolysosomal	MyD88	Flagellin	Flagellated bacteria
TLR12	Endolysosomal	MyD88	Profilin	Apicomplexan parasites
TLR13	Endolysosomal	MyD88	23S RNA	Bacteria

Table 3. **Key components of innate and adaptive immunity in mammals and bony fish** (132) (134)**.**



Structure/function	Vertebrate lineage	
	Mammalia	Osteichthyes (bony fish)
Hematopoiesis	Bone marrow	Head kidney
Secondary lymphoid organs	Spleen, thymus, lymph nodes	Spleen, thymus
Innate immunity		
Phagocytes		
Macrophages, dendrites, neutrophils	+	+
Toll-like receptors (TLR)	TLR1–13	TLR1–3, 5, 8–9, 14, 18–23
Complement system	+	+
Interleukins	+	+
Chemokines	+	+
Adaptive immunity		
B cells	+	+
T cells	+	+
MH I		
Classical	HLA-A/-B/-C	U
Non-classical	HLA-E/-F/-G, CD1, MICA/B	U, Z, L, S, P
MH II		
Classical	HLA-DP/-DQ/-DR	DA
Non-classical	HLA-DM/-DO	DB, DE
TCR, CD4, CD8	+	+
Immune memory	+++	+
Immunoglobulins	IgM, IgG, IgA, IgD, IgE	IgM, IgD, IgT, IgZ
RAG-1, RAG-2	+	+

In the jawed vertebrates, T and B lymphocytes are the acknowledged cellular pillars of adaptive immunity. T lymphocytes are primarily responsible for cell-mediated immunity, and B lymphocytes are responsible for humoral immunity, but they work together and with other types of cells to mediate effective adaptive immunity (135). Specificity of recognition, memory of previous antigen encounter and lack of self-reactivity are the physiological hallmarks of T lymphocytes. These features are acquired during the maturation of T cells, which takes place in the thymus and later on in secondary lymphoid organs (spleen, lymph nodes and mucosal-associated lymphoid tissue) (136). T cells are categorized into two general populations according to their function, cytotoxic T cells (CTLs) and helper T (Th) cells. CTLs express CD8 molecules involved in the interaction with MHC class I, while helper T cells express CD4 that interacts with MHC class II. Specificity of recognition is achieved by the T cell receptor (TCR). Like mammals, two main functionally distinct lineages of T cells exist in jawed vertebrates, one expressing an αβ TCR and the other expressing a γδ TCR (137). Cartilaginous fishes are an exception to this rule, as they have been found to additionally express several unique types of antigen receptors [NAR-TCR, a variant δ chain (138), and chimeric Ig/TCR forms (139)] whose exact roles in adaptive immunity remain to be established. CTLs and Th cells are present in many fish species and have been identified as

$CD8^+$ and $CD4^+$ cells using monoclonal antibodies (140). Th cells assist other cells such as B cells and macrophages, CTLs kill virus-infected cells and transplanted allogeneic cells and tissues. B cells express antigen receptors on their cell surface as B cell receptors (BCRs) and upon antigen encounter can secrete them as immunoglobulins (Igs or antibodies). The immunoglobulins, which are the principal indicators of acquired immunity to pathogens have evolved toward producing highly diversified molecules that recognize a remarkably large number of different antigens. Resistance to and recovery from infections are the results of complex interactions between non-specific and specific defence mechanisms (141). Recent evidence supports the existence of at least two functionally and genetically distinct B cell lineages in fish. Teleost B lymphocytes primarily present immunoglobulin (Ig) of the IgM class, with a heavy chain that is quite similar to the mammalian mechanism (142). Recently a further two heavy chain isotypes have been identified, IgD and IgZ/T (143) (144) (145). IgD is thought to be located in the cell membrane of B cells, where it might act as a receptor. IgZ/T-expressing lymphocytes are thought to constitute the B cell type associated with the intestinal immune system or mucosal immunity (146). The lymphocytes mediate the acquired immunity to reinfection and affects mainly through antibodies which can neutralize viruses, facilitate phagocytosis of pathogens through opsonisation, and activate classical complement (147).

MHC (major histocompatibility complex) are proteins which initiate the generation of adaptive immunity to pathogens after T cells detect intracellular pathogens and displayed on the cell surface. The MHC (major histocompatibility complex) receptors are immunoglobulin superfamily member proteins that are responsible for the recognition and presentation of foreign antigens (148). MHC loci have traditionally been subdivided into classical loci and non-classical loci (149). Classical MHC class I molecules (MHC I) are located on all nucleated cells and are activated following the binding of antigens synthesized within the host (e.g., viruses). These proteins are then presented on the cell surface to cytotoxic CD8 T cells (CTLs), which become activated and destroy the infected cell, a process known as cell-mediated specific immunity (150). MHC I molecules can also recognize and present exogenous antigens to cytotoxic T cells through a process known as cross presentation (151). Classical MHC class II molecules (MHC II), in contrast, are restricted to professional antigen-presenting cells (APCs). The presentation of pathogen-derived antigens by MHC II on the surface of APC B cells and phagocytes is essential to the elicitation of CD4 T cell (Th) binding (i.e., humoral immunity), which activates B cell differentiation into plasma cells, producing antibodies specific to the invading pathogen, and memory cells, preserving a record of past infection. Th cells themselves then differentiate into effector cells, which activate B cells to produce cytokines and memory cells. Memory B and Th cells allow the body to respond more rapidly to secondary infection, promoting a higher affinity and accelerated immune response, and providing enhanced immunoprotection (152). These MHC molecules are highly polymorphic in nature, both types of MH receptors, i.e. class I (for intracellular pathogens) and class II (for extracellular pathogens) are present in teleost. (153). The functions of the MHC I were involved in anti-virus responses regulated by type I IFN in grass carp (154). The role of MH molecules as APCs has been noticed (155). Although MHC II and the related humoral immunity have been sequenced in several fish species (156) (157), both gadiform (cod and its allies) and syngnathiform fishes (seahorses, pipefish, and seadragons) have independently lost classical MH II loci challenge, suggesting that teleost may have evolved alternative mechanisms of immune protection.

Altogether, teleost fish have a diversity in their innate and adaptive immune system. There are relevant differences between fish and mammals, for instance fish possess many more TLRs but less immunoglobulin types (158). Future studies on fish immune molecules could help to understand structural and functional aspects of mammalian immune system, as well as being key to the development of protective measures such as vaccines for diseases in aquaculture.

Mucosal immunity

Mucosal immunity is the study of immune responses occurred on the mucosal surfaces. When the mucosal barriers aware the danger signals, an immediate innate immune response is triggered. Next, the highly specific adaptive immune system is established by T and B lymphocytes (20). The innate and adaptive immune systems are both present in mucosal barriers. At these barriers, epithelial cells and antigen-presenting cells (dendritic cells and macrophages) constitute physical and innate defense systems while B and T lymphocytes form a dynamic network for the induction and regulation of secretory antibodies and cytotoxic T lymphocyte (CTL) responses (159). The mucosal immune system of vertebrates comprises a unique array of innate and adaptive immune cells and molecules that act in concert to protect the host against pathogens. Fish live in aquatic environments where fish continuously expose to a microbial-rich environment. This situation may pose additional challenges to the mucosal immune system of aquatic vertebrates versus their terrestrial counterparts. As a consequence, some of the principles of mammalian mucosal immunity may not be necessarily applicable to aquatic vertebrates (160). Therefore, the study of fish mucosal immunity appears to be extremely important. In teleost, the main mucosa-associated lymphoid tissues (MALT) are the gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), the gill-associated lymphoid tissue (GIALT) and the recently discovered nasopharynx-associated lymphoid tissue (NALT) (161).

Mucus is one of the most important innate defense mechanisms at mucosal surfaces. Mucins, ions, and lipids construct the mucus as an ideal niche for microbiota. Mucus composition determines its adhesiveness, viscoelasticity, transport and protective capacity. The study of composition and structure of intestinal mucins in carp (*C. carpio*) reveals that fish mucins are similar with mammalian mucins

(162). The mucin-encoding genes (Muc) identified in carp (Muc2 and Muc5B) also showed highly similarity to mammalian counterparts (163). Carp Muc2 is mostly expressed in fish intestine while mammalian Muc2 is secreted from goblet cells residing in the epithelial lining into the lumen of the large intestine. Carp Muc5B is mostly expressed in the skin (163). In mammals, Muc5B is a major contributor to the lubricating and viscoelastic properties of whole saliva, normal lung mucus and cervical mucus (164). Interesting, carp skin mucus appear to shift the compositions in response to the increase of bacterial load in the water (165). Similarly, the gut mucus characteristics changed upon myxozoan parasite infection in seabream (*Sparus aurata*) (166). From another study, Atlantic salmon and brown trout (*Salmo trutta*) both exhibit a whole-body mucus response to amoebic gill disease (AGD), whereas rainbow trout (*O. mykiss*) exhibits only a local response in the gills (167).

Fish mucosal secretions also carry a wide range of innate immune molecules including cytokines, complement proteins, and antimicrobial peptides (AMPs) (168). Current opinion of cytokines in mucosal immunity is that commensals induce the expression of anti-inflammatory cytokines such as TGF-β whereas pathogens trigger pro-inflammatory responses such as IL-1β and IL-17 (169). Complement system is important in killing pathogens in the mucosal surfaces. In mammals, the complement is responsible for several functions including the modulation of adaptive immune responses, the promotion of inflammatory reactions, the elimination of apoptotic and necrotic cells and most importantly, the destruction of pathogens. Moreover, the complement protein C3 is essential for the regulation of intestinal tolerance and thereby for the establishment of commensals (170). All three complement activation pathways (classical, lectin and alternative pathways) as well as the cytolytic pathway are present in teleosts (171) (172). *I. multifilii* infection could increase the expression of a factor B-homologue in carp skin and C3 in the gill of rainbow trout (173) (174). In zebrafish, expression of some complement genes was slightly induced in the skin (C3-2, C8b, B/C2-A1, B/C2-B, MASP2, I) and gills (C1q, C4, C3, C6, C7, B/C2-A1, B/C2-B) after stimulation with poly I:C (175). C3, C8, C9 and factor B were induced in the skin of zebrafish infectied with *Citrobacter freundii* (176). C6 was up-regulated in the gut but down-regulated in the skin of grass carp after challenge with *A. hydrophila* (177). Fish, like mammals, produce different AMPs that shaped the composition of microbiota on the mucosal sufaces and limit the extent of microorganism colonization (178). Teleost skin is a major source of AMPs with approximately 70% of all AMPs expressed, compared to around 52% and 29% expressed in the gills and the gut, respectively (160). The molecular and structural studies of teleost AMPs are starting to be coupled to investigations on their specific role in mucosal immunity and their effects on commensals and pathogens.

On mucosal surfaces, epithelial cells are the first sensors of commensals and pathogens due to its structural characteristics. Epithelial cells express PRRs including lectins, nod- like receptors (NLRs) and toll-like receptors (TLRs). In fish, as in mammals, intestinal epithelial cells express intestinal alkaline phosphatase. This enzyme could dephosphorylate and detoxify LPS and prevent intestinal inflammation in response to the resident microbiota in the gut of zebrafish (179). Intestinal epithelial cells (enterocytes) are also known to be responsible for antigen uptake in teleosts (180). In addition, rainbow trout skin epithelial cells were demonstrated to play a role in innate immune response by phagocytizing or clearing bacteria from the skin epidermis (181). Mast/eosinophilic granule cells (EGCs) are also believed innate immune cells in mucosal immunity. In fish, mast cells/EGCs are most abundant in the gills, gut and skin. It was reviewed that mast cells/EGCs were recruited to the inflammation sites in many teleost mucosal tissues (182). Additionally, mast cells responded to copepode parasite infections in gill of striped trumpeter (*Latris lineata*) and seabream (*S. aurata*) (183) (184). Mucosal dendritic cells (DCs) are one of the most important components of the mucosal immune system of mammals. Mucosal DCs are instrumental for the containment of mucosal immune responses avoiding systemic immune responses (185). In mammals, mucosal DCs are able to directly sample antigens from the gut lumen and uptake both commensals and pathogens (186). Dendritic cells have been characterized in zebrafish and rainbow trout as antigen-presenting cells (38) (39). Macrophages and granulocytes are also present in teleost mucosal lymphoid tissues. In mammals, mucosal macrophages are essential for local homeostasis and in keeping a balance with the commensal microbiota. In vitro studies in rainbow trout comparing mucosal versus non mucosal leucocyte activities show some parallelism with mammalian mucosal macrophages (187). There are available zebrafish lines [eg. Tg(mpx) (188) and Tg(mpeg1) (189)] where neutrophils and macrophages can be visualized by fluorescence microscopy which offering chances to study particular cells on the mucosal surfaces.

Adaptive immunity is also present on the mucosal surface and differences exist between systematic adaptive immunity and mucosal adaptive immunity in vertebrates. For example, regarding to antibodies, exposure of mucosal surfaces to antigens results in the secretion of antigen-specific IgA at these locations while IgM represents the main Ig in systemic immune responses. IgM is also present in mucosal secretions and is involved in responses against several pathogens (34). However, mucosal secreted IgM may different with the systematic one. In common carp, the monoclonal antibodies (mAb) derived skin mucus IgM recognized IgM heavy (H) chain of the skin mucus but not that of the serum (32). Three immunoglobulin isotypes have been reported so far in teleost: IgM, IgD, IgZ/T. IgD is known to be expressed in all immune tissues (190), its involvement in mucosal responses has not been clarified. IgZ/T is a new type immunoglobulin H chain class in teleost. In some species, like zebrafish (145) and common carp (191), it is called IgZ, but in rainbow trout (143) and Atlantic salmon (192) it is termed IgT . IgT, similar to mammalian IgA, was suggested to be the Ig isotype with a specialized mucosal function in the gut of rainbow trout (146). Among the two IgZ isotypes in carp, IgZ2 has a preference for mucosal tissues, while IgZ1 is associated with systemic organs (193). In addition, IgT is also present in the skin mucosa of rainbow trout and a specific anti-Ich IgT-response is found in the skin mucus (194). Polymeric Immunoglobulin Receptor (pIgR) directly involved in the transport of the immunoglobulin molecules. The pIgR was reported in associated with IgM in the skin mucus of fugu and IgT and IgM in gut mucus of rainbow trout (195) (146).

The main role of B cells in adaptive immunity is to recognize antigens in their native form and produce Igs against those antigens. Some teleosts like the channel catfish possess three B cell subsets (196), whereas two populations have been characterized in rainbow trout (146). Additionally, IgM^+ B cell populations have been found in many other teleost species (32) (197) (198). The role of IgT and IgT^+ B cells in gut mucosal immunity was indicated by the high percentage of IgT^+ B cells in total B cells in the gut and the increase of IgT^+ B cells, but not IgM^+ cells, in the lamina propria of the gut after a parasite infection (146). IgT^+ B cells are located in the gill epithelium whereas IgM^+ B cells are located in the gill arterioles and capillaries of rainbow trout after infection with Ich (174), supporting the difference between systemic and mucosal immunity. Importantly, another study demonstrated that IgT^+ B cells are also the main B cell population involved in adaptive immunity in the rainbow trout skin epithelia (194). T cells play an essential role in cell-mediated immunity and as they interact with the bacteria present in mucosal surfaces. T cells are abundant in mucosal tissues (the gut, the gills and the skin) of teleosts (140). The teleost gut has been showed that present a T cell-like population in a number of species including seabass, carp, Atlantic salmon, and rainbow trout (180) (199). In seabass, the intestine contains clearly more CD8α than CD4 T cells and the number of such cells increases from the foregut to the hindgut (200). In rainbow trout, an anti-CD8α mAb has shown that cytotoxic T lymphocytes (CTLs) constitute around 55% and 25% of all lymphocytes from gut and gills, respectively (199). Additionally, $CD3\varepsilon^+$ T cells are found in the gut and abundantly in the interbranchial tissue within the gills of Atlantic salmon (201). The presence of a putative $CD8^+$ T cell population has been found in the intraepithelial lymphoid tissue of rainbow trout at the base of gill filaments, and it accumulates in that area after Ich infection (174). In carp, the intestinal mucosal T cells were identified by a specific T cell mAb (202). Teleost skin T cells have not been studied in detail. It is likely that putative T cells are present in Atlantic salmon skin by the expression study of TCRα (203). In summary, teleost mucosal T cells express CD3ε, TCRα, TCRβ, TCRγ, CD4, CD28, TCRζ, CD8α, CD8β, and RAG-1.

Cytokines as adjuvants

Cytokines are small soluble proteins which play an important role in the regulation of immune responses. In mammals, upon inflammation, cytokines are released in a signaling cascade from tumor necrosis factor α (TNFα), then interleukin-1β (IL-1β) to IL-6. Chemokines downstream induce migration of neutrophils and microphages to the corresponding inflammatory sites (63). Cytokines have been studied the ability of vaccine adjuvants due to their functional effects on the immune system. In recent years, many cytokine genes were cloned and sequenced in different fish species. However, not many studies focused on the application of cytokines as adjuvant in fish. This may due to the lacking of systematic studies of cytokines and their immunological role. Still, some attempts of using cytokines as adjuvant have been explored and some positive studies have showed in some fish species.

TNFα is a member of the β-jellyroll family of cytokines. It is produced mainly by activated macrophages, NK cells and T cells (mainly Th1 cells) as a type II transmembrane protein or glycoprotein (204). Unlike mammals, similar observation in various fish concluded that teleost have a duplicated TNF gene (205). TNFα were found in Japanese flounder (*P. olivaceus*) (206), rainbow trout (*O. mykiss*) (207), gilthead seabream (*S. aurata*) (208), common carp (*C. carpio*) (209), and catfish (*I. punctatus*) (71). Multiple isoforms These cloned TNF genes from fish possess a well-defined transmembrane domain, two conserved cysteines important for disulphide-bond formation, a well-defined cleavage site for the release of the mature protein, high conservation of the TNF signature, and a similar genomic structure which are typical in mammalian TNFα. The functional study in rainbow trout macrophages showed recombinant trout TNFα enhanced leukocyte migration and phagocytic activity (72). Regarding to the adjuvant application, the recombinant tumor necrosis factor α (TNFα) from European sea bass (*D. labrax L.*) used as adjuvant significantly extended the protection of fish against *Vibrio anguillarum* challenge when orally immunized with vaccine (210). In a previous study of our research group, when recombinant cytokine TNFα from rainbow trout (*O. mykiss*) was i.p. injected to zebrafish, the animals showed significantly higher survival when challenged lethally with *Pseudomonas aeruginosa* (211).

IL-1β is a member of the β-trefoil family of cytokines. In mammals it is produced as an inactive precursor molecule that is processed by interleukin converting enzyme (ICE) to give a biologically active 'mature' peptide. IL-1β has diverse physiological functions and its roles in regulating the inflammatory process are conserved in fish (60). IL-1β has been cloned in a number of teleost species: rainbow trout (*O. mykiss*) (49) and carp (*C. carpio*) (212), seabass (*D. labrax*) (58), seabream (*S. aurata*) (213), turbot (*Scophthalmus maximus*) (214), and dogfish (*Scyliorhinus caniculus*) (215). The study of interleukin-1β (IL-1β) as an adjuvant was reported in carp (*C. carpio*) which were either i.p. injected with formalin-killed *A. hydrophila* plus recombinant carp IL-1β peptide or formalin-killed *A. hydrophila* cells. The result exhibited that the agglutinating antibody titre was significantly higher when fish injected with formalin-killed *A. hydrophila* plus recombinant carp IL-1β peptide at 3 weeks post vaccination (216). Moreover, IL-1β-derived peptide could induce the migration of peritoneal leucocytes and phagocytosis when i.p. administered in *O. mykiss*. *In vivo* experiments showed the fish injected with this peptide were more resistant to VHSV infection (217). In addition, the recombinant IL-1β from sea bass (*D. labrax*) was able to induce IL-1β gene expression in head kidney cells and could have immune-adjuvant effects

(218). In Japanese flounder (*P. olivaceus*), IL-1β has also been shown to enhance antibody production, suggesting it may be exploited as an immune-adjuvant for improving vaccine efficacy (219). Some other members of interleukin family have been studied as potential adjuvant in fish species. Rainbow trout interleukin-8 (IL-8) was able to modulate early cytokine immune response in *O. mykiss* when co-injected with the glycoprotein gene of VHSV, suggesting it might be a potential immune adjuvant (220). In the same species, the recombinant IL-8 produced in *Escherichia coli* showed the ability of inducing the migration of head kidney leukocytes in cells and attracting leukocytes into the peritoneal cavity when intraperitoneally (i.p.) injected (221). In channel catfish (*I. punctatus*), IL-8 was used as adjuvant and co-vaccinated with subunit vaccine encoding α-enolase (rENO) of *Streptococcus iniae*. The results showed that the co-vaccinated fish has higher relative percent survival than the fish vaccinated with rENO alone after *S. iniae* infection at both 4 and 8 weeks post vaccination (222). In amberjack (Seriola dumerili), recombinant amberjack IL-12 was investigated the adjuvant effect. Fish vaccinated with IL-12 and formalin-killed *N. seriolae* (FKC) vaccine showed much higher survival rate in challenge test compare to the PBS (control) and FKC only (223). The immune adjuvant effects of recombinant IL-6 (rIL-6) and pcDNA3.1-IL-6 (pcIL-6) from flounder (*P. olivaceus*) were evaluated. Fish vaccinated subunit vaccine recombinant outer membrane protein V (rOmpV) plus rIL-6 or pcIL-6 showed significantly higher survivals against *E. tarda* infection compare to the fish vaccinated with the subunit vaccine alone. In addition, the rOmpV plus rIL-6 could induce significant higher levels of specific serum antibodies, immunoglobulin-positive lymphocytes and relevant genes expressions than rOmpV plus pcIL-6, demonstrating the adjuvant effect of IL-6 (224).

Interferons (IFNs) are a family of multi-functional cytokines which involved in antiviral defense. In recent study, type I IFNs from Atlantic salmon were used as adjuvants in a DNA vaccine model based on the hemagglutinin-esterase (HE) gene of infectious salmon anemia virus (ISAV) as antigen. The results showed that all three IFN plasmids delivered together with DNA vaccine potently enhanced protection of salmon against ISAV and stimulated an increase in IgM antibodies against the virus (225). The IFNs genes are regulated by interferon regulatory factors (IRFs) which form a large family of transcription factors (226). IRF-1 has been shown to have a role as potential adjuvant in host defense against pathogens. The study of interferon regulatory factor-1 (IRF-1) of Japanese flounder (*P. olivaceus*) as a potential adjuvant showed an antiviral ability and protect fish cells against hirame rhabdovirus (HIRRV) and viral hemorrhagic septicemia virus (VHSV) infection (227). Moreover, the recombinant Japanese flounder IRF-1 was investigated as adjuvant in *P. olivaceus*. The results showed that the IRF-1 modulated the early immune response when intramuscularly vaccinated with a DNA vaccine but did not show significant difference with the fish vaccinated with the DNA vaccine alone (228).

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Review

Nanodelivery Systems as New Tools for Immunostimulant or Vaccine Administration: Targeting the Fish Immune System

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Abstract: Fish disease treatments have progressed significantly over the last few years and have moved from the massive use of antibiotics to the development of vaccines mainly based on inactivated bacteria. Today, the incorporation of immunostimulants and antigens into nanomaterials provide us with new tools to enhance the performance of immunostimulation. Nanoparticles are dispersions or solid particles designed with specific physical properties (size, surface charge, or loading capacity), which allow controlled delivery and therefore improved targeting and stimulation of the immune system. The use of these nanodelivery platforms in fish is in the initial steps of development. Here we review the advances in the application of nanoparticles to fish disease prevention including: the type of biomaterial, the type of immunostimulant or vaccine loaded into the nanoparticles, and how they target the fish immune system.

Keywords: nanoparticles; fish; immunostimulation; alginate; liposome; chitosan; PLGA; carbon nanotubes

Introduction

The use of vaccines has been essential in aquaculture and has been under development for over 70 years since the first successful fish vaccine was formulated [1]. Vaccines stimulate the immune system to mount a defence against a pathogen and as such to protect the host from infection by this pathogen. While they are extremely important to control infectious diseases in farmed fish, there are still some hurdles affecting the development of effective vaccines against viruses, parasites, and intracellular pathogens. One of these bottlenecks is the vaccine administration system [2,3]. Different approaches have been employed in aquaculture to improve the vaccine efficacy and to explore alternative routes of immunization. Traditional adjuvants such as mineral oils have been routinely used for vaccine injection, the most common examples are Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA) and more recently Montanide. Although adjuvants are very effective in potentiating the immune response against the pathogen, they present different side effects. There are three main methods for vaccine administration in fish: orally, by immersion or by injection. Vaccination by injection is the most reliable and effective system for vaccine delivery in fish and the protection is by far the most effective and long lasting [4,5]. However, the injectable vaccines are usually prepared with oil/water adjuvant formulations (FCA or FIA) which result in adverse effects such as the appearance of granulomas [2,6] adhesions between organ and peritoneal wall [7], injection site lesions [8,9], reduced appetite and growth [10], or deformations of the skeleton [11]. Added to this, the anaesthesia, handling, and injection may cause occasional mortality. Importantly, there are also logistical challenges to inject fish of less than 20 g in large numbers, but these fish need vaccination the most because they are the most susceptible to disease [12]. The disadvantages of vaccination by immersion are: the large amounts of vaccine required [13], the difficulty to measure the efficiency of the uptake [14] and the degradation of the compound in the water. Like for immersion, oral vaccines offer the advantages of being stress free and easy to administer to large numbers of fish but it is also difficult to determine the dose of antigen received. Also depending whether fish are gastric or agastric the intact antigen has to pass through the digestive system to reach the second segment of the hindgut where antigens are absorbed [15,16]. In this context, the use of nanodelivery systems has been proposed as an alternative strategy to address not only the above mentioned problems, but also to enhance the efficacy since some of these delivery systems may act also as a potent adjuvant, which is extremely important for anti-viral vaccines. Therefore, searching for new delivery systems is required to improve the administration and the efficacy of vaccines and immunostimulants.

Delivery systems are those materials used for the administration of pharmaceuticals in a controlled manner aimed to achieve a therapeutic effect. These systems provide: cell or tissue targeted delivery of active compounds, improved bioavailability, improved solubility of hydrophobic drugs, sustained release and protection of the therapeutic agent from degradation [17]. Nanoencapsulation involves forming drug loaded particles with diameters ranging from 1 to 1000 nm, although other stricter definitions refer only to structures in the 1–100 nm range (*US National Nanotechnology Initiative, What is nanotechnology?*). This size property enables the nanoscale devices to readily interact with biomolecules, such as enzymes and receptors, both on the surface and inside the cells.

Since 1960, when the first liposomes for drug delivery were described, a variety of other organic and inorganic biomaterials were developed for drug delivery [17]. In 1980 more complex drug delivery systems capable of responding to pH changes to trigger drug release or the first examples of cell specific targeting of liposomes were described [18]. Nowadays, nanoparticles can be easily tuned to have unique physical characteristics in size, shape, surface chemistry, or targeted surface ligand/receptor. The benefits of nanoparticles as delivery tools are the reduction of the doses, tissue specific targeting, reduction of the toxic or secondary effects of the drug and increase in the delivery efficiency [19]. The encapsulated molecules will generally have completely different properties (e.g., solubility or circulating half-life) compared to the non-encapsulated ones. Thus, it is very important to understand and control the *in vivo* behaviour on cells or tissues of these bioactive compounds once encapsulated, to know their efficacy and side effects. As mentioned, the size of the nanoparticle is not only important for the interaction with biomolecules but also because it will influence its biodistribution *in vivo*. In mammals it has been extensively studied that particles of less than 5 nm are cleared from the circulation through extravasation or renal clearance, whereas bigger nanoparticles (up to 15 μm) accumulate in the spleen, liver, and bone marrow [20]. The particle size also influences the preferred mechanism of cellular internalization, such as phagocytosis, macropinocytosis, caveolae-mediated-endocytosis, or others. Of note, the fate of the internalized material will be different in each case [21,22].

In teleosts, there are few reports on how the nanoparticles spread throughout the organism through the circulatory system, gills, gut, spleen, liver, or brain depending on the administration route [23,24]. Current findings indicate that particle shape and rigidity are also key factors for the kinetics and fate of the nanoparticles, mainly affecting the endocytosis. The vast majority of nanoparticles have a spherical shape, however similar volumes with different shapes are internalized at different rates [25,26]. Increased nanoparticle rigidity is related to enhanced phagocytosis by macrophages [27]. Finally, the nanoparticle surface charge critically affects how they interact with serum proteins and cell membranes. Highly charged particles fix more complement proteins [28], a process that can only be inhibited by addition of a hydrophilic coating. The surface charge will also determine the interaction with cell membranes. In general, neutral and anionic nanoparticles will be less internalized than positively charged ones [22,29]. Different studies using the same nanoparticle with different surface charges have shown that those with cationic groups were internalized more efficiently, [30,31] mostly due to the high affinity for the negatively charged proteoglycans present on the surface of cells [32]. The use of nanoparticles does also have some limitations. For example, their small size and large surface area can lead to particle aggregation and result in limited drug loading and burst release, making physical handling of nanoparticles difficult in liquid and dry forms [33]. Another issue that should be addressed in the future is the safety, for both human and animals, not only of the delivery system itself but also of the degradation products of the nanoparticles. These biosafety issues should be carefully addressed to avoid environmental contamination that can provoke detrimental effects on animal and human health.

In this review, we summarized different nano- and micro-sized delivery systems that have been described as delivery tools for fish vaccination or immunostimulation. Calcium phosphate nanoparticles, carbon nanotubes, chitosan nanoparticles, liposomes, poly-lactic-glycolic acid nanoparticles, or alginate micro-particles are described in detail below.

Nanodelivery Systems

Alginate

Alginate is found naturally in brown algae, such as *Laminaria hyperborea*, *Laminaria digitata*, *Macrocystes pyrifera*, and *Lessonia nigrescens*. It can also be found as a polysaccharide in some bacteria such as *Azotobacter vinelandii* and *Pseudomonas* [34]. Alginate is a generic name used to define a complex molecule made of repeated units of the unbranched polyanionic polysaccharides α-L-guluronic acid (G) and β-D-mannuronic acid (M). Alginate is built by combination of G-G, G-M, and/or M-M blocks. These blocks can be found in different G/M composition and chain arrangements, which gives them its differential physico-chemical properties [35,36]. The mechanical and the physical stability of alginate mainly depend on the G content, the greater the G content, the more rigid and brittle the matrix [37]. Alginate-microparticles (alginate-MPs) are eroded at neutral and basic pH allowing the release of the cargo by diffusion, while at low pH values they are extremely stable [38]. This stability at low pH makes alginate-MPs suitable for oral administration, since in the fish stomach (pH between 2 and 4) the release will be low while the release in the foregut or hindgut at neutral-basic pH (pH 7 and 8.3, respectively) will be high [39,40]. Notably, alginate is mucoadhesive allowing the adhesion to the epithelial mucus (e.g., intestinal mucosa) and making it very attractive for oral administration. Other important features of alginate-MPs are the high biocompatibility and the low cost of production.

Alginate-MPs can be produced by classical techniques such as air atomization, emulsification, gelation, and complexation with counterion polymers, or by new methods, such as spray-drying, electrohydrodynamic atomization, impinging aerosols, and inkjet/drying process, that enable a better control of the size [37]. For application in fish, alginate-MPs are generally produced by emulsification [39–42] that is one of the fastest methods for nanoparticle preparation and is readily scalable [43], and to a lesser extent by other methodologies such as the orifice-ionic gelation and the spray method [44,45] (Table 1).

In mammals, alginate nanoparticles have been used for the delivery of different drugs [46–49], but to date there are no alginate nano-sized particles routinely used for delivery of active compounds in fish. Nevertheless, micro-sized alginate particles are one of the most common delivery systems assayed in fish with promising results for viral diseases.

2.1.1. Encapsulation of Bacterial Antigens in Alginate Microparticles

To date, the main bacterial antigens encapsulated into alginate-MPs have been formalin-killed bacteria (FKB) from different species (Table 1). FKB have been widely used as antigens for fish vaccination in some diseases, mainly those caused by Gram-negative bacteria. In general, FKB vaccines provide excellent levels of protection by itself or in combination with an adjuvant (e.g., FCA) [40,50]. In general, oral administration of FKB encapsulated in alginate-MPs does not work very well alone, and only when combining the alginate-MPs with the FKB vaccine they obtained a longer lasting protection. The oral administration of alginate-FKB from *Lactococcus garviaeae* in rainbow trout (*O. mykiss*) provided low levels of protection against *L. garviaeae* infection (35% Relative percent survival (RPS) at 30 days) compared with the naked FKB vaccine administrated intraperitoneally (100% RPS at 30 days and 40% at 90 days) (Table 1). These results were improved when fish were immunized orally a second time with the alginate-vaccine three months later (61% RPS at 180 days) [44]. Altun and coworkers observed similar result with this alginate-construct administrated orally in rainbow trout (Table 1). It did not provide better protection against *L. garviaeae* infection (53% RPS at 30 days and 38% RPS at 60 days) than the naked vaccine (95% RPS at 30 days and 82% RPS at 60 days). The protection was again increased when fish was immunized a second time with alginate-FKB-LG at day 61 (67% RPS at 90 days and 62% RPS at 120 days) or with a first administration of naked vaccine and then a second administration of the alginate-construct at day 61 (86% RPS at 90 days and 81% RPS at 120 days) [45].

Leal *et al.* [40] evaluated the alginate-MPs formulated with FKB from *Flavobacterium columnare* in nile tilapia (*Oreochromis niloticus*) (Table 1). Alginate-vaccine and naked vaccine administrated orally did not provide protection against *F. columnare* challenge (0% of RPS at 21 days in both cases) and did not stimulate the production of specific antibodies against *F. columnare* in immunized fish [40].

2.1.2. Encapsulation of Viral DNA in Alginate Microparticles

For viral diseases, alginate-MPs have been used to encapsulate DNA vaccines made with plasmids coding for viral proteins. The alginate-MPs loaded with DNA vaccines are smaller (≤ 10 μm) than the alginate-MPs loaded with bacterial antigens (10–30 μm) [44] and this seems to favor the targeting of different organs, such as spleen, kidney, liver, pyloric caeca, heart, intestine, or gills [41,42]. Alginate-MPs containing the plasmid coding for the major capsid protein (MCP) of Lymphocystis Disease Virus (LCDV) increased the titer of specific antibodies against LCDV in olive flounder (*Paralichthys olivaceus*) serum after oral administration (Table 1). The results showed a progressive increase until week 11 while the naked DNA vaccine did not stimulate any increase in the antibody titer. The naked DNA vaccine might thus be hydrolyzed in the gastrointestinal tract while the alginate-MPs can reach the tissues [41].

Alginate-MPs with a plasmid coding for VP2, one of the major structural proteins of Infectious Pancreatic Necrosis Virus (IPNV) stimulated the production of specific neutralizing antibodies in *O. mykiss* until eight weeks after oral administration (Table 1). In infection experiments with this virus, alginate-MPs orally administrated to *O. mykiss* and *Salmo trutta* increased the protection levels nearly to 80% RPS at 15 and 30 days post-vaccination [42]. These levels of protection were comparable with a commercial subunit vaccine (e.g., Microtek) administrated by intraperitoneal injection [51].

Table 1. Microparticles used as delivery systems in fish.

Microparticle	Size	Production Technique and Composition	Encapsulated Molecule	Administration	Species	Fish Size	RPS	Reference
Alginate	30 µm	Spray method, sodium alginate, 0.5% (*w/v*)	FKB from *Lactococcus garviaeae*	Oral	*Onchorhynchus mykiss*	22 g	35% E and 100% N at 30 DPV; 5% E and 40% N at 90 DPV; 61% first V with N and second with E at 180 DPV	[44]
	n.d.	Orifice-ionic gelation, Sodium alginate, 4% (*w/v*)	FKB From *Lactococcus garviaeae*	Oral	*Onchorhynchus mykiss*	20 g	53% E and 95% N at 30 DPV; 38% E and 82% at 60 DPV; 67% first V with N and second with E at 90 DPV; 62% first and second V with E at 120 DPV	[45]
	n.d.	Emulsification, sodium alginate, 3.5% (*w/v*)	FKB from *Flavobacterium columnare*	Oral	*Oreochromis niloticus*	15.7 g	0% E and 0% N at 21 DPC	[40]
	≤ 10 µm	Emulsification, sodium alginate, 3% (*w/v*)	Plasmid DNA: MCP from LCDV	Oral	*Paralichthys olivaceus*	40–60 g	n.d.	[41]
	10 µm	Emulsification, sodium alginate, 3% (*w/v*)	Plasmid DNA: VP2 from IPNV	Oral	*Salmo trutta*	1.5 g/3 cm	At 15 DPV: 78% E and 0% empty plasmid at 30 DPC, At 30 DPV: 79% and 0% empty plasmid at 30 DPC (*)	[42]
	10 µm	Emulsification, sodium alginate, 3% (*w/v*)	Plasmid DNA: VP2 from IPNV	Oral	*Onchorhynchus mykiss*	1 g/3.5 cm	At 15 DPV: 80% E and 5% empty plasmid at 30 DPC; At 30 DPV: 67% and 0% empty plasmid at 30 DPC (*)	[42]
Chitosan	≤ 10 µm	Emulsification, 3% chitosan (*m/v*)	Plasmid DNA: MCP from LCDV	Oral	*Paralichthys olivaceus*	50–100 g and 13–15 cm	n.d.	[52]
	< 5 µm	Spray drying, 240 mg of PVMMA and 250 mg of chitosan Seacure 210 HCl	Surface antigens (Ag) from *Philasterides dicentrarchi*	i.p. injection	*Scophthalmus maximus*	50 g	68% E, 58% Ag in FCA and 43% FCA at 20 DPC	[53]

Table 1. *Cont.*

Microparticle	Size	Production Technique and Composition	Encapsulated Molecule	Administration	Species	Fish size	RPS	Reference
Chitosan	4.28 ± 0.4 µm	Spray drying, 240 mg of GantrezAN119 and 250 mg of chitosan Seacure 210 HCl	Surface antigens (Ag) from *Philasterides dicentrarchi*	*In vitro*, anterior kidney leukocytes	*Scophthalmus maximus*	n.d	n.d.	[54]
	1.101 ± 0.0103 µm	TPP ionic gelation, 5 mg/mL chitosan in sodium alginate solution at concentration of 10 mg/mL	FKB from *Aeromonas hydrophila*	Oral	*Labeo rohita*	Juveniles	13% alginate and chitosan E, 13% chitosan E, 16% alginate and chitosan, 0% N at 15 DPC (*)	[55]
PLGA	1.12 µm	D.E., PLGA 50:50, MW: 30–70 kDa	OMP from *Aeromona hydrophila*	Parenteral	*Labeo rohita*	30–40 g and 250–300 g	n.d.	[56]
	< 10 µm	D.E., L:G = 75:25, MW:50 kDa	Plasmid DNA: MCP from LCDV	Oral	*Paralichthys olivaceus*	500–1000 g	n.d.	[57]
	1 µm	Emulsion, PLGA 50:50	γ-globulins from human blood	Oral	*Onchorhynchus mykiss*	100–200 g	n.d.	[58]
	n.d.	D.E., PLGA 50:50	i-antigen from *Uromena marinum*	i.p. injection	*Epinephelus bruneus*	31.4 ± 2.3 g	78% E and 66% N at 30 DPC (*)	[59]
PLGA/Liposome	5–10 µm	Film dispersion method, PS, PC, and Chol (molar ratio 1:10:5)	FKB from *Aeromonas hydrophila*	Oral	*Cyprinus carpio*	30 g	64% E at 12 DPC	[60]
	n.d.	D.E., PLGA 50:50	ODN1668	i.p. injection	*Epinephelus bruneus*	36.7 ± 2.8 g	78% PLGA E, 83% Liposome E, 83% PLGA/Liposome E and 78% N at 30 DPC (*)	[61]

Chol: Cholesterol; D.E.: double emulsion; DPC: days post-challenge; DPV: days post-vaccination; E.: encapsulated antigen; FCA: Freund's complete adjuvant; FKB: formalin killed bacteria; GantrezAN119: methyl vinyl ether-co-maleic anhydride; i.p.: intraperitoneal; IPNV: Infectious pancreatic necrosis virus; MCP: major capsid protein; N: naked antigen; n.d.: not described; LCDV: Lymphocystis disease virus; ODN1668: oligodeoxynucleotide 1668; Omp: outer membrane protein; PC: Phosphatidylcholine; PLGA: poly(lactic-co-glycolic acid); PS: Phosphatidylserine; PVMMA: Poly (methyl vinyl ether)-co-(maleic anhydre); RPS: Relative percent survival; V: vaccination; VP2: Viral protein 2; (*): calculated RPS.

Carbon nanotubes

Carbon nanotubes (CNTs) were discovered in 1991 by Iijima [62]. CNTs are allotropes of carbon with a cylindrical nanostructure and this network of carbon atoms can reach several micrometers in length with a nanosized diameter. CNTs can be produced at large scale by three methods: discharge, laser ablation, and chemical vapor deposition. During the production process with all these methods impurities are formed, thus requiring an additional purification step [63]. Pure CNTs are not soluble in aqueous solutions because they have highly hydrophobic surfaces and an additional functionalization step is needed. There are two main types of carbon nanotubes, single-walled, and multi-walled. Single-walled CNTs are flexible but require catalytic synthesis making its bulk production difficult and leading to poor levels of purity. Multi-walled CNTs are formed by several concentric layers and thus are more rigid. They can be produced without catalyst, which allows bulk synthesis and high purity [64]. CNTs are chemically stable, relatively inert, non-immunogenic, and non-toxic. Additionally, CNTs have a large surface area available and are able to absorb or to be conjugated to a wide variety of antigens, presenting high stability *in vivo* [65,66]. In mammals, CNTs are being investigated as a delivery system for genes, peptides, oligonucleotides, antimicrobial agents, and cytotoxic drugs [67–70]. In fish, the study of CNTs as delivery systems has recently started, focusing on its functionalization with chemical groups and proteins and on the encapsulation of DNA vaccines [71–73] (Table 2).

2.2.1. Functionalization of CNTs

As mentioned above, functionalization is required to solubilize the CNTs and to make them biocompatible. This process can be divided in two different approaches, depending on the covalent/non-covalent nature of the linked antigens [64]. The covalent attachment of different chemical groups (e.g., sulfonate) and proteins (e.g., bovine serum albumin) has been used to design nanoparticles for fish [71–73]. Different studies warn about the potential for these manufactured nanomaterials to contaminate the aquatic environment. To evaluate immunotoxicity, functionalized single-walled and multi-walled CTNs with chemical groups, such as sulfonate, sulfonic acid, and polyethylene glycol were tested for toxicity in head kidney macrophages isolated from *O. mykiss* (Table 2). The CNTs formulations did not decrease the cell viability after 24 h treatment [72]. None of these formulations stimulated the expression of interferon alpha (IFNα) gene, however CNTs with and without functionalization stimulated interleukin 1 beta (IL-1β) gene expression in trout macrophages indicating that they can be pro-inflammatory if they gain entry to the body. Multi-walled CNTs containing anionic groups (sulfonate groups) caused the highest IL-1β stimulation, while single-walled CNTs containing neutral groups (polyethylene glycol groups) caused the least reaction. The functionalized CNTs were also more potent in stimulating gene expression than the non-functionalized counterparts [72].

The functionalized CNTs thus produce a stimulation of the immune system by themselves without any loaded antigen [71,72], although there is no information about the levels of protection that they may provide in a challenge. Fluorescent multi-walled CNTs functionalized with bovine serum albumin (BSA) were tested in zebrafish (*Danio rerio*) embryos by microinjection into the circulation at 72 h post fertilization (Table 2). These CNTs distributed all along the blood circulation and then moved to the muscle, brain ventricle and notochord, being finally cleared out at 96 h after injection. The immune response of the embryos was studied by *in situ* hybridization of Matrix Metalloproteinase 9 (MMP9). At early stages, the injected embryos showed an increase in MMP9 expression levels and changes in the expression pattern. These results suggest that embryos may generate an innate immune response when being injected with CNTs at the 1-cell stage. The injected zebrafish embryos had normal primordial germ cells and were able to produce a new generation at the adult stage. However, the larvae of the second generation showed lower survival rates as compared with the untreated group, suggesting a negative effect on the reproduction potential [71].

2.2.2. Encapsulation of Viral DNA in CNTs

To date only one work has evaluated CNTs as a DNA delivery system in fish, but with promising results. Single-walled CNTs were loaded with a plasmid encoding the VP7 protein of Grass Carp Reovirus (GCRV). The plasmid expression after intramuscular injection in grass carp (*C. idella*) was detected at high levels in muscle at 28 days post-injection. At the level of the humoral response, specific VP7 antibody production was detected during eight weeks with a peak titer at four weeks post-vaccination. Other immune parameters such as respiratory burst, serum lysozyme activity, complement activity, or superoxide dismutase activity were also stimulated. Importantly, in a challenge against GCRV, the treated fish showed good protection levels even at low plasmid doses (1 µg: 73% RPS, 5 µg: 91% RPS and 10 µg: 100% RPS) when compared with the naked plasmid (1 µg: 9% RPS, 5 µg: 27% RPS and 10 µg: 44% RPS) [73] (Table 2).

Chitosan

Chitin is a natural, biodegradable, biocompatible, and nontoxic biopolymer derived from the shells of crustaceans, insects, and some microorganisms. It can be converted to chitosan, a linear polysaccharide compound of β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine obtained from the *N*-deacetylated derivative of chitin by enzymatic or chemical processes. Chemical methods are used extensively for chitosan preparation for commercial purposes because of their low cost and scalability, but have a high energetic cost and produce a concentrated alkaline waste solution. In contrast, enzymatic methods offer the possibility of a controlled process, resulting in the production of well-defined chitosan [74]. Chitosan nanoparticles are prepared by ionic gelation [75], followed by freeze-drying (or spray-drying) to recover these particles.

The solubilization of chitosan occurs by protonation of the $-NH_2$ group on the C-2 position of the D-glucosamine repeat unit, whereby the polysaccharide is converted to a polyelectrolyte in acidic media. Being soluble in aqueous solutions, chitosan and its derivatives are largely used in medical and pharmaceutical applications like artificial matrices for tissue engineering, targeted drug delivery, drug transport, protein delivery or gene transfer [76–79]. They can be functionalized to display antimicrobial activity against many bacteria, filamentous fungi and yeasts [80], hemostatic potential [81], and antioxidant activity [82].

The beneficial applications of chitosan on fish have been demonstrated in different studies in which chitosan nanoparticles were administrated through diet. Diets supplemented with chitosan for rainbow trout [83], olive flounder [84], koi [85], kelp grouper [86], turbot [87], gibel carp [88], mrigal carp [89], and Asian seabass [90] have proved that the chitosan could enhance growth, the innate immunity, disease, and stress resistance, improve haematological parameters and improve water quality. From fish immunological perspective, chitosan nanoparticles have been used for the delivery of vitamin C [91], RNA [92], or DNA [93–95] due to their positive charge and solubility in aqueous solution. In addition, chitosan protects encapsulated active compounds from the harsh conditions in the gastrointestinal tract and enhances their absorption [96]. Therefore, chitosans can be used for delivering immunostimulants or vaccines to fish in aquaculture.

2.3.1. Encapsulation of Compounds in Chitosan Nanoparticles

The DNA that encodes for the 38 kDa protein of the external membrane (OMP38) of *Vibrio anguillarum* was encapsulated in chitosan and administered with food to Asian sea bass (*Lates calcarifer*). It induced a significant antibody immune response and was able to give moderate levels of protection (RPS 46%) against experimental challenge with *V. anguillarum* [94] (Table 2). Another pDNA vaccine constructed with the outer membrane protein K of *Vibrio parahaemolyticus* was encapsulated in chitosan nanoparticles and mixed with dry fish food powder and used to feed blackhead seabream (*A. schlegelii*). The outer membrane protein K gene and protein were expressed in muscle, liver, kidney, and mid-intestine of the vaccinated animals. Furthermore, blackhead seabream were protected from *V. parahaemolyticus* challenge with 72.3% RPS after 21 days post-vaccination [95] (Table 2). Ramos and coworkers also clearly showed that chitosan is an excellent DNA delivery system through oral administration, either by feeding with plasmid DNA-chitosan incorporated into the food, or by direct intrabuccal delivery [93] (Table 2).

In a different setup, dietary RNA (*i.e.*, nucleotides derived from yeast) was loaded into chitosan nanoparticles at a chitosan/RNA ratio of 2:1 and were fed during 60 days to fingerlings of *Labeo rohita*. The body composition in terms of protein and lipid content was not affected by RNA-loaded chitosan nanoparticles (chitosan-NPs) while the growth, performance, immunity, and survival following a bacterial challenge (*A. hydrophila*) were significantly increased compared to only chitosan or bare RNA. Unaffected glucose and serum uric acid levels, and decreased transaminases and dehydrogenases, coupled with improved performance, indicated an enhanced energetic efficiency for anabolic processes and the safety of RNA-loaded chitosan-NPs as a nutraceutical [92] (Table 2).

Finally, chitosan-NPs are very suitable to encapsulate Vitamin C. In the gastrointestinal tract of rainbow trout (*O. mykiss*), the release of Vitamin C was regulated by the chitosan encapsulation up to 48 h. The innate immunity indices (lysozyme and complement proteins) were considerably increased in the treated rainbow trout and even the non-specific defense mechanisms were stimulated as a result of the synergistic effects caused by Vitamin C and the chitosan nanoparticle itself [91] (Table 2). Vitamin C was also administered in this way to post-metamorphic larvae of *Solea senegalensis* and rotifers (*Brachionus plicatilis*). The NPs were stable in seawater and *in vitro* assays with a zebrafish liver cell-line showed a statistically significant increase in total antioxidant capacity. In addition, the nanoparticles were able to penetrate through the intestinal epithelium in *S. senegalensis* larvae and could be used as an enriching additive for rotifers [97] (Table 2).

2.3.2. Encapsulation of Compounds in Chitosan Microparticles

Not only chitosan-NPs but also the larger chitosan microparticles (chitosan-MPs) are intensively studied. A plasmid containing the major capsid protein (MCP) gene of Lymphocystis Disease Virus (LCDV) was encapsulated in chitosan-MPs using an emulsion-based methodology. Oral administration led to an increase in the immune response in Japanese flounder (*Paralichythys olivaceus*) compared to injection immunization with naked plasmid DNA [52] (Table 1). The surface antigens (Ag) of the parasite *Philasterides dicentrarchi* were encapsulated and covalently linked to a polymeric microparticle formulation composed of two biodegradable polymers (chitosan and Gantrez). Poly (methyl vinyl ether)-co-(maleic anhydride) (Gantrez AN119) is a polymer belonging to the vinyl ether group that it is widely used for pharmaceutical purposes and has also been used to prepare ligand-nanoparticle conjugates for eliciting immune responses [98]. These chitosan and gantrez MPs encapsulated vaccine induced higher level of antibody than that induced by the same vaccine emulsified in FCA [53] (Table 1). These MPs could also significantly stimulate the phagocytic activity of leukocytes and the levels of the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) and also increased the production of reactive oxygen and nitrogen species in the anterior kidney of turbot (*Scophthalmus maximus*) [54] (Table 1). Finally, alginate-coated chitosan-MPs were evaluated through oral dietary administration. A potent humoral and innate immune response was elicited but it was not sufficient to induce protection against *A. hydrophila* infection under these conditions [55] (Table 1).

Liposomes

Liposomes are spherical, closed structures, composed of phospholipid bilayers, which enclose part of the surrounding solvent into their interior [99]. They are self-sealing and have the capacity to incorporate both hydrophilic and lipophilic drugs. Since the early 1980s, liposomes have been extensively studied as a drug carrier transport to target cells or tissues [100–103]. The drug delivery properties of liposomes are largely determined by factors such as the lipid composition, the particle size, the net charge and the loaded compound [104]. The liposome charge needs to be considered when administering molecules to fish, since fish gills contain a high level of mucin. In rainbow trout fry, a mechanism of acute toxicity after liposome treatment was suggested to be an interaction between the cationic liposomes and anionic components of gill mucin [105]. However, no toxicity was reported in zebrafish after immersion administration of nanoliposomes [23].

There is a wide variety of techniques that can be used to produce liposomal formulations, such as the Bangham method, detergent depletion method or extrusion [106]. All methods for producing liposomes require lipids to be combined by some means with an aqueous phase [107]. The extrusion technique is the most common method to prepare liposomes because it allows a better control of the size and the polydispersity index [108,109]. Extrusion is a process in which micrometric liposomes are structurally modified to large unilamellar vesicles or nanoliposomes depending on the pore-size of the filters used [108,110,111]. Compared to micro-liposomes, nanoliposomes provide more surface area and have the potential to increase solubility, enhance bioavailability, improve controlled release, and enable precision targeting of the encapsulated material to a greater extent [112].

2.4.1. Encapsulation of Bacterial Antigens in Liposomes

FKB vaccines composed of liposomes entrapping *Vibrio harveyi* were tested in *E. bruneus.* In *in vivo* infection assays, the cumulative mortality was 10%, 15%, and 65% lower in this immunized group compared to treatment with *V. harveyi* alone, liposome alone and non-immunized groups, respectively [113] (Table 2). In another study in carp (*C. carpio*), the oral administration of liposomes containing *A. salmonicida* antigen was investigated. The survival of carp after the challenge was 83% when they were immunised with *A. salmonicida* antigen-containing liposomes, whereas non-immunized carp showed 66% survival. Furthermore, the development of skin ulcers was significantly inhibited in carp immunized with liposomes containing *A. salmonicida* antigen [114].

Lipopolysaccharide (LPS) from *A. salmonicida* was also incorporated into liposomes in order to enhance the immune response in rainbow trout (*O. mykiss*). LPS incorporated into multilamellar vesicles or large unilamellar vesicles prolonged the period of serum anti-LPS antibody levels to 6–14 weeks comparing to free-LPS (2–4 weeks) when administered intraperitoneally [115] (Table 2). Also in rainbow trout, the immune efficacy of vaccine containing liposome particles with vaccine alone against furunculosis was compared [116]. Results indicated that the protection level was significantly enhanced when the vaccine also contained liposomes (Table 2). In addition, vaccinated fish appeared to be significantly larger than control fish.

Lastly, the *A. hydrophila* antigens entrapped in liposomes were developed for oral administration to immunize common carp (*C. carpio).* The levels of antibodies in the serum rose at two and three weeks post-vaccination and the vaccination protected the fish after injection with live *A. hydrophila* at 22 days post-vaccination [60] (Table 2).

2.4.2. Encapsulation of Viral Antigens in Liposomes

Formalin-inactivated koi herpesvirus entrapped within liposomes was used for oral vaccination of common carp (*C. carpio*). Specific antibody titer was significantly increased and challenge experiments revealed that orally vaccinated fish were protected from infection with two different isolates of koi herpesvirus (NKC03 and IKC03) showing high RPS (75% and 65%, respectively) [117] (Table 2).

Distinct from classical vaccines, immunostimulant-loaded liposomes are also developed to protect fish against bacterial and viral infections. For example, polyinosinic-polycytidylic acid (Poly I:C) is a synthetic analog of double-stranded RNA and is a typical molecular pattern associated with viral infections. When combined with LPS, it is a strong stimulus to the innate immune system. Liposomes encapsulating both Poly I:C and LPS elicited a pro-inflammatory and anti-viral response in zebrafish hepatocytes and trout macrophages. When administrated *in vivo* they accumulated in immune tissue and specifically in macrophages. Of interest, they protected zebrafish against otherwise lethal bacterial (*Pseudomonas aeruginosa* PAO1) and viral (Spring Viraemia of Carp Virus) infections regardless of whether they were administered by injection or by immersion. No stimulation of innate immunity was observed in the treatment with empty liposomes or with the free immunostimulants [23,118] (Table 2).

Table 2. Nanoparticles used as delivery system in fish.

Nanoparticle	Size	Production Technique and Composition	Encapsulated Molecule	Administration	Species	Fish Size	RPS	Reference
Calcium phosphate	224.98 ± 14.62 nm	n.d.	S-layer protein from *Aeromonas hydrophila*	i.p. injection	*Labeo rohita*	100–150 g	97% E, 13% N and 94% E with FIA at 15 DPC (*)	[119]
Carbon nanotubes	d: 10-20 nm; l: 1–2 µm	n.d., SWCNTs and MWCNTs	Sulfonate group, polyethyleglycol and sulfonic acid	*In vitro*, head kidney monocytes	*Oncorhynchus mykiss*	0.5–1 kg	n.d.	[72]
	d: 19.9 ± 8.25 nm; l: 0.8 ± 0.5 µm	n.d., MWCNTs	BSA	Microinjection	*Danio rerio*	embryos/ larvae	n.d.	[71]
	n.d.	n.d., SWCNTs	Plasmid DNA: VP7 from grass carp reovirus	i.p. injection	*Ctenopharyngodon idella*	25–30 g	73% E (1 µg), 91% E (5 µg) 100% E (10 µg), 9% N (1 µg), 27% N (5 µg) and N (10 µg) at 15 DPC	[73]
Chitosan	n.d.	0.02% chitosan in sodium acetate buffer	Plasmid DNA: OMP38	Oral	*Lates calcarifer*	Juveniles	46% E at 14 DPC	[94]
	218.9 nm	TPP ionic gelation, 2 mg/mL chitosan in 3% (*v*/*v*) acetate	Plasmid DNA: OMPK	Oral	*Acanthopagrus schlegelii*	15–16 cm	72.3% E and 0% N 14 DPC	[95]
	n.d.	Complex coacervation, 0.02% (*w*/*v*) powdered chitosan	Plasmid DNA: βgalactosidase	Oral	*Oreochromis niloticus*	5–10 cm and 33–40 g	n.d.	[93]
	287.1 ± 1.49 nm	Complex coacervation, chitosan to RNA ratio: 1:1, 2:1, and 3:1	Bare RNA	Oral	*Labeo rohita*	2.7–3.1 g	83% E (2:1) and 33% N at 15 DPC (*)	[92]
	185.4 ± 2.1 nm	TPP ionic gelation, chitosan in 1% (*w*/*v*) acetic acid solution	Vitamin C	Oral	*Onchorhynchus mykiss*	Adult	n.d.	[91]
	253–258 nm	Ionotropic gelation, chitosan at concentration of 2.4 mg/mL in acetic acid solution (0.4% *v*/*v*)	Vitamin C	Oral	*Solea senegalensis*	Larvae	n.d.	[97]

Table 2. *Cont.*

Nanoparticle	Size	Production Technique and Composition	Encapsulated Molecule	Administration	Species	Fish size	RPS	Reference
Liposomes	n.d.	Film dispersion method. DPPC, DPPS, Chol (molar ratio 1:10:5)	FKB *Vibrio harveyi*	i.p.injection	*Epinephelus bruneus*	29.5 ± 2.1 g	75% E, 65% N and 60% liposome at 30 DPC	[113]
	n.d.	Film dispersion method. DPPC (0.5 µmol), DPPS (0.5 µmol) and Chol (1 µmol)	*Aeromonas salmonicida* total extract	Oral	*Cyprinus carpio*	350 g	54% E at 30 DPC (*)	[114]
	200 nm	Extrusion method. PC:Chol: PG or PC:Chol:SA in a 6:3:1 molar ratio	LPS from *Aeromonas salmonicida*	i.p.injection	*Onchorhynchus mykiss*	40 and 80 g	n.d.	[115]
	n.d.	Film dispersion method. 600 mg of phosphatidylcholine in 25 mL chloroform	FKB *Aeromonas salmonicida*, inactivated toxin and LPS	Immersion	*Salmo gairdneri*	Fry	70% E and 59% N at 126 DPC (*)	[116]
	n.d.	Film dispersion method. PS, PC, and Chol (molar ratio 1:10:5)	Koi herpesvirus whole extract	Oral	*Cyprinus carpio*	30 g	74% E (NKC03) and 65% E (IKC03) at 23 DPC	[117]
	125 nm	Extrusion method. DOPA, DLPC, Chol, Cholesteryl and Chol-PEG600	LPS and Poly I:C	*In vitro*, zebrafish hepatocytes and head kidney macrophages	*Danio rerio* and *Onchorhynchus mykiss*	Zebrafish hepatocytes, trout macrophages	n.d.	[118]
	125 nm	Extrusion method. DOPA, DLPC, Chol, Cholesteryl and Chol-PEG600	LPS and Poly I:C	Injection and immersion	*Danio rerio*	Adult	33% E, 21% N and 20% liposome at 15 DPC	[23]
	n.d.	High-pressure homogenization. 6% (*wt/v*) cinnamaldehyde, 10% (*v/v*) lecithin and 0.5% (*v/v*) α-tocopherol	Cinnamaldehyde	Immersion	*Danio rerio*	Adult	58% E at 11 DPC (*Vibrio Vulnificus*), 35% E at 8 DPC (*Aeromonas hydrophila*) and 31% E at 8 DPC (*Streptococcus agalactiae*) (*)	[120]

Table 2. *Cont.*

Nanoparticle	Size	Production Technique and Composition	Encapsulated Molecule	Administration	Species	Fish size	RPS	Reference
Liposome	n.d.	Lipid film hydration, lipid:peptide ratio of 1:50	Melittin	*In vitro,* EPC cell line	*Pimephales promelas*	EPC cell line	n.d.	[121]
	n.d	Film dispersion method, DPPC (0.5 µmol), DPPS (0.5 µmol) and Chol (1 µmol), or DPPC (3.5 µmol) and Chol (1 µmol)	BSA	Oral	*Cyprinus carpio*	350 g	n.d.	[122]
PLGA	125–225 nm	D.E., PLGA: 50:50 (40–75 kDa); PLA (85–160 kDa)	OMP from *Aeromonas hydrophila*	i.p. injection	*Labaeo rohita*	50 ± 10 g	75% PLA, 55% PLGA and 38 % N at 42 DPV	[123]
	320–500 nm	D.E., n.d.	Plasmid DNA: Firefly luciferase gene	i.m. injection	*Salmo salar*	30 g	n.d.	[124]
	< 500 nm	D.E., n.d.	Plasmid DNA: MCP from LCDV	Oral	*Paralichthys olivaceus*	50–100 g	n.d.	[125]
	n.d.	D.E., 5% of PLGA/methylene chloride and 5% of PVA/water soluble	Plasmid DNA: protein-G from IHNV	Oral	*Onchorhynchus mykiss*	5 g	11% E low dose, 22% E high dose and 82% N at 180 DPC; 0% E low dose, 19% E high dose and 55% N at 300 DPC	[126]
	300–400 nm	D.E., PLGA : 50:50 (5–15 kDa; 40–75 kDa); 75:25 (66–107 kDa); PLA (24–47 kDa)	Hemocyanin from *Limulus polyphemus*	i.p. injection	*Salmo salar*	29 ± 3.1 g	n.d.	[127]
	300–400 nm	D.E., PLGA: 50:50 (5–15 kDa; 40–75 kDa); 75:25 (66–107 kDa); PLA (24–47 kDa)	β-glucan	i.p. injection	*Salmo salar*	29 ± 3.1 g	n.d.	[127]

Table 2. *Cont.*

Nanoparticle	Size	Production Technique and Composition	Encapsulated Molecule	Administration	Species	Fish size	RPS	Reference
PLGA	300–400 nm	D.E., PLGA: 50:50 (5–15 kDa; 40–75 kDa); 75:25 (66–107 kDa); PLA (24–47 kDa)	β-glucan	i.p. injection	*Salmo salar*	29 ± 3.1 g	n.d.	[127]
	< 1000 nm	D.E., n.d.	γ-globulins from human blood	i.p. injection	*Salmo salar*	30 g	n.d.	[128]
	< 1000 nm	D.E., n.d.	β-glucan	i.p. injection	*Salmo salar*	30 g	n.d.	[128]
SLN	141–335 nm	n.d.	6-Coumarin	*In vitro*, SAF-1 cell line and HK leukocytes	*Sparus aurata*	100 g	n.d.	[129]

BSA: Bovine serum albumin; Chol: Cholesterol; D.E.: double emulsion; DLPC: 1,2-didodecanoyl-sn-glycero-3-phosphocholine; DOPA: 1,2-dioleoyl-sn-glycero-3-phosphoric acid monosodium salt; DPC: days post-challenge; DPPC: Dipalmitoylphosphatidylcholine; DPPS: Dipalmitoylphosphatidylserine; E: encapsulated antigen; FIA: Freund`s incomplete adjuvant; FKB: formalin killed bacteria; i.m.: intramuscular injection; i.p.: intraperitoneal; IHNV: Infectious haematopoetic necrosis virus; LCDV: Lymphocystis disease virus; LPS: Lipopolysaccharide; MCP: major capsid protein; MWCNTs: Multi-Walled Carbon Nanotubes; N: naked antigen ; n.d.: not described; NKC03 and IKC03: two koi herpesvirus isolates; OMP: outer membrane protein; OMP38: outer membrane protein of *Vibrio anguillarum*; OMPK: Outer membrane protein K; PC: Phosphatidylcholine; PG: Phosphatidylglycerol; PLA: Polylactic acid; PLGA: poly(lactic-co-glycolic acid); Poly I:C: Polyinosinic:polycytidylic acid; PS: Phosphatidylserine; PVA: polyvinyl alcohol ; RPS: Relative percent survival; SA: Stearylamine; SWCNTs: Single-Walled Carbon Nanotubes; βgal: β-galactosidase; VP7: Viral protein 7; (*): calculated RPS.

2.4.3. Encapsulation of Other Compounds in Liposomes

Cinnamaldehyde, a natural compound extracted from cinnamon, was encapsulated in liposomes. These liposomes displayed antimicrobial activity *in vitro* against aquatic pathogens such as *Streptococcus agalactiae*, *A. hydrophila*, and *Vibrio vulnificus*, as well as the antibiotic resistants *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. The *in vivo* results using an immersion treatment demonstrated an increased survival rate and bacterial growth inhibition in zebrafish infected with *S. agalactiae*, *A. hydrophila* and *V. vulnificus* [120] (Table 2).

Also melittin, an antimicrobial peptide, was loaded into liposomes with covalently attached antibodies directed against Viral Haemorrhagic Septicemia Rhabdovirus (VHSV) glycoprotein G (Table 2). These melittin-immunoliposomes were capable of inhibiting the VHSV infectivity by 95% via direct inactivation of the virus. To our knowledge, this is the first report on fish pathogen targeted liposomes. However, the characterization of this formulation was not described nor the size or the charge of this formulation [121].

Finally, humoral immune responses were analyzed in a study of oral administration of liposome-entrapped BSA in carp (*C. carpio*). The BSA-containing liposomes were stable in carp bile and induced significant antibody responses against BSA in serum as well as in intestinal mucus and bile. BSA-specific antibody secreting lymphocytes were detected in the spleen and head kidney of immunized fish. In contrast, no serum antibody responses were observed when fish were orally immunized with BSA-containing unstable liposomes or BSA alone [122] (Table 2).

Poly (Lactic-co-Glycolic Acid) (PLGA)

PLGA, Poly (Lactic-co-Glycolic Acid) is a biodegradable polymer and probably the most extensively investigated carrier for drug delivery in mammals [130,131]. PLGA is a copolymer synthesized by two different monomers, lactic acid and glycolic acid. The forms of PLGA depend on the monomer ratio used during the polymerization process. PLGA nanoparticles (PLGA-NPs) are degraded by hydrolysis and the degradation time depends on the monomer ratio and on the molecular weight of the polymers [132]. The PLGA-NPs were approved for human use by the Food and Drug Administration (FDA, USA) and the European Medicines Agency (EMA), because they are highly biodegradable and biocompatible [133]. When the polymer is hydrolyzed the glycolic and lactic acid monomers are released and are eventually removed from the body through the citric acid cycle [134]. For this reason, there is minimal toxicity associated with the use of PLGA as a nanodelivery system since these components are present in different metabolic pathways. The most common PLGA-NPs preparation method used in fish is the double emulsion method [123–128] which is based on the dissolution of an appropriate amount of polymer

(PLGA) in an organic solvent (oil phase) such as dichloromethane, chloroform or ethylacetate [135]. Hydrophobic drugs can be added directly to the oil phase, whereas hydrophilic drugs must be first emulsified with the polymer solution prior to the formation of particles [136]. Then, the solution is emulsified by the addition of an aqueous solution containing a surfactant or an emulsifying agent (e.g., polyvinyl alcohol). By reducing the pressure or by continuous stirring the organic solvent evaporates and this results in the formation of solid nanoparticles. The encapsulation efficiency and the particle size can be controlled by the solvent choice and the stirring rate [135].

The internalization of PLGA-NPs by cells involves different uptake mechanisms. In mammals, the PLGA-NPs are able to avoid the endo-lysosomal system and are maintained in the cytoplasm. The uptake mechanisms of PLGA-NPs in teleosts is poorly understood and few published work have addressed this issue [118]. Despite this, it is known that nanoparticles of less than 500 nm in size are able to enter the bloodstream and subsequently they are cleared by phagocytes in the head kidney, spleen, and/or liver [137]. PLGA-NPs are highly versatile loading bioactive compounds. For applications in fish, γ-globulins from human blood, β-glucan [127,128], DNA vaccines [124–126] or the bacterial outer membrane complex [123] have been encapsulated (Table 2).

2.5.1. Encapsulation of Bacterial Antigens in PLGA-NPs and -MPs

PLGA-NPs were compared with polylactic acid-NPs (PLA-NPs), which also have good mechanical strength, to encapsulate the outer membrane complex from *A. hydrophyla* [138,139]. This complex consists mainly of lipopolysaccharide, phospholipids, and a group of outer membrane proteins. The encapsulation efficiency was higher in PLGA-NPs compared with PLA-NPs (59% and 44%, respectively) but the release *in vitro* was slower from PLA-NPs than from PLGA-NPs (50% at 24 h and 4 h, respectively). This might be explained by the higher hydrophilic nature of the PLGA- compared to PLA-NPs. The non-specific and specific immunity were stimulated in *L. rohita* by both PLGA- and PLA-NPs and this at higher levels than the naked antigen (Table 2). Finally, in a challenge against *A. hydrophyla*, the PLA-vaccine provided higher levels of protection compared with the PLGA-vaccine (75% RPS and 55% RPS, respectively) and with the naked antigen (38% RPS) [123]. In addition, the encapsulation of the same antigen in PLGA-microparticles (PLGA-MPs) was evaluated [56]. These PLGA-MPs were studied in *L. rohita* administrated parenterally (Table 2). Encapsulation efficiency of PLGA-MPs was lower compared to PLGA-NPs (25% and 50%, respectively). Both the microparticles and the nanoparticles significantly stimulated non-specific (myeloperoxidase, respiratory burst activity, haemagglutination, *etc.*) and specific immune response parameters at similar levels at 21 and 42 days after vaccination. Finally, in a challenge study PLGA-NPs provided protection (55% RPS) against *A. hydrophila* infection, while no data was reported for PLGA-MPs [123].

Oligodeoxynucleotides (ODNs) are short single-stranded synthetic DNA molecules that contain unmethylated CpG motifs. These motifs are highly abundant in bacterial DNA and extremely rare in vertebrates, and they are classified as a type of *Pathogen Associated Molecular Pattern* (PAMP). In mammals, they are recognized by Toll-like receptor 9 leading to strong immunostimulatory effects and also fish are able respond to CpG binding to TLR9 [140]. This antigen was encapsulated in PLGA/liposome-MPs and used to stimulate the immune system of *E. bruneus* using intraperitoneal injection [61] (Table 2). Superoxide dismutase, respiratory burst, and complement activity were mainly stimulated by the PLGA/Liposome microparticles. In contrast, the adaptive immune response and the specific *V. alginolyticus* serum antibody levels were significantly higher with the PLGA-MPs. Finally, the treatment with the PLGA-, Liposome- and PLGA/Liposome-MPs encapsulating ODNs provided good protection levels (78%, 83%, and 83% of RPS, respectively) against a *V. alginolyticus* infection [61] but they are not significantly different from the naked ODN (78% RPS).

The alginate-MPs mentioned in section 2.1.1 were compared with PLGA-MPs as vehicles for the delivery of FKB from *L. garvieae* (Table 2). The PLGA-NPs provided similar protection levels than alginate-FKB-LG (about 63% RPS at 30 days), but lower when compared with the conventional FKB vaccine intraperitoneally injected (95% RPS) [45].

Hølvold *et al.* [124] encapsulated in PLGA-NPs a plasmid containing the firefly luciferase gene under the control of the CMV-IEP promoter. Despite the fact that this formulation does not contain a specific bacterial antigen, the plasmid itself is from bacterial origin and acts as an immunostimulant (bacterial CpG). The PLGA-NPs showed a fast release of the plasmid (80% after 1 h), induced a significant increase in IL-1β and IFNα gene expression in muscle at the injection site in comparison with naked plasmid and stimulated TNFα expression in head kidney. The PLGA-NPs labeled with [^{125}I]-fluorescein were detected until day 70 in trunk kidney, muscle and organ package (liver, heart, gastrointestinal tract and interstitial adipose tissue) [124]. The performance of these NPs was here also compared to MPs. The PLGA-MPs showed a lower release of antigen than the PLGA-NPs (49% at 1 h and an accumulative release of 69% at day 70; 81% at 1 h and an accumulative release of 96% at day 70, respectively). Additionally, PLGA-MPs had a higher retention than PLGA-NPs at the injection site, contributing to the onset of severe histopathological inflammation. This suggests that nanoparticles are more suited to avoid potential tissue damage. Both PLGA-MPs and PLGA-NPs showed better performance than naked plasmid DNA for the induction of pro-inflammatory and antiviral immune responses [124].

2.5.2. Encapsulation of Viral Antigens in PLGA-NPs and -MPs.

PLGA-NPs have been mainly used to encapsulate DNA vaccines aiming to protect against viral diseases. Lymphocystis Disease Virus (LCDV) infection is not lethal, but infected fish are more susceptible to secondary microbial infection [141]. The progression of the disease correlates with an increase in the presence of nodules. A plasmid coding for the major capsid protein (MCP) of LCDV was encapsulated in PLGA-NPs and PLGA-MPs and tested in *P. olivaceus* [125]. The encapsulation efficiency was 64% and full release (100%) was achieved after 60 h at pH 2.0 and after 90 h at pH 9.0. MCP gene expression was detected in gills, intestine, spleen, and kidney from 10 to 90 days after oral administration. Specific serum antibody titers against LCDV reached a maximum at 30 days post-administration. Importantly, in a challenge against LCDV, the presence of nodules was significantly lower in PLGA-vaccinated fish compared to naked DNA vaccinated fish (17% *versus* 100%, respectively) [125]. Of note, Tian and coworkers showed that the encapsulation efficiency in PLGA microparticles was more stable than in the nanoparticle system (78%–88% and 64%–96%, respectively) [57,125]. PLGA-NPs and PLGA-MPs displayed similar performance except that the nanoparticles showed higher release characteristics. The study concluded that PLGA-MPs were also effective oral carriers for the transfer of plasmid DNA [57].

Other PLGA-NPs containing a DNA vaccine against Infectious Haematopoetic Necrosis Virus (IHNV) were used to vaccinate *O. mykiss* (Table 2). In this case, the release of plasmid DNA was not clearly pH dependent, nor were there significant differences between the number of fish expressing the plasmid gene compared to the naked plasmid treatment. This PLGA-vaccine was also not able to confer protection against IHNV [126].

2.5.3. Encapsulation of Other Antigens into PLGA-NPs and -MPs

As mentioned above, PLGA-NPs allow maximal versatility in encapsulating molecules of different nature. Other representative examples of this are immunostimulants such as γ-globulins from human blood, β-glucan from *L. hyperborea* and hemocyanin from *Limulus polyphemus* (Table 2). Three different loaded PLGA-NPs (β-glucan, hemocyanin and both combined) were administrated by intraperitoneal injection in *S. salar* (Table 2). The gene expression profile showed that even PLGA-NPs alone induced a mild inflammatory response in *S. salar* having potential as an adjuvant in salmon vaccine [127]. In a subsequent study, the same group assessed different formulations of PLGA-NPs and -MPs at different monomer ratios. The release of the antigen was similar (around 10%) for all formulations, however the nanoparticles co-encapsulating γ-globulins and β-glucan induced the highest specific antibody response [128]. In another study, PLGA-MPs loaded with γ-globulins were also investigated in *S. salar* by oral administration. The encapsulation in PLGA-MPs allowed its stability in the stomach for longer periods of time, slowing down the passage into the intestine and increasing the levels of intact antigen reaching the blood stream. Also, the PLGA-MPs stimulated the antibody titer in serum but not in cutaneous mucus, gut mucus, or in bile [58].

Parasitic protozoa have developed sophisticated evasion mechanisms to evade the host's innate immune defenses and currently, there are no anti-parasitic vaccines commercially available for farmed fish. A unique study aimed to design a specific delivery system for parasite disease prevention [59]. Formalin-killed parasite (i-antigen) from *Uronema marimun,* an opportunistic pathogen infecting flounder (*P. olivaceus*) and grouper (*E. bruneus*), was encapsulated into PLGA-MPs. The PLGA-MPs were administrated to *E. bruneus* by intraperitoneal injection and different innate immune response parameters such as respiratory burst activity, serum lysozyme activity, or complement activity were evaluated. All of them were stimulated by PLGA-MPs and were sustained from one to four weeks, whereas the treatment with the free i-antigen was detected only at week four and at lower levels. The specific i-antigen antibody levels were stimulated both by the free i-antigen and the PLGA-i-antigen, but again at higher levels by the PLGA-MPs [59]. Interestingly, the levels of protection from the loaded PLGA-MPs against *U. marinum* infection were notably high (only 20% of cumulative mortality after 30 days), but only slightly different from the empty PLGA-MPs or the free i-antigen [59].

Other Nanodelivery Systems

Although the amount of research done in mammals and fish is not comparable, there exists a large effort to discover new nanodelivery systems in teleost to cover the different needs for the prevention of diseases in aquaculture. Here we mention two additional approaches aiming to develop new nanomaterials for *in vivo* delivery in fish: calcium phosphate nanoparticles (CaP-NPs) [119] and solid lipid nanoparticles (SL-NPs) [129].

Calcium phosphate is a natural, inorganic, and biocompatible material. CaP-NPs are synthesized using different methods such as mechanochemical synthesis, combustion preparation, wet chemistry techniques, and others [142]. CaP-NPs can be produced in different morphologies, such as spheres, plate-like crystals, needles, or blades [142,143], however, the size and the stability of CaP-NPs are very difficult to control [143]. CaP-NPs are a potential nanodelivery system due to their high bioactivity, biocompatibility, biodegradability and strong adsorption ability under physiological conditions. In mammals, they have been used as nanodelivery system for drugs, vectors, antibacterial agents, or as a vaccine adjuvant [142]. In *L. rohita* CaP-NPs loaded with the S-layer from *A. hydrophila* was assessed by intraperitonal injection (Table 2). The non-specific immune responses (superoxide dismutase, myeloperoxidase, respiratory burst, *etc.*) and the specific immune response (antibody titers) were stimulated and detected at 21, 42, and 63 days post-vaccination. When fish were challenged with *A. hydrophila*, loaded CaP-NPs were able to provide good levels of protection 15 days post-vaccination (97% RPS) with a significant difference in comparison with non-encapsulated S-layer (13%

RPS), but with similar level (94% of RPS) when compared with CaP-NPs with only Freund`s incomplete adjuvant [119].

Solid lipid nanoparticles are produced in solution using solid lipidic materials with surfactants that confer stability and co-surfactants that confer specific ligand properties [144]. SL-NPs can be prepared by different techniques, such as high-pressure homogenization, high-shear mixing, ultrasound, or solvent emulsification/evaporation methods [143,145]. Additionally, SL-NPs present a range of characteristic advantages, such as biocompatibility, non-toxicity, high bioavailability, high-antigen loading ability, controlled release, physical stability, and protection of encapsulated antigens. Finally, SL-NPs can be easily scaled-up for industrial purposes [145,146]. A preliminary *in vitro* characterization of SL-NPs was performed in fish [129] (Table 2). The loaded SL-NPs had a mean diameter of 235–335 nm depending on the amount of cargo, with a net surface charge between −12.5 and −16.5 mV. These nanoparticles were loaded with a fluorescent molecule (6-Coumarin) and were tested for uptake and toxicity in a cell line (SAF-1) and in leukocyte primary cell culture of *S. aurata* head kidney. Release of 6-Coumarin from SL-NPs was around 1% over the course of 48 h at 22 °C and both the cell line and the primary leukocytes were able to internalize these SL-NPs without affecting the cell viability. SL-NPs internalization was dose- and time-dependent. The uptake in SAF-1 cells decreased over time indicating that the SL-NPs in SAF-1 cells are likely processed in the endolysosomal compartment, while the fluorescent signal was stable over the time in primary leukocytes [129].

Discussion

Nowadays there is a large variety of materials that can be used as delivery systems for vaccine/immunostimulant administration in fish. This diversity provides a wide range of options to respond to the high number of different farmed species and the challenge to achieve a good health status in the presence of different potentially harmful microorganisms. However, the study of nanoparticles for aquacultural use is still in its early stage. Research shows variable efficiency of protection depending on the nature of the nanomaterials, the method to produce the nanoparticles, the antigens encapsulated or the fish species assessed. Often there is a lack of information about the manufacturing process as well as the physico-chemical characteristics of the nanoparticles and the properties of the antigens after the encapsulation process, hampering a correct analysis and comparison between delivery systems. For instance, not all publications mention the size of the particles, the efficiency of encapsulation or the release of the antigens in *in vitro* conditions (see Tables 1 and 2). In many cases, the starting point is the “recycling” of particles for mammalian use that are applied directly in fish, without considering the evident differences between mammals and fish. The researcher working on fish health should make a strong effort to design or adapt nanoparticles in order to reach optimal compatibility with the fish characteristics. However, this is difficult because the fish immune system has several differences with the mammalian immune system regarding cell types, cell biology, tissues involved in immune response, *etc.* Also, the fish immune system is not well known in many aspects such as how the adaptive system memory works, which cell types are involved or the role of mucosal immunity.

In general, the use of microparticles is more frequent than the use of nanoparticles even when the surface area/volume ratio is much more advantageous in nanoparticles. An explanation of this is that some materials are not easy to manipulate in the nanosize range or there are not protocols to nanosize such materials. An example is alginate that has been mainly used to produce microparticles and only recently has it started to be used to produce sub-200 nm particles [147]. Again, the characterization of the manufactured nanoparticles should be detailed in the publications in order to compare between the administration routes, the adjuvant properties, the potential degradation of the loaded compound, the efficacy of the system protecting against infection, and the targeted cells or tissues. Different fish species have different responses to vaccination [148] and this fact should be considered when choosing an encapsulation system because they may not be transferable from one species to another. Additionally, the encapsulated antigens modify the physico-chemical characteristics of the nanodelivery system so that the results of the assays on stability, size, surface charge, and organ biodistribution cannot be extrapolated from one molecule to another using the same encapsulating particle. Similarly, the characteristics of the antigen can be changed when it is encapsulated, and thus the functional structure, stability, and immunogenicity of the antigen need to be verified. For example, the size and the surface charge are extremely important for interaction with cells and should be characterized in the loaded system because they can change easily [118]. Of note, in some cases the encapsulation did not provide any protection [40] or did not improve the protection with respect to conventional immunoprotective therapies [55]. Overall, the administration of nanoparticles by intraperitoneal injection achieves good protection levels against infections while the oral administration is at this moment less efficient. One of the exceptions is the system developed with alginate or chitosan to encapsulate DNA vaccines [41,42]. DNA vaccines are still under development and only one commercial vaccine has been licensed in Canada. They are the most promising tools to fight viral infections and thus, the development of novel encapsulation systems to improve their administration and the efficiency is very important. Several new nanomaterials such as carbon nanotubes or solid lipid NP are still in the early steps of development but have shown promising results. For example, CNTs have been very effective for encapsulating a DNA vaccine and to confer protection against infection even at low DNA concentrations [73]. It is important to mention that in some studies, the adjuvant effect of the nanodelivery system is almost as potent as the loaded antigen itself. The adjuvant effect of the system itself has been extensively reported in mammals (e.g., liposomes) and it is also clearly observed in some fish species [124] but not in others [23].

Also an important point that should be taken into consideration is the final cost of the encapsulation system for industrial production. Some of the systems developed under research conditions are expensive and may not be affordable for the fish farmers. Finally, all of the nanodelivery systems included in this review have been characterized as non-toxic for cells (*in vitro* viability assays) and similarly *in vivo*. However, the toxicity of nanomaterials and, more importantly, the toxicity of the nanomaterial degradation products that could be detected in water should be addressed carefully [149,150].

Conclusions

Altogether, nano-encapsulation is a very promising strategy with a potential to substantially improve the development of effective vaccines for farmed fish. The research on the delivery of viral vaccines using nanoparticles will be the more important milestone in fish vaccinology. In this context, more traditional biomaterials such as alginate and chitosan have shown good results but new materials such as CNTs or solid lipid NP could improve the delivery of DNA vaccines. More research is still needed to specifically design encapsulation systems adapted to the fish immune system and to decipher the basis of the fish immune system.

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Author Contributions

Jie Ji mainly wrote the chitosan and liposome sections and the Table 1, Debora Torrealba mainly wrote the alginate, PLGA and CNTs sections and Table 2, Àngels Ruyra and Nerea Roher wrote the introduction and discussion and Nerea Roher revised and corrected the whole manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Aims and objectives

The overall aim of this thesis is to understand the principles of Toll-like receptors in immunology and develop vaccine adjuvants as an alternative approach to improve the prevention and control diseases in aquaculture. Therefore, the main objectives are:

1) The study of the Toll-like receptors family of *Branchiostoma lanceolatum* and their annotation by phylogenetic analysis.
2) The comprehensive study and annotation of one novel TLR, Bl_TLRj, including full-length cDNA cloning, sequence analysis, and phylogenetic analysis.
3) The establishment of heterologous transitory and stable expression of Bl_TLRj in HEK293 cells.
4) The cellular localization of Bl_TLRj in HEK293 cells and its specific ligand.
5) The *in vivo* study of $\text{IBs}^{\text{TNF}\alpha}$ uptake and immunomodulation in adult zebrafish intestine administrated by oral intubation.
6) The establishment of an adult zebrafish bacterial infection model by i.p. injection of a fish pathogen, *M. marinum*.
7) The evaluation of the protection conferred by $\text{IBs}^{\text{TNF}\alpha}$ against a *M. marinum* lethal infection.
8) The evaluation of the protection conferred by NL_c against a *M. marinum* lethal infection.
9) The establishment of a zebrafish larvae infection model by bath immersion of a Gram-positive and Gram-negative fish pathogen, *M. marinum* and *A. hydrophila*.
10) The *in vivo* study of the tissue biodistribution and the immunomodulation of $\text{IBs}^{\text{TNF}\alpha}$ and NL_c in zebrafish larvae
11) The assessment of the protection conferred by the $\text{IBs}^{\text{TNF}\alpha}$ and NL_c in zebrafish larvae against either *M. marinum* or *A. hydrophila* infection.

Chapter 1

The Toll-like Receptor system in amphioxus (*Branchiostoma lanceolatum*) and the full characterization of a novel TLR in *B. lanceolatum*

Chapter 1: The Toll-like Receptor system in amphioxus (*Branchiostoma lanceolatum*) and the full characterization of a novel TLR in *B. lanceolatum*

Abstract

Toll-like receptors (TLRs) are important for raising innate immune defense. Here we identified 28 new putative TLR genes of *Branchiostoma lanceolatum* from genomic data and we demonstrated by RT-PCR that all of them are expressed in adult amphioxus. Phylogenetic analysis showed that the repertoire of the *B. lanceolatum* TLRs consists in both non-vertebrate- and vertebrate-like TLRs. One of these novel *B. lanceolatum* genes, Bl_TLRj, was cloned and we propose, based on sequence and functional analysis, that it might be classified as an antiviral receptor belonging to the TLR13 subfamily. Phylogenetic analysis showed that it clusters with the vertebrate TLR11 family and might be homologous of TLR13 or TLR22 based on sequence alignments. Transitory and stable expression in HEK293 cells showed that Bl_TLRj localized in the cytoplasm, but it did not recognize most of the common ligands for TLRs. However, when we fused the ectodomain of Bl_TLRj to the TIR domain of human TLR2, it could induce NF-κB transactivation in response to Poly I:C treatment, indicating that in amphioxus, specific accessory proteins bind to the TIR domain and are needed for downstream activation. In summary, *B. lanceolatum* has a full set of TLR receptors both non-vertebrate- and vertebrate-like TLRs and we propose that Bl_TLRj may be a receptor that responds to viral infection as part of the innate immune system and could be an ancestor of fish specific TLR13 subfamily.

Introduction

In vertebrates there are two types of immunity to fight infections: innate and adaptive. The innate immune system is genetically programmed to detect invariant features of invading microbes. In contrast, the adaptive immune system employs antigen receptors that are not encoded in the germ line but are generated *de novo* in each organism (1). The innate immune system is the first line of defense against infectious diseases (2). Immediately after infection, the innate response is activated to combat pathogens and synthesize inflammatory mediators and cytokines (3). However, the primary challenge of the innate immune system is how to discriminate a countless number of pathogens using a restricted number of receptors (2). In response a variety of receptors that can recognize conserved motifs on pathogens have evolved (4). These conserved motifs are known as Pathogen-Associated Molecular Patterns (PAMPs) (5) and their recognition partners, are called Pattern Recognition Receptors (PRRs) (6).

Toll-like receptors (TLRs), one of the most widely studied PRRs, are type-I transmembrane proteins consisting of several extracellular leucine-rich repeat (LRR) motifs, one transmembrane (TM) domain, and one intracellular Toll/interleukin-1 receptor (TIR) domains (7). The ectodomain, which mediates the recognition of PAMPs, is arranged in tandem LRRs, from 1 to many depending on the receptor type. Each LRR contains a conserved 11-residue segment with the consensus sequence LXXLXLXXNXL, where X can be any amino acid, L is a hydrophobic residue (leucine, valine, isoleucine or phenylalanine) and N can be asparagine or cysteine (8). The TIR domain is present in the cytoplasmic region and required for downstream signal transduction (9). Upon PAMPs recognition, TLRs recruit TIR domain-containing adaptor proteins such as MyD88 and TRIF, which initiate signal transduction pathways that culminate in the activation of NF-κB, IRFs, or MAP kinases to regulate the expression of cytokines, chemokines, or type I interferons (IFNs) (10).

Prior to the identification of Toll-like receptors, the first Toll receptor was described in *Drosophila* in 1985 (11). It was involved in the dorsoventral polarity of the developing embryo and in antifungal responses in adults (11) (12). *Drosophila* Toll is composed of an extracellular LRR domain, a transmembrane domain, and an intracellular TIR domain. To date, nine family members (Toll and Toll2-9) have been identified in the *D. melanogaster* genome (11) (13) (14). These Tolls do not bind any PAMPs directly and they need the assistance of accessory proteins, such as peptidoglycan-recognition proteins (PGRPs) and Gram-negative-binding proteins (GNBPs) which cooperate to sense the Gram-positive bacteria and to activate proteolytic enzyme(s) leading to the cleavage of Spaetzle, and thus activate the signaling cascade (13). Ancient homologs of TLRs are present within the genomes of the cnidarians, including a single TLR in *Nematostella vectensis* that structurally resembles Toll and four Toll-related proteins in *Hydra magnipapillata* that play a role in epithelial immunity (15) (16). In the mollusk, *Crassostrea gigas*, four TLRs were reported that could constitutively activate NF-κB signal pathway in HEK293 cells (17). In another species such as *Chlamys farreri* the TLR ectodomain fused with the TIR domain of human TLR2 could activate NF-κB in response to multiple ligands in a heterologous system (18). In hemichordates, an acorn worm, *Saccoglossus kowalevskii*, was reported to have eight TLRs (19). In echinoderms, 222 TLRs candidates were described in purple urchin *Strongylocentrotus purpuratus* according to the genome survey (20). In another echinoderm species, *Lytechinus variegatus*, the TLR gene family found in the genome is notably smaller (68 TLR sequences) (19). Function and cellular localization of sea urchin TLRs await further investigation. In cephalochordata, genome investigations have revealed that *Branchiostoma floridae* has 48 TLRs (21). An amphioxus TLR was identified in another species *Branchiostoma belcheri tsingtauense*. Experimental data support the immunological function of this TLR that together with a MyD88 accessory protein were shown to be involved in the NF-κB signal pathway (22). In tunicates, two functional TLRs were identified in *Ciona intestinalis* and both can stimulate NF-κB in response to multiple pathogenic ligands in a mammalian system, indicating a broader pathogen-associated molecular pattern recognition than vertebrate TLRs (23).

In vertebrates, at least 16 TLR types were discovered in teleosts by exploring the available draft genomes of five bony fish species (24). In particular, 14 and 11 distinct TLR types were identified respectively in zebrafish (*Danio rerio*) and pufferfish (*Takifugu rubripes*) by genomic and phylogenetic analysis (25). In jawless vertebrates, 16 TLRs were annotated in the genome of Japanese lamprey *L. japonicum* (26). In birds, 10 TLRs were found in the chicken (*Gallus gallus*) (24). In humans and mice, 10 and 12 TLRs were characterized respectively and their ligands were studied (7) (27). According to the protein structure and the ligands of human and mouse TLRs, vertebrate TLRs can be divided into six families: TLR1, 3, 4, 5, 7 and 11. Family 1 includes

TLR1/2/6/10/14/18/24/25 as well as TLR27; family 3, 4 and 5 only include TLR3, 4 and 5 by itself; family 7 includes TLR7/8/9; family 11 includes two subfamilies: TLR11/12/16/19/20/26 and TLR13/21/22/23 (27) (9).

To date, the known functions of TLRs in these families are as follows: In family 1, TLR1/6/10 are able to form functional heterodimers with TLR2/24, and together recognize hydrophobic molecules such as lipids and lipoproteins (28) (29) (30). The TLR3 homodimer recognizes double-stranded RNA (dsRNA) mainly in the endosomes (31) (32). TLR4 binds the MD-2 protein. When this form homodimerizes it recognizes lipopolysaccharide (LPS) on the cell surface (33). As a homodimer, TLR5 specifically detects bacterial Flagellin on the cell plasma membrane (34). In family 7, Both TLR7 and TLR8 mediate the recognition of single-stranded RNA (ssRNA) (35); TLR9 homodimers recognize agonistic unmethylated CpG-containing DNA from bacteria (36). In family 11, Mouse TLR11 is localized in the endolysosomes and recognizes Flagellin (37) or an unknown proteinaceous component of uropathogenic *Escherichia coli* (UPEC) as well as a profilin-like molecule derived from *Toxoplasma gondii* (38) (39). TLR12 is predominantly expressed in myeloid cells and is highly similar to TLR11 and recognizes profilin from *T. gondii* (40). TLR12 functions either as a homodimer or a heterodimer with TLR11 (41). TLR13 in mice recognizes a conserved 23S ribosomal RNA (rRNA) of bacteria in endolysosomal compartment (42). In fish, Miiuy croaker TLR13 showed the immune response after *Vibrio anguillarum* and Poly(I:C) stimulation (43). TLR20-23 are “fish-specific” TLRs which were suggested to be duplicated genes in piscine genomes. The expression of grass carp toll-like receptor 20.2 fluctuated in head kidney cells stimulated by LPS, FLA-ST and poly(I:C) (44). The expressions of TLR20a and TLR22 were up-regulated when zebrafish *in vivo* infected with *M. marinum* (45). The large yellow croaker TLR21 could specifically recognize three CpG-oligodeoxynucleotides but not poly(I:C), LPS, and LTA-SA (46). The expressions of the TLR21 gene was induced to up-regulate in isolated peripheral blood lymphocytes of yellow catfish after stimulation with LPS, PGN, and Poly I:C (47). Peptidoglycan and Poly(I:C) were found to induce the expression of the TLR22 gene in Japanese flounder (48). The expression of rainbow trout TLR22 was induced by the Gram-negative bacterium *A. salmonicida in vitro* (49). The pufferfish TLR22 seems to be located on the cell surface recognizing long dsRNA sequences (50). TLR22 of common carp was highly expressed when fish challenged with poly(I:C) or *A. hydrophila* (51). The Fugu TLR23 may participate in LPS recognition (52).

In this work we have focused on TLRs from amphixous, also called lancelet. This is an ancient chordate lineage which shares key anatomical and developmental features with vertebrates and tunicates (also known as urochordates) (53). The vertebrates, urochordates and lancelets (also known as cephalochordates) together, constitute the phylum Chordata. All chordates have a similarly organized genome though amphioxus has relatively little duplication (54). Thus amphioxus, with its phylogenetic position at the base of the chordata and genomic simplicity is a good non-vertebrate model to help understand the evolution of vertebrates (55). *B. lanceolatum* (the Mediterranean amphioxus) has been extensively studied together with other amphioxus species such as *B. belcheri* (the Chinese amphioxus), *Branchiostoma japonicum* (the Japanese amphioxus), and *B. floridae* (the Florida amphioxus) (56). However, the genome data of *B. lanceolatum* has not been published and no TLRs have been reported in this species. To date, TLRs have been found from cnidarians to mammals which implies a conserved evolution. Therefore, the study of TLRs and their corresponding signaling pathways in *B. lanceolatum* could contribute to a better understanding of the origin and evolution of vertebrate TLRs. In this study, we investigated the total number of TLRs and deduced their protein structure in *B. lanceolatum, B. belcheri,* and *B. floridae* using the available genomic data. We studied the basal expressions of the whole TLR family of *B. lanceolatum* and annotated them by phylogenetic analysis and through their similarities to known TLRs. Moreover, we cloned and characterized a TLR from *B. lanceolatum* and we further investigated the ligands of this TLR in a mammalian expression system. With phylogenetic analysis, we explored the evolutionary relationships of this novel TLR with known vertebrate TLRs.

Materials and methods

Amphioxus culture in the laboratory

Adult Mediterranean amphioxus (*B. lanceolatum*) were kindly provided by Dr. Hector Escrivà from Observatoire Océanologique de Banyuls, Banyuls sur Mer, France. The amphioxus were kept in 60 l glass tanks with approximately 5 cm height of sand covered on the bottom in the laboratory. The water temperature was maintained around 17 ℃ and the salinity was controlled to be between 4.0-4.5 g per 100 g. One third of water was replaced every month. The photoperiod was set to 14 h light/10 h dark. The animals were not fed with extra food during the whole experiment.

RNA isolation and first-strand cDNA construction

Amphioxus were collected and immediately frozen in liquid nitrogen, and then stored at -80 ℃. Total RNA was extracted from the whole animal using TRI reagent (Sigma-Aldrich, T9424) according to the manufacturer’s protocol. The homogenization was performed on Polytron PT1600E instrument (Switzerland). The quality of the RNA was assessed with the Bioanalyzer (Agilent Technologies, G2946-90004) and the concentration was measured with a Nanodrop 1000 (Thermo scientific) according to the user’s manual. The first-strand cDNA was synthesized with reverse transcriptase (RT) reaction using 1 µg of total RNA with SuperScript III first-strand synthesis system (Thermo Fisher scientific, Cat. No. 18080051). The RT reaction was performed in 3 steps: first, mix dNTP (Biotools, Cat. No. 20.038-4186), RNA and oligo(dT)$_{15}$ (Promega, Cat. No. C1101) and denature the mixture for 5 min at 65 ℃, followed by 1 min on ice. Second, add 5X first-strand buffer, 0.1 M DTT, and Superscript III RT and anneal for 60 min at 50 ℃. Third, the reaction was stopped by incubating 15 min at 70 ℃. The cDNA was stored at -20 ℃ until use.

Cloning the full-length cDNA

A DNA BLAST search of NCBI database was conducted using the sequence of Toll-like receptor 1 from *B. Belcheri* (GenBank: DQ400125.2). We obtained a cosmid

MPMGc117K0348 sequence (GenBank: AF391294.1) from *B. floridae* showing 82% identity. Moreover, a BLAST search with *B. belcheri* TLR1 was performed in the genome scaffold of *B. lanceolatum* (Dr. Hector Escrivà) and we identified the ContigAmph29716 sequence showing 83% identity. The forward primer was designed based on the sequence of *B. belcheri* and *B. floridae* and the reverse primer was designed based on the sequence of ContigAmph29716 (Table 1). Subsequently, a partial sequence of 2000 bp was cloned using amphioxus cDNA and the primers mentioned above. The PCR product was separated by electrophoresis and stained with GelGreen Nucleic Acid Gel Stain (Biotium, Cat. No. 41005) and the DNA fragment was recovered using the NucleoSpin Gel and the PCR clean-up kit (Macherey-Nagel, Cat. No. 740609.250). The fragment was then cloned into the pGEM-T Easy Vector (Promega, Cat. No. A1360) and the fragment was sequenced using the sequencing primers T7 and SP6 (provided by Servei de Genòmica i Bioinformàtica, IBB-UAB). The 5'-end fragment was obtained by rapid amplification of cDNA ends (5' RACE: Invitrogen, Cat. No. 18374058) through using gene specific primers (5'-GAGTGAAGAACAGTGA-3', reverse and 5'-GTCATTCCCTCCAAGGTTCAAAGAAGTC-3', reverse). A fragment of approximately 600 bp was amplified and subcloned into pGEM-T Easy Vector for sequencing. The 3'-end fragment was obtained by rapid amplification of cDNA ends (3' RACE: Invitrogen, Cat. No. 18373019) using gene specific primer (5'-CGAAGACAGGCGATGGGTT-3', forward). A fragment of approximately 1000 bp was amplified and subcloned into pGEM-T Easy Vector for sequencing. Finally to obtain the full-length a PCR was carried out with the Expand high fidelity PCR system (Roche, Cat. No. 11732650001) with the primers (5'-AGAGAGAGAAAACTGCCAGCC-3', forward and 5'- TTTCTGTCTCGACGGTCCTT-3', reverse) designed in the non-coding regions of 5' and 3'-ends. The open reading frame (ORF) has a final length of 2913 bp.

Table 1. **Primers used for cloning and RT-qPCR**

Category	**Primer**	**Sequence (5'-3')**	**Product size (bp)**
Clone	Bl_TLRj_For	GGGACGATCCAGTCACGCTG	2190
	Bl_TLRj_Rev	GACACCAACGGCTGCGCAG	
5'RACE	5'RACE_GSP1_Rev	GAGTGAAGAACAGTGA	684
	5'RACE_GSP2_Rev	GTCATTCCCTCCAAGGTTCAAAGAAGTC	
3'RACE	3'RACE_GSP_For	CGAAGACAGGCGATGGGTT	1119
RT-qPCR	qPCR_Bl_TLRj_For	TCACACGCTTTCTACGGCTT	

qPCR_Bl_TLRj_Rev	AGGCTTAGGTCCAGTACGGT	200

Bioinformatic analysis

The obtained nucleotide sequence of Toll-like receptor from *B. lanceolatum*, named Bl_TLRj, was translated into protein using the EMBL-EBI translate tool (http://www.ebi.ac.uk/Tools/st/). Molecular weight was calculated with ProtParam (http://web.expasy.org/protparam/) and the sequence was examined for the presence of a signal peptide using SignalP (http://www.cbs.dtu.dk/services/SignalP/) and transmembrane domains were predicted using TMHMM Server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Individual LRRs were identified with the LRRfinder software (http://www.lrrfinder.com/). The domains of the protein Bl_TLRj were predicted by the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/). The phylogenetic and evolutionary tree of the vertebrates TLRs and Bl_TLRj protein sequences was built with MrBayes version 3.2 (57) (http://mrbayes.sourceforge.net/) by using the LG+G+I model selected by ProTest 3.4.2 software. MrBayes software runs around 20,000,000 generations to make the average standard deviation of split frequencies less than 0.01 and abandon the first 25% of burn-in samples to summary the parameter values. The alignment of amino acid sequences was performed by running MUSCLE in MEGA7. The N-linked glycosylation sites were predicted by NetNGly 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). The similarity of the protein sequences were blasted in National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi). The ectodomain architecture of Bl_TLRj was predicted according to Wang J. *et al*., 2016 (58).

LPS and Poly (I:C) treatment

Adult amphioxus were immersed in 10 µg/ml bacterial lipopolysaccharide (LPS) from *E. coli* O111:B4 strain (Sigma-Aldrich, Cat. No. L2630) or 10 µg/ml Poly (I:C), a synthetic analogue of dsRNA viruses (Invivogen, Cat. No. tlrl-pic-5) in 6 cm diameter glass plates, respectively. LPS and Poly (I:C) stock solutions at 1 mg/ml were prepared in 1X Phosphate Buffered Saline (PBS; Sigma-Aldrich, Cat. No. p5493-1L) and diluted to the indicated working concentrations with filtered sea water. Sea water filtration was performed with 0.22 µm sterile filter under vacuum (Millipore, Cat. No. SCGPU02RE).

1% PBS in sea water (v/v) treated animals were used as a negative control. After 3 h, 6 h, 12 h, and 24 h post-treatment (immersion), 3 animals of each group were sampled. Treated animals were immediately frozen into liquid nitrogen and stored in -80ºC until use. The total RNA was extracted using TRI reagent and the first-strand cDNA was synthesized with reverse transcriptase reaction using SuperScript III first-strand synthesis system as described above in Materials and Methods.

Quantitative PCR of Bl_TLRj after LPS and Poly I:C treatments

The RT-qPCR was carried out to analyse the relative transcription levels of Bl_TLRj after LPS and Poly (I:C) treatments. The RT-qPCR was performed in the CFX384 Touch Real-Time PCR Detection System (Bio-Rad) using the iTaq universal SYBR Green Supermix kit (Bio-Rad, Cat. No. 1725121) following manufacturer's protocol. RT-qPCR primers of Bl_TLRj (Table 1) were designed for specific gene amplification with a product size of 121 bp. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) gene (forward: 5'-CCCCACTGGCCAAGGTCATCA-3' and 5'-GCTGGGATGATATTCTGGTGGGC-3') was used as the reference gene. 10^{-1} and 10^{-2}-fold diluted cDNA from pooled cDNA were used for Bl_TLRj and GAPDH gene expression analysis, respectively. Each PCR mixture consisted of 5 µl of SYBR green Supermix, 0.5 µM of primers, 2.5 µl of diluted cDNA and 1.5 µl sigma water (Sigma-Aldrich, Cat. No. W4502-1L) in a final volume of 10 µl. All samples were run in triplicate using the following steps: initial denaturation at 95 ℃ for 3 min, 39 cycles of 95 ℃ for 10 s and 60 ℃ for 30 s, and finally, 95 ℃ for 10 s, increase every 0.5 ℃ for 5 s from 65 ℃ to 95 ℃. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (59). All the data was analyzed in GraphPad and significant difference was analyzed by one-way analysis of variance (ANOVA) using the value of ΔCt (Normalize each technical repeat's gene-specific Ct value by subtracting from it the reference gene Ct value).

Plasmids

The full-length of Bl_TLRj cDNA was cloned into pMA-T vector (Invitrogen) between XhoI and AflII restriction enzyme sites. For testing the cellular localization in mammalian cells (HEK293), the Bl_TLRj cDNA was cloned into pIRES2-EGFP vector (Clontech, Cat. No. 6029-1) with two HA-tags (YPYDVPDYA) at 3’ end (named pBl_TLRj-HA) using XhoI and EcoRI as a restriction sites. For testing the ligands of ectodomain of Bl_TLRj in HEK293 cell, the ectodomain and transmembrane domain (amino acids 1-774) of Bl_TLRj fused with human TLR2 cytoplasmic region (amino acids 611-784; NCBI accession number: NP_001305716.1) was cloned into pIRES2-EGFP vector with a HA-tag at 5´ end named pHAbTLRjECDhTLR2TIR (chimeric TLRj) between SacII and EcoRI restriction sites. The eukaryotic expression vector pIRES2-EGFP was purchased from BD Biosciences. The NF-κB-dependent luciferase reporter vector (pNFκB) which included several NF-κB binding sites in tandem repeat and the Renilla luciferase vector (pRenilla) were kindly given to us by Dr José Miguel Lizcano de Vega (Dept. of Biochemistry, Universitat Autònoma de Barcelona). All the recombinant plasmids were confirmed by sequencing and agarose gel electrophoresis digestion pattern with the corresponding restriction enzymes. All the plasmids were prepared at large scale using NucleoBond Maxi endotoxin-free plasmid preparation kit (Fisher Scientific, Cat. No. 74042410) and stored at -20°C until use.

HEK293 cell culture, transient transfection and stable cell lines

Human embryonic kidney cells 293 (HEK293) were grown in complete medium: Dulbecco's Modified Eagle's medium (DMEM: Life Technologies, Cat. No. 31966), supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS: Gibco, Cat. No.10500064) and 1% (v/v) penicillin and streptomycin (AA: Gibco, Cat. No. 15240) at 37 °C and 5% CO_2. The first passage of HEK293 cells stored in liquid nitrogen was carried out by defrosting cells at 37ºC and the cells were cultured overnight in 25 cm^2 flask (Falcon Corning, Cat. No. 353109). The cells were transferred to 75 cm^2 cell culture flask with vented cap (Falcon Corning, Cat. No. 353136) and when the cells reach 80 to 90% density (after 2-3 days), the cells were trypsinized with 2 ml of TrypLE express enzyme (Gibco, Cat. No. 12605010) for 5 min. The trypsin activity was neutralized by adding 4 ml DMEM complete medium. The cells were recovered and the clumps were broken up by pipetting up and down for 20-30 times. The cell suspension was then subcultured in complete medium at a passage ratio of 1:8.

The HEK293 cell lines stably expressing Bl_TLRj (named HEK293_BlTLRjHA) and chimeric TLRj (named HEK293_chimericTLRj) were generated by using Geneticin

(G418, Invitrogen, Cat. No. ant-gn-1) selection. In brief, Twenty four hours before the transfection experiment, HEK293 cells were seeded from the culture flask into 6-well plate (Thermo Scientific, Cat. No. 140675) at the passage ratio of 1:8. HEK293 cells were transiently transfected with pBl_TLRjHA and chimeric TLRj using linear polyethylenimine (PEI: CliniScience, Cat. No. 23966-1) in DMEM. The transfection was performed by incubating the plate 6 h at 37 °C in a CO_2 incubator before replacing the transfection medium with DMEM complete medium. 24 h after transfection, the culture medium were substituted with selective culture medium containing 1000 µg/ml G418. Selective medium was refreshed every 2-3 days until the G418-resistant foci could be identified and all non-transfected cells (control) were dead (around 2 weeks). The colonies were picked and expanded to 75 cm^2 cell culture in selective culture medium containing 1 mg/ml G418 for the following 2 weeks. Finally, the HEK293 stable cells lines were cultured in DMEM complete medium at 37 °C and 5% CO_2.

Flow cytometry

Transfection efficiency was assessed by cytometry using a FACS Canto (Becton Dickinson, USA). Twenty four hours before the transfection experiment, HEK293 cells were seeded from the culture flask into 6-well plate (Thermo Scientific, Cat. No. 140675) at the passage ratio of 1:8. The cells were transiently transfected with empty vector (pIRES2-GFP) and pBl_TLRj-HA using PEI in DMEM according to the manufacturer's protocol. The volume of PEI used was based on a 3:1 ratio of PEI (µg) to total plasmids (µg). The non-transfected cells were used as negative control. The transfection was performed by incubating the plate 6 h at 37 °C in a CO_2 incubator before replacing the transfection medium with DMEM complete medium. 24 h, 48 h, and 72 h after transfection, cells were washed by 500 µl PBS 1X and trypsinized with 500 µl TrypLE express enzyme for less than 5 min. The trypsin activity was terminated by adding 500 µl DMEM complete medium. The cells were harvested and the supernatant was removed by centrifugation (400 ×g, 5 min) in Eppendorf tubes and the cell pellets were resuspended in 500 µl PBS. All the samples were immediately analyzed in a FACS Canto cytometer (Becton Dickinson, USA) to detect the FITC fluorescence signal. The cytometer was set as follow: the mean flow rate was set to moderate speed, cells were visualized on dot plot with SSC height in Y-axis and FSC height in X-axis, an appropriate gate was set to eliminate debris and 10,000 events were recorded, a histogram analysis was generated with FITC fluorescence. The raw data were analyzed with Flowing software (Finland) and GraphPad software (USA).

We also generated two HEK293 cell lines stably expressing Bl_TLRj (named HEK293_BlTLRjHA) and a chimeric TLRj fused to human TLR2 TIR domain (named HEK293_chimericTLRj). Stable expression was confirmed by cytometry detecting GFP signal. Non-transfected HEK293 cells were used as control. The cells preparations for cytometry analysis were described above. The stable cell lines were used for further experiments when the percentages of fluorescent cells remain a constant.

Western blot analysis

HEK293 cells were transiently transfected with empty vector (pIRES2-GFP) and pBl_TLRj-HA as described above. 24 h, 48 h, and 72 h after transfection, cells were washed twice with PBS 1X, lysed in 200 µl 2.5 X home-made cell lysis buffer, and dislodged on ice by using cell scraper (BD Falcon, Cat. No. 353086). The lysed cells were subjected to sonication in a 10 seconds program: 1 s on, 0.5 s off, for 3 times. The cell extracts were loaded into 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Cat. No. ISEQ00010) using Mini-protean Tetra (Bio-Rad). After 1 h blocking with freshly prepared 5% (w/v) BSA (Sigma-Aldrich, Cat. No. A7906) Tris-buffered saline with 0.1% (v/v) Tween-20 (TTBS), the membrane was incubated with mouse anti-HA primary antibody (Covance, Cat. No. MMS-101P) diluted 1:1000 (1 µg/ml of antibody) overnight at 4 ℃ with agitation. Goat anti-mouse IgG (H+L) horseradish peroxidase conjugate (Bio-Rad, Cat. No. 1706516) at 1:3000 dilution in TTBS was used as the secondary antibody. The protein bands were visualized adding the chemiluminescent substrate (Thermo Fisher scientific, Cat. No. 34080).

Immunofluorescence and confocal imaging

The 24×24 mm cover glasses (Labbox, Cat. No. COVN-024-200) were cleaned and treated with Poly-D-lysine hydrobromide (Sigma-Aldrich, Cat. No. P7280). The treated cover glasses were placed on the bottom of 6-well plates. HEK293 cells were seeded on cover glasses in 6-well plate. At the density of 60-70%, the transfections of empty vector and pBl_TLRj-HA were performed as described above in Materials and Methods. 48 h after transfection, cells were washed with PBS 1X and fixed with 4% (w/v) paraformaldehyde (Sigma-Aldrich, Cat. No. 158127) for 15 min at room temperature. The cells were either permeabilized with 0.2% (v/v) Triton X-100 (Sigma-Aldrich, Cat. No. 9002-93-1) or washed with PBS 1X for 15 min. Then, the cells were blocked with 2% (w/v) BSA in PBS 1X for 1 h at room temperature, followed by incubation with the primary antibody (Mouse anti-HA, 1:1000 dilution; 1 µg/ml of antibody) overnight at 4 ℃ under agitation. After incubation, the cells were washed with PBS 1X and then incubated with secondary antibody (anti-mouse Alexa Fluor 555, Invitrogen, Cat. No. A-31570) at 1:1000 dilution in PBS 1X for 2 h at room temperature. The cover glasses were moved with tweezers and placed on SuperFrost Plus slides (Thermo scientific, Cat. No. 10149870) covered with Fluoroshield with DAPI mounting medium (Sigma-Aldrich, Cat. No. F6057). Confocal imaging was performed using a Zeiss LSM 700 confocal laser-scanning microscope with Plan-Apochromat 63× objective. The excited 488-nm laser line and the 555-nm laser line were selected for the green and red range, respectively. The images were analyzed with Fiji software (60) and Imaris software (Bitplane).

Ligand stimulation and dual luciferase activity assay

Human TLR1-9 agonist kit (Cat. No. tlrl-kit1hw) and murine TLR13 agonist (Cat. No. tlrl-orn19) were purchased from Invivogen. Ligands and working concentrations are listed in Table 3. In order to reach a 60-70% density during transfection, the HEK293_BlTLRjHA and HEK293_chimericTLRj stable cells were seeded from one 75 cm^2 flask at 100% density into 24-well plates one day before the experiment. Two plasmids (0.5 µg/ml pNFκB, and 0.05 µg/ml pRenilla) were co-transfected in HEK293 cells using PEI as a transfection vehicle. The pRenilla were used as internal control to normalize the differences in the reporter due to different transfection efficiencies. 24 h after transfection, each ligand was added into the cell medium with the recommended concentrations (Table 3) and incubated for an additional 24 h. 20 ng/ml human tumor necrosis factor-α (TNFα, Sigma-Aldrich, Cat. No. H8916) prepared in DMEM supplemented with 1% FBS was used as positive control. Luciferase activities were performed with the Dual-luciferase reporter assay system (Promega, Cat. No. E1910) using the Victor3 (PerkinElmer) with two auto-injectors according to the manufacturer’s instructions. Briefly, 20 µl of each cell lysate was mixed with 100 µl of Luciferase Assay Reagent II, then the signal of firefly luciferase was collected for 10 s reading, followed by addition of 100 µl Stop&Go reagent, 10 s reading of the renilla signal. The luciferase activities were expressed as fold-changes relative to the controls after the standardization of renilla luciferase activity.

Table 3. **TLRs ligands tested in this study**

TLR Ligands	Working concentrations	TLR agonist
Pam3CSK4, synthetic tripalmitoylated lipopeptide	1 µg/ml	TLR1/2
HKLM, heat-killed *Listeria monocytogenes*	10^8 cells/ml	TLR2
Poly (I:C), synthetic analog of double-stranded RNA, 1.5-8 kb	10 µg/ml	TLR3
Poly (I:C) LMW, synthetic analog of double-stranded RNA, 0.2-1 kb	10 µg/ml	TLR3
LPS, Lipopolysaccharide from *E. coli* K12	10 µg/ml	TLR4
Flagellin from *S. typhimurium*	1 µg/ml	TLR5
FSL-1, synthetic lipoprotein	1 µg/ml	TLR6/2
Imiquimod, imidazoquinoline amine analogue to guanosine	1 µg/ml	TLR7
SsRNA40, single-stranded RNA oligonucleotide	1 µg/ml	TLR8
ODN2006, synthetic oligonucleotides containing unmethylated CpG dinucleotides	5 µM	TLR9
LPS, Lipopolysaccharide from *E. coli O111:B4* (Sigma)	10 µg/ml	TLR4

Bioinformatics of TLR sequences in *B. floridae, B. belcheri*, and *B. lanceolatum*

The *B. floridae, B. belcheri,* and *B. lanceolatum* genomic sequences were obtained from the websites of JGI (http://genome.jgi.doe.gov/Brafl1/Brafl1.home.html), the database LanceletDB (http://genome.bucm.edu.cn/lancelet/index.php), and Dr. Jordi García-Fernandez (Department of Genetics, Universitat de Barcelona), respectively. All the vertebrates Toll-like receptor sequences (Nucleotide and protein) were obtained from National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). Genome sequences were translated and open reading frames were identified with ExPASy (http://web.expasy.org/translate/). Transmembrane regions were predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Individual LRRs were identified with the LRRfinder (http://www.lrrfinder.com/). The protein domain structure was predicted with the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). The phylogenetic analysis and the evolutionary tree of the vertebrates TLRs and three *Branchiostoma* species TLRs were built with MrBayes version 3.2 (57) (http://mrbayes.sourceforge.net/) by using protein sequences. MrBayes software runs around 20,000,000 generations until the average standard deviation of split frequencies was less than 0.01 and then we discarded the first 25% of burn-in samples to summarize the parameter values. The alignment of protein sequences was performed with MUSCLE in MEGA7.

Identification of TLR genes in *B. laceolatum*

TLR sequences were obtained using Bl_TLRj cDNA sequence as query and blast in the *B. laceolatum* genomic sequences kindly provided by Dr. Enrique Navas (Department of Genetics, University of Barcelona). Primers were designed using NCBI primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) and showed in Table 2. Total RNA was extracted from the whole amphioxus and cDNA was synthesized as described above in Materials and Methods. RT-PCR reactions were performed with primers specific for each *B. laceolatum* TLR under following conditions: initial denaturation at 94 ℃ for 5 min, followed by 35 cycles of denaturation at 94 ℃ for 45 s, annealing at 60 ℃ for 45 s, and extension at 72 ℃ for 50 s, and final extension at 72 ℃ for 7 min. GAPDH gene was used as a reference gene. PCR products were separated by electrophoresis on a 1% agarose gel and stained with GelGreen Nucleic Acid Gel Stain. Photos of agarose gel were taken by GelDoc XR system (Bio-Rad, 170-8170).

Table 2. **Primer sequences used for RT-PCR analysis**

Gene ID	**Primer sequence (5'-3')**		**T_m (ºC)**	**Amplicon size (bp)**
BL10262	For	CCACCAATGAAAGAGCTGCG	68.0	245
	Rev	GATGGCAGACCACCAAATGC	67.7	
BL09440	For	AAACCGCTTATCCTCCGTGG	67.1	200
	Rev	TTGCATATCTCCGTGGCGTT	67.5	
BL22164	For	AGAACTGCACGGACGACATT	64.7	217
	Rev	GTAGAACTGAACACGGGCGA	65.2	
BL05337v2	For	GACACTGCCGACTCTCACAT	63.1	173
	Rev	GTGGTAGAGTCACTTGCGCT	62.1	
BL07821	For	CCCGAGAAAACGTACGACAT	63.8	191
	Rev	AGTGTTCTCCTGCTGCTGGT	64.1	
BL52875_d	For	GTAGGTGGTGTGCCGATCTT	63.9	169
	Rev	GAACCAGGCTCACTCTACGC	63.9	

BL52875_c	For	GTCCTGGGCTGATGATCACC	67.0	159
	Rev	AAAGAGGTCGTTATGGCGCA	66.7	
BL52875_b	For	TGGTTCATATCCTGGGCTGC	67.1	167
	Rev	AAAGAGGTCGTTATGGCGCA	66.7	
BL52875_a	For	TGCGTGAACTAACTCTGTCGG	65.3	209
	Rev	AAGTGCCTCTTGCAAGTTTGG	65.4	
BL72403	For	ACTACGAAATGCGAGCGTCA	65.8	183
	Rev	ATAGCGGCCTACCCTTCTCA	64.7	
BL19440	For	TCTTGGAGAAGATTCCTCGGC	66.7	188
	Rev	TTCGTCTCCCATTCCACCATC	68.2	
BL21810	For	GGGAAGCGTCTCATTCTCGT	65.6	274
	Rev	TGGGGCCATGGATCTGTACT	66.6	
BL12652	For	CCACTTTTCTCGCAGTCGGT	66.5	215
	Rev	ACTCAGATCCAGGGTGACGA	64.9	
BL56664	For	CAGCCATCGAAGAGAACGGA	67.7	256
	Rev	AATCGTACAAGAGGCCGGAG	65.2	
BL20861	For	AAACGTCAAGAAGGGCTCGT	64.9	190
	Rev	CAAACCTCGCAGACTCCACA	66.5	
BL48785	For	AGAATTTCGTGGACAGCGAGT	64.9	263
	Rev	GTCAATGTGCTCAGAGTCGGT	64.3	
BL24356	For	AGTTGAACTTGACCCCAGGC	65.3	195
	Rev	AGGTGCCGTAAGTGTTCTGG	64.0	
BL18798_a	For	AGTGCAAATCCACAGGTTGG	64.9	286
	Rev	ATCCCGTAGGTTCAGCAATGTT	65.5	
BL18798_b	For	GGCGTTTGTATCGATTCCG	65.2	254
	Rev	AGACCGCGAGTTTATGCAGT	63.7	
BL24343	For	AATTGTAGCCAGACGAGCCC	65.3	190
	Rev	ATGCTGGGAGGATGTCGAAC	66.4	
BL19922	For	ACATAATCACTCGGGCTCGG	66.1	260
	Rev	TGCAGCTACAGTCAAAGGGG	65.3	
BL17405	For	CTGGGTCATTCTGCTGGGAT	66.3	178
	Rev	TGGCTAGTTTGCGAATCCTGT	65.7	
BL04519_a	For	GGAGACCGAAGATTGGTGAA	64.0	160
	Rev	GCAAAGTCCCGTTCGTGTAT	63.8	
BL08928_a	For	GACGTGAGGAAACGACCGC	68.2	244
	Rev	GACCATCTTCACCATCTCCAAAC	65.6	
BL08928_b	For	AGCATGTTCTTCGGCAAGGA	67.0	250
	Rev	TGACGTCCCGGTTGTTGTAG	65.9	
BL08928_c	For	CGTCTGTTCGGGTTGAGGAA	67.4	189
	Rev	CCAGTTCGCTACCATGTCGT	65.1	
BL30396	For	TTCTTACAACGACGCCTGCT	64.8	226
	Rev	GGGCTATCGGGGTGTGTATG	66.2	

Results

Cloning and analysis of the Bl_TLRj protein sequence

The full-length cDNA of Bl_TLRj (GenBank Accession number: MG437061) and its 5'and 3'-UTRs were obtained by RACE methodology based on three analogous sequences in the genus of *Branchiostoma* that were used to design primers. The length of Bl_TLRj cDNA is 3,772 bp, containing a 5'UTR of 227 bp, an ORF of 2,913 bp encoding 970 putative amino acid residues, and a 3'UTR of 616 bp with a putative polyadenylation signal (AATAAA) which is 17 nucleotides upstream of the poly (A) tail (Figure 1). The SMART predicted that the Bl_TLRj protein has a C-terminal Toll/interleukin-1 receptor (TIR) domain (800-947 amino acid residues), a transmembrane (TM) domain (752-774 amino acid residues), an N-terminal signal peptide (1-27 amino acid residues), and 23 tandem extracellular leucine-rich repeats (LRRs) which include a leucine rich repeat C-terminal domain (LRRCT) and a leucine-rich repeat N-terminal domain (LRRNT) (Figure 2). The LRRs in the ectodomain of Bl_TLRj are flanked by LRRCT and LRRNT domains. TLRs with this type of extracellular domain are known as single cysteine cluster TLRs (sscTLRs) (61). The Bl_TLRj belongs to sscTLRs. The highly conserved segment (LxxLxLxxNxL: "L" is Leu, Ile, Val or Phe and "N" is Asn, Thr, Ser or Cys and "x" is any amino acid) of Bl_TLRj was predicted and identified by LRRfinder (Figure 2). 10 potential N-linked glycosylation sites were predicted by NetNGly 1.0: N^{101}-N^{114}-N^{154}-N^{163}-N^{276}-N^{375}-N^{393}-N^{522}-N^{573}-N^{632} (Figure 1). The full-length CDs showed the highest 42.08% identifies with *Salmo salar* TLR3. The full-length protein showed the highest 30.43% identities with TLR22 of *Squaliobarbus curriculus*. The deduced molecular weight of Bl_TLRj protein is 111.27 kDa.

ACATTAAGGTATACACTAACTTGTAAGCTCTACACAGTTCAATTTATCATTCAAATAACCAAAAACAACC
TGGGTACTGTTACAGTAGTACAGTCCTAGAAGCATCAAGAGTTCAACTTCTGCAACAGAGAGAGAAAACT
GCCAGCCATAAAGACTTGTATATCATCCAGATTCACCTCAATCATAAGGAAGGAAAATAGATGTGAACAA
CACCCTGACAAGTAACA
ATGGAAAACCCACCCAAGTCAACATCAACTTGTATCTACAGTTTACTGTGTCTGTGCTTG
M E N P P K S T S T C I Y S L L C L C L
TTTCTACTGTCGGTCAAGGGTGACCACGTAGCGAACCCTTATCAGTGTCAAGAGTGGACC
F L L S V K G D H V A N P Y Q C Q E W T
ACCCTGCACATTACATGTACCAAACTACAACTCAGCAAAGTGCCAGACAATATTCCCCCA
T L H I T C T K L Q L S K V P D N I P P
TCTACTCTTCATTTAGACCTACATGACAACAGCATCACAGAGCTACAGCAAGAGGACTTT
S T L H L D L H D N S I T E L Q Q E D F **LRR1 61-84**
AAGACATTGATCAACCTTCAGTACCTAGATCTGAGGTGGAACAAGATAGACCACATCGAA
K T L I N L Q Y L D L R W N K I D H I E **LRR2 85-108**
AATGCAACGTTTGCTCCACTAGCCAACTTGAAGACACTAAACGTGTCTGGAAACAAAATC
N A T F A P L A N L K T L **N** V S G N K I **LRR3 109-133**
CATGTTTCTCTGCTGCCTCAATTAGTGGATTTTCTACCTTCTCTTGAACACCTTGAGATA
H V S L L P Q L V D F L P S L E H L E I
TCCGTCAACTGGAAATGGGACGATCCAGTCATGCTGGGGAATATGACAAGCTTTAAAGGC
S V N W K W D D P V M L G **N** M T S F K G
TTGGGAAACCTGACTTCTTTGAACCTGGGAGGAAATGACATTGTTGACGTACAAGAGAAC
L G **N** L T S L N L G G N D I V D V Q E N **LRR4 163-186**
TCCTTTGATGGACTGGACAAGCTACAGAGTCTCAATCTTAGGGACAATCTCATTTCAAAC
S F D G L D K L Q S L N L R D N L I S N **LRR5 187-210**
ATCAACGAAGCATCCTTCTCCCCGCTCAAAGAATTAGAACACTTGGTTCTCTCTAACAAT
I N E A S F S P L K E L E H L V L S N N **LRR6 211-237**
TATCTTACCGACGACGTCCTACAAGTTGATAAACTTTGGTCACCAGTGGTGAAGCTGACA
Y L T D D V L Q V D K L W S P V V K L T **LRR7 238-262**
TCTCTTTACTTGTCTGAAAACTTGCTGTCTTTCGCCCGCTTTCCGTCAGTGTTTCAAAAC
S L Y L S E N L L S F A R F P S V F Q N
TTCTCCCTCCTCCACACCCTGGACCTCTCCAGAAATCAGCTAATAAACCTGACTACAGAT
F S L L H T L D L S R N Q L I **N** L T T D **LRR8 263-288**
GATTTTGCGTCACTGTTATTTACTCCACTGCAAATCCTTCAGTTAGAACGGAACTCTATC
D F A S L L F T P L Q I L Q L E R N S I **LRR9 289-312**
AGTCACATCGACCAAGGGTTGCTGGCATCTTTGGCGAACCTCAAGTCTCTAAAACTGCAG
S H I D Q G L L A S L A N L K S L K L Q **LRR10 313-336**
TCCAATCCCATCCTGTTTTCCCAGCTGAAAGATAAACTGGTCGGACTACAGATTGAAGAG
S N P I L F S Q L K D K L V G L Q I E E **LRR11 337-361**
CTGACACTGGGAGGAAGCCCTGATTTAGACATCATCCGTAGTGATACGTTTCCTTCACTT
L T L G G S P D L D I I R S D T F P S L
CCTTCTCTAAAACACCTAACAATGAGTCTTCTTTATGACTGGAACCCTTCAATCAAATCA
P S L K H L T M S L L Y D W **N** P S I K S
AGCAGACTTATGGGAGGAAGCTTCCTCAACCTGCCAAATCTGACACAACTCAACTTGGAA
S R L M G G S F L N L P **N** L T Q L N L E **LRR12 393-416**
GACTATTCCATCAGCTCAGTTGAACCATACACTTTTACTGGCTTGGAATATTTAGAAAGG
D Y S I S S V E P Y T F T G L E Y L E R **LRR13 417-440**
CTAGAGTTGGGAGAAAATAACATAGCAGACTTCCCCACGCATGCCTTTGATGGTTTGTCA
L E L G E N N I A D F P T H A F D G L S
TCACTCACACATCTAGACCTGGGCCACAACAGCCTCACAGCAGTCAAGTCACACTACTTT
S L T H L D L G H N S L T A V K S H Y F **LRR14 441-464**
CACAGCCTGAAGAATCTGGTTTGGCTGAACCTACAGAACAATGACATCTACCTCATTGAA
H S L K N L V W L N L Q N N D I Y L I E **LRR15 465-488**
GAAACAGCCTTCAAAGATCTTGAAAGCCTTCAGTTTCTTATCTTGACGTCGAACCATCTC
E T A F K D L E S L Q F L I L T S N H L **LRR16 489-511**
ACCACAGTGGCAGGCTTACAGCTGGGTCTTTCTAACTTACGACACCTGGACTTGGAAAGA
T T V A G L Q L G L S N L R H L D L E R **LRR17 512-535**
AACAACTTCACGTCTATTAAAACAGGTTCCTTCAGCAGACTGGAGAGCCTAACACACCTG
N **N** F T S I K T G S F S R L E S L T H L **LRR18 536-559**
ACTCTCGCTCATAACTGGATCAGAAAAATAGAGAAGGAAGCTTTCTCTGAACTCGCAAGG
T L A H N W I R K I E K E A F S E L A R
TTAAAGCGGCTAAATCTGGCGGATAACAGACTTACTAATCTGACGTCCTGGGCATTCGAT

L K R L N L A D N R L T **N** L T S W A F D **LRR19 560-583**

GGGCTGTCGGAATTAGAGGAAATAAAGTTACAACATAACCTGATTGTGGTGGTTGAATCA

G L S E L E E I K L Q H N L I V V V E S **LRR20 584-607**

CACGCTTTCTACGGCTTAGAACAGATGACAAAACTCAACCTGAAAGGGTTCAGCATTGCA

H A F Y G L E Q M T K L N L K G F S I A

ACAATCCCTGACAATGCCTTCATGGGTCTACACAACTTAACCGTACTGGACCTAAGCCTT

T I P D N A F M G L H **N** L T V L D L S L **LRR21 632-655**

AACCAAATCAAGACATTTGGGAAGAAAGCTTTCAACGGTTTAGATAACCTAAGAGTTTTA

N Q I K T F G K K A F N G L D N L R V L

CAGCTGCAGAAAAATGAGATAACCTTCTTGGATGAAACTGTCTTCAAAGAAGTCTTAGAT

Q L Q K N E I T F L D E T V F K E V L D

CGTGTATGGAAAATGGATATACAGGACAATCCTTTATTCTGTGACTGTGATCTGCTATGG

R V W K M D I Q D N P L F C D C D L L W

CTTGTTTTCAAAGCAAACAGCCAACCAAAGAAGGTTGTAGGGTGGAACACAAGCTCCTTC

L V F K A N S Q P K K V V G W N T S S F

AAATGTGCTGCACCCCCTAAGGACCAAGGGAAGTCTTTACAAATCCTTCCTTCCCAGTGT

K C A A P P K D Q G K S L Q I L P S Q C

GAATACGTCTTTATGCCAAACTTATGGCTGGCCAGCTTGCTTTCTTCTTTAGGGATCTTC

E Y V F M P N L W L A S L L S S L G I F

CTCTTTGTGATGACCACGTTCTGTGTCAACTACTACACCTGGAAGTTACGTGACCTGTGG

L F V M T T F C V N Y Y T W K L R D L W

TTTAGGATAAGGCACAGGGACAGGGTGGTGCAAGTCTGTGACAACGATCATAGGTTCGTC

F R I R H R D R V V Q V C D N D H R F V

TTCGACGCCTTCATCGCTCATCACAACGAAGACAGGCGATGGGTTGAGCGGGACCTTTGC

F D A F I A H H N E D R R W V E R D L C

CGGAATCTAGAATGTTCCGAGAACTGCCCAAACTACCGACTGTGTCTTCACCAGCGAGAC

R N L E C S E N C P N Y R L C L H Q R D

TTCCAAGCTGGCGTTCCAATCATCACGAACATCCGTACAGCCGTGGACAGCAGCAGGAAG

F Q A G V P I I T N I R T A V D S S R K

ATTGTCTGTGTCATCACCAGGAGCTTCCTGCGCAGCCGTTGGTGTCAGTTTGAGTTCCAG

I V C V I T R S F L R S R W C Q F E F Q

CTGGCCCAACACACCATGGTGGAGGAGGGAGGGGGGATACGTCTCATCCTGGTGTTCCTG

L A Q H T M V E E G G G I R L I L V F L

GAGGACATCCCGCGCCACCTGGTACGACAGTACCGCCACCTGCAGGCCGTTGTGGACAGG

E D I P R H L V R Q Y R H L Q A V V D R

GACACGTACCTGGAGTGGCCGGGGGACCCGAGGGAACGCCCCCTGTTCTGGAGGAGGCTG

D T Y L E W P G D P R E R P L F W R R L

AGAGCTGCGTTAGGACAGCCATTAGATCAGCAGCCTGACGATAAGGACTCTGAGCCTGAC

R A A L G Q P L D Q Q P D D K D S E P D

CAGCATGGCTTCATGGCATTGGTGGAGGTG**TGA**

Q H G F M A L V E V -

CAGGAAATGTAAAATTGCTATTCTCTCTGAACTAAGAGTAGATAAGGACCGTCGAGACAGAAAATAATTC

TTAAGGCTCCTTTATGAAGAATCAAATCATGTACGAATATGTGTAAATGGTTGGTGAATCAGGACATAAC

TTGTGATCTGCCAAAATGTACTACGGCATTATCATAAACATTTTAGAGGGGAAAGATTCAAATGATAAAC

TCTTTTTCTATTTCTTTAAGTTAATAGTATAAGTCTGCAACTATCATGCAAGTGTGTAGAAGGGATCGTT

TATGAATGACTTTCTCAAAACTAAATGACATCCCTTAATTTGCAAAGGCTAACAGGTAGTTGAAGTGTGA

CAAAACAAGCATGTGTTAACAAGAAGAGGAACTGTTTGAGCAATGCTGCATCCTAATGCCTTTCATCAAA

ATTAAAAGTACTGGCCTGTTTTTGGACACAATGATGAATAACTCAATAAGACACAACTAAACAAAGCTAA

GTATAAGGTGTGTTTAACCTGAAGCAGAACTGTTTTAGTGTATTACTGCCTTTCAGTGAATTTGAAAGAT

CTGGCCTGTTTTTGTACACAATGATGAATAACA**AATAAA**GAAACAAAGCTTGAAGTAAAAAAAAAAAAAA

AA

Figure 1. **Nucleotide and the deduced amino acid sequences of Bl_TLRj**. The start codon (ATG), the stop codon (TAA), and the polyadenylation signal sequence

(AATAAA) are in bold. The signal peptide and the transmembrane region are underlined. LRR domains are highlighted in grey. The potential N-linked glycosylation sites were underlined and in bold.

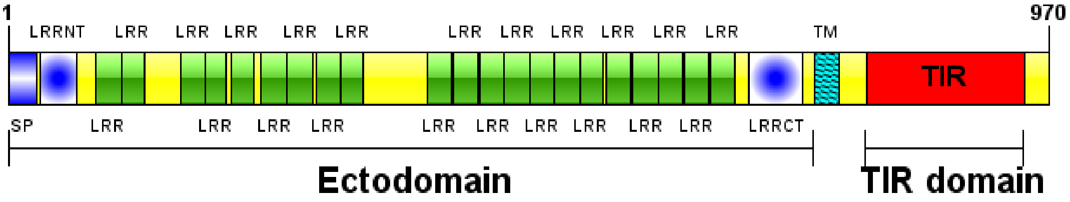


Figure 2. **Predicted domain architecture of Bl_TLRj protein.** The domain structure was predicted using SMART program. LRRs were predicted by LRRfinder. Signal peptide (SP), LRRNT, LRR, LRRCT, Transmembrane domain (TM) and TIR domain are indicated in the figure. Figure was made by IBS (62).

Phylogenetic analysis of Bl_TLRj and vertebrate TLRs

To explore the phylogenetic relationship between Bl_TLRj and vertebrate TLRs (human, mouse, chicken, zebrafish, xenopus, salmo, carp, fugu, etc), a phylogenetic analysis of the protein sequences was constructed based on the MUSCLE by MEGA7 and using MrBayes 3.2 software, fruitfly TLRs were used as outgroup. The substitution model of LG+G+I was selected by considering the lowest value of Bayesian Information Criterion (BIC) score as the best model using ProTest 3.4.2 software. MrBayes software ran 20,000,000 generations to make the average standard deviation of split frequencies less than 0.01 and abandon the first 25% of burn-in samples to summary the parameter values. The amino acid sequences used in this analysis retrieved from GenBank and UniProt are showed in Table 3.

In the phylogenetic tree, all amino acid sequences of the vertebrate TLR genes were clustered into six branches and fruitfly TLRs were clustered into one branch which is away from the six branches. This result indicated that the phylogenetic tree was reliable. The phylogenetic tree showed that the Bl_TLRj, together with TLR1 and TLR13 in *B. belcheri*, is grouped with TLR11 family which including TLR11, TLR12, TLR13, TLR19, TLR20, TLR21, and TLR22 of vertebrates (Figure 3). The probability of the divergence between Bl_TLRj and TLR11 family is 86%. The phylogenetic tree indicates that Bl_TLRj may be a member of TLR11 family.

Table 3. **Protein sequences used in the phylogenetic analysis.**

Gene name	**Species**	**GenBank ID/NCBI reference**
TLR1	*Homo sapiens*	AAC34137.1
	Mus musculus	AAG35062.1
	D. rerio	AAI63271.1
	G. gallus	BAD67422.1
	B. belcheri	ABD58972.2
TLR2	*D. rerio*	AAQ90474.1
	H. sapiens	AAC34133.1
	M. musculus	AAF04277.1
TLR3	*H. sapiens*	AAC34134.1
	M. musculus	AAK26117.1
	S. salar	AKE14222.1
TLR4	*H. sapiens*	AAC34135.1
	G. gallus	AJR32867.1
	M. musculus	AAD29272.1
	Labeo rohita	AOM81178.1
TLR4a	*D. rerio*	NP_001315534.1
TLR5	*H. sapiens*	ACM69034.1
	Rattus norvegicus	ACN60145.1
	Cirrhinus mrigala	AHI59128.1
	Larimichthys crocea	KKF22099.1
	T. rubripes	AAW69374.1
TLR6	*H. sapiens*	ABY67133.1
	M. musculus	AAG38563.1
	D. rerio	NP_001124065.1
	G. gallus	NP_001075178.3
TLR7	*G. gallus*	ACR26243.1
	H. sapiens	AAF78035.1
	Xenopus tropicalis	NP_001120883.1
	D. rerio	XP_003199309.2
TLR8	*H. sapiens*	AAF64061.1
	M. musculus	AAK62677.1
	D. rerio	XP_001920594.4
TLR9	*H. sapiens*	NP_059138.1
	D. rerio	NP_001124066.1
	M. musculus	AAK28488.1
TLR10	*H. sapiens*	AAK26744.1
	R. norvegicus	ACN78428.1
TLR11	*M. musculus*	AAS37672.1

	R. norvegicus	ACL80330.1
TLR12	*M. musculus*	AAS37673.1
TLR13	*M. musculus*	AAS37674.1
	S. salar	NP_001133860.1
	B. belcheri	XP_019646902.1
	X. tropicalis	XP_002935047.2
TLR14	*P. olivaceus*	BAJ78225.1
	T. rubripes	XP_003970412.2
TLR15	*G. gallus*	ABB71177.1
	Coturnix coturnix	ADL14379.1
TLR16	*G. gallus*	ABQ85926.1
TLR18	*D. rerio*	NP_001082819.1
	I. punctatus	AEI59674.1
TLR19	*C. carpio*	BAU98390.1
	S. salar	CDH93609.2
	D. rerio	F1Q6F4
TLR20	*C. idella*	AHN49762.1
	C. carpio	AHH85805.1
TLR21	*D. rerio*	AAI63075.1
	G. gallus	NP_001025729.1
	E. coioides	ADM34974.2
TLR22	*L. rohita*	AGW43270.1
	E. coioides	AGA84053.1
	D. rerio	NP_001122147.2
Toll C	*D. melanogaster*	NP_001262995.1
Toll D	*D. melanogaster*	NP_733166.1

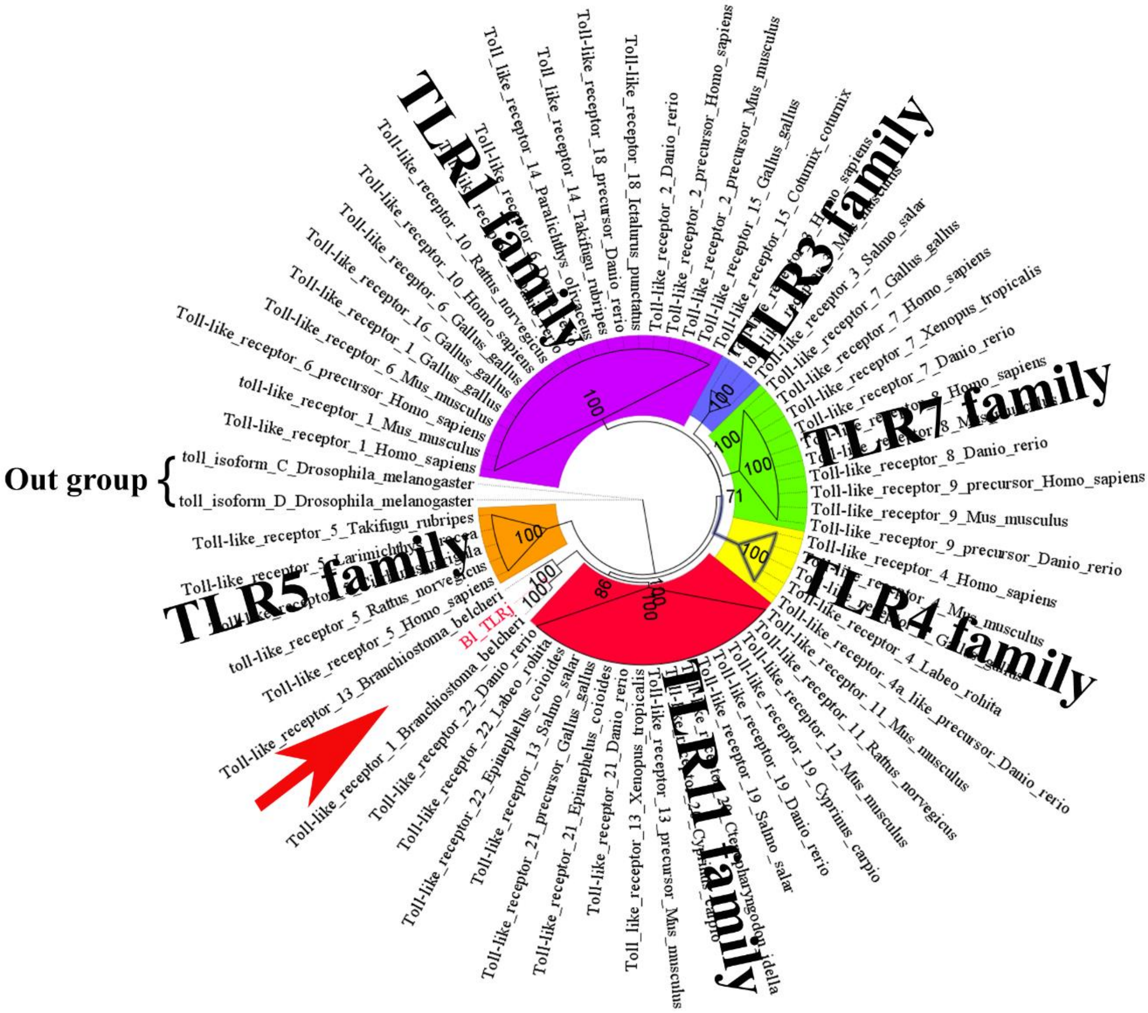


Figure 3. **Phylogenetic tree of the protein sequences of Bl_TLRj and the representative vertebrates TLRs**. *D. melanogaster* Toll sequences were used as out-group. Accession numbers of the sequences are shown in Table 3. Protein sequences were aligned using MUSCLE and phylogenetic trees were constructed in MrBayes using LG+G+I as substitution model. Values on the nodes are probability of the divergence obtained with MrBayes. The tree was generated in FigTree. Six families of vertebrates TLRs are showed in different colors. Bl_TLRj is highlighted with a red arrow.

Expression of Bl_TLRj after LPS and Poly I:C treatments

Amphioxus were treated with 10 µg/ml LPS or 10 µg/ml Poly (I:C) prepared in sea water using immersion method in order to mimick the natural infection route. The expression of Bl_TLRj in response to LPS and Poly (I:C) was analyzed by RT-qPCR at 3, 6, 12, 24 h post immersion (Figure 4). No significant differences in the Bl_TLRj gene expression were detected between control and treated groups indicating that gram negative bacteria and dsRNA virus do not induce up- or down-regulation of the Bl_TLRj in adult animals.

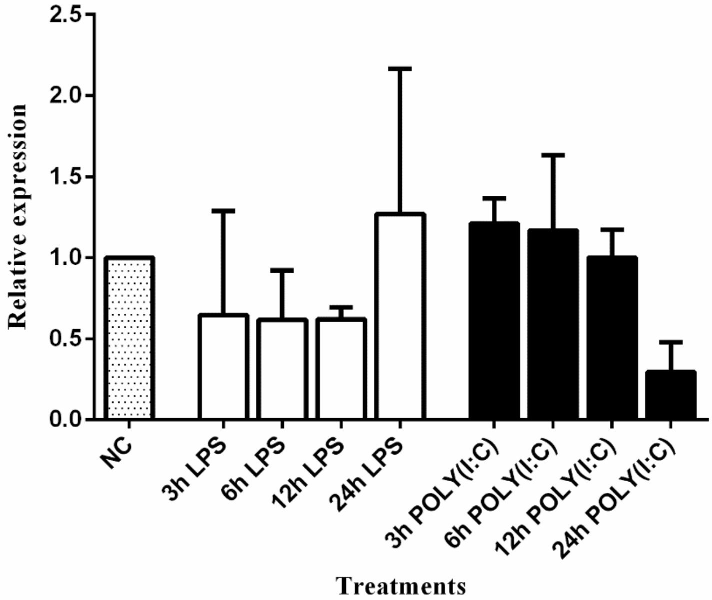


Figure 4. **Expression of Bl_TLRj in amphioxus at 3 h, 6 h, 12 h, and 24 h post immersion in LPS and Poly (I:C)**. GAPDH gene expression was used as reference gene. The bars indicate mean expression of 3 independent experiments (3 individuals each) ±SE.

Transient transfection assay of pBl_TLRj in HEK293 cells

First, we determined the optimal PEI transfection conditions and the optimal transfection times for pBl_TLRj using HEK293 cells. The cells were transiently transfected with empty vector (pIRES2-GFP) and pBl_TLRj and the GFP signal was detected by flow cytometry at 24, 48, and 72 h post transfection. Figure 5 shows that the percentage of fluorescent cells was around 28% compared to the negative control (0.01%) 24 h after transfection, and it increased to 62% at 48 h and remained stable (60% GFP positive cells) at 72 h post transfection while the negative groups stabilized at less than 0.1%. The results suggested that 48 h or 72 h are optimal times for further transfection experiments.

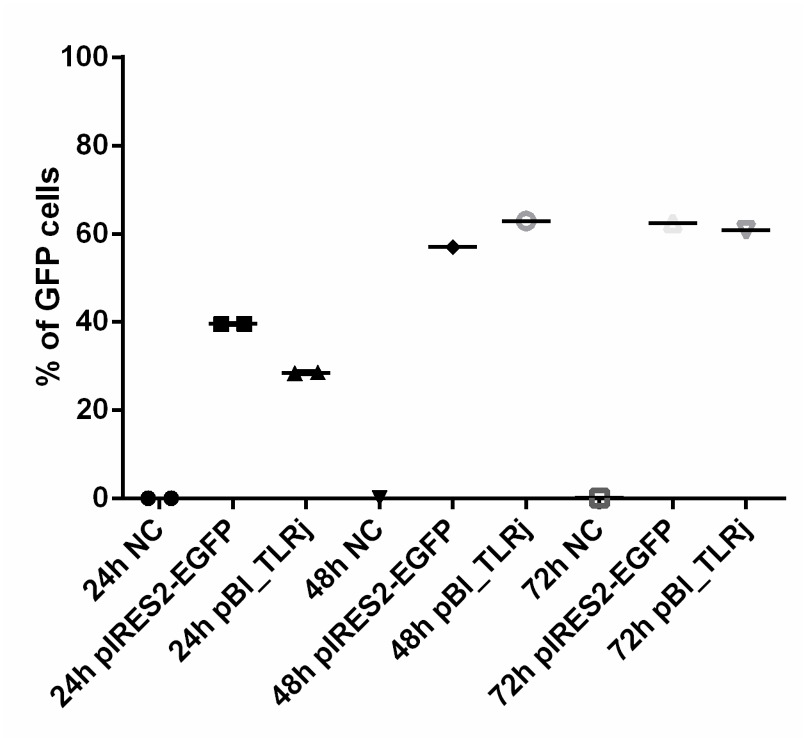


Figure 5. **Transient transfection efficiency of pBl_TLRj by flow cytometer.** HEK293 Cells were analysed 24 h, 48 h, and 72 h post transfection. The non-transfected cells were used as controls. The transfection efficiencies of the empty vector pIRES2-EGFP and pBl_TLRj were evaluated by the percentage of GFP cells in the total gated cells.

Second, we perform a Western blot analysis to confirm that after transfection the Bl_TLRj protein was properly expressed in HEK293 cells and was not degraded by intracellular proteases. The expression of the Bl_TLRj protein was detected in HEK293 cells at 24 h, 48 h, and 72 h post transfection using an antibody against the HA-tag. The expression levels at 48 h and 72 h were much higher than at 24 h post transfection which confirms the results obtained by flow cytometery. The Bl_TLRj protein was detected to run at 135 kDa which is bigger than the theoretical molecular weight (111.27 kDa). This may due to post translational modifications such as glycosylation, phosphorylation, ubiquitination, ubiquitin-like modifications or S-nitrosylation among others.

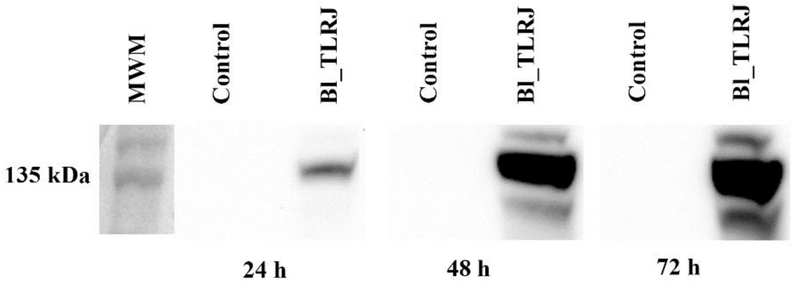


Figure 6. **Western blot analysis of Bl_TLRj protein in HEK293 cells**. The empty vector pIRES2-EGFP was used as controls. Lanes from left to right: protein molecular standard (Niborlab, PLE-1), 24 h control, 24 h Bl_TLRj protein, 48 h control, 48 h Bl_TLRj protein, 72 h control, and 72 h Bl_TLRj protein.

Subcellular localization of Bl_TLRj protein in HEK293 cells

In mammals, TLRs can be divided into two groups according to the localization of protein on the cell surface or intracellular compartments (ER, endosomes, lysosomes, and endolysosomes) (63). To explore the subcellular localization of BL_TLRj we used a heterologous mammalian system: HEK293 cells because these cells are efficiently transfected and they have been extensively used for receptor localization studies immunofluorescence and confocal microscopy observation of HA-tagged BL_TLRj were performed. The successful transfection was confirmed by the GFP expression of the vector pIRES2-EGFP and the nuclei were stained with the DAPI. Confocal microscopy imaging of Bl_TLRj-HA expressed in HEK293 cells showed that the HA tagged protein (labelled with BL_TLRj_P) was localized intracellularly probably in the endosomal membranes (red signal) (Figure 7).

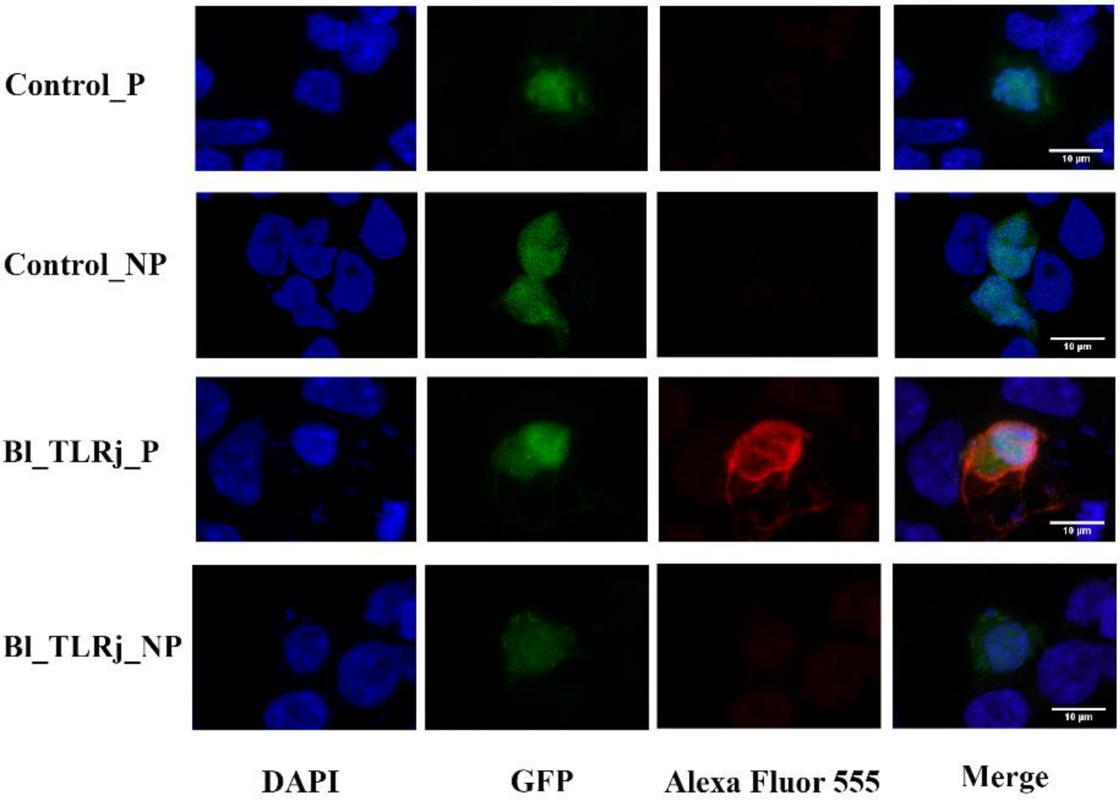
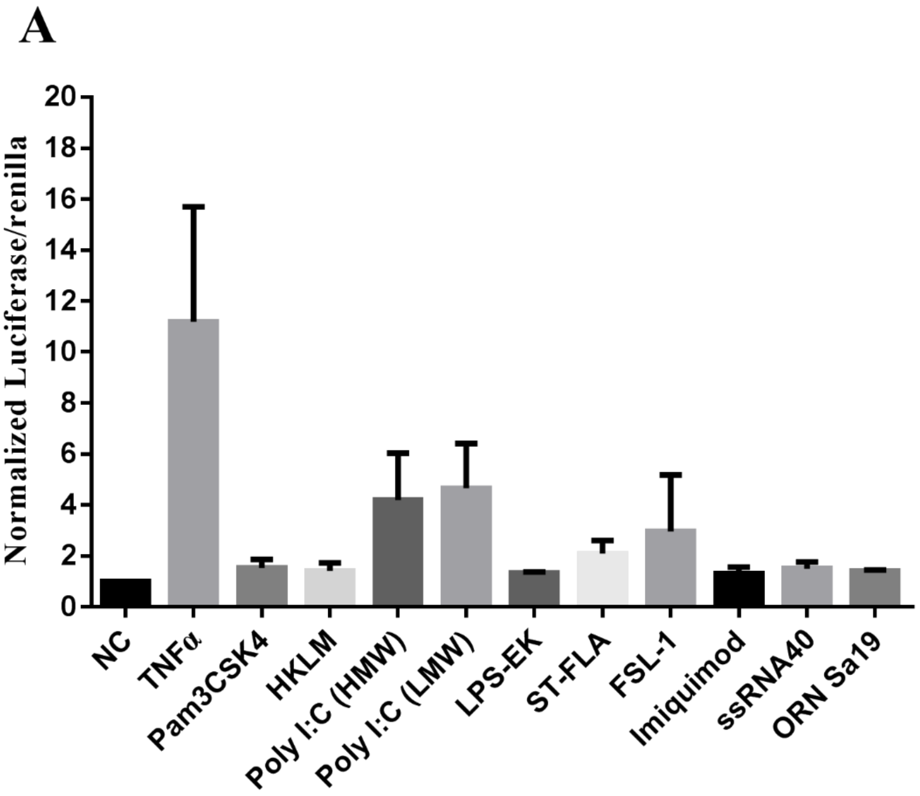


Figure 7. Sub**cellular localization of Bl_TLRj in HEK293 cells.** Empty vector pIRES2-EGFP was used as control. Control_P and Bl_TLRj_P mean the cells were permeabilized with Triton X-100. Control_NP and Bl_TLRj_NP mean the cells were not permeabilized. The red color shows the HA tagged Bl_TLRj located in the cytoplasm.

NF-κB reporter assay

Mammalian TLRs can transactivate the transcriptional factor NF-κB in response to specific ligand binding. Usually each TLR has a restricted ligand preference and reporter assays allow to functionally discriminate between TLR types. To shed light on the role of Bl_TLRj in PAMPs recognition, HEK293 cell lines stably expressing Bl_TLRjHA (HEK293_BlTLRjHA) and chimeric TLRj (HEK293_HAbTLRjECDhTLR2TIR) were generated. The activation of the receptor was analyzed using NF-κB luciferase reporter and the renilla luciferase reporter was used as internal control. However, the HEK293_BlTLRjHA stable cells could not activate the NF-κB promoter stimulated by any of the most common mammalian PAMPs (Table 2) (data not shown). In order to further study the receptor activity, we design a chimeric receptor made with the TIR domain of human TLR2 receptor. This approach has been used before with other aquatic animals to ensure a correct downstream signaling avoiding the differences in the set of adaptors and accessory proteins between vertebrates and non-vertebrates (18). The chimeric TLRj stable cells could respond to mammalian TLR3 ligands, Poly I:C (LMW) and Poly I:C (HMW). While other ligands, including Pam2CSK4 for TLR1/2, HKLM for TLR2, LPS for TLR4, Flagellin for TLR5, FSL-1 for TLR2/6, Imiquimod for TLR7, ssRNA for TLR8, ODN2006 for TLR9, failed to induce NF-κB transactivation in this cells (Figure 8A). Human recombinant TNFα was used as a positive control since it is a well know NF-κB activator. The NF-κB luciferase signals were significantly up-regulated in this stable cells respect to non-transfected HEK293 cells both, treated with Poly I:C (LMW) and Poly I:C (HMW) (Figure 8B). No significant difference on the luciferase signals were found between the stable cells and non-transfected HEK293 cells both treated with Flagellin and FSL-1 (Figure 8B).



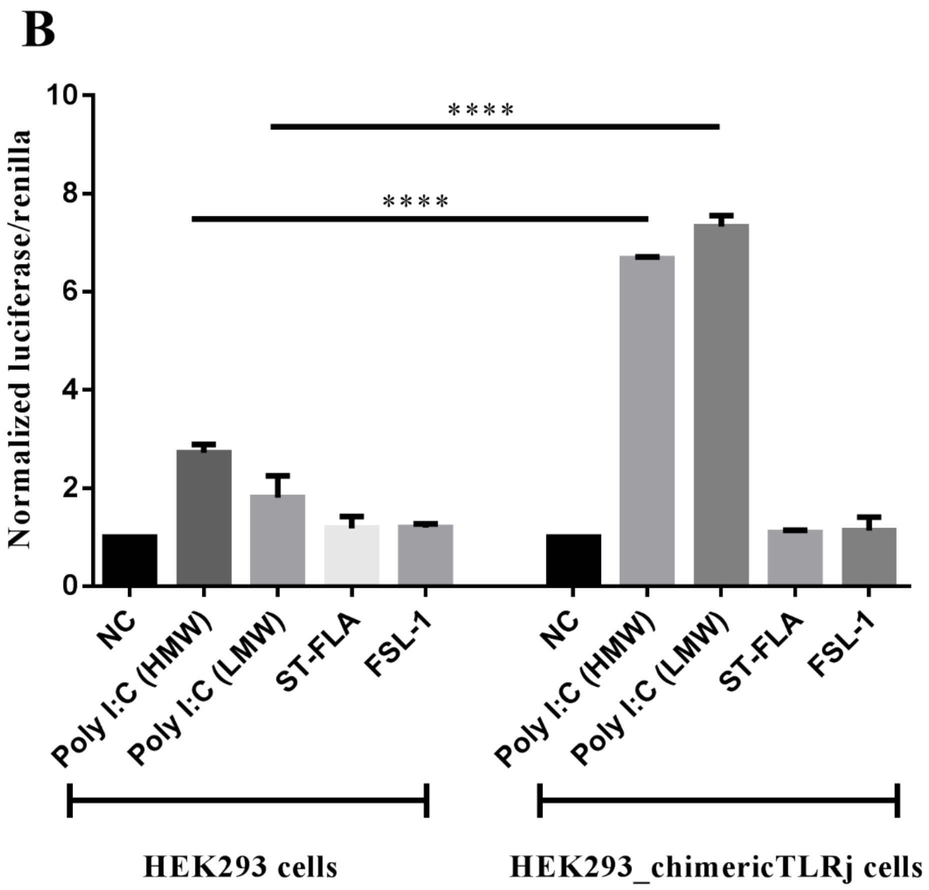


Figure 8. **Dual luciferase report assay.** (A) HEK293_chimericTLRj stable cells were treated with most common mammalian ligands. The stable cells without treatment were used as negative control and cells treated with TNFα (20 ng/ml) were used as positive control. Data were normalized to the value of negative control group. Bars represented mean ± S.D. (B) Chimeric TLRj non-transfected HEK293 cells and chimeric TLRj (HEK293_chimericTLRj) stable cells were treated with four potential ligands. Data were normalized to the value of negative control, respectively. Bars represented mean ± S.D.

Bioinformatics analysis of TLRs in *B. floridae*, *B. belcheri*, and *B. lanceolatum*

There are two structural types of TLRs according to the sequence analysis of TLR ectodomains: Single cysteine cluster TLRs (sccTLRs) and multiple cysteine cluster TLRs (mccTLRs). The sccTLRs are characterized by the presence of a single cysteine cluster on the C-terminal end of LRRs (a CF motif), which is juxtaposed to the plasma membrane. Most TLRs found in deuterostomes have this domain organization. The mccTLRs are characterized by an ectodomain with two or more CF motifs and another cysteine cluster on the N-terminal side of the LRRs (NF motif). They are systematically found in protostomes, but have also been recently identified in the invertebrate deuterostome *S. purpuratus* and in *N. vectensis* (61). However, both sccTLR and mccTLR share the same TLR pattern of "LRR+TM+TIR". Therefore, according to this pattern, we identified 22 TLRs in *B. floridae* (Table 4), 37 TLRs in *B. belcheri* (Table 5), and 28 TLRs *B. lanceolatum* (Table 6). We also discriminated sccTLR and mccTLR in these three species according to the domain structure and phylogenetic analysis (Table 4, 5, 6). The phylogenetic analysis was performed using protein sequences of all TLRs of *Branchiostoma* and representative vertebrate TLRs. The constructed tree revealed the presence of several TLR subfamilies (Figure 9). At least one TLR is grouped with the vertebrate TLR family 1, 4, and 11 but no TLR is clustered with family 3, 5, and 7. There is also what seems to be a distinct cluster of *Branchiostoma* TLRs, which is not grouped with any vertebrate TLR family.

Table 4. **TLRs in *B. floridae***

Gene ID in database	**LRRs**	**sccTLR/ mccTLR**	**Domain structure**	**First annotated sequence by blastp in NCBI**
68489	19	sccTLR	LRR+TM+TIR	TLR 22 *Seriola lalandi*
88412	13	sccTLR	LRR+TM+TIR	TLR 22 *E. coioides*
89467	25	sccTLR	LRR+TM+TIR	TLR 22 *Megalobrama amblycephala*
89468	14	sccTLR	LRR+TM+TIR	TLR 22 *M. amblycephala*
89511_a	6	sccTLR	LRR+TM+TIR	TLR 1 *B. belcheri*
89511_b	5	sccTLR	LRR+TM+TIR	TLR 22 *M. amblycephala*
89513	14	sccTLR	LRR+TM+TIR	TLR 22 *D. rerio*
89514	4	sccTLR	LRR+TM+TIR	TLR 22 *D. rerio*
92915	3	sccTLR	LRR+TM+TIR	TLR *Portunus trituberculatus*
94576	16	sccTLR	LRR+TM+TIR	TLR 13 *S. salar*
97448	16	sccTLR	LRR+TM+TIR	TLR 3 *Anas platyrhynchos*
105256	18	sccTLR	LRR+TM+TIR	TLR 3 *A. platyrhynchos*
126412	16	sccTLR	LRR+TM+TIR	TLR 22 *D. rerio*
100709_a	12	sccTLR	LRR+TM+TIR	TLR 21 *Lissotriton montandoni*
213613	13	sccTLR	LRR+TM+TIR	TLR 3 *D. labrax*
236291_a	14	sccTLR	LRR+TM+TIR	TLR 13 *Tupaia chinensis*
73275_a	5	mccTLR	LRR+TM+TIR	Toll *Apostichopus japonicus*
82252_v1	12	sccTLR	LRR+TM+TIR	TLR 22 *C. idella*
82677_a	12	sccTLR	LRR+TM+TIR	TLR 21 *Miichthys miiuy*
85671_a	7	mccTLR	LRR+TM+TIR	TLR *P. trituberculatus*

88496_v1	16	sccTLR	LRR+TM+TIR	TLR 3 *Haliaeetus albicilla*
99056a	7	sccTLR	LRR+TM+TIR	TLR 4 *Leopoldamys sabanus*

Table 5. **TLRs in *B. belcheri***

Gene ID in database	**LRRs**	**sccTLR/ mccTLR**	**Domains structure**	**First annotated sequence by blastp in NCBI**
304600F	14	sccTLR	LRR+TM+TIR	TLR22 *D. rerio*
294010R	4	sccTLR	LRR+TM+TIR	TLRII *O. mykiss*
115530R	2	sccTLR	LRR+TM+TIR	TLR13 *L. crocea*
308500F	4	sccTLR	LRR+TM+TIR	TLR22 *I. punctatus*
008380R	2	sccTLR	LRR+TM+TIR	TLR22 *M. amblycephala*
292100F	9	sccTLR	LRR+TM+TIR	TLR22 *D. rerio*
020090R	5	sccTLR	LRR+TM+TIR	TLR22 *Scleropages formosus*
024630R	7	sccTLR	LRR+TM+TIR	TLR13 *Myotis brandtii*
320220F	5	sccTLR	LRR+TM+TIR	TLR22 *I. punctatus*
112940R	4	sccTLR	LRR+TM+TIR	TLR22 *I. punctatus*
207490F	7	sccTLR	LRR+TM+TIR	TLR22 *S. formosus*
020250R	7	sccTLR	LRR+TM+TIR	TLR22 *S. formosus*
020150F	4	sccTLR	LRR+TM+TIR	TLR22 *C. carpio*
088530F	1	sccTLR	LRR+TM+TIR	TLR22 *C. carpio*
091830F	4	sccTLR	LRR+TM+TIR	TLR22 *C. carpio*
020140F	7	sccTLR	LRR+TM+TIR	TLR22 *D. rerio*
112920R	7	sccTLR	LRR+TM+TIR	TLR22 *S. formosus*
010410F.t1	6	sccTLR	LRR+TM+TIR	TLR22 *I. punctatus*
010410F.t2	7	sccTLR	LRR+TM+TIR	TLR22 *I. punctatus*
032660R	5	sccTLR	LRR+TM+TIR	TLR22 *M. amblycephala*
134830R	4	sccTLR	LRR+TM+TIR	TLR22A *L. montandoni*
300960R	5	sccTLR	LRR+TM+TIR	TLR22 *M. amblycephala*
044020R	16	sccTLR	LRR+TM+TIR	TLR3 *Chiloscyllium griseum*
202930F	21	sccTLR	LRR+TM+TIR	TLR22 *Scophthalmus maximus*
122860F	2	sccTLR	LRR+TM+TIR	TLR21 *E. lanceolatus*
112820F	6	sccTLR	LRR+TM+TIR	TLR22 *S. formosus*
210680R	8	sccTLR	LRR+TM+TIR	TLR22d *Gadus morhua*
205510F	4	sccTLR	LRR+TM+TIR	TLR *Carassius auratus*
131620F	13	sccTLR	LRR+TM+TIR	TLR13 *M. davidii*
210690F_a	9	sccTLR	LRR+TM+TIR	TLR22 *Siniperca chuatsi*
210690F_b	1	sccTLR	LRR+TM+TIR	TLR13 *Rhincodon typus*
267200F	4	sccTLR	LRR+TM+TIR	TLR13 *L. crocea*
025770R	6	mccTLR	LRR+TM+TIR	TLR *P. trituberculatus*
025760F	6	mccTLR	LRR+TM+TIR	TLR *P. trituberculatus*
041610R.t1	5	mccTLR	LRR+TM+TIR	TLR *P. trituberculatus*
113680F.t1	1	sccTLR	LRR+TM+TIR	TLR *Anthopleura buddemeieri*
081670R.t1	6	sccTLR	LRR+TM+TIR	TLR *Biomphalaria glabrata*

Table 6. **TLRs in *B. lanceolatum***

Gene ID in database	**LRRs**	**sccTLR/ mccTLR**	**Domains structure**	**First annotated sequence by blastp in NCBI**
TLR_J	22	sccTLR	LRR+TM+TIR	TLR22 *S. chuatsi*
BL10262	8	sccTLR	LRR+TM+TIR	TLR13 *S. kowalevskii*
BL09440	16	sccTLR	LRR+TM+TIR	TLR3 *S. kowalevskii*
BL22164	14	sccTLR	LRR+TM+TIR	TLR13 *S. kowalevskii*
BL05337v2	5	mccTLR	LRR+TM+TIR	TLR *P. trituberculatus*
BL07821	9	mccTLR	LRR+TM+TIR	TLR *P. trituberculatus*
BL52875_d	16	sccTLR	LRR+TM+TIR	TLR3 *Chelonia mydas*
BL52875_c	15	sccTLR	LRR+TM+TIR	TLR3 *S. kowalevskii*
BL52875_b	19	sccTLR	LRR+TM+TIR	TLR3 *C. mydas*
BL52875_a	4	sccTLR	LRR+TM+TIR	TLR3 *S. kowalevskii*
BL72403	20	sccTLR	LRR+TM+TIR	TLR3 *C. griseum*
BL19440	20	sccTLR	LRR+TM+TIR	TLR22 *D. rerio*
BL21810	7	sccTLR	LRR+TM+TIR	TLR13 *R. typus*
BL12652	18	sccTLR	LRR+TM+TIR	TLR22 *S. chuatsi*
BL56664	13	mccTLR	LRR+TM+TIR	TLR *P. trituberculatus*
BL20861	17	sccTLR	LRR+TM+TIR	TLR13 *Poecilia formosa*
BL48785	14	mccTLR	LRR+TM+TIR	TLR *B. glabrata*
BL24356	15	mccTLR	LRR+TM+TIR	TLR *P. trituberculatus*
BL18798_a	18	sccTLR	LRR+TM+TIR	TLR22 *E. coioides*
BL18798_b	20	sccTLR	LRR+TM+TIR	TLR3 *P. olivaceus*
BL24343	16	mccTLR	LRR+TM+TIR	TLR *P. trituberculatus*
BL19922	10	sccTLR	LRR+TM+TIR	TLR13 *Xiphophorus maculatus*
BL17405	15	sccTLR	LRR+TM+TIR	TLR22 *S. chuatsi*
BL04519_a	15	sccTLR	LRR+TM+TIR	TLR22 *T. rubripes*
BL08928_a	6	sccTLR	LRR+TM+TIR	TLR13 *Astyanax mexicanus*
BL08928_b	17	sccTLR	LRR+TM+TIR	TLR9 *P. mexicana*
BL08928_c	15	sccTLR	LRR+TM+TIR	TLR9 *P. mexicana*
BL30396	17	sccTLR	LRR+TM+TIR	TLR13 *P. latipinna*

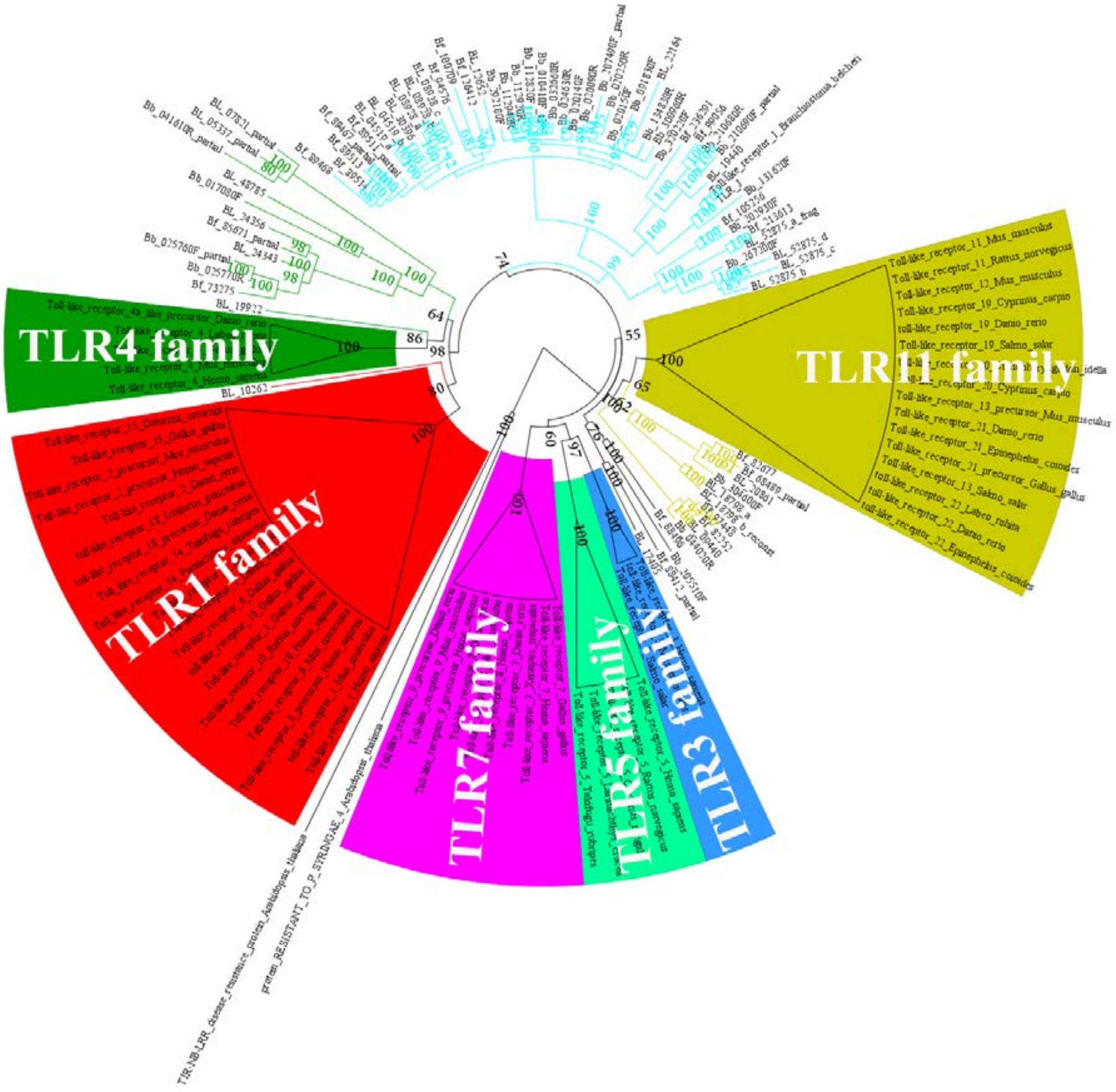


Figure 9. **Phylogenetic tree of *Branchiostoma* and vertebrate TLRs.**

The phylogenetic analysis was performed using the protein sequences of *Branchiostoma* TLRs and different representative vertebrates TLRs with MrBayes using the LG+G+I model. MrBayes software ran 20,000,000 generations to make the average standard deviation of split frequencies less than 0.01 and abandon the first 25% of burn-in samples to summary the parameter values. Vertebrates TLRs were divided into 6 families which were labelled with different background colors. *Branchiostoma* TLRs were labelled with the same color when cluster to vertebrate TLRs family.

Expression analysis of TLRs genes in *B. lanceolatum*

To access the expression of TLRs in *B. lanceolatum*, we performed RT-PCR analysis. The cDNA library was constructed from the whole animal. Each of the TLR primer pairs was derived from the nucleotide sequences reconstructed from genomic sequences of *B. lanceolatum*. We found out expression of all TLR sequences in amphioxus in basal conditions, but the expression of BL07821, BL52875_a, BL48785, BL18798_a, BL18798_b, BL04519_a, BL08928_a, and BL08928_b was quite weak whereas the other genes were strongly expressed in the whole animal.

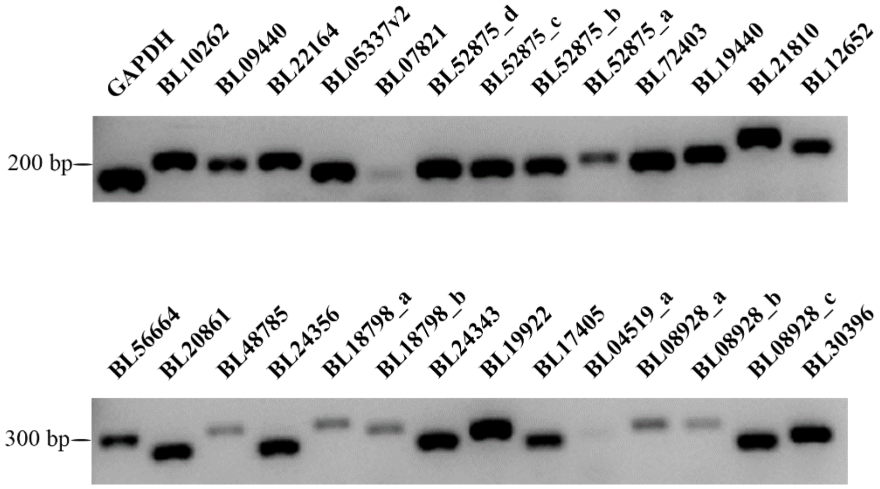


Figure 9. **RT-PCR detection of TLRs genes in *B. lanceolatum* from the whole adult animal.** The *B. lanceolatum* GAPDH was used as a reference gene. RT-PCR reactions were accomplished using equal number of cycles and the PCR products were loaded equally on one 1 % agarose gel.

Discussion

TLRs play crucial roles in the innate immune system by recognizing different PAMPs. In addition to innate immunity, TLRs have multiple functions ranging from developmental signaling to cell adhesion. The study of TLRs may help to understand the role of TLR-mediated responses which could increase our range of strategies to treat infectious diseases and manipulate immune responses by drug intervention (64). From the evolutionary point of view, TLRs are conserved across invertebrates to vertebrates and absent from non-animal phyla (plants and fungi). However, there are vast structural and functional divergences in TLRs between invertebrates and vertebrates (61). Within three subphylum of chordates, vertebrates such as humans and mice have 10 and 12 TLRs, urochordates like *C. savignyi* and *C. intestinalis* show no expansion of TLRs (having between 3 and 7 TLR genes each), but cephalochordates like *B. floridae* have 48 TLRs according to Huang *et al*. (21). This expansion of TLRs in invertebrate deuterostomes remains to be understood by comprehensive and thorough study of the evolution of TLRs. Amphioxus is a good model to study the invertebrate-chordate to vertebrate transition and the evolution of vertebrates due to the advantage of its phylogenetic position, as well as its simple and evolutionarily conserved genome. Therefore, studying TLR functions in such organism could improve our understanding of the ancestral innate immune system of vertebrates.

In this study, we identified 22 TLRs in *B. floridae*, 37 TLRs in *B. belcheri*, and 28 TLRs in *B. lanceolatum* according to the basic TLR protein structure which shares the same pattern of "LRR+TM+TIR". Differences in the total number of *B. floridae* TLRs between Huang *et al.* and our data probably reflects discrepancies in the consensus of what is the basic structure of TLRs. Our rule includes only those putative receptors with a TIR domain, a transmembrane domain (TM) and at least 1 LRR. Our available genomic and transcriptomic data (from Dr. Jordi Garcia-Fernandez, UB) maybe do not include all the possible TLRs. Probably, the total number of TLRs in the 3 species of lancelet should be similar. Among them, we identified 2 mccTLRs in *B. floridae*, 3 mccTLRs in *B. belcheri*, 6 mccTLRs in *B. lanceolatum*. The expression of 28 TLRs in *B. lanceolatum* was confirmed by RT-PCR with cDNA from the whole animal. These findings approximately match the observation by Huang *et al*. (2008) concerning amphioxus genomics: that it has a high rate of domain combination acquisition and therefore a high number of TLRs (prediction of 36 sccTLRs and 12 mccTLRs) (21). Note Bányai and Patthy in 2016 provide evidence to dispute that the rate of protein innovation is exceptionally high in lancelets. They surmise these high rates are likely due to gene prediction errors (65). This may explain why there are less TLRs found in our study than the genomic prediction. Our work shows that amphioxus and vertebrates share a conserved TLR framework in terms of gene number, family classification, protein architecture, etc. On the other hand, amphioxus shares some features of non-vertebrates, like the mccTLRs which are mainly found in protostomes (61). Our analyses suggested that amphioxus is a critical organism to understand the function and evolution of TLRs.

We cloned the full-length sequence of Bl_TLRj cDNA from Mediterranean amphioxus (*B. lanceolatum*). The full-length CDs showed the highest 42.08% identifies with *Salmo salar* TLR3. The full-length protein showed the highest 30.43% identities with TLR22 of *Squaliobarbus curriculus*. Like most of the vertebrate TLRs, the domain analysis of Bl_TLRj amino acid sequence showed that it has 21 LRR domains, a LRRCT, a LRRNT, a transmembrane domain, and a TIR domain. The phylogenetic analysis of Bl_TLRj protein sequence and representative vertebrates' TLR protein sequences reveal that Bl_TLRj is grouped with the TLR11 family of vertebrates. Interestingly, the phylogenetic analysis of *B. floridae* TIR domain and vertebrate TLRs has indicated that 33 variable-type TLRs show a paraphyletic relationship with the vertebrate TLR11 lineage (21). The TLR11 family is represented in humans only by a pseudogene and the major divisions of the TLR11 family are clearly very ancient (27). On the other hand, according to the ectodomain architecture analysis of vertebrate TLRs (58), the TLR11 family can be divided into two subfamilies, TLR11 and TLR13 (Table 7). TLR11 subfamily has a trans-three-domain architecture while TLR13 subfamily has a single-domain architecture. The Bl_TLRj has a single-domain architecture which makes it more likely to be a member of TLR13 subfamily.

Table 7. **Ectodomain architecture of vertebrate TLRs and Bl_TLRj.**

Family name	TLR	LRR number	Architecture	Ligands
Family 1	TLR1, 2, 6, 10, 14, 18, 24, 25	19	Three-domain	Hydrophobic: lipids and lipoprotein
	TLR15	19	Single-domain	Virulence-associated fungal and bacterial proteases
Family 3	TLR3	23	Single-domain	dsRNA
Family 4	TLR4	21	Three-domain	LPS
Family 5	TLR5	21	Single-domain	Bacterial Flagellin
Family 7	TLR7, 8, 9	25	Single-domain	ssRNA, CpG-DNA
Family 11	Subfamily 11: TLR11, 12, 16, 19, 20, 26	23, 24	Trans-three-domain	Profilin from *T. gondii*
	Subfamily 13: TLR 13, 21, 22, 23	25	Single-domain	ssRNA, CpG-DNA, dsRNA
	Bl_TLRJ	21	Single-domain	dsRNA

The location of TLRs affects ligand recognition. Among the mammalian TLRs, the ones located at the plasma membrane (TLR1, 2, 4, 5, and 6) recognize microbial pathogenic components of the cell wall, while the others (TLR3, 7, 8, 9, and 13) located intracellularly in endosomes or lysosome recognize nucleic acids and their derivatives (66). Regarding the TLR11 family, TLR11 is localized on the cell surface, whereas TLR13 is an endosomal receptor. In our study, immunofluorescence and confocal microscopy showed that Bl_TLRj was localized in the cytoplasm. The above results may also imply that Bl_TLRj has a close relationship to the TLR13 subfamily and it may recognize nucleic acid-like ligands.

N-linked glycosylation is reported to be involved in trafficking, binding activity and PAMP recognition (67) (68) (69). In vertebrates, TLRs located on the plasma membrane have less N-linked glycosylation sites (mean value less than 10) than those in the cytoplasm (mean value more than 10) according to our statistical analysis (Figure 11). For instance, the TLR1 family has a mean of 8.1 N-linked glycosylation sites compared to 15.8 in TLR7 family. TLR11 family could be divided into two subfamilies (TLR11 and TLR13) according to the ligand types and ectodomain. TLR11 subfamily which mainly recognizes the components of bacteria and parasites has less numbers of N-linked glycosylation sites (mean=7.8) than the TLR13 subfamily (mean=14.3) which recognizes nucleic acids and their derivatives. The glycosylation may reflect the function of the ectodomain in terms of PAMPs recognition. Hence, TLRs may need more N-linked glycosylation sites to bind nucleic acid-like PAMPs, than to bind bacterial and parasite components. Interestingly, Bl_TLRj was predicted to have 10 N-linked glycosylation sites which may recognize nucleic acids and their derivatives. It follows that Bl_TLRj could belong to the TLR13 subfamily.

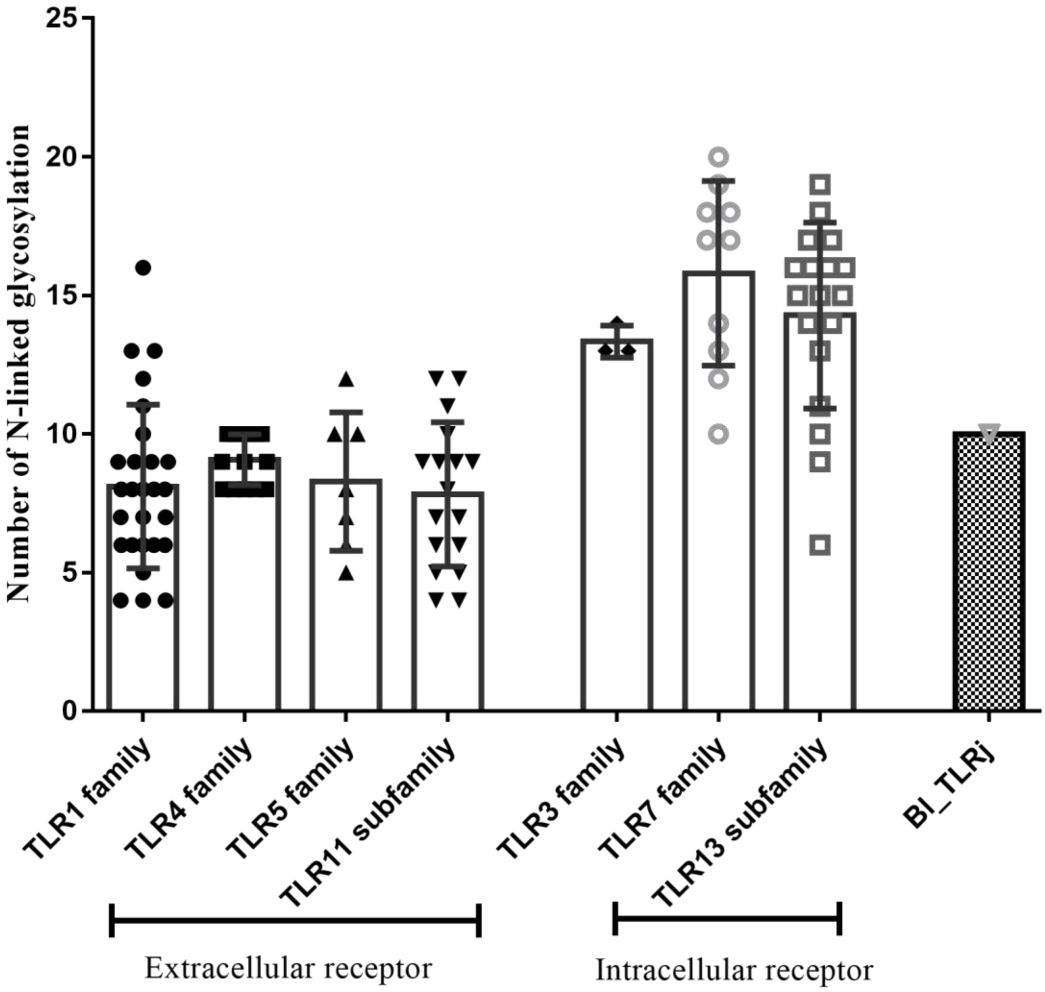


Figure 11. **Comparison of N-linked glycosylation sites of TLRs in vertebrates and Bl_TLRj.**

In mammals, TLRs can specifically recognize specific PAMPs, with high levels of sensitivity (70). To test B1_TLRj specificity, we performed different assays with commercially available mammalian TLRs ligands, using NF-κB promotor activity as indicator. We did not observe activation of the NF-κB promoter with any of the ligands when we treated Bl_TLRj-expressing HEK293 cells. There are several possible explanations but after discarding problems with protein expression levels, intracellular degradation or incorrect trafficking, the two most likely reasons are that: **1)** Bl_TLRj could not directly recognize the PAMPs tested and the recognition process might require the assistance of other proteins that are specific for amphioxus and are not present in a mammalian system. For instance, the *D. melanogaster* Tolls do not bind any PAMPs directly (13) and mammalian TLR4 cannot recognize LPS without the assistance of MD2 and CD14 proteins (71); **2)** Bl_TLRj has a TIR domain that interacts with a specific partner not present in mammalian cells. To directly test the second possibility we designed a chimeric protein containing the ectodomain of Bl_TLRj fused to the human TLR2 TIR domain. We tested to see whether this construct could respond to ligand stimulation or not when stably transfected in HEK293 cells. Indeed, the chimeric Bl_TLRj in HEK293 cells activated NF-κB reporter in response to Poly I:C (LMW) and Poly I:C (HMW) which are specific ligand for mammalian TLR3, ligand for TLR13 of Miiuy croaker (43), and ligand for TLR22 of Japanese flounder (48) and pufferfish (50). Taken together, we suggest that Bl_TLRj could be classified as TLR13 subfamily, and it may be an ancestor of the vertebrate TLR13 subfamily. Furthermore, our methods using heterologous expression and a chimeric protein are an innovative way of studying Mediterranean amphioxus for which to date there is no cell line or effective genetic techniques.

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Chapter 2

Adult zebrafish as a model to study the fish immune system: evaluation of two strategies for immunomodulation.

Chapter 2: Adult zebrafish as a model to study the fish immune system: evaluation of two strategies for immunomodulation.

Abstract

Most diseases invade organism through their mucosa. This is particularly true in fish as they are continuously exposed to a microbial-rich water environment. Developing effective mucosal vaccines for fish, which stimulate the immune system against infectious diseases is highly desirable in aquaculture. However, the number of mucosal vaccines for fish is still limited. This is partly due to the lack of safe and effective mucosal adjuvants. Here we test the potential of recombinant cytokine TNFα as an immunomodulator. Our recombinant TNFα is nanostructured as bacterial inclusion bodies (IBs$^{TNF\alpha}$), which are highly stable, non-toxic, and low-cost protein-based biomaterials. We explored the *in vivo* uptake by the intestinal mucosa and the immunomodulation capacity at a local site via oral intubation, which experimentally mimics oral vaccination. Combining flow cytometry, histology, and confocal microscopy, we show that IBs$^{TNF\alpha}$ are able to cross the intestinal mucosal epithelial barriers, pass through the lamina propria, and reach the muscle layer. In RT-qPCR analysis, the expression of innate immune-related genes (IL-1β, IL-6, COX2, and MMP9) were significantly up-regulated after orally intubation with IBs$^{TNF\alpha}$ in the zebrafish intestine. Furthermore, we established a zebrafish *Mycobacteria marinum* infection model and investigated the protection evoked by IBs$^{TNF\alpha}$ in zebrafish against this natural fish pathogen. Importantly, we showed that i.p. injected IBs$^{TNF\alpha}$ could protect fish against a lethal infection. This zebrafish *M. marinum* infection model was also used to test LPS and Poly I:C encapsulated into nanosized liposomes (NL_c), which was previously designed in our lab. The results show that NL_c liposomes can protect zebrafish against *M. marinum* infection by i.p. injection. Our study suggests that IBs$^{TNF\alpha}$ can be further used as a potential mucosal adjuvant for the application in aquaculture.

Introduction

Prevention and control of infectious diseases by vaccination is an effective and ethical method in aquaculture. Vaccine production has advanced from traditional methods using whole-killed or attenuated-live pathogens to applying only the relevant antigen as a DNA or protein sub-unit vaccine. Traditional methods can induce undesirable side effects due to their toxicity and even re-emerging virulence Current methods decrease such vaccine-associated complications but the immune responses elicited is weaker (1). These antigens alone are unstable due to their fragile nature and may not provide secondary immunostimulatory signals (1). Adjuvants (from the Latin *adjuvare* meaning "to help") are a group of structurally heterogeneous compounds which increase the intrinsic immunogenicity of an antigen (2).

Adjuvants are divided into immunostimulants and delivery systems. Immunostimulants interact with specific receptors, like TLRs and others, while delivery systems increase the immune response by multiple mechanisms, such as antigen delivery, particle size-dependent tissue penetration and access to the lymphatic system, depending on their particular characteristics (3) (4). Traditional adjuvants such as mineral oils had proved to be effective in the immune response but cause different side effects depending on the route of administration (5). Therefore, new, efficient adjuvants are needed to overcome these drawbacks. Nanoparticles or nanodelivery systems can either be co-delivered with adjuvants or act as adjuvants themselves to enhance vaccine efficacy and protect the immunogen (5). Recent work has shown their potential as adjuvants in aquaculture (5) (6). Yet, despite progress, the major bottleneck for the development of aquaculture remains: the lack of efficient vaccines against infectious diseases. New adjuvants are required to enhance the immunogenicity of vaccines, to provide stability to antigens and to improve targeted delivery.

Liposomes have been extensively studied as vaccine carriers and adjuvants. They have a hydrophilic core, a hydrophobic bilayer and self-sealing allows encapsulation of both lipophilic and hydrophilic compounds (7). Nanoliposomes, liposomes on a nanoscale, provide more surface per unit volume, and thus have the potential to increase solubility, enhance bioavailability, improve controlled release, and enable precision targeting of the encapsulated material (8). Nowadays, the use of liposome or liposome-derived nanovesicles has increased as adjuvants or carriers in aquaculture. In *E. bruneus*, a formalin killed bacteria (FKB) vaccine composed of *Vibrio harveyi* whole cells entrapped in liposomes improved the cumulative survival after *V. harveyi* infection when immunized by i.p. injection (9). In *C. carpio*, oral administration of liposomes containing *A. salmonicida* antigen improved the survival of carp after challenged with *A. salmonicida* and significantly inhibited the skin ulcers (10). In the same species, oral administration of liposomes encapsulating *A. hydrophila* antigens enhanced the levels of antibodies and protected the fish against *A. hydrophila* infection (11). In rainbow trout (*O. mykiss*), lipopolysaccharide (LPS) from *A. salmonicida* was incorporated into positively- or negatively-charged liposomes. The results showed that both formulations prolong humoral immune responses against LPS when i.p. administered (12).

Besides the encapsulation of bacterial antigens, some attempts have been made in aquaculture to encapsulate viral antigens in liposomes. Experiments with formalin-inactivated koi herpesvirus entrapped within liposomes elicited a significant increase in specific antibody titer and high protection levels in common carp (*C. carpio*) against this infection when orally vaccinated (13). In our previous study, liposomes encapsulating both polyinosinic-polycytidylic acid (Poly I:C) and lipopolysaccharide (LPS) elicited a pro-inflammatory and anti-viral response in zebrafish hepatocytes and trout macrophages. *In vivo*, these loaded liposomes protected zebrafish against a lethal bacterial (*Pseudomonas aeruginosa* PAO1) and viral (spring viraemia of carp virus) infection regardless of whether they were administered by injection or by immersion (14) (15).

Mucosa is the first biological barrier to fight infections. When the mucosal barriers perceive the danger signals, an immediate innate immune response is triggered. Next, the highly specific adaptive immune response is established by T and B lymphocytes (16). The innate and adaptive immune system are both present in mucosal barriers. At these barriers, epithelial cells and antigen-presenting cells (dendritic cells and macrophages) constitute physical and innate defense systems while B and T lymphocytes form a dynamic network for the induction and regulation of secretory antibodies and cytotoxic T lymphocyte (CTL) responses (17). In mammals, it is well documented that exposure of mucosal surfaces to antigen results in a local response, finally resulting in secretion of antigen-specific IgA at mucosal surfaces. Therefore, mucosal vaccines would be an effective tool to protect the organism at exposed sites due to the specific response of immune cells in the mucosa-associated lymphoid tissues (MALT) (18) (19). In aquaculture, mucosal vaccines have obvious advantages compared to injectable vaccines.

They are practical for mass vaccination, less labor-intensive, are less stressful to the fish, and can be administered to young fish (20). Compared to immersion or bath administration, vaccines for oral administration also have advantages. For example, they do not require extensive purification of bacterial by-products, as the gut is already heavily populated by bacteria. One of the major challenges in developing highly protective mucosal vaccines for fish is the choice of an antigen delivery system or adjuvant. The main factors that influence the choice of adjuvant include safety, ability to evoke a strong innate and adaptive immune response, the ability to deliver vaccines to the second gut segment without denaturing in the acidic environment (oral vaccines), and ability of antigens to cross mucosal barriers in order to gain access to antigen presenting cells (APCs) to induce local and/or systemic responses (20).

Cytokines are small soluble proteins mediating and regulating immune and non-immune cells and have been used as adjuvants, immunostimulants and therapeutic agents in the treatment of different human diseases (21) (22) (23). During the past years, a great number of cytokine genes have been identified in many fish species (24) (25) and some cytokines were reported as vaccine adjuvants in fish. The study of interleukin-1β (IL-1β) as an adjuvant was reported in carp (*C. carpio*). Carp were either i.p. injected with formalin-killed *A. hydrophila* plus recombinant carp IL-1β peptide or formalin-killed *A. hydrophila* cells. The results showed that the agglutinating antibody titre was significantly higher in the fish injected with formalin-killed *A. hydrophila* plus recombinant carp IL-1β peptide at 3 weeks post vaccination (26). In addition, IL-1β-derived peptide could induce the migration of peritoneal leukocytes and phagocytosis when i.p. administered in *O. mykiss*. *In vivo* experiments demonstrated that the fish injected with this peptide were more resistant to VHSV infection (27). Further, the immunoadjuvant effect of recombinant IL-1β from sea bass (*D. labrax*) was evaluated in head kidney cells (28). However, the role of IL-1β as an adjuvant was not totally elucidated. Interleukin-8 from rainbow trout (IL-8) was able to modulate the early cytokine immune response in *O. mykiss* when co-injected with the glycoprotein gene of VHSV, suggesting IL-8 could be a potential immune adjuvant (29). In the same species, recombinant IL-8 produced in *Escherichia coli* induced head kidney leukocytes to migrate in cells and when intraperitoneally (i.p.) injected, attracted leukocytes into the peritoneal cavity (30). In channel catfish (*I. punctatus*), IL-8 was used as an adjuvant and co-vaccinated with a subunit vaccine encoding the α-enolase (rENO) of *Streptococcus iniae*. The results showed that the co-vaccinated fish had a higher relative percentage survival than the fish vaccinated with rENO alone, after *S. iniae* infection at both 4 and 8 weeks post vaccination (31).

Interferon regulatory factors (IRFs) form a large family of transcription factors. Interferon regulatory factor-1 (IRF-1) of Japanese flounder (*P. olivaceus*) used as a potential adjuvant showed an antiviral activity and protected fish cells against hirame rhabdovirus (HIRRV) and viral hemorrhagic septicemia virus (VHSV) infection (32). In addition, the recombinant Japanese flounder IRF-1 was investigated as an adjuvant in *P. olivaceus*. The results showed that the IRF-1 modulated the early immune response when intramuscularly vaccinated with a DNA vaccine. However, there was no significant difference in serum neutralizing antibody levels when the fish was vaccinated with the DNA vaccine and IRF-1, or with the vaccine alone(33).

There is only one study using a cytokine as an adjuvant for oral vaccination. Recombinant tumor necrosis factor α (TNFα) from European sea bass (*D. labrax L.*) when used as an adjuvant, significantly extended the protection of fish against a *Vibrio anguillarum* challenge when orally immunized with vaccine (34). A limitation of most studies is only mortality was monitored, and little attention was paid to the uptake mechanisms and subsequent local or systemic immune responses. A detailed understanding of uptake and bio-distribution of cytokines in mucosal vaccination is still lacking. In our previous studies, the recombinant cytokine TNFα nanostructured in inclusion bodies showed suitable features as a mucosal adjuvant such as no toxicity both in cells and *in vivo,* as well as high stability under harsh physicochemical conditions *in vitro* (pH and temperature). Finally when was i.p. injected to zebrafish, the animals showed significantly higher survival percentages when challenged lethally with *Pseudomonas aeruginosa* compared to untreated control (35). However, the ability of deliver to the second segment of the gut and cross mucosal barriers in order to gain access to antigen presenting cells (APCs) has not been fully investigated.

The zebrafish has been widely used as a teleost model to study the vertebrate immune system. Zebrafish has a complete set of genes required for the establishment of a fully functional innate and adaptive immune system (36). Innate immune mechanisms like cytokine and interferon production, complement activation, and stimulation of cellular effectors, such as cells with cytotoxic and macrophage-like activity were found in zebrafish (37). Three classes of immunoglobulins, M, D, and Z (functional equivalent of mammalian mucosal IgA) contribute to the adaptive immune system (38). APCs like macrophages, dendritic cells (DCs), and B lymphocytes connect the innate and adaptive immune system, although it is unclear if they fulfill similar roles in the initiation of adaptive immunity as in mammals (39).

Here, we studied the uptake and innate immune regulation of nanostructured trout TNFα ($IBs^{TNFα}$) as a mucosal adjuvant in zebrafish by oral intubation. We used oral intubation to experimentally mimic oral vaccination. We show that $IBs^{TNFα}$ are able to cross the intestinal mucosal epithelial barriers, pass through the lamina propria, and reach the muscle layer. We also demonstrate that innate immune-related genes in the zebrafish intestine are significantly up-regulated after oral intubation. Further, we investigated the protection of zebrafish against a natural fish pathogen (*Mycobacteria marinum*) and we showed that $IBs^{TNFα}$ injected i.p. could protect fish against a lethal infection. Our results suggest that the recombinant cytokine $IBs^{TNFα}$ could be used as a potential mucosal adjuvant or immunostimulant in aquaculture.

Materials and methods

Ethics statement

All experimental procedures involving zebrafish (*Danio rerio*) were submitted and authorized by the Ethics Committee of the Autonomous University of Barcelona (CEEH number 1582) who agree with the International Guiding Principles for Research Involving Animals (EU 2010/63).

Zebrafish husbandry

Wild type (wt) zebrafish (*D. rerio*) were housed in the aquaria system at fish densities of one fish in 1-3 l. The room temperature was maintained between 27-28 ºC and the water temperature between 26-28 ºC. The photoperiod of the aquarium was set at 14 h light/10 h dark. Adult fish were fed twice a day (once at weekends) at a rate of 2% of biomass, once in the morning and once in the early evening. Any uneaten food and detritus from the bottom of the tank were removed every day. Ammonia, nitrite, pH, and nitrate levels were measured once a week. Ammonia and nitrite levels were maintained to be undetectable, pH was kept between 6.8 and 8.0, and nitrate level was controlled to be less than 100 mg/l. The conductivity of dechlorinated water was maintained between 180-350 µS.

Preparation and lyophilization of NL_c liposomes

The NL_c liposomes were prepared by the thin film hydration method (40) with some modifications as previously described (14). Briefly, 1,2-didodecanoyl-sn-glycero-3-phosphocholine, 1,2-dioleoyl-sn-glycero-3-phosphoric acid monosodium salt, Cholesterol, Cholesteryl and Cholesterol-PEG_{600} were dissolved in chloroform solutions (100 mg/ml) and mixed at the desired molar ratios (0.5:0.35:0.1:0.05). The organic solvent was then evaporated by rotary evaporation to obtain a lipid film (NL_s). For the preparation of NL_c liposomes, the dry lipid film was hydrated with a solution containing 0.5 mg/ml poly (I:C) and 1.0 mg/ml LPS in PBS. The co-encapsulation of poly (I:C) and LPS was done with an immunostimulant:lipid ratio of 1:30 and 1:15, respectively. The resulting lipid suspensions were then vigorously shaken, and the liposomes obtained were homogenized by means of an extruder (Lipex Biomembranes, Canada) through 2 stacked polycarbonate membranes (200 nm pore size, Avanti Polar Lipids) to finally obtain unilamellar liposomes. In all cases, non-encapsulated immunostimulants were removed from liposome preparations by ultracentrifugation at 110,000 ×g for 30 min at 10 °C. Liposome integrity was checked by DLS. The particle size distribution and zeta potential (ζ) of the final liposomal formulations were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, UK). For long-term conservation, the cryoprotectant trehalose was incorporated into the procedure. The dry lipid film was hydrated with a solution containing the immunostimulants and trehalose at a lipid/carbohydrate ratio of 1:5 (2.7%, w/v). The resulting NL_c liposomes were frozen in liquid nitrogen, lyophilised (48 h at -80 °C) and finally, stored at RT for several weeks. When needed, the lyophilised samples were resuspended in PBS (15).

Protein nanoparticles ($IBs^{TNF\alpha}$) preparation and purification

The $IBs^{TNFα}$ were prepared and purified as described before (41). In briefly, *E. coli* strains carrying the protein expression plasmids were cultured in LB medium supplemented with ampicillin (100 µg/ml). Bacterial cultures were started at an optical density at 550 nm (OD_{550}) of 0.05 and incubated aerobically (250 rpm) at 37°C until they reached an OD_{550} of 0.5. Then, 1 mM isopropyl-D-thiogalactoside (IPTG) was added and protein expression was induced for 3 h. For purification of IBs, the bacterial cultures were processed through a combination of enzymatic and mechanical disruption steps. First, lysozyme (1 µg/ml) and phenylmethanesulfonyl fluoride (PMSF, 0.4 mM) were added to cell cultures and incubated at 37 °C for 2 h and 250 rpm. Then, the cells were frozen and thawed, followed by addition of Triton X-100 (0.2% (v/v)) and incubation at room temperature (RT) for 1 h with gentle agitation. IBs were harvested by centrifugation and re-suspended in PBS using one tenth of the original culture volume. Next, samples were treated with 0.6 µg/ml DNase at 37°C for 1 h under agitation. Freeze/thaw cycles were repeated until no viable bacteria were detected. For this, 100 µl of the culture was seeded in LB plates without antibiotic and cultivated overnight at 37°C. Samples were centrifuged at 15,000 ×g for 15 min, and pellets containing purified IBs were stored at -80°C until use.

Preparation of fluorescent $IBs^{TNFα}$ and zebrafish oral intubation

In order to detect *in vivo* $IBs^{TNFα}$, fluorescent $IBs^{TNFα}$ were prepared by conjugating $IBs^{TNFα}$ with Atto-488 NHS ester (Sigma-Aldrich, Cat No. 41698) at a molar ratio 1:2 (protein/dye) following manufacturer's instructions. Labeling efficiency was calculated using Nanodrop ND-1000 (USA).

Fluorescent $IBs^{TNFα}$ were orally administrated to adult zebrafish using the method described by Collymore et al. (2013) (42) with some modifications. Briefly, the zebrafish were starved for 48 h before intubation to empty the intestine. Anestesiathed zebrafish (150 mg/l MS-222) were places on a wet tray and a silicone tube (SILASTIC, Cat. No. 508-001) was inserted approximately 1 cm into the mouth, ensuring that the needle passes the gills and esophagus. The home-made oral intubation device and a brief experimental flow chart is shown in Figure 1. The silicon tube was coupled to the needle of a 100 µl syringe (Hamilton, Cat. No. 81020). 50 µl of each fluorescent $IBs^{TNFα}$ at 0.2 mg/ml, 0.4 mg/ml, 2 mg/ml and PBS were directly intubated into zebrafish gastrointestinal system.

The zebrafish were immediately placed into fresh water and observed until they recover completely. 1 h, 5 h, 24 h, and 48 h after intubation, zebrafish were sacrificed by over-anesthesia (300 mg/l MS-222) and the intestine was sampled for cytometry analysis, histology and RNA extraction (see sections 11, 12 and 13).

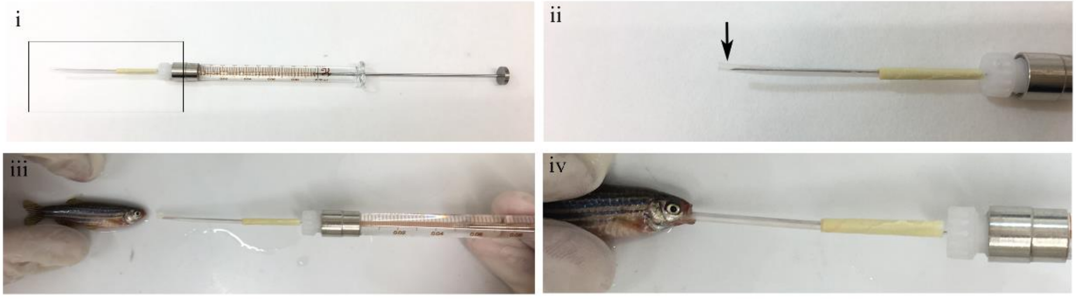


Figure 1. **The home-made device for zebrafish oral intubation.**

(i) 100 µl Hamilton syringe coupled with a silicone tube. (ii) An enlarged image of the needle part (black rectangle in Figure 1. i). The black arrow indicates where the tube exceeds the needle. (iii) Zebrafish before intubation. (iv) Zebrafish undergoing intubation.

Flow cytometry analysis

Zebrafish intestine were dissected from 0.2 mg/ml, 0.4 mg/ml, 2 mg/ml IBs$^{TNF\alpha}$ and PBS treated groups at 5 h, 24 h and 48 h after intubation. After removing the adhesive tissue using tweezers and cleaning the intestinal lumen with PBS, the intestines were immediately incubated in 0.15% collagenase solution (Gibco, Cat No. 17104019) for 1 h under rotation at room temperature. The intestinal cells were collected in the 50 ml centrifuge tubes using 100 µm cell strainer (Falcon, Cat No. 352360) by smashing and washing with PBS. Mucus and debris were removed from the supernatant by centrifugation at 400 ×g for 10 min. All isolated cells were re-suspended in 500 µl PBS and immediately analyzed in a FACSCanto cytometer (Becton Dickinson, USA) to detect the fluorescent signal. The cytometer was set as follow: the mean flow rate was set to moderate speed, cells were visualized on dot plot with SSC height in Y-axis and FSC height in X-axis, an appropriate gate was set to eliminate debris and an excess limit of 10,000 events was recorded, a histogram analysis was generated with FITC fluorescence. The raw data were analyzed with flowing software (Finland) and GraphPad (USA).

Confocal microscopy and Histology analysis

The intestines of zebrafish were dissected from 0.4 mg/ml $IBs^{TNF\alpha}$ and PBS treatment groups at 1 h, 5 h and 24 h after intubation. The samples were quickly embedded in Tissue-Tek O.C.T. compound (SAKURA, Cat. No. 4583) (see Figure 2), snap frozen in dry ice and stored at -80ºC. Sections of 10 µm were collected on SuperFrost Plus slides (Thermo scientific, Cat. No. 10149870) by using the cryostat (Leica CM3050S, Nussloch, Germany) at -20ºC. The slides were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature. After fixation, the slides were washed 3X with PBS for 10 min.

For confocal microcopy imaging, the slides were dried for 15 min in the hood and mounted with Fluoroshield with DAPI (Sigma-Aldrich, Cat. No. F6057). Confocal imaging was performed using a ZEISS LSM 700 confocal laser-scanning microscope with Plan-Apochromat 40× objective lens. The images were analyzed with Fiji software and Imaris software (Bitplane).

For light microscopy imaging, the hematoxylin and eosin staining method (HE) was performed manually according to the standard protocol with some modifications. Shortly, the slides were stained with Harris modified hematoxylin solution (Sigma-Aldrich, Cat. No. HHS16) for 1.5 min. Then, after 2 min washing with tap water, the slides were dipped in acid alcohol (1% hydrochloric acid in 70% ethanol) for 5 s and washed with tap water. The slides were stained with 1% eosin for 1.5 min (Sigma-Aldrich, Cat. No. HT110216). Then, the samples were dehydrated in an ethanol gradient (70%-100%) and cleaned with three changes of xylene PRS (Panreac, Cat. No. 141769). Finally, the slides were mounted with DPX mounting medium (Sigma-Aldrich, Cat. No. 06522) and covered with cover glasses and left to air dry for a few days. The images were taken with a Nikon Eclipse 80i microscope with 20X objective. The images were analyzed with Fiji software (43).

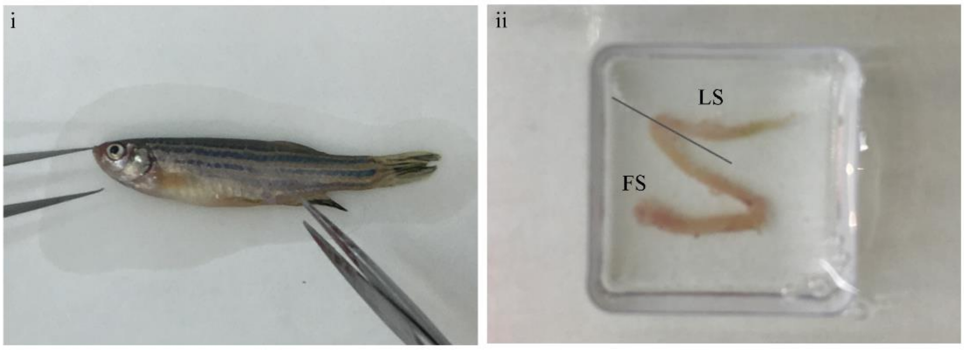


Figure 2. **Zebrafish intestine sampling and intestine anatomy.** (i) Zebrafish was anestesiathed and prepared for intestinal necropsy. (ii) An isolated zebrafish intestine *in vitro* after removal of the surrounding tissues and embedded into OCT compound. For cytometry analysis, the intestine was divided into two segments, first segment (FS) and last segment (LS), as indicated by the black line.

RNA preparation, cDNA synthesis and RT-qPCR

Zebrafish were intubated with 50 µl of 0.4 mg/ml $\text{IBs}^{\text{TNF}\alpha}$ (20 µg/fish) and PBS, respectively. 1 h, 5 h, and 24 h post intubation, the intestines were dissected and frozen rapidly in liquid nitrogen. Total RNA was extracted using Maxwell RSC simplyRNA Tissue Kit (Promega, Cat. No. AS1340) according to the manufacturer's instructions. The concentration of RNA was determined by a Nanodrop 1000 (Thermo scientific) and the quality was assessed using Agilent 2100 Bioanalyzer system (Agilent Technologies, G2946-90004). The cDNA was synthesized with 1 µg of total RNA using iScript cDNA synthesis kit (Bio-Rad, Cat. No. 170-8891) according to the manufacturer's instructions.

The RT-qPCR was performed in the CFX384 Touch Real-Time PCR Detection System (Bio-Rad) using the iTaq™ universal SYBR Green Supermix kit (Bio-Rad, Cat. No. 1725121) following manufacturer's instructions. In briefly, Each PCR mixture consisted of 5 µl of SYBR green supermix, 0.5 µM of primers, 2.5 µl of diluted cDNA and 1.5 µl sigma water (Sigma-Aldrich, Cat. No. W4502-1L) in a final volume of 10 µl. All samples from 3 independent experiments were run in triplicate using the following settings: initial denaturation at 95 ℃ for 3 min, 39 cycles of 95 ℃ for 10 s and 60 ℃ for 30 s, and finally, 95 ℃ for 10 s, increase every 0.5 ℃ for 5 s from 65 ℃ to 95 ℃. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (44).

Mycobacterium marinum culture

M. marinum on 7H10 agar plates were a gift from Prof. E. Julián Gómez, Department of Genetics and Microbiology, UAB. The bacteria were cultured in Middlebrook 7H9 broth (Becton Dickinson, Cat. No. 271310) liquid medium supplemented with ADC in 250 ml glass bottles with screwed cap in an orbital shaking incubator at 30 ℃. The bacteria were cultured in the dark by covering the glass bottle with aluminum foil. Briefly, a single colony from 7H10 agar plate was inoculated into 20 ml 7H9 liquid medium supplemented with 2 ml ADC. The 7H9 liquid medium supplemented with ADC was loaded into the glass bottle and warmed up in the incubator at 30 ℃. The bottle was tightly sealed with continuous shaking at 200 rpm. In order to draw the growth curve, OD600 was examined with a spectrophotometer (Pharmacia LKB, England) every 24 h. The subculture was carried out when the OD600 reaches ~1.2-1.5. For short-term storage, 500 µl bacteria in 7H9 medium were stored in 500 µl glycerol (Sigma-Aldrich, Cat. No. G9012) at -20℃. For long-term storage, the bacteria on the 7H10 agar plate were collected and stored in 1.5 ml milk solution (See recipe) at -80 ℃.

M. marinum frozen aliquots preparation

The *M. marinum* were cultured in 7H9ADC medium until the OD600 reaches ~1.5 after 5 days. The bacteria were collected by centrifugation for 15 min at 4,000 ×g. The bacterial pellets were then re-suspended with 7H9OADC. In order to break up aggregate forms of *M. marinum*, the suspension was passed through 29-G syringes (BD medical, Cat. No. 320924) for approximately 10 times until no clear aggregation was observed. The dispersed bacteria were aliquoted into 1.5 ml tubes with 7H9OADC (20 µl/tube) and kept at -80 ℃ until use.

Zebrafish infected with *M. marinum* and bacterial enumeration

The zebrafish (average weight: 0.37±0.08 g) were settled into isolated infection tanks one day before infection. After lightly anesthetized with 150 mg/L Ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma-Aldrich, cat. No. E10521-50G), the zebrafish (group of 12 fish) were infected by intraperitoneal (i.p.) injection using 100 µl syringe (Hamilton, Cat. No. 81020) with 20 µl of diluted *M. marinum* suspensions (serial 10 fold dilutions: 10^{-2} to 10^{-5}) in phosphate-buffered saline (PBS) to obtain the optimal infection range. The zebrafish injected (i.p.) with the same volume of PBS were used as negative control. The experiments were performed in triplicate and the survival was recorded for 24 days after infection. The survival curve was analyzed by GraphPad prism version 6 (USA). To verify the bacterial dose, 100 µl of each 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} dilutions of bacterial suspension were inoculated onto Middlebrook 7H10 agar plates (Becton Dickinson, Cat.

No. 254520) and the bacteria colonies was counted. During the experiment, dead fish were removed and stored for detection of infection.

Detection of *M. marinum* infected zebrafish by RT-PCR

Genomic DNA from: cultured *M. marinum*, internal organ tissues of 2 dead zebrafish from *M. marinum* injected group and 1 alive zebrafish from PBS injected group, was extracted with Wizard Genomic DNA purification kit (Promega, Cat. No. A1120) according to the manufacturer's instructions. DNA from cultured *M. marinum* was extracted following the gram positive and negative bacteria protocol with modifications. In briefly, 2 ml of *M. marinum* cultured medium was centrifuged 2 min at 16,000 ×g and the bacteria pellet were resuspended with 480 µl EDTA solution (50 mM, pH=8). After adding 120 µl lysozyme (10 mg/ml) and 20 µl proteinase K (20 mg/ml), bacteria were incubated for 40 min at 37 ºC, centrifuged for 5 min at 16,000 ×g, re-suspended in 600 µl nuclear lysis solution, added to a 2 ml screw-top vial with 0.5 g of 500 µm glass beads, shaking for 5 min at 5.5 m/s, incubated again for 1 h at 65 ºC, shaking again for 3 min at 5.5 m/s, incubated for 5 min at 80 ºC. The following procedure was performed according to the protocol. DNA from fish tissues was extracted following the animal tissue protocol. 0.5 µg genomic DNA from each sample, 1 U DNA polymerase (Biotools, Cat. No. 10.013), 1 µM primers, 1.5 mM $MgCl_2$, and 200 µM of each dNTPs were used in the PCR reaction. Two pairs of primers, 16S-23S internal transcribed spacer (ITS) gene (Forward: 5'-CACCACGAGAAACACTCCAA-3' Reverse: 5'-ACATCCGAAACCAACAGAG-3') (45) and 16S rRNA gene (Forward: 5'-AGGACCACGGGATTCATGTCC-3' Reverse: 5'-GTAGGAGTCTGGGCCGTATCTCAG-3') (46), were used to detect the *M. marinum* in zebrafish tissues. The RT-PCR was carried out in a Thermal cycler T100 (Bio-Rad) with the following settings: 3 min 94 ℃, 35 cycles of 45 s 94 ℃, 45 s 60 ℃, and 1 min 72 ℃, followed by final extension at 72 ℃ for 7 min. 20 µl of each reaction mixture was analyzed on 1% agarose gel (Sigma-Aldrich, Cat. No. A9539) stained with GelGreen Nucleic Acid Gel Stain (Biotium, Cat. No. 41005) for visualization of the PCR products on a Geldoc XR (USA).

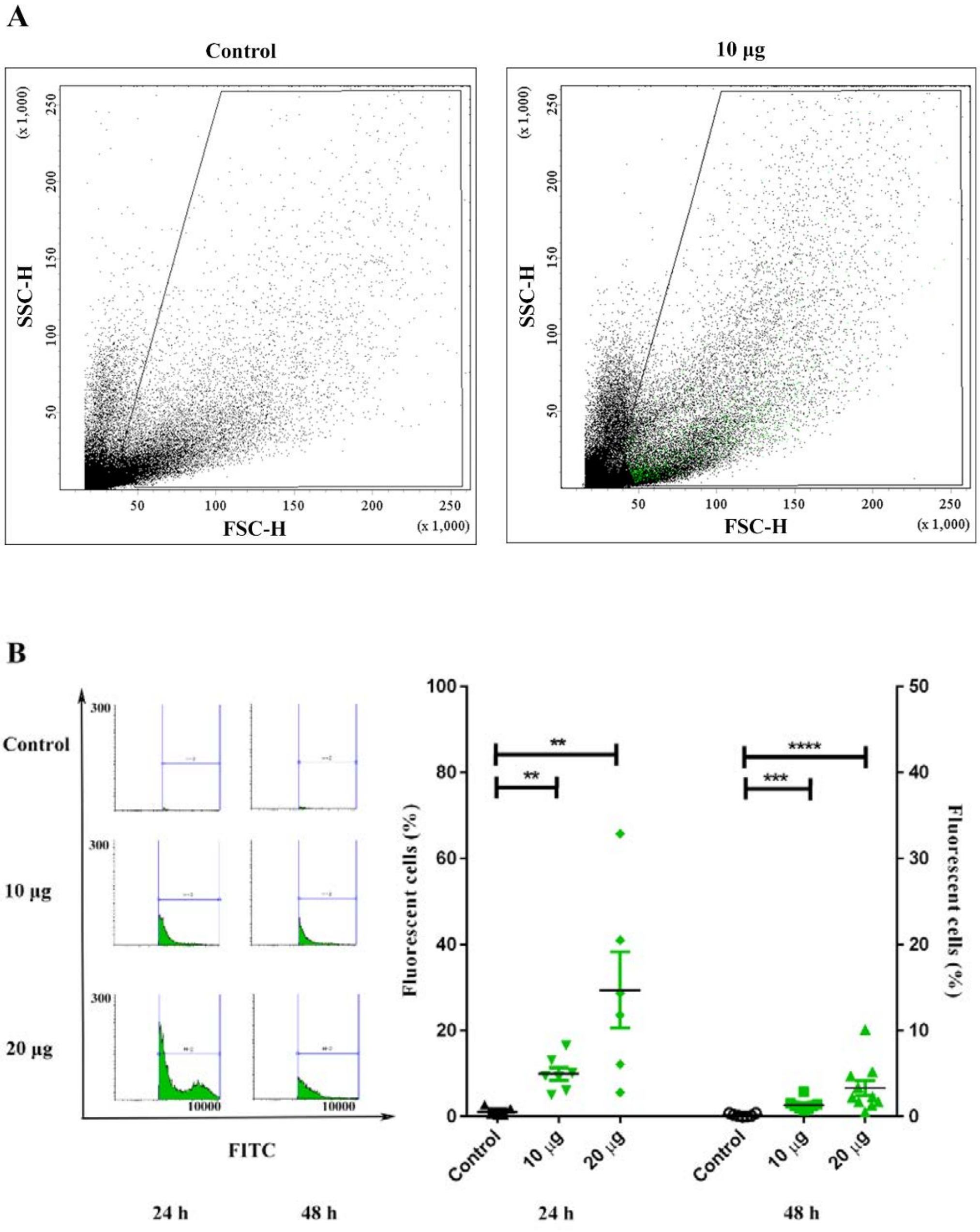
M. marinum challenge in zebrafish after NL_c liposomes and $IBs^{TNF\alpha}$ administration

Adult zebrafish (average weight: 0.46 ± 0.11 g) were settled in isolated tanks one night before administrations. For the NL_c liposomes experiments: zebrafish were injected (i.p.) with 10 µl of either NL_c liposomes (15 mg/ml liposomes containing 0.5 mg/ml poly (I:C) and 0.25 mg/ml LPS), NL_s (15 mg/ml liposomes), a mixture of the free immunostimulants (0.5 mg/ml Poly (I:C) and 0.25 mg/ml LPS) or PBS. For $IBs^{TNF\alpha}$ experiment: zebrafish were injected (i.p.) with 20 µl of either $IBs^{TNF\alpha}$ (300 µg/fish) or PBS. At 7 and 40 days post injection (dpi), the fish were challenged with *M. marinum* (approximately 10^6 cfu/fish) and the survival was assessed for 15 days or when all the fish died in *M. marinum* injected group. *M. marinum* were prepared and diluted to working concentrations in PBS from the aliquots stored at -80 ºC (see Material and Methods, section 4). 12 zebrafish were used in each group and all the experiments were done in triplicate. Survival curves were analyzed using the Kaplan–Meier method and the statistic differences were evaluated using the log-rank test (GraphPad Prism v6, USA).

Results

In vivo fluorescent $IBs^{TNF\alpha}$ uptake in zebrafish intestine

In previous studies, $IBs^{TNF\alpha}$ were administrated by intubation to rainbow trout and the uptake was analyzed by cytometry. The results showed that fluorescent cells were detected in the pyloric caeca and the midgut 24 h after intubation at the percentage of 20.2 ± 7.4 of positive cells (35). To better understand the uptake of $IBs^{TNF\alpha}$ in zebrafish intestine, different doses of fluorescent $IBs^{TNF\alpha}$ were administrated by intubation to zebrafish. The zebrafish intestine was dissected at different time points and the total intestinal cells were prepared for flow cytometry analysis. The gated cells for analysis (right angle trapezoidal area, R1) and FITC histograms are shown in Figure 3A and B. Fluorescent signals were significantly detected in the zebrafish intestinal cells at 24 h and 48 h post intubation when 10 µg and 20 µg were administrated per fish. The percentages of total positive cells in the 10 µg and 20 µg groups were 15.0% and 29.0% at 24 h, 9.3% and 13.2% at 48 h, compared to the PBS group (4.3% and 1.5%), respectively (Figure 3B). Furthermore, in order to identify the main uptake segment of $IBs^{TNF\alpha}$ in zebrafish intestine, three zebrafish from the 10 µg, 20 µg, and PBS groups at 48 h post intubation were sacrificed and the intestines were dissected into two segments, first segment (FS) and last segment (LS) (Figure 2). The intestinal cells were isolated and processed for flow cytometry. Figure 3D showed that the fluorescent signals were predominantly detected in the last segment of the intestine with a percentage of 11.1% (10 µg) and 15.3% (20 µg). Less than 10% of fluorescent cells were found in the first segment of intestine. When we increased the quantity of IBs$^{\mathrm{TNF}\alpha}$ to 100 µg per fish, fluorescent signals were increased and the percentages were significantly higher at 5 h (43.5%) and 24 h (38.1%) post intubation (Figure 3C). The rapid uptake of IBs$^{\mathrm{TNF}\alpha}$ in zebrafish compared to trout could be due to anatomical differences on the gastrointestinal system. Rainbow trout has stomach while zebrafish is a stomach-less organism. Thus, probably IBs$^{\mathrm{TNF}\alpha}$ reach the absorptive mucosa section much faster in zebrafish than in trout.



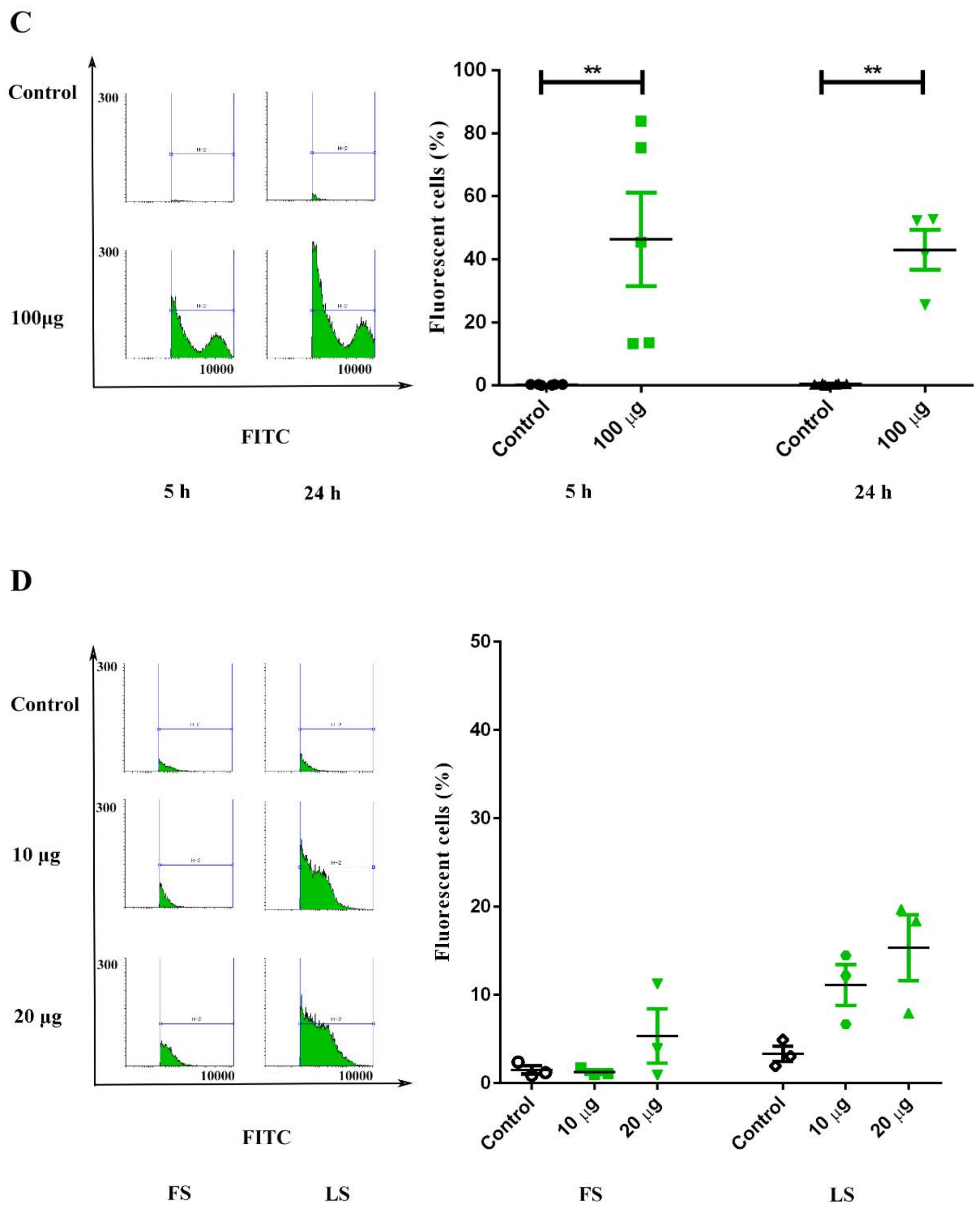


Figure 3. **Flow cytometry analysis of fluorescent IBs$^{TNF\alpha}$ in zebrafish intestinal cells.** (A) Representative flow cytometry plots (control and 10 µg/fish) and the gated intestinal cells for analysis (R1). The green dots represent the fluorescent cells. (B) Representative histograms and the quantification of fluorescent cells in zebrafish intestines treated with PBS, 10 µg, and 20 µg fluorescent IBs$^{TNF\alpha}$ for 24 h and 48 h, respectively. n ≥ 5, each dot represents one adult, pooled from two experiments or more. (C) Representative histograms and the quantification of fluorescent cells in zebrafish intestines treated with PBS and 100 µg fluorescent IBs$^{TNF\alpha}$ for 5 h and 24 h, respectively. n ≥ 4, each dot represents one adult. (D) Representative histograms and the quantification of fluorescent

cells in two segments of zebrafish intestines (FS and LS) treated with PBS, 10 µg, and 20 µg fluorescent $IBs^{TNF\alpha}$ for 48 h. $n \geq 3$, each dot represents one adult.

Hematoxylin and eosin staining and confocal microscopy

To study the morphology of adult zebrafish intestine and better understand which mucosal layer is involved to the $IBs^{TNF\alpha}$ uptake, we examined the intestinal architecture of the first segment (FS, Figure 2) in Hematoxylin and Eosin (HE) stained samples. Hematoxylin and eosin (HE) staining of zebrafish intestine cross-sections reveals that the intestine has a basic structural morphology consisting of mucosa and muscularis. The mucosa consists of a layer of columnar-shaped enterocytes and intercalated mucous-secreting goblet cells and an underlying lamina propria layer that is connected with the muscularis layer (Figure 4). These findings are in line with previous reports in which the anterior segment (S1-S5) has a similar molecular and structural characteristics (47).

To understand whether the $IBs^{TNF\alpha}$ could cross the intestinal mucosa and target the deeper mucosal layer like lamina propria, oral intubation experiments were performed. 20 µg/fish (or 0.04 mg/ml, 50 µl) of $IBs^{TNF\alpha}$ orally administrated to zebrafish by intubation. 1 h, 5 h, 24 h after intubation, the intestines were prepared by frozen tissue method and observed with confocal microscope. At 1 h post intubation, the fluorescent signals were mainly found in the intestine lumen, in epithelial cells, in lamina propia, and in goblet cells. Furthermore, fluorescence was observed in epithelial cells, lamina propia, goblet cells and muscular cells at 5 h samples. However, no signal was observed in the samples at 24 h post intubation by confocal microscopy (Figure 3B).

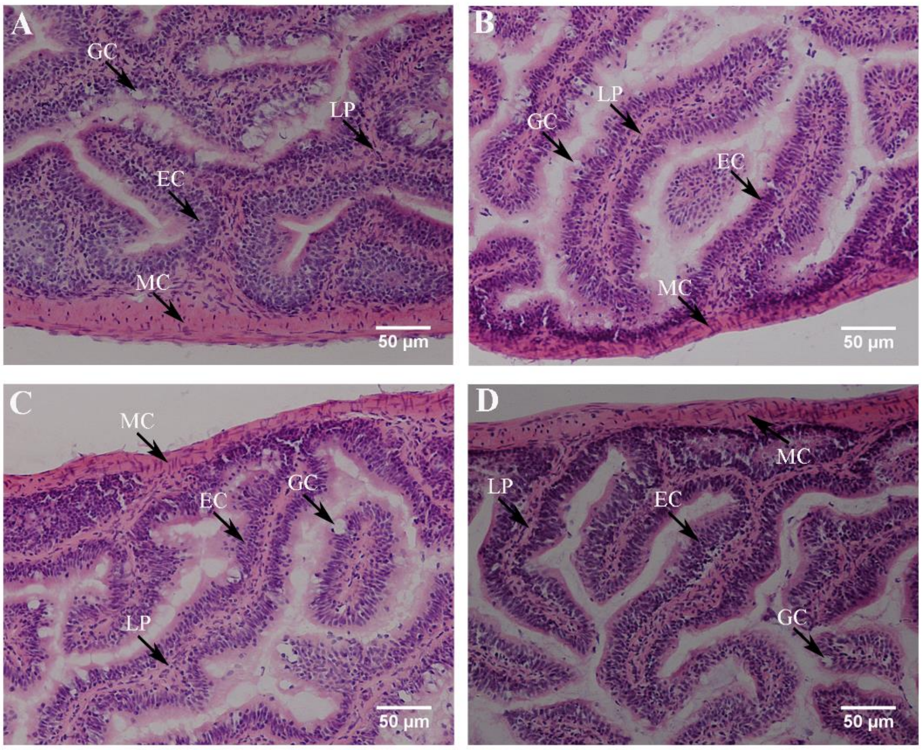
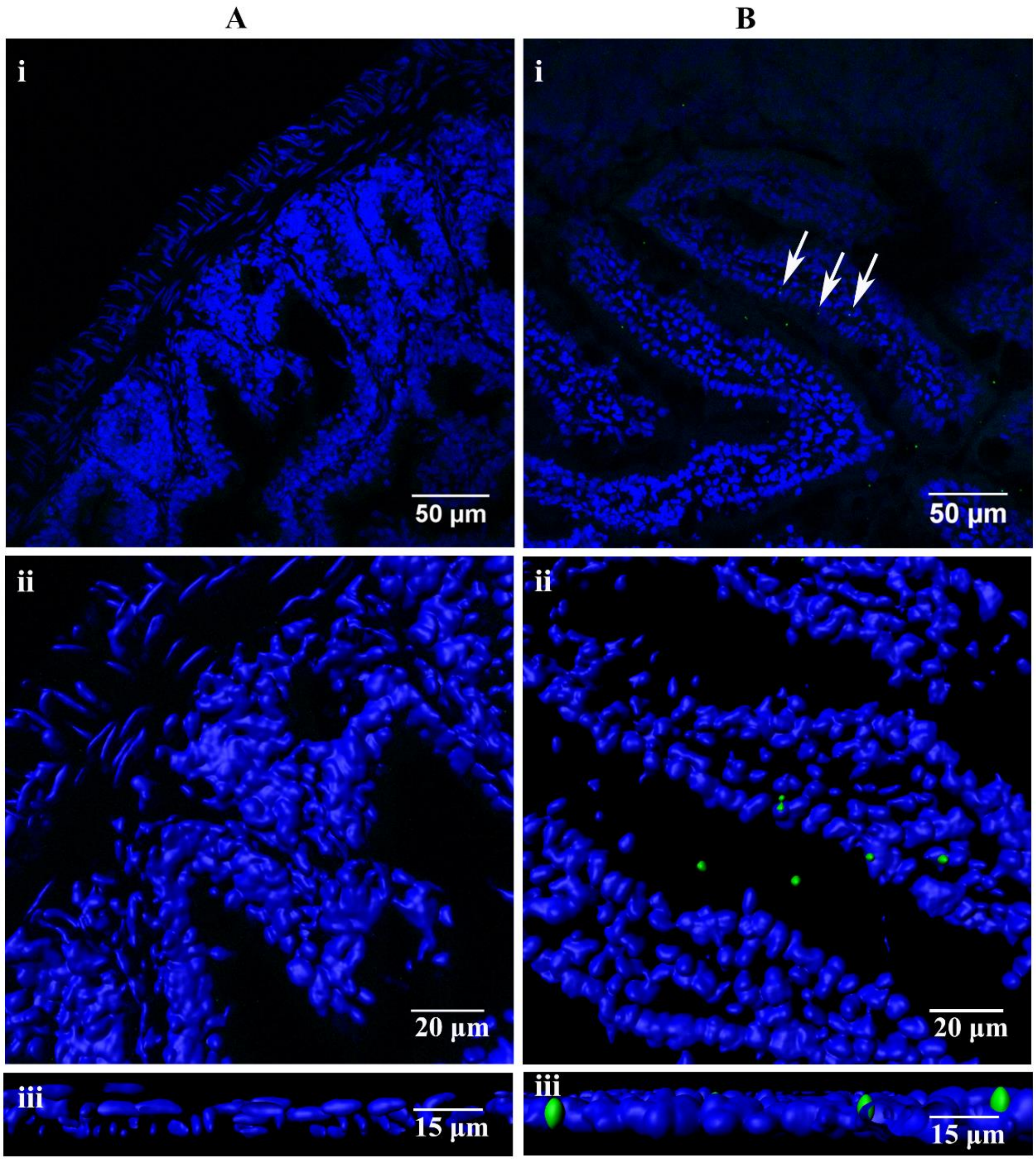


Figure 4. **Representative Hematoxylin and Eosin (HE) staining on longitudinal sections of zebrafish intestine prepared with the frozen tissue method**. A, B, C, and D are representative images of the morphology of the FS of the zebrafish intestine. Black arrows indicate different cell types. GC, goblet cells; LP, lamina propria; EC, epithelial cells; MC, muscle cells.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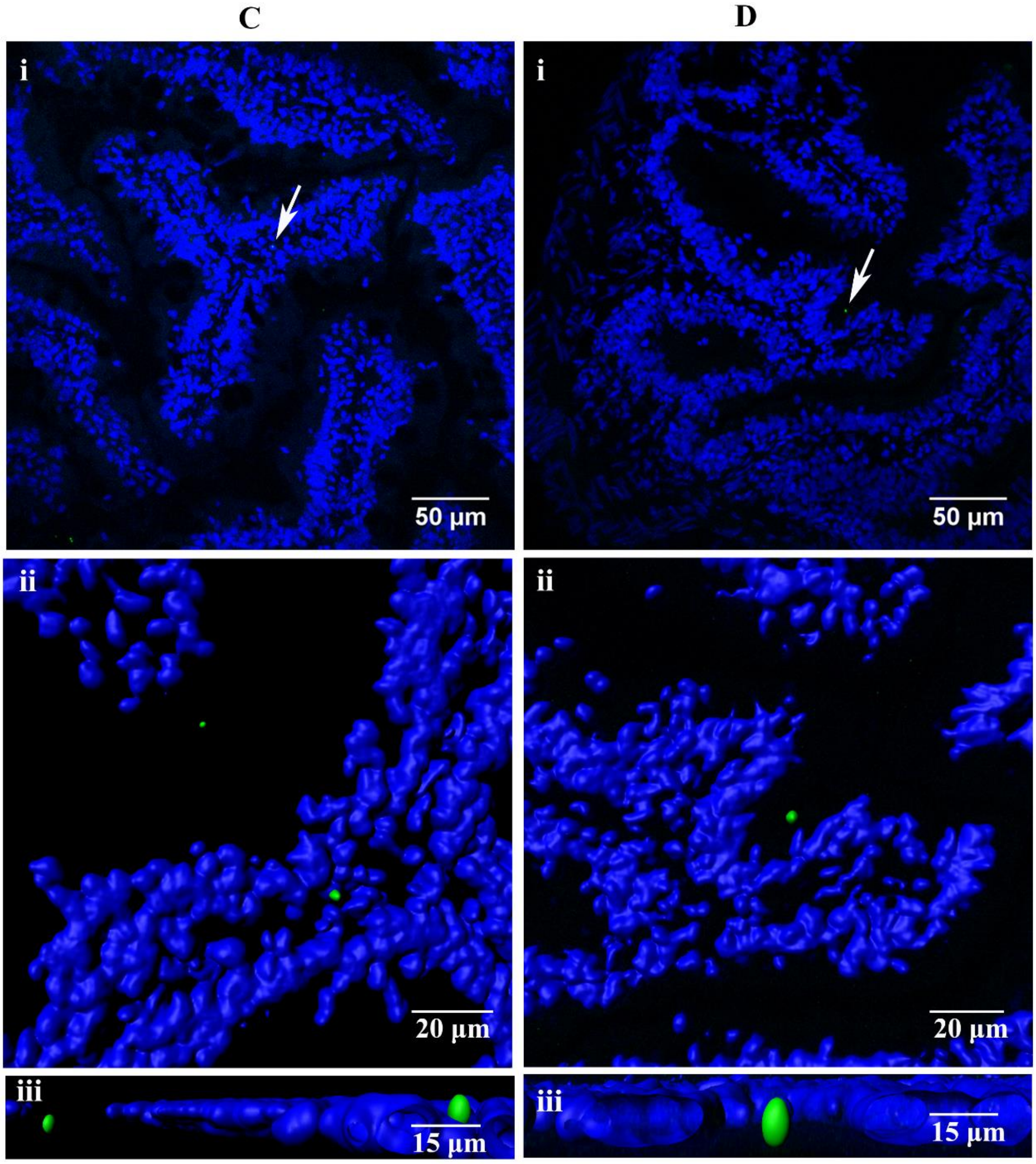
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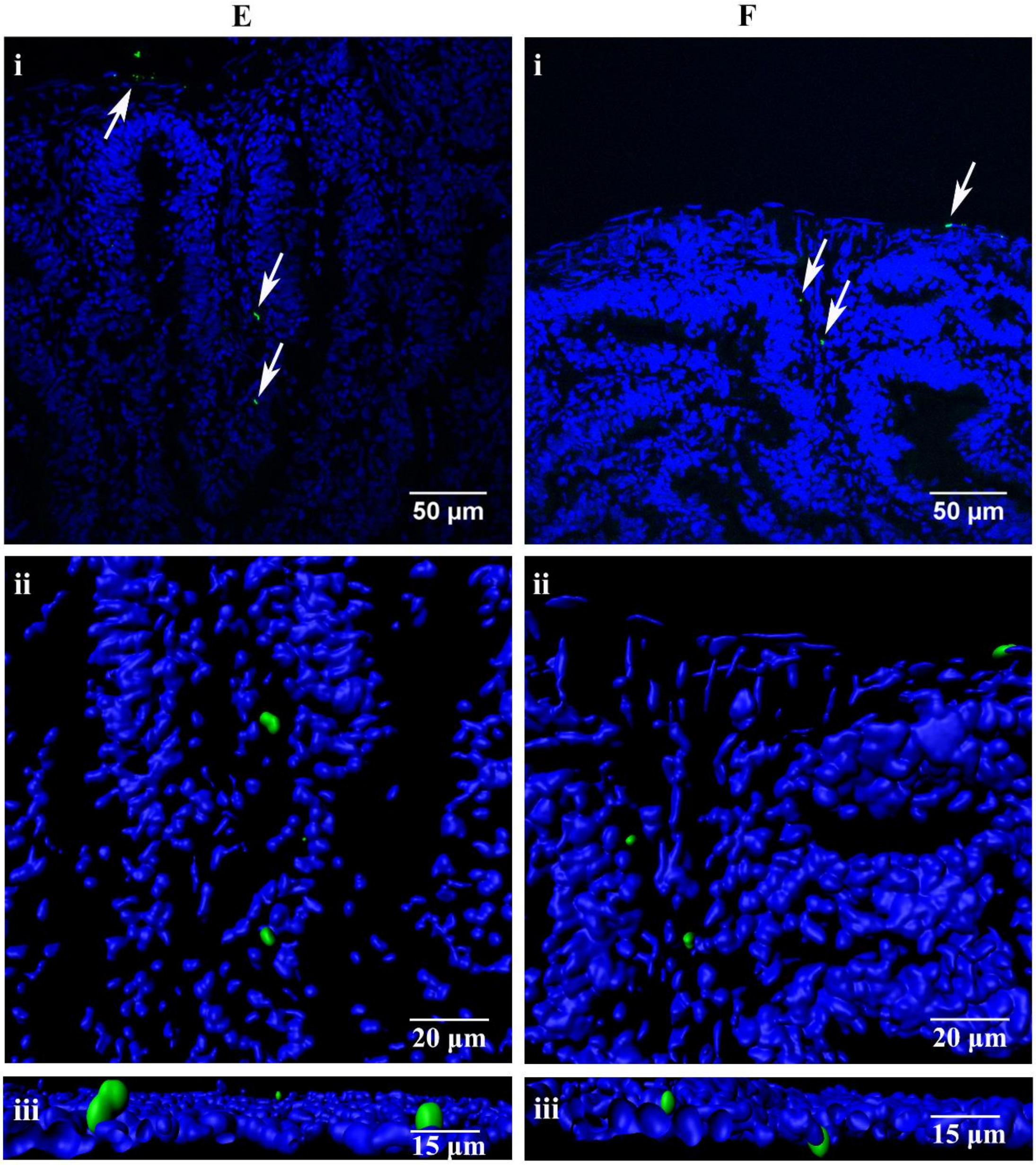
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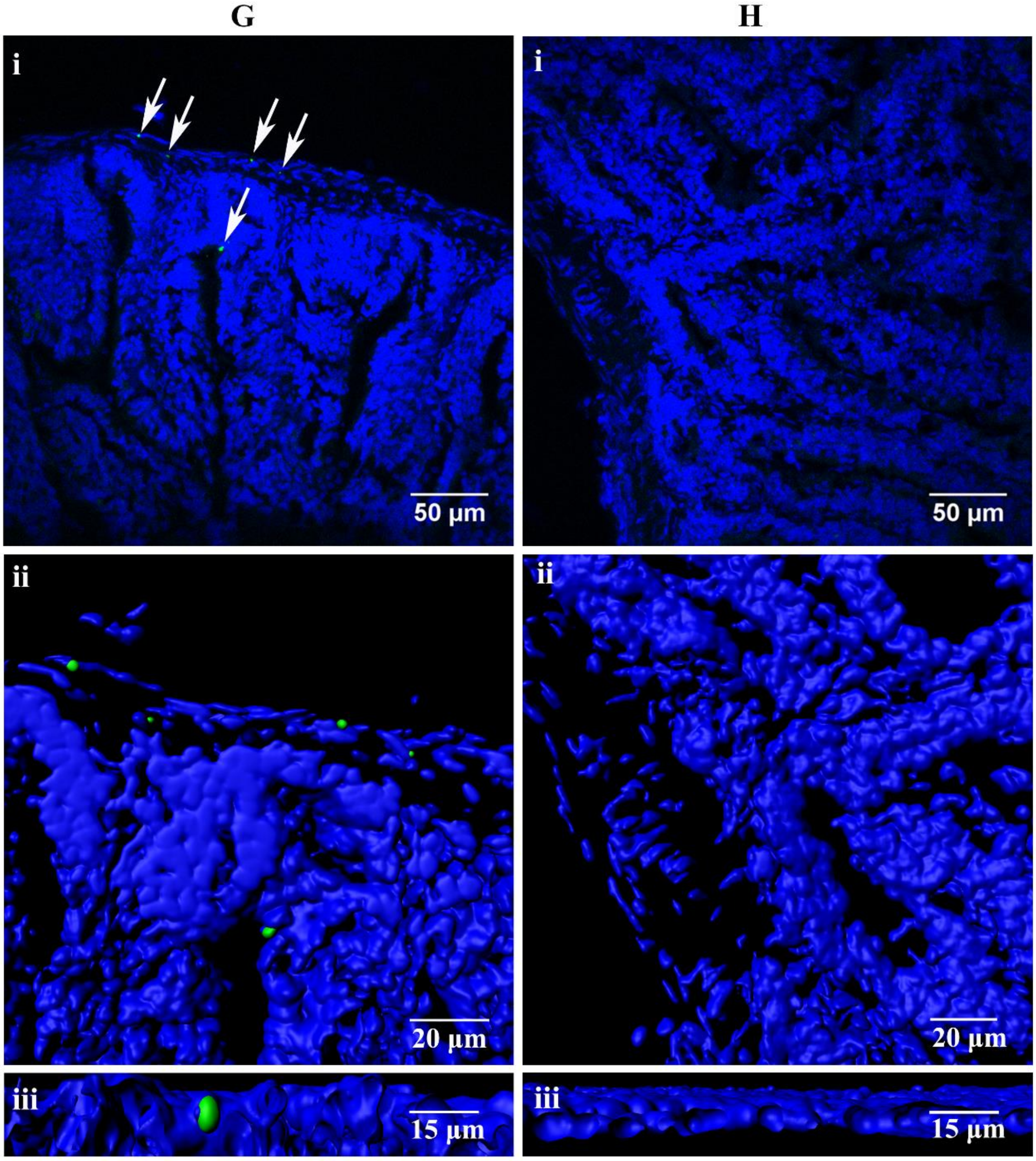


Figure 5. **Uptake of fluorescent IBs$^{TNF\alpha}$ in the zebrafish intestine after 1 h, 5 h, and 24 h oral intubation.** (A) Confocal images of the zebrafish intestine after oral intubation with PBS. (B-D) Confocal images of the zebrafish intestine after 1 h oral intubation with fluorescent IBs$^{TNF\alpha}$. (B) 1 h after oral intubation, the fluorescent IBs$^{TNF\alpha}$ were mostly found in the lumen, a part of fluorescent IBs$^{TNF\alpha}$ were found in the epithelial cells and lamina propria (white arrows). (C) 1 h after oral intubation, the fluorescent IBs$^{TNF\alpha}$ was found in lamina propria (white arrow). (D) 1 h after oral intubation, the fluorescent IBs$^{TNF\alpha}$ was found in goblet cells (white arrow). (E-G) Confocal images of the zebrafish intestine after 5 h oral intubation with fluorescent IBs$^{TNF\alpha}$. (E) 5 h after oral intubation, the fluorescent IBs$^{TNF\alpha}$ were found in the epithelial cells, lamina propria, and muscular cells (white arrows). (F) 5 h after oral intubation, the fluorescent IBs$^{TNF\alpha}$ were found in

the lamina propria and muscular cells (white arrows). (G) 5 h after oral intubation, the fluorescent IBs$^{\mathrm{TNF\alpha}}$ were found in the epithelial cells and muscular cells (white arrows). (H) Confocal images of the zebrafish intestine after 24 h oral intubation with fluorescent IBs$^{\mathrm{TNF\alpha}}$. No fluorescent IBs$^{\mathrm{TNF\alpha}}$ was found in the sample. In all graphs, fluorescent IBs$^{\mathrm{TNF\alpha}}$ are shown in green and nuclei in blue. (i) Confocal images prepared with Fiji. (ii) Confocal images prepared with Imaris with high magnification of representative area. (iii) Confocal images prepared with Imaris and z-stack view of the representative area.

Stimulation of immune-related genes in the intestine

In order to evaluate the capacity of immune stimulation in zebrafish mucosal intestine, zebrafish were orally intubated with 20 µg IBs$^{\mathrm{TNF\alpha}}$ per fish as previously described (Materials and Methods section 5). 1 h, 5 h, 12 h post oral intubation, zebrafish intestines were prepared for RT-qPCR analysis. IBs$^{\mathrm{TNFa}}$ were able to stimulate zebrafish mucosal response by increasing the gene expression of cytokines and regulators of inflammation. The results showed that IBs$^{\mathrm{TNF\alpha}}$ could stimulate the expression of pro-inflammatory cytokines such as IL-6 and IL-1β. IL-6 was significantly up-regulated at 24 h post intubation but not at 1 h and 5 h post intubation. IL-1β was significantly up-regulated at 5 h post intubation but not at 1 h and 24 h post intubation (Figure 6A and B). Next, we also analyzed genes such as COX2 and MMP9 involved in the modulation of the inflammatory process. The expression of COX-2 was significantly up-regulated at 5 h post intubation but not at 1 h and 24 h. The expression of MMP9 was significantly up-regulated at 24 h post intubation but not at 1 h and 5 h (Figure 6C and D). The results indicated that IBs$^{\mathrm{TNF\alpha}}$ could modulate the immune genes expression in the mucosa of zebrafish intestine. No significant differences in the expressions of other innate immune related genes (TNFα and IL22) and adaptive immune related genes (IgM, IgZ, and MHC-II) were observed in our experimental time frame (data not shown).

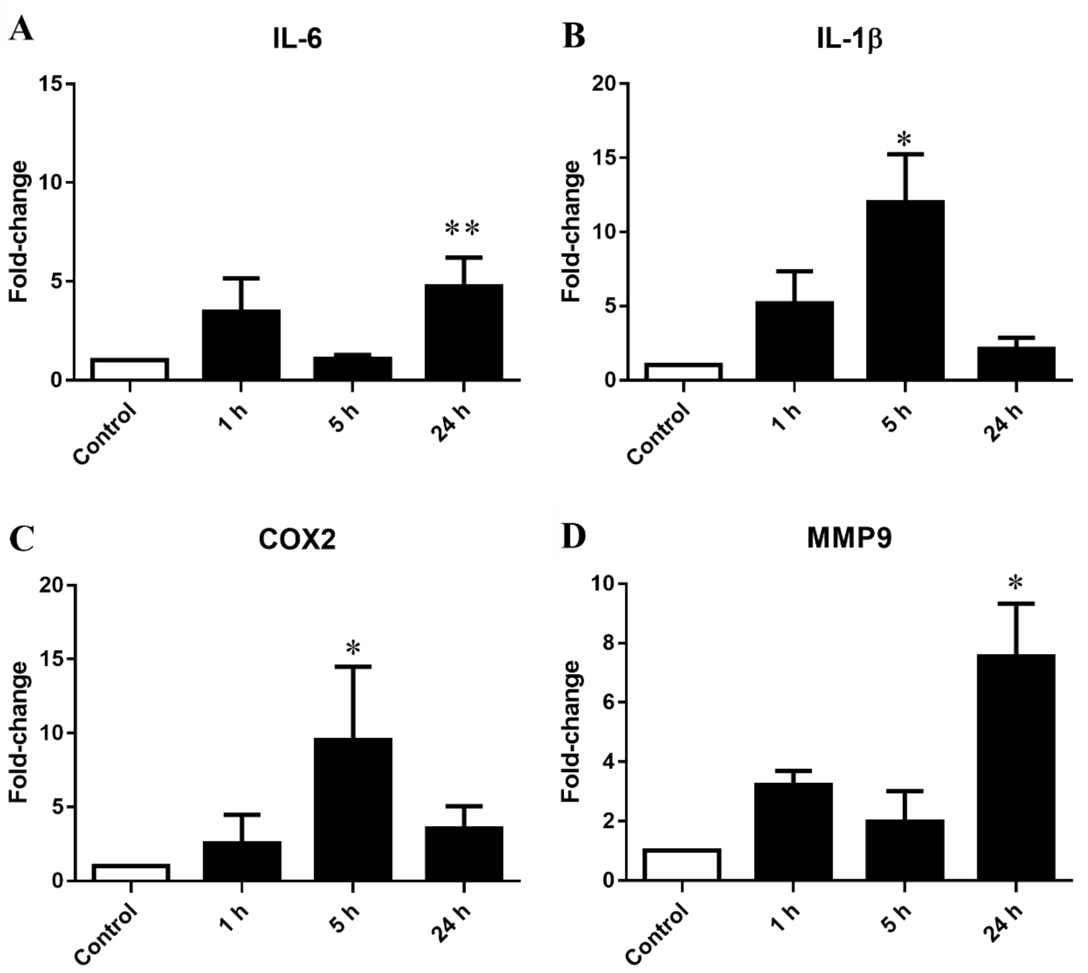


Figure 6. **Immune genes expression analysis in the zebrafish intestine stimulated with IBs$^{TNF\alpha}$.** Zebrafish intestines were dissected at 1 h, 5 h, 24 h post intubation with IBs$^{TNF\alpha}$ (20 µg per fish). The gene expression was analyzed by RT-qPCR. Data represent mean ± SEM (n=3). Differences were analyzed using one-way ANOVA. Significant differences with respect to control (*, $p < 0.05$; **, $p < 0.01$)

Establishing the *M. marinum* infection model in zebrafish

The growth curve for *M. marinum* was obtained to understand the growth of this strain and to calculate the exponentially phase and the corresponding OD600 values. The growth curve of *M. marinum* is shown in Figure 7. The OD600 value reached ~1.2 on day 4 and increased exponentially until day 6. After 6 days a very slight increase was observed and the *M. marinum* culture reach the maximal OD600 value (1.99) on day 8. After day 8 the OD600 did not change significantly and even we observed a small decline on day 9. Therefore, we considered that from day 6 we are in the stationary phase. The bacterial stock for subsequent zebrafish infection experiments were prepared at day 5 when the OD600 value reaches ~1.5. The results of bacterial count on agar plates showed that the colony-forming units (CFU) of the stock aliquots were $2.17 \pm 0.53 \times 10^{10}$ cfu/ml.

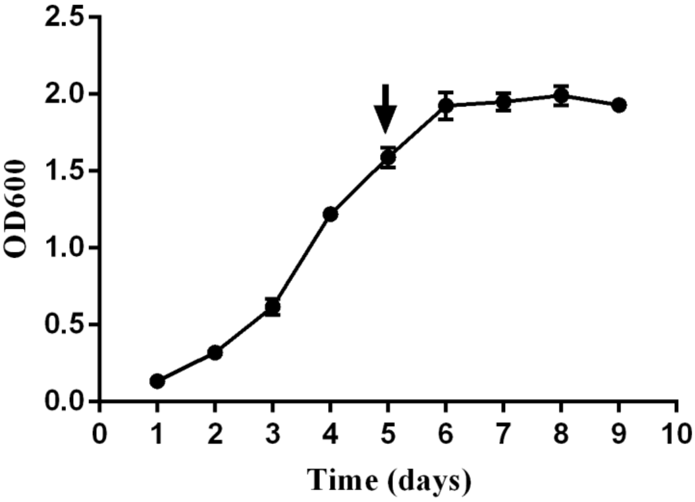


Figure 7. ***M. marinum* growth curve.** *M. marinum* was grown in 7H9 medium supplemented with ADC. Optical density at 600 nm was measured every 24 h. Data represents mean ± SEM. The black arrow indicates the time point when *M. marinum* stock aliquots for infection experiments were obtained.

Adult zebrafish were intraperitoneally injected with four different doses of *M. marinum* and the survival was recorded during 24 days. PBS injected fish were used as a control. The survival curves are shown in Figure 8A. All fish infected with 3.2×10^6 cfu of *M. marinum* died within 7 days whereas only 50% of fish infected with 3.2×10^3 cfu died within 24 days. The fish infected with 3.2×10^4 and 3.2×10^5 cfu shown an intermediate mortality pattern reaching 83.3% and 91.7% mortality, respectively. No mortality was found in the PBS injected group throughout the entire duration of the experiment (24 days, Figure 8A). A dose-dependent mortality was recorded during the 24 days monitoring period. Dead fish showed external red lesions on the ventral trunk compatibles with lesions caused by *M. marinum* (Figure 8B). To demonstrate that dead fish were dying from a *M. marinum* infection, RT-PCRs were carried out using 16S-23S ITS and 16S rRNA gene specific primers (Figure 8C). The suitability and specificity of the primers was described before by Parikka M et al. and Pourahmad F et al. (45) (46). The results showed that *M. marinum* was detected both in the two dead fish and in the positive control (*M. marinum* culture) and the size of the amplifications is the same in all samples. In addition, no amplification was observed in non-infected fish. Meanwhile, we also tested the infection of adult zebrafish by oral intubation. Three different doses of *M. marinum* (3.5×10^8 cfu/fish, 3.5×10^7 cfu/fish, and 3.5×10^6 cfu/fish) were used in the experiment, no mortality was observed during one month of monitorization with this *M. marinum* strain (data not shown).

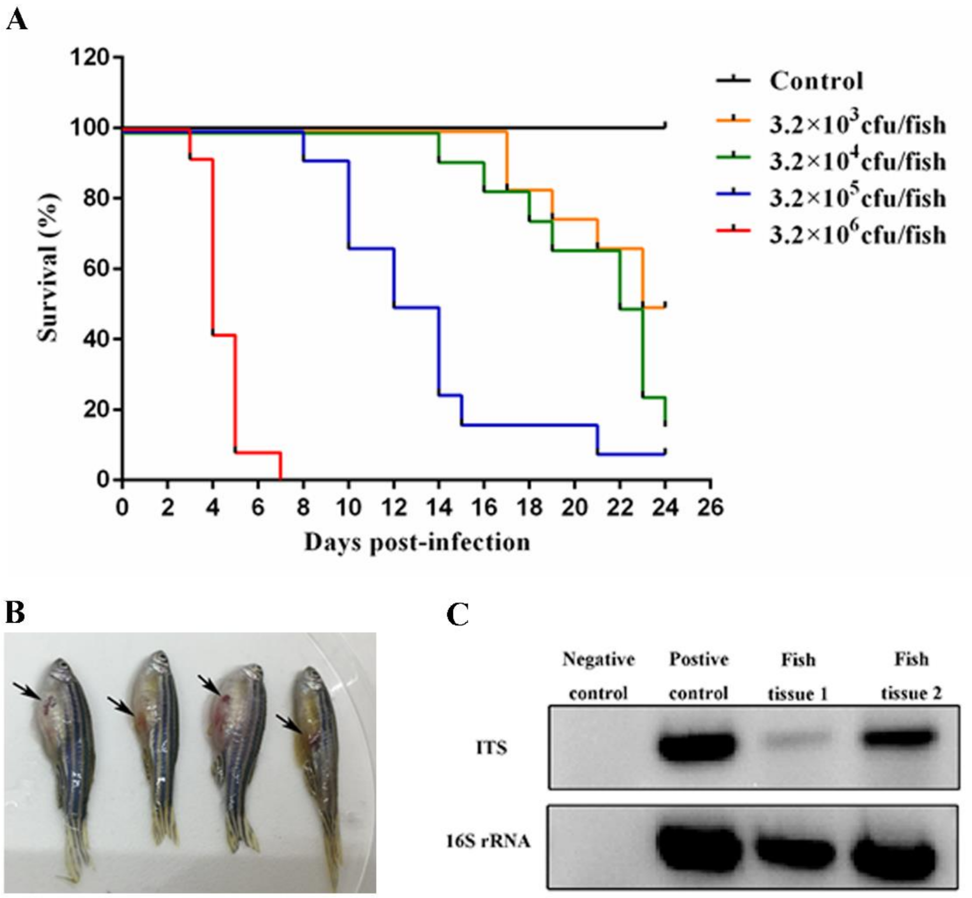
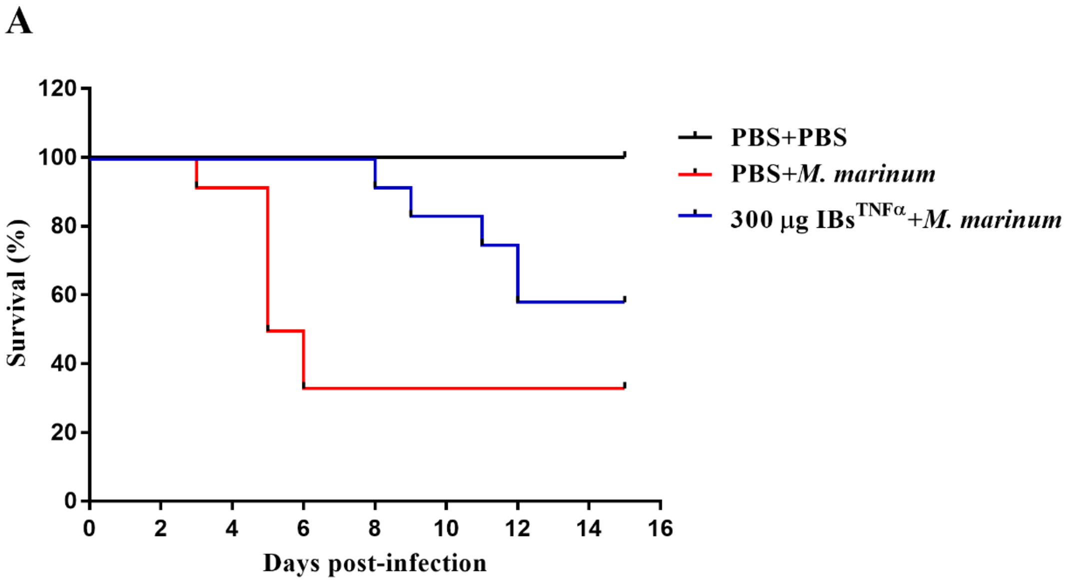


Figure 8. ***M. marinum* infection model**. (A) Dose-dependent survival curves of adult zebrafish intraperitoneally injected with *M. marinum*. 12 fish were injected in each group and PBS was used as control. Cumulative survival was calculated in 24 days.

(B) Dead fish infected with *M. marinum*. The arrows showed the external red lesions on the ventral trunk. (C) PCR amplification of genomic DNA with specific primers for ITS and 16S rRNA genes. (Negative control: genomic DNA extracted from non-infected zebrafish tissues; Postive control: genomic DNA extracted from 7H9ADC medium cultured *M. marinum*; Fish tissue pool 1 and 2: genomic DNA extracted from dead zebrafish tissues after infection by *M. marinum*).

IBs^{TNFα} protect zebrafish against a *M. marinum* lethal infection

The IB^{TNFα} was obtained following the protocol previously described in our group (35). IB^{TNFα} have been used to protect zebrafish against a lethal bacterial infection of *P. aeruginosa*. To perform the IB^{TNFα} protecting experiments against a natural fish pathogen, zebrafish were treated with IBs^{TNFα} by i.p. injection and challenged with *M. marinum* i.p. injection at 7 and 40 days post-treatment. ~10^{6} cfu/fish of *M. marinum* was injected in the experiments and *M. marinum* infection in zebrafish was demonstrated using PCR and agarose gel electrophoresis as describe before (data not shown). The dead fish were recorded every day and the survival curves are shown in Figure 9A and Figure 9B. IBs^{TNFα} treated zebrafish displayed a higher survival rate at 7 days (58.3%) and a significantly higher survival rate (33.3%) at 40 days than the controls, 33.3% and 0%, respectively. No mortality of zebrafish was observed in the PBS groups. The results showed that IBs^{TNFα} were able to protect zebrafish against *M. marinum* lethal infection.



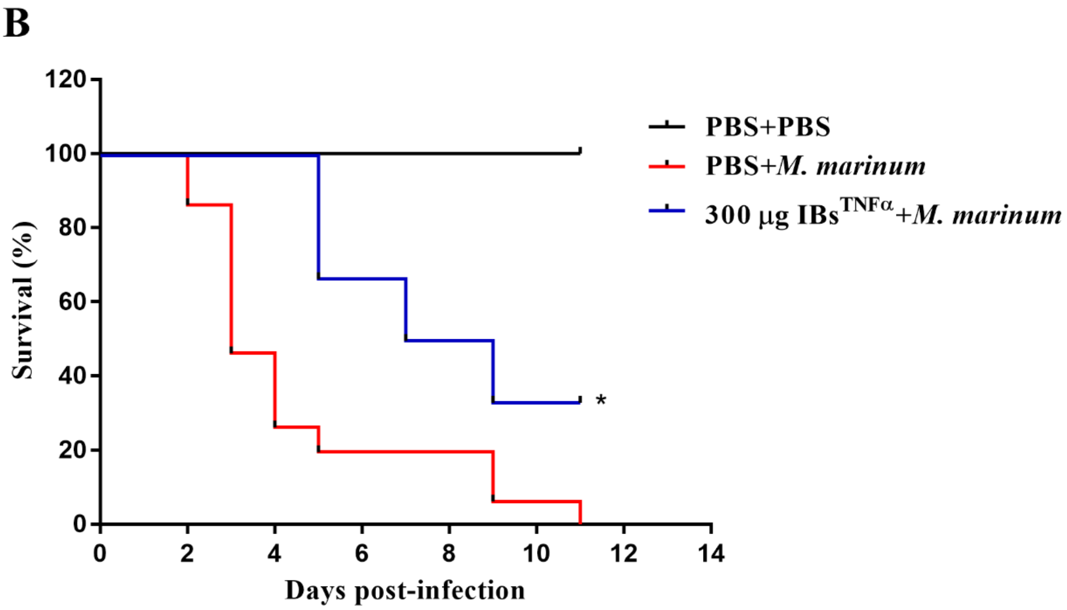
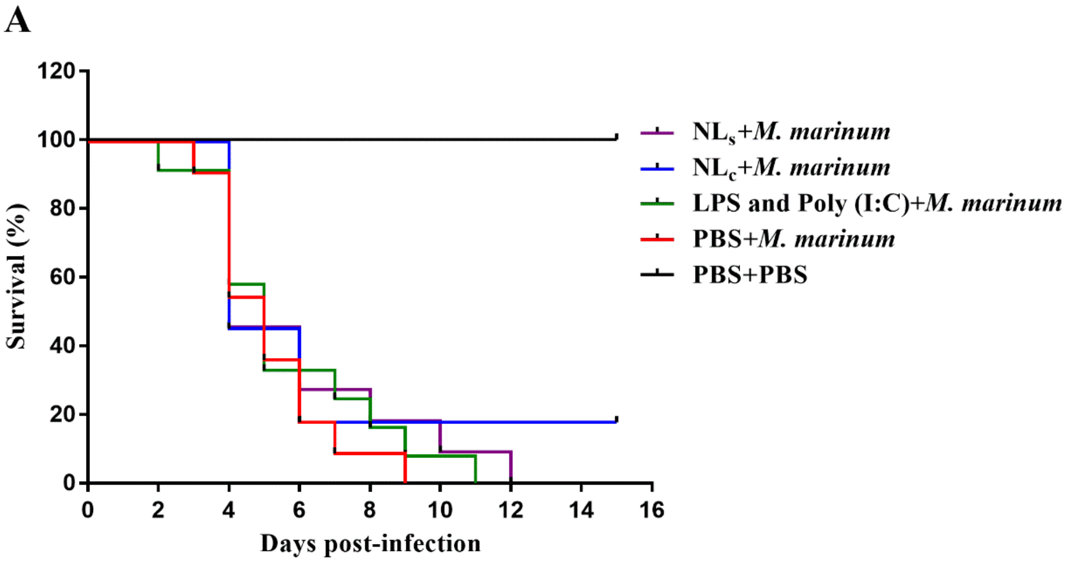


Figure 9. **Survival of adult zebrafish after i.p. injection of IBs$^{TNF\alpha}$ and challenge with *M. marinum*.**

(A) Fish were i.p. injected with IBs$^{TNF\alpha}$ and PBS 7 days before challenged with *M. marinum* at the dose of 4.1×10^5 cfu/fish. (B) Fish were i.p. injected with IBs$^{TNF\alpha}$ and PBS 40 days before challenged with *M. marinum* at the dose of 1.0×10^6 cfu/fish. Fish injected with PBS followed by *M. marinum* challenge were used as mortality control. Cumulative survival rate was measured in 15 days or survival in mortality control group dropped to 0%. Significant difference was analyzed using Log-rank (Mantel-Cox) Test. *p<0.05.

NL$_C$ protect zebrafish against *M. marinum* lethal infection

NL$_C$ liposomes and empty liposomes were obtained by the protocol previously described in our group and NL$_C$ liposomes were lyophilized using trehalose at 2.7%, w/v as cryoprotectant (14). Liposomes containing immunostimulants (LPS and Poly I:C) have been demonstrated to protect zebrafish against model viral and bacterial infections (15). In this study, we develop an infection model in adult zebrafish using a natural pathogen, *M. marinum*. We showed that adult zebrafish could be infected by *M. marinum* by intraperitoneal injection and the mortality was dose-dependent. Next, we studied the protective effect of NL$_C$ liposomes in zebrafish against *M. marinum* lethal infection. We selected the dose ~10^6 cfu/fish because 100% mortality can be obtained in a short time frame (7 days). The detection of *M. marinum* infection in dead zebrafish was demonstrated using conventional PCR and agarose gel electrophoresis as describe before (data not shown). The zebrafish were treated with NL$_c$ liposomes and the corresponding controls by i.p. injection, followed by i.p. injection of *M. marinum* at 7 and 40 days post-treatments. The survival curves are shown in Figure 10A and Figure 10B. NL$_c$ liposomes treated animals exhibited higher survival rates (18.2%) at 7 days post-immunization and significant higher survival rates (50%) at 40 days post immunization compared to the mortality control group (0%). In contrast, neither the NL$_s$ nor the mixture of LPS and Poly (I:C) treated zebrafish showed the protection against *M. marinum* infection at both 7 days and 40 days post immunization. No mortality of zebrafish was reported in the PBS groups. Thus, NL$_c$ liposomes protect zebrafish against *M. marinum* lethal infection and would be suitable as adjuvant in combination with specific vaccines to achieve full protection against bacterial infections.



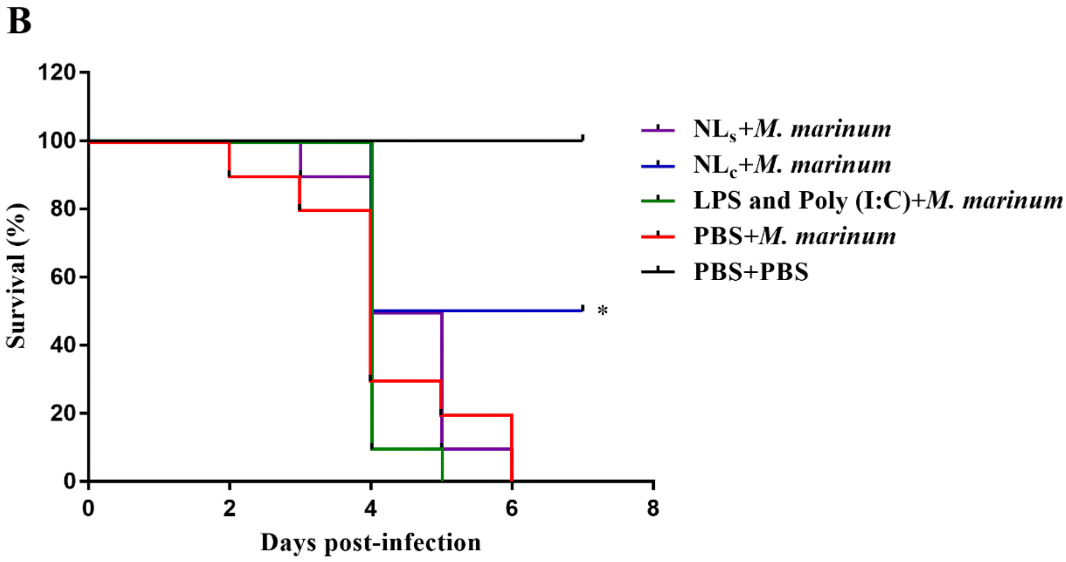


Figure 10. **Survival of adult zebrafish after i.p. injection of NL$_c$ liposomes and injection challenge with *M. marinum***. (A) Fish were i.p. injected with NL$_c$, NL$_s$, free LPS and Poly (I:C), and PBS 7 days before challenged with *M. marinum* at the dose of 1.9×10^6 cfu/fish. (B) Fish were i.p. injected with NL$_c$, NL$_s$, free LPS and Poly (I:C), and PBS 40 days before challenged with *M. marinum* at the dose of 3.7×10^6 cfu/fish. Fish injected with PBS followed by *M. marinum* challenge were used as mortality control. Cumulative survival rate was measured in 15 days or survival in mortality control group dropped to 0%. Significant difference was analyzed using Log-rank (Mantel-Cox) Test. *$p<0.05$.

Discussion

The emerging field of mucosal vaccination is now one of the main areas in fish vaccinology. Mucosal vaccines surpass injectable vaccines in terms of practicality. They are less labor intensive and require less handling of individual fish. However, to date, there is only one licensed mucosal vaccine which has achieved superior protection to an injectable vaccine. This is the live-attenuated Cyprinid herpesvirus 3 (CyHV-3) vaccine administered by immersion in carp (*C. carpio*) (48). Mucosal vaccination systems need to overcome the challenge of delivering vaccines to the second gut segment without denaturation, crossing the mucosal barrier to access the APCs, and most importantly, evoking a strong immune response. Adjuvant design is one strategy in mucosal vaccination which could protect the antigen and meanwhile serve as an immunostimulant.

Here, using an adult zebrafish oral intubation model which mimics oral vaccine administration and allows us to control doses, we analyzed a potential cytokine adjuvant. We tested whether recombinant protein nanoparticle IBs$^{TNF\alpha}$ could be taken up by the intestinal mucosa, cross the mucosal barrier targeting immune-related cells and stimulate the local immune system. IBs$^{TNF\alpha}$ had been previously studied in our lab, administered to rainbow trout by oral intubation. These results demonstrated that intestinal uptake was possible, as well as immune stimulation. This nanoparticle also retained stability and functionality in extreme pH and temperature making it suitable for oral vaccine (35). Nevertheless, its role as a mucosal adjuvant was not investigated in detail. Here, we have shown that IBs$^{TNF\alpha}$ cross the zebrafish intestine mucosal barrier and stimulate local immune responses. When intraperitoneally injected, the nanoparticle protected zebrafish against a lethal infection by the fish pathogen *M. marinum,* significantly increasing survival. In addition, the zebrafish oral intubation model is a simple procedure that can be easily performed and maybe further applied as a comprehensive test model in the screening of oral vaccines. In the zebrafish intestine, we observed how fluorescent IBs$^{TNF\alpha}$ penetrated the epithelium, lamina propria and accumulated in the deeper muscle cell layer at different time points post oral intubation (Figure 5). In teleosts, intestinal epithelial cells (enterocytes) are known to be responsible for antigen uptake. A large number of intraepithelial lymphocytes (IELs) are also present in the intestinal epithelium. Most of them are CD3-ε^+/CD8-α^+ and have cytotoxic and/or regulatory function (49). It has been reported that IELs contribute to mucosal immunity, producing cytokines in rainbow trout in response to bacterial infection (50). Our results showed the IBs$^{TNF\alpha}$ appears very quickly in the epithelium at 1 h post oral intubation (data not shown). This indicates that epithelial cells or IELs may be responsible for the initial recognition and uptake of IBs$^{TNF\alpha}$. 5 h post intubation, the IBs$^{TNF\alpha}$ was mainly found in the lamina propria. The lamina propria (LP) is a layer of tissue beneath the epithelial cells of the villi that contains several different types of immune cells, including B and T lymphocytes, macrophages, mast cells, and dendritic cells (reviewed in Gomez et al. (2013) (51). The main role of B cells is to recognize antigens in their native form and produce Igs against those antigens. Indeed, a high percentage of IgT and IgT$^+$ B cells were found in the lamina propria of the rainbow trout gut after a parasite (*Ceratomyxa Shasta*) infection (38). Zebrafish intestine cryosection showed that CD4-1$^+$ and CD4-1$^-$ T cells are abundant in the lamina propria of the gut mucosa and are dispersed among overlying epithelial cells (52). Intraepithelial macrophages were shown to efficiently take up and transport antigens especially when antigens were coupled to enteric adhesion molecules in carp (53). In addition, mucosal dendritic cells in the zebrafish intestine were investigated as APCs and efficiently took up surfactant-free polymeric nanoparticles (55). We also observed the $\text{IBs}^{\text{TNF}\alpha}$ in the deeper muscle cells layer at 5 h post intubation. This layer has the distribution of blood capillaries which indicate that the $\text{IBs}^{\text{TNF}\alpha}$ may enter in the bloodstream through these vessels and target the systemic immune system. However, the way that $\text{IBs}^{\text{TNF}\alpha}$ are taken up from mucosa barrier to the muscle layer remains unknown and should be further studied. All the above observations confirm that $\text{IBs}^{\text{TNF}\alpha}$ are very efficiently uptaken and cross the mucosal barrier of the zebrafish intestine where could reach immune cells.

To further demonstrate whether $\text{IBs}^{\text{TNF}\alpha}$ were able to induce local immune responses in the zebrafish intestine we assessed the expression profile of relevant immune related genes. Some cytokine (IL-6 and IL-1β) and inflammation-related genes (COX2 and MMP9) were significantly upregulated in a short time frame. These results indicate $\text{IBs}^{\text{TNF}\alpha}$ stimulate an immediate response and concord with our previous results showing that $\text{IBs}^{\text{TNF}\alpha}$ stimulated the gene expression of cytokines and regulators of inflammation in rainbow trout macrophages (35). IgT/Z is the only immunoglobulin marker for mucosal immunity in the fish gut and the IgM response also exists in the gut, although is typically very low (56) (57). However, the expression of mucosal adaptive immune-related genes did not show any significant difference between those fish treated with $\text{IBs}^{\text{TNF}\alpha}$ and control fish. This may be due to the short monitoring period of time after oral intubation. For example, Galindo-Villegas et al. reports IgT transcripts were significantly upregulated 28 day post priming and 156/15 days post priming/booster by oral administration of vaccine with a TNFα adjuvant compared to controls (34).

The main objective when prophylactic or immunostimulatory strategies are under development is to test how effective these strategies are. The development of infection models is an important topic in fish vaccinology since we need to test the prophylactic systems under development. The adult zebrafish *M. marinum* infection model had been developed as described previously (58). *M. marinum* causes tuberculosis in poikilothermic species and is a close genetic relative of *Mycobacterium tuberculosis*. It has become a model for the study of *Mycobacterium* pathogenesis (59) (60). We selected *M. marinum* because it invades the organism through the mucosal surfaces. *M. marinum* resides and multiplies within host macrophages, evading the immune system by using host macrophages. In its natural range *M. marinum* infects fish probably via the oral-intestine route (61) (62). The route of administration both of prophylactic systems and infective pathogens is a very important issue when developing infection models. Immersion and oral administration are preferable as they involve less handling costs and stress. However, we were unable to develop a *M. marinum* infection model mimicking the natural infection routes. We did not observe any mortality when we orally intubated animals with a high dose of *M. marinum* followed by a one month's monitoring period. On the other hand, we successfully set up the *M. marinum* infection model by i.p. injection and modified the protocol so it can be easily and repeatably performed in the lab. Injection by i.p. remains the most widely used route to administer pathogens, although there are disadvantages, such as stress and side-effects at the injection site. By immunizing zebrafish by i.p. injection with $\text{IBs}^{\text{TNF}\alpha}$, we evoked efficient protection in terms of increasing survival after *M. marinum* lethal infection. The *M. marinum* infection model has been shown to be also useful to screen for other prophylactic methods.

We used an adjuvant immunostimulant, nanoliposome encapsulating LPS and Poly I:C (NL_c), to test the infection model. NL_c liposomes can protect zebrafish against *P. aeruginosa* infection when i.p. injected, as described previously by our lab (15). Here we further demonstrate that NL_c liposomes can protect zebrafish against *M. marinum* infection by i.p. injection. At the same time, with these experiments we evidence the viability and versatility of our infection model.

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Chapter 3

Zebrafish larvae models to study the fish innate immune system: evaluation of two novel strategies for immunomodulation.

Chapter 3: Zebrafish larvae models to study the fish innate immune system: evaluation of two novel strategies for immunomodulation.

Abstract

Zebrafish larvae have become an important *in vivo* model to study the innate immune system with view to immunostimulant screening and pathogen infections. Infection achieved by microinjection into the Duct of Cuvier is the most common method used in larval bacterial infection. However, this method achieves relatively low throughput, is labor-intensive and is very different from the natural infection routes. Alternatively, infection by immersion is an easier, more convenient method that mimicks the natural route of infection. In this study, we explored the infective possibilities of two fish pathogens, *Mycobacteria marinum* and *A. hydrophila*, by immersion. Using zebrafish larvae, we examined the biodistributions of two potential immunostimulants: nanoliposome (NL_c) and TNFα protein nanoparticle ($IBs^{TNF\alpha}$), and explored their capacity to induce immune protection. In setting up the model, mortality of larvae after immersion with *M. marinum* showed no significant differences from controls in a 4 days' monitoring period and are varied between replicates. Zebrafish larvae infected with *A. hydrophila* by immersion showed significant differences compared to controls in a 5 days monitoring period in a dose-dependent manner. When zebrafish larvae were immersed with the fluorescent NL_c and $IBs^{TNF\alpha}$, the particles localized in pharynx and intestine at 3 dpf and 5 dpf, respectively. RT-qPCR analysis showed that the expression of the immune-related genes, TNFα, iNOS, TLR9, and COX2, were up-regulated after 48 h immersion with NL_c in 2 dpf larvae while IL-1β and IRF1α were significantly up-regulated. RT-qPCR analysis shows that the expression of the immune-related genes, TNFα, IL-1β, IL-6, COX2, IL-22, and IL-10 were not significantly up-regulated after 3 h, 12 h, and 24 h immersion with $IBs^{TNF\alpha}$ in 5 dpf larvae. $IBs^{TNF\alpha}$ could not protect zebrafish larvae against *A. hydrophila* lethal infection.

Our assay provides a useful reference to set up future bacterial infection models by immersion and for the *in vivo* study of potential immunostimulants.

Introduction

With fish capture production relatively static since the late 1980s, the aquaculture industry has grown impressively to meet the demand of fish for human consumption. Globally, aquaculture provided only 8 % of aquatic food in 1974, but this had increased to 53 percent by 2015 (FAO) (1). However, the effective commercial cost of fish larvae production is still a bottleneck in most aquaculture species. The main symptoms observed are poor growth of individuals, a sudden decrease in survival and malformations (2). Pathogenic bacterial infections in larvae are one of the main causes. Larvae have an immature immune system and are an easy target for pathogens. Therefore, the prevention of infection and the maintenance of optimal water quality parameters is of utmost importance to reduce mortality and to ensure the production of quality larvae and fry in the hatcheries (3). However, disinfection of the entire hatchery facility including tanks as well as routine monitoring of all the parameters is extremely labor intensive and may not be feasible from an economic point of view (4). Meanwhile, pathogens may be hidden in the aquatic environment or in fish feed. It is very difficult to prevent larvae from becoming infected from these sources. Some pathogenic bacteria have been identified in the fish larvae of both fresh and marine water. For example: *Vibrio anguillarum* in zebrafish (*Danio rerio),* Turbot (*Scophthalmus maximus),* halibut (*Hippoglossus hippoglossus)* and cod (*Gadus morhua)* (5) (6); *Carnobacterium sp.* in Atlantic cod (*G. morhua)* (7); *Endozoicomonas sp.* in sharpsnout seabream (*Diplodus puntazzo)* (8); *A. hydrophila* in carp *(C. idellus)* (9); *Photobacterium damselae* and *A. salmonicida* in Atlantic halibut (*Hippoglossus hippoglossus*) (10); and *Lactococcus garvieae* in rainbow trout (*O. mykiss)* (11). Some antibiotics provide a broad-spectrum antimicrobial activity but their frequent use has led to the development of resistant strains. This is an increasing problem for veterinary treatment in aquaculture and even a matter for human health (12).

In fish larvae, innate immunity plays a continuous role in orchestrating quick immune responses and protects larvae against the hostile environment, even when their own immunological capacity is still limited. Fish innate immunity is activated just after egg fecundation and becomes fully functional by the time of hatching (2). The fish larval innate immune system includes, but is not limited to, physical barriers, humoral factors, cellular defenses, and inflammatory processes (13) (14) (15) (16). The mucus, epithelial cells, and dendritic cells lining the skin, gills and digestive tract of fish larvae are the first immune barrier against microorganism invasion (17) (18). If bacteria are successful in crossing the physical barriers, the humoral factors, such as antibacterial peptides, complement proteins, lectins and pentraxins, may prevent further spread of the infection (13). If the bacteria gain entry into the tissue of the fish larvae, the innate cellular defenses play a role. Innate cellular defenses involve a variety of leukocytes, such as mononuclear phagocytes, polymorphonuclear leukocytes, and natural-killer cells (19). The responses of these immune cells are driven by a diverse array of pattern recognition receptors (PRRs), such as Toll-like receptors (TLR), discussed in Chapter 1 of this thesis. These receptors recognize a variety of highly conserved pathogen-associated molecular patterns (PAMPs). With these PRRs, immune cells activate signaling cascades leading to the expression of inflammatory cytokines and chemokines and to the activation of antimicrobial host defense mechanisms (2). Therefore, the innate immune system plays a pivotal role in fish larvae against invading pathogens. Vaccination is one of the most effective prophylactic tools for disease control in the fish industry. However, vaccination may not provide the same protection in immunologically immature individuals as in adults. Moreover, effective commercial vaccines against many fish pathogens are not available (20) (21). There is an urgent need for vaccines adapted to the different life cycle stages: larval, juvenile and adult stages, as well as to develop novel and effective adjuvants for aquaculture.

Adjuvants are a group of structurally heterogeneous compounds able to modulate the intrinsic immunogenicity of an antigen (22). They are divided into immunostimulants and delivery systems. Immunostimulants interact with specific receptors, like TLRs and others, while delivery systems increase the immune response by multiple mechanisms, depending on their particular characteristics (23) (24). Nanodelivery systems can either co-deliver with adjuvants or act as adjuvants themselves to enhance vaccine efficacy and protect the immunogens (25). The immunomodulation of larvae has been proposed as a potential method for improving larval survival by increasing the innate responses of the developing animals until its adaptive immune response is sufficiently developed to mount an effective response to the pathogen.

Liposomes are promising as vaccine carriers and/or adjuvants. They have a hydrophilic core, and an interior, self-sealing, hydrophobic bilayer allows encapsulation of both lipophilic and hydrophilic compounds (26). Nanoliposomes, liposomes on a nanoscale provide more surface area per volume than larger liposomes. They thus have the potential for greater solubility, enhanced bioavailability, improved controlled release and better precision targeting of the encapsulated material (27). Liposomes have been used as adjuvants, delivering bacterial or viral antigens to adult fish species, achieving good results (28) (29) (30) (31) (32) (33) (34). However, no report was found which used liposomes as an adjuvant or immunostimulant for fish larvae. The most relevant study was carried out more than 20 years ago and the liposome containing vaccine significantly enhanced the protection of rainbow trout fry against furunculosis (35).

Cytokines are small soluble proteins mediating and regulating immune and non-immune cells and have been used as adjuvants, immunostimulants and therapeutic agents in the treatment of different human diseases (36) (37) (38). During the last years, a great number of cytokine genes have been identified in many fish species (39) (40). Some of these cytokines, such as interleukin-1β (IL-1β), interferon regulatory factors (IRFs), and tumor necrosis factor α (TNFα) were reported as vaccine adjuvants in adult fish species (41) (42) (43) (44) (45) (46) (47). However, there is no study that used cytokines as an immunostimulant in fish larvae.

Zebrafish (*Danio rerio*) has been widely used as a vertebrate model to study vertebrate developmental patterns and different human diseases such as cancer and the ontology of the immune system (48) (49) (50) (51). During the last decade, zebrafish has also been a good model for aquaculture purposes (52), being an excellent organism to test prophylactic approaches adapted to different life cycle stages. Concerning immunology and particularly the innate immune system, zebrafish larvae are a perfect model because only the innate immune response is present. The innate immune system of zebrafish is active by day 1 of embryogenesis (53), whereas the adaptive immune system is not mature until 4–6 weeks later when the lymphocytes become functional (54). There is a clear temporal gap between both innate and adaptive immune responses in zebrafish. Moreover, another advantage is the body transparency during the early life stages which allows real-time visualization. The rising number of markers of immune cells and transgenic lines contribute to the study of the innate immune system in zebrafish larvae. In our study, we use zebrafish larvae as a model to study the biodistribution and the immune-stimulating capability of two different types of potential immunomodulators, nanosized liposomes and recombinant TNFα protein nanoparticles. By setting up two bacterial infection models appropriate for bath administration, we have further investigated the protection provided to zebrafish larvae by novel immunomodulators.

Materials and methods

Zebrafish breeding

Wild type (wt) zebrafish (*D. rerio*) were housed in the aquaria system described in Chapter 2. The adult zebrafish (approximately 5 months old) were separated by gender and fed with a rich supply of food (blood worm or artemia) in two different tanks (30 l) one week before reproduction. One day before reproduction, one female and three males were mixed in the reproduction tank (3 l) in the late afternoon. Embryos were collected in the morning and cultured in E3 medium in 90 × 14 mm petri dish (Deltalab, Cat. No.200200). Debris and chorions were removed as soon as possible during the experiments.

Preparation of fluorescent NL_c and $IBs^{TNF\alpha}$

The fluorescent NL_c were prepared by incorporating Fluorescein-DHPE (ThermoFisher Scientific, Cat. No. F362) into the lipid mixture (at 0.01 molar ratio). Briefly, 1,2-didodecanoyl-sn-glycero-3-phosphocholine, 1,2-dioleoyl-sn-glycero-3-phosphoric acid monosodium salt, Cholesterol, Cholesteryl, Cholesterol-PEG_{600}, Fluorescein-DHPE were dissolved in chloroform solutions (100 mg/ml) and mixed at the desired molar ratios (0.5:0.35:0.1:0.05:0.1). The organic solvent was then evaporated by rotary evaporation to obtain a lipid film (fluorescent NL_S). The fluorescent NL_c was made by hydrating the dry lipid film with 0.5 mg/ml poly (I:C) and 1.0 mg/ml LPS prepared in PBS as describe before (28).

The fluorescent IBs$^{TNF\alpha}$ was prepared by conjugating IBs$^{TNF\alpha}$ with Atto-488 NHS ester (Sigma-Aldrich, Cat No. 41698) following manufacturer's instructions. Briefly, the IBs$^{TNF\alpha}$ were resuspended in 0.1 M sodium bicarbonate buffer (Sigma-Aldrich, Cat No. S5761) at 2 mg/ml. The Atto 488 NHS ester was dissolved in amine-free DMSO at 2 mg/ml. the IBs$^{TNF\alpha}$ and Atto 488 NHS ester were mixed at a molar ratio of 1:2 (protein/dye) by shaking in the dark. The labeled IBs$^{TNF\alpha}$ were washed with sodium bicarbonate buffer 5X and the supernatant was discard by centrifugation at 8000 ×g for 10 min at room temperature. The aliquots of 100 µl (2 mg/ml) were stored in -80 °C until use.

Biodistribution of fluorescent NL$_c$ and IBs$^{TNF\alpha}$ in zebrafish larvae by immersion

The *in vivo* biodistribution of the fluorescent NL$_c$ and IBs$^{TNF\alpha}$ in zebrafish larvae were studied after bath immersion administration. The transparent zebrafish larvae were generated using 75 µM 1-phenyl 2-thiourea (PTU) as described in Karlsson J *et al*. (55). Immersion in fluorescent NL$_c$ solution: 2 days post fertilization (dpf) zebrafish larvae were immersed with 3 different concentrations (0.75 mg/ml, 1.0 mg/ml and 1.5 mg/ml) of fluorescent NL$_c$ in E3 water. E3 water immersion group and 10% (v/v) PBS of E3 water group were used as controls. 48 h post immersion, the larvae were anaesthetized with 160 mg/l MS-222 and imaged in a Nikon Eclipse 80i fluorescent microscope (Japan). Immersion in fluorescent IBs$^{TNF\alpha}$ solution: zebrafish larvae (5 days post-fertilization) were immersed in 0.01 mg/ml and 0.05 mg/ml fluorescent IBs$^{TNF\alpha}$ prepared in E3 water. E3 water immersion group and 2.5% (v/v) PBS of E3 water were used as controls. 1 h, 3 h, and 5 h post immersion, the larvae were anaesthetized with 160 mg/l MS-222 and imaged with a Nikon Eclipse 80i microscope.

RNA extraction and cDNA synthesis

For fluorescent NL$_c$ immersion: zebrafish larvae (2 days post fertilization or 0 hour post hatching) were immersed with 10% PBS, mixture of 0.5 mg/ml poly (I:C) and 1.0 mg/ml LPS, fluorescent NL$_s$ (0.75, 1.0, and 1.5 mg/ml) and fluorescent NL$_c$ (0.75, 1.0, and 1.5 mg/ml). 48 h post immersion, 20 larvae of each group were sampled for RNA extraction.

For fluorescent IBs$^{TNF\alpha}$ immersion: larvae (5 days post-fertilization) were immersed in 2.5% PBS and 0.01 mg/ml fluorescent IBs$^{TNF\alpha}$ in E3 medium. 3 h, 12 h, and 24 h post immersion, larvae were sampled for RNA extraction.

Total RNA samples were extracted from homogenized zebrafish larvae using polytron (KINEMATICA, Switzerland) and the Maxwell RSC simplyRNA Tissue Kit (Promega, Cat. No. AS1340) according to the manufacturer's instructions. The concentration of RNA was determined with a Nanodrop 1000 (Thermo scientific) and the quality was assessed using Agilent 2100 Bioanalyzer system (Agilent Technologies, G2946-90004). The cDNA was synthesized with 0.5 µg of total RNA by using iScript cDNA synthesis kit (Bio-Rad, Cat. No. 170-8891).

Real time quantitative PCR (RT-qPCR)

The RT-qPCR was performed in the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad) using the iTaq™ universal SYBR® Green Supermix kit (Bio-Rad, Cat. No. 1725121) following manufacturer's instructions.

For fluorescent NL$_c$ immersion: zebrafish larvae (2 dpf) were treated with 10% PBS, fluorescent NL$_s$, and fluorescent NL$_c$ for 48 h. 0.5 µg cDNA of each treatment was used as a template in RT-qPCR analysis. The housekeeping gene EF1α was used as a reference. 10^{-1} and 10^{-2}-fold diluted cDNA from pooled cDNA were used for immune-related genes and EF1α gene analysis, respectively. The primers used in RT-qPCR analysis are shown in table 1.

For fluorescent IBs$^{TNF\alpha}$ immersion: 5 dpf zebrafish larvae were treated with 2.5% PBS and fluorescent IBs$^{TNF\alpha}$ for 3 h, 12 h, and 24 h. 0.5 µg cDNA of each was used as a template in RT-qPCR. 10^{-1} and 10^{-2}-fold diluted cDNA from pooled cDNA were prepared for immune-related genes and EF1α gene, respectively. The primers used in RT-qPCR analysis are shown in Table 1.

Each PCR mixture consisted of 5 µl of SYBR green supermix, 0.5 µM of primers, 2.5 µl of diluted cDNA and 1.5 µl sigma water (Sigma-Aldrich, Cat. No. W4502-1L) in a final volume of 10 µl. All samples from 3 independent experiments were run in triplicate using the following settings: initial denaturation at 95 ℃ for 3 min, 39 cycles of 95 ℃ for 10 s and 60 ℃ for 30 s, and finally, 95 ℃ for 10 s, increase every 0.5 ℃ for 5 s from 65 ℃ to 95 ℃. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (56).

Table 1. **Primers for RT-qPCR analysis**

Gene name	Primers	Product size
EF1α	Forward: CTTCTCAGGCTGACTGTGC Reverse: CCGCTAGCATTACCCTCC	358
TNFα	Forward: TGCTTCACGCTCCATAAGACC Reverse: CAAGCCACCTGAAGAAAAGG	232
INOS	Forward: GAGCAGGCCCAATGCATTT Reverse: TGCGCTGCTGCCAGAAAC	186
COX2	Forward: ACACATGGCATCCGCAACAT Reverse: TGGGCAGCCAGATCTTTGTC	224
TLR9	Forward: ATGCCCAAACAACCAGTCTC Reverse: GTAAAAGGTGCCGTTTTGGA	196
IL1β	Forward: CATCAAACCCCAATCCACAG Reverse: CACCACGTTCACTTCACGCT	111
IRF1α	Forward: GAGACACGGCTGGAACATCG Reverse: ACCCTGAAGGCGTTGTGGC	198

M. marinum culture and zebrafish larvae infection

M. marinum was cultured in 7H9 broth liquid medium supplemented with ADC and stored at -80 ºC for infection experiment as previously described in Materials and Methods of Chapter 2. The infection was performed by immersing the larvae with different doses of *M. marinum* prepared in E3 water. One larvae per well was placed 96 well plate with 200 µl E3 medium or diluted bacteria. 12 zebrafish larvae (4 dpf) of each group was immersed with around 10^8, 10^7, 10^6, 10^5, 10^4 cfu/ml *M. marinum* and the mortality was recorded every day until death was observed in the control (around 4 days). In parallel, the tail fin injured larvae immersing with *M. marinum* were also evaluated according to the infection model of *A. hydrophila* (57). The death was determined by observation of absence of heartbeat for 3 seconds under the microscope. To verify the bacterial doses, 100 µl of each

10^{-6}, 10^{-7}, 10^{-8} dilutions of bacterial suspension from the highest concentrated aliquot were inoculated onto Middlebrook 7H10 agar plates and the bacteria colonies were counted.

A. hydrophila culture and zebrafish larvae infection

The *A. hydrophila* (AH-1) strain was kindly provides by Dr. J. Tomas (University of Barcelona, Barcelona). The bacteria were grown on LB agar plates at 30 ℃. For larval infection, the bacteria from an overnight culture on LB plates were washed with PBS (3x) and resuspended in PBS to obtain the stock solution (OD_{620}=1.3). The infection model by bath immersion using injured and non-injured larvae was described in Saraceni *et al*. with some modifications (57). Zebrafish larvae (6 dpf) injured in the tail fin were used for immersion infection. To obtain injured larvae, animals were anesthetized and placed on a Petri dish. A small transection of the tail fin was done with a sterile surgical blade (Albion) under a stereomicroscope. Groups of 12 injured larvae were distributed into 96-well plates (Thermo Fisher, Cat. No. 167008) with one larvae per well, containing 200 µl E3 water or diluted bacteria suspensions in a final volume of 200 µl E3 water. Bacteria were diluted from stock solution with E3 water in serial 100-fold dilutions from 10^{-1} to 10^{-5}. The bacteria dilutions were kept in each well throughout the whole experiment at 28 ºC. The control condition were non-injured and injured larvae in 10 % (v/v) PBS in E3 water. 100 µl of each 10^{-7} and 10^{-8} dilutions were inoculated on LB plate and incubated overnight at 37 ºC for CFU enumeration. The survival curves were analyzed with the GraphPad software.

A. hydrophila challenge zebrafish after $IBs^{TNF\alpha}$ immersion

Zebrafish larvae (5 dpf) were used in immersion experiments according to the uptake results as previously described in Materials and Methods for nanosized NL_c liposomes. Larvae were immersed in 2.5% PBS, 0.01 mg/ml and 0.05 mg/ml $IBs^{TNF\alpha}$ resuspended in E3 medium. The $IBs^{TNF\alpha}$ immersion experiment was performed in 6 well plates with 50 larvae per group. After 5 h immersion, the larvae were cleaned with E3 water and kept in E3 water for 12 h. At 6 dpf, the larvae were challenged with *A. hydrophila* (10^{8}) and the survival was assessed during 5 days. *A. hydrophila* was inoculated overnight on LB plate one day before infection experiments. The CFUs were counted by culturing 100 µl of PBS diluted 10^{-7} and 10^{-8} bacterial suspension on LB plates overnight at 37ºC. All the experiments were done in triplicates and 12 larvae were used in each condition. Survival curves were analyzed using the Kaplan–Meier method and the statistic differences were evaluated using the log-rank test (GraphPad, USA).

Results

Biodistribution of fluorescent NL_c and $IBs^{TNF\alpha}$ in zebrafish larvae

Zebrafish larvae (2 dpf) were immersed with 0.75, 1.0, and 1.5 mg/ml NL_c for 48 h and larvae immersed in 10% PBS and E3 water were used as controls. After anesthetized with 160 mg/l of MS-222, the larvae were observed and the photos were taken with a fluorescent stereomicroscope. The results showed that the NL_c liposome is mainly gathered to the intestine of zebrafish larvae after 48 h of immersion (Figure 1). Interestingly, the liposomes were also observed in the pharynx. No fluorescence is visible in the PBS treated and non-treated zebrafish larvae after 48 h of immersion (Figure 1). Moreover, no fluorescence was observed in the zebrafish larvae after 24 h of immersion (data not shown). These results suggested that the NL_c liposomes are taken through the oral system and then absorbed in the digestive system. The zebrafish mouth is widely open at 72 hpf during the development that strongly supports our results (58).

For immunostimulation with nanostructured TNFα, 5 dpf zebrafish larvae were immersed with 0.01 and 0.05 mg/ml $IBs^{TNF\alpha}$ for 1, 3, 5, and 7 h and PBS treated larvae were used as a control. After anesthetized with 160 mg/l with MS-222, the larvae were examined under the fluorescent stereomicroscope. The photos were taken with transmitted light and green fluorescence. The results demonstrate that the $IBs^{TNF\alpha}$ are found in the gastrointestinal system (pharynx and intestine) of zebrafish larvae after 1, 3, 5, and 7 h of immersion (Figure 2). No fluorescent signal is observed in the PBS treated zebrafish larvae.

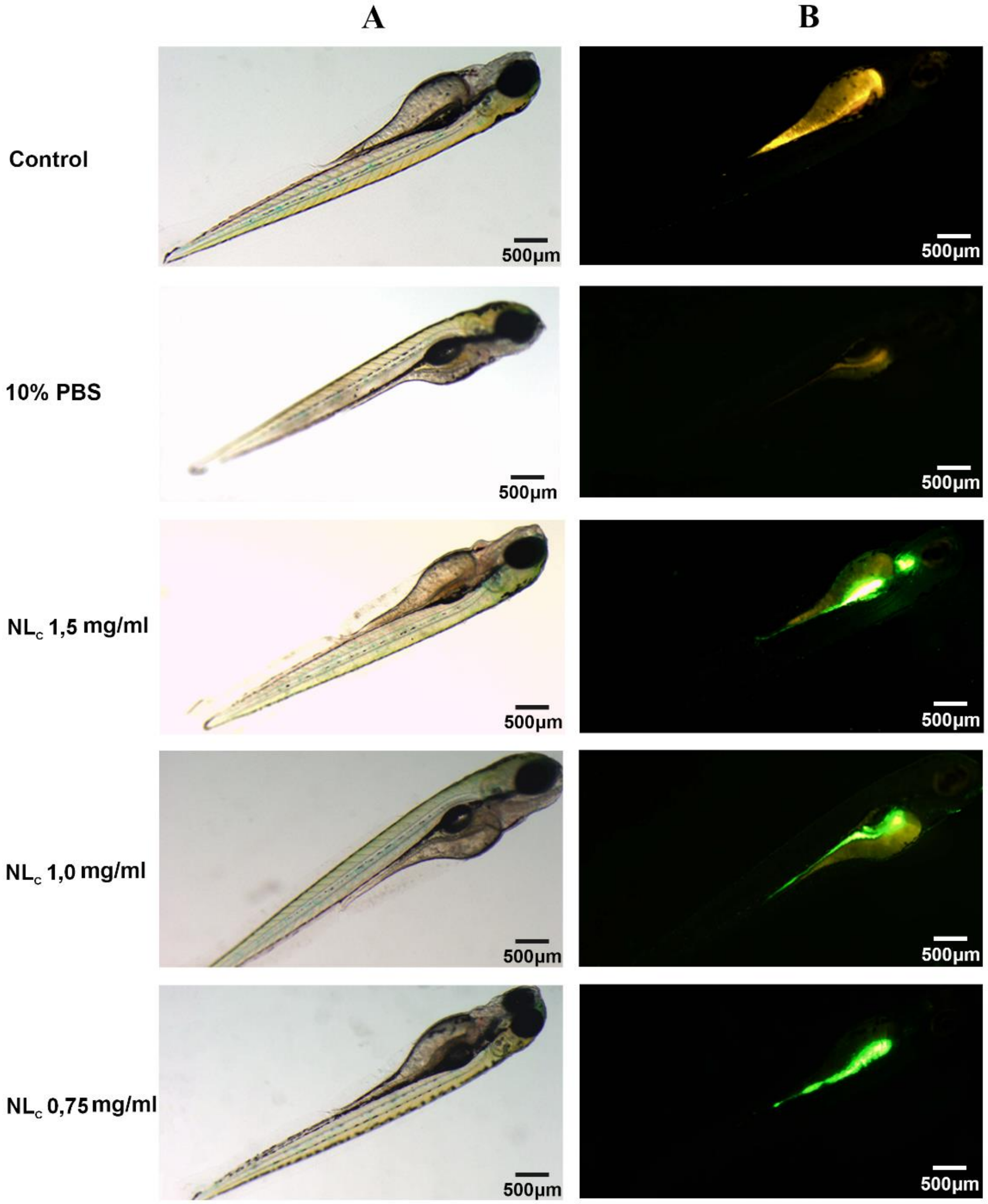
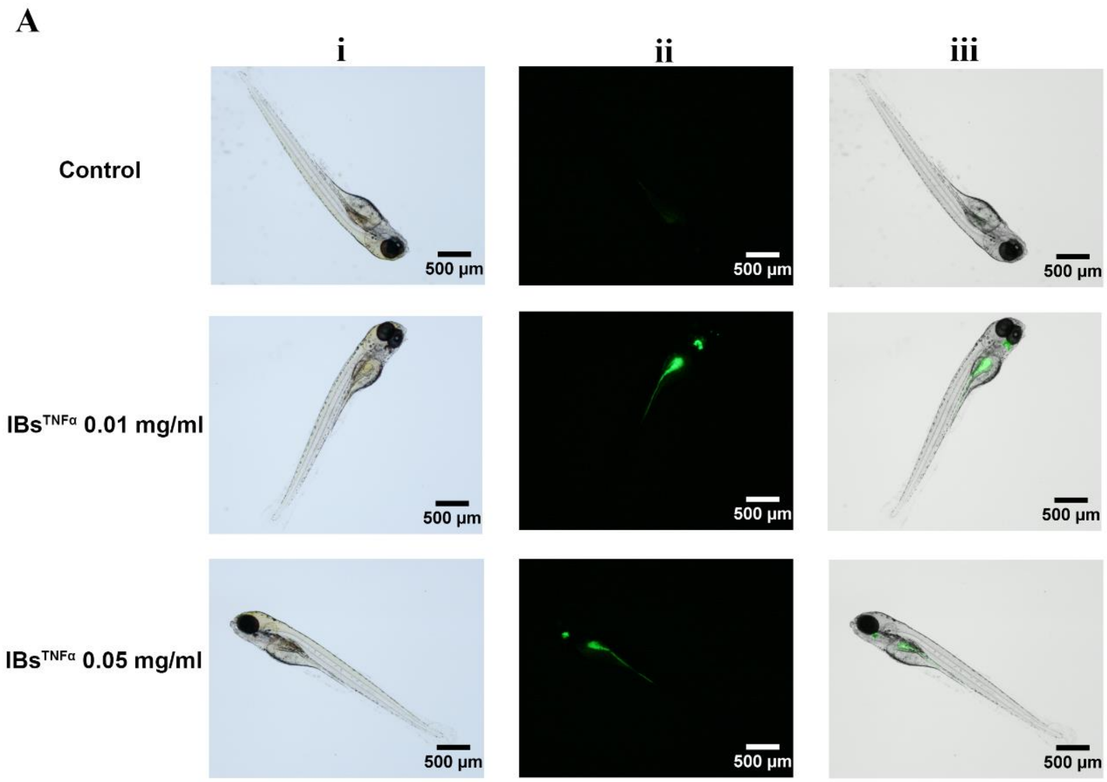
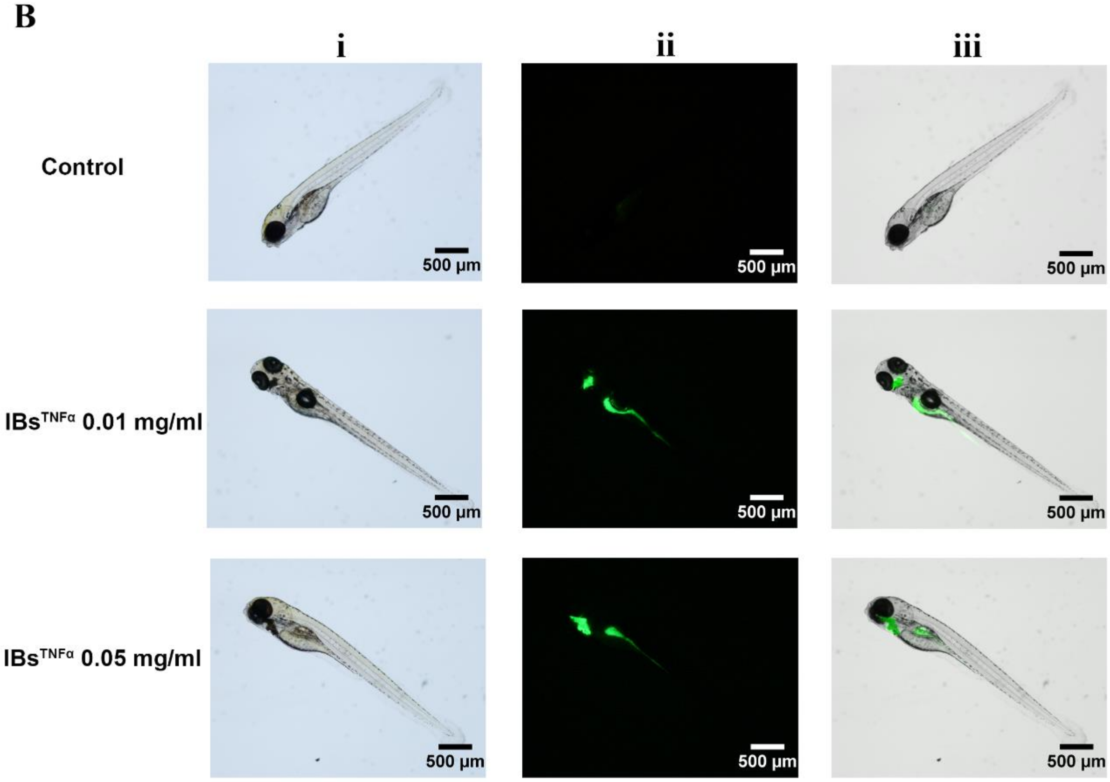


Figure 1. **Biodistribution of fluorescent NL$_c$ in zebrafish larvae.** Zebrafish larvae (2 dpf) were immersed with 0.75, 1.0, and 1.5 mg/ml NL$_c$ for 48 h. 10% PBS and E3 water immersion groups were used as controls. Fluorescence signal was detected in the pharynx and intestine among NL$_c$ treated groups. Fluorescence signal was not detected in controls. (A) Transmitted images. (B) Fluorescent images.


A
i
ii
iii
Control
IBs^{TNFα} 0.01 mg/ml
IBs^{TNFα} 0.05 mg/ml
500 µm


B
i
ii
iii
Control
IBsTNFα 0.01 mg/ml
IBsTNFα 0.05 mg/ml
500 µm

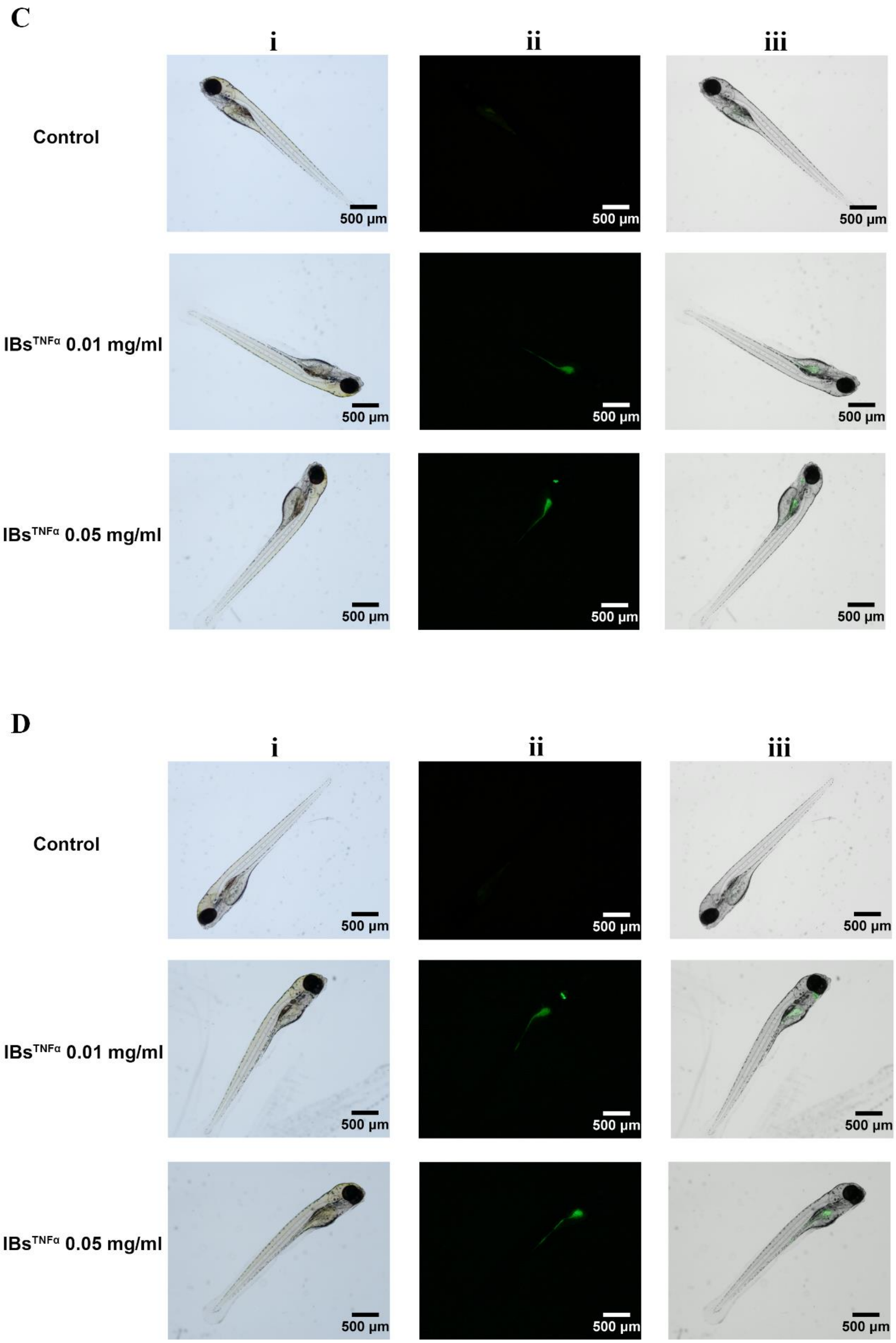

C
i
ii
iii
Control
IBsTNFα 0.01 mg/ml
IBsTNFα 0.05 mg/ml
500 µm
D
i
ii
iii
Control
IBsTNFα 0.01 mg/ml
IBsTNFα 0.05 mg/ml
500 µm

Figure 2. **Biodistribution of fluorescent IBs^{TNFα} in zebrafish larvae.** 5 dpf zebrafish larvae were immersed with 0.01 and 0.05 mg/ml IBs^{TNFα} for 1, 3, 5, and 7 h. PBS treated larvae were used as a control. Fluorescence signal was detected in the pharynx and intestine among IBs^{TNFα} treated groups. Fluorescence signal was not detected in control. Transmitted image (i), fluorescent image (ii) and merged image (iii) of zebrafish larvae at 1 h (A), 3 h (B), 5 h (C) and 7 h (D) post-immersion.

Immune gene expression analysis after NL$_c$ treatment

Zebrafish larvae (2 dpf) were immersed in PBS, mixture of poly (I:C) and LPS, NL$_s$ and NL$_c$ for 48 h and non-treated larvae were used as the control. RT-qPCR results are shown in Figure 3. There is a higher expression of TNFα gene (200-fold upregulation) when treated with 0.75 and 1.0 mg/ml NL$_c$ liposome. However, the fold-change is around 50 in the 1.5 mg/ml NL$_c$ liposome treated group. The expression of iNOS gene shows a similar fold-change pattern regarding TNFα gene, the fold-changes are around 150, 120, and 30 in the samples of 0.75, 1.0, and 1.5 mg/ml, respectively. In the expression of IL-1β gene, it presents moderate high fold-change with the mean value of 40, 14, and 17 in the samples of 0.75, 1.0, and 1.5 mg/ml, respectively. The expression of COX2 and TLR9 genes in all the NL$_c$ treated samples display around 5 fold-change which is relatively lower compared to the genes mentioned above.

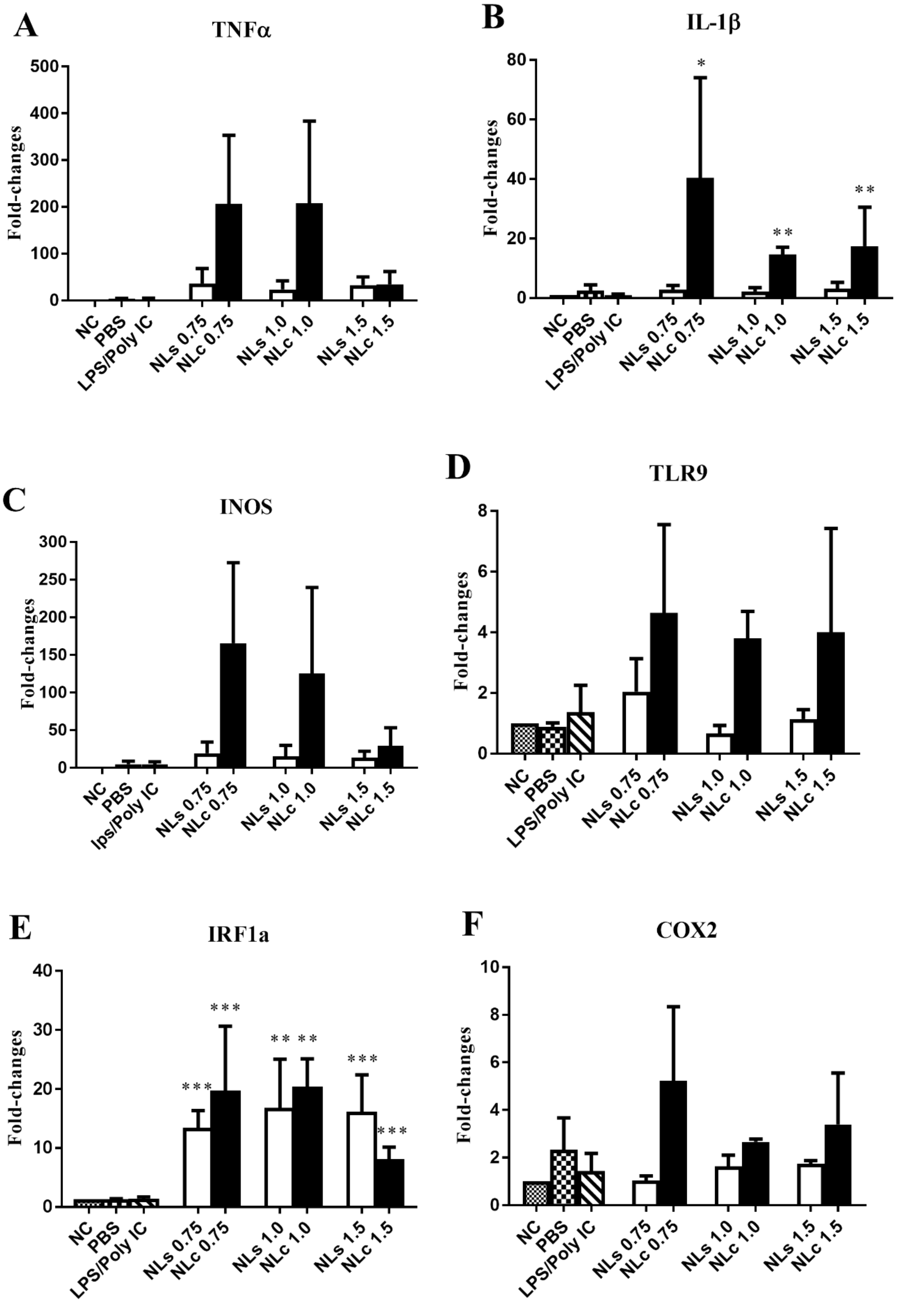
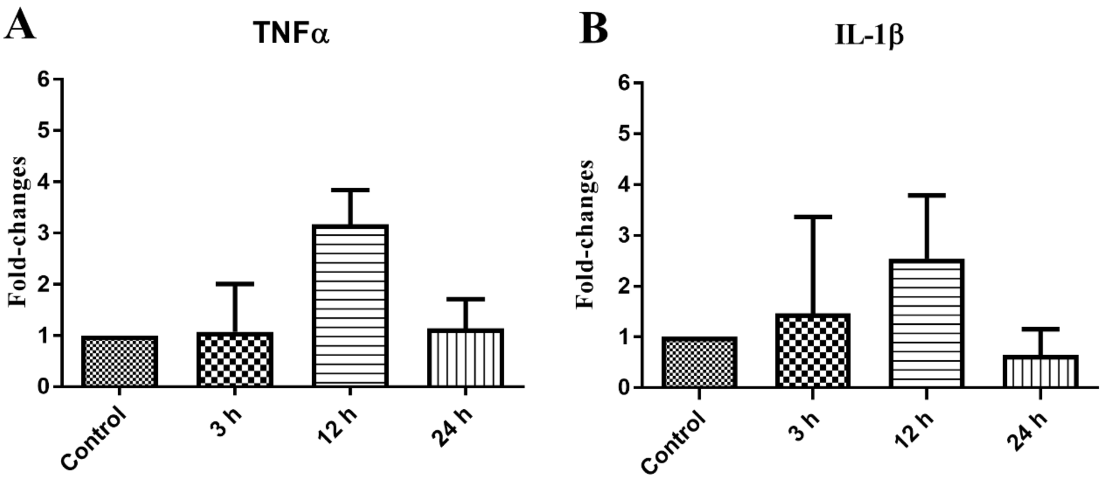

A
TNFα
Fold-changes
500
400
300
200
100
0
NC
PBS
LPS/Poly IC
NLs 0.75
NLc 0.75
NLs 1.0
NLc 1.0
NLs 1.5
NLc 1.5
B
IL-1β
Fold-changes
80
60
40
20
0
*
**
**
NC
PBS
LPS/Poly IC
NLs 0.75
NLc 0.75
NLs 1.0
NLc 1.0
NLs 1.5
NLc 1.5
C
INOS
Fold-changes
300
250
200
150
100
50
0
NC
PBS
lps/Poly IC
NLs 0.75
NLc 0.75
NLs 1.0
NLc 1.0
NLs 1.5
NLc 1.5
D
TLR9
Fold-changes
8
6
4
2
0
NC
PBS
LPS/Poly IC
NLs 0.75
NLc 0.75
NLs 1.0
NLc 1.0
NLs 1.5
NLc 1.5
E
IRF1a
Fold-changes
40
30
20
10
0
***
***
** **
***
***
NC
PBS
LPS/Poly IC
NLs 0.75
NLc 0.75
NLs 1.0
NLc 1.0
NLs 1.5
NLc 1.5
F
COX2
Fold-changes
10
8
6
4
2
0
NC
PBS
LPS/Poly IC
NLs 0.75
NLc 0.75
NLs 1.0
NLc 1.0
NLs 1.5
NLc 1.5

Figure 3. **Gene expression analysis by RT-qPCR.** 2 dpf zebrafish larvae were immersed with PBS, mixture of poly (I:C) and LPS, NL_S (0.75, 1.0, and 1.5 mg/ml of each) and NL_C (0.75, 1.0, and 1.5 mg/ml of each) for 48 hours. The gene expression level was showed as fold change using the Livak method (2−ΔΔCt). Statistical significance was determined by one-way ANOVA using Prism software (GraphPad).

Gene expression analysis after IBs$^{TNF\alpha}$ treatment

5 dpf zebrafish larvae were either immersed with PBS or 0.01 mg/ml IBs$^{TNF\alpha}$. 3 h, 12 h, and 24 h post immersion, larvae were sacrificed and RNA was extracted for RT-qPCR analysis. The results showed that the expression of both two cytokine genes, TNFα and IL-1β were upregulated at 12 h post immersion, but not at 3 h and 24 h (Figure 4). The gene expression of IL-6 and IL-22 were slightly upregulated at 12 h post immersion, the expression of IL-10, and COX2 did not show differences between control and treatments in three time points.



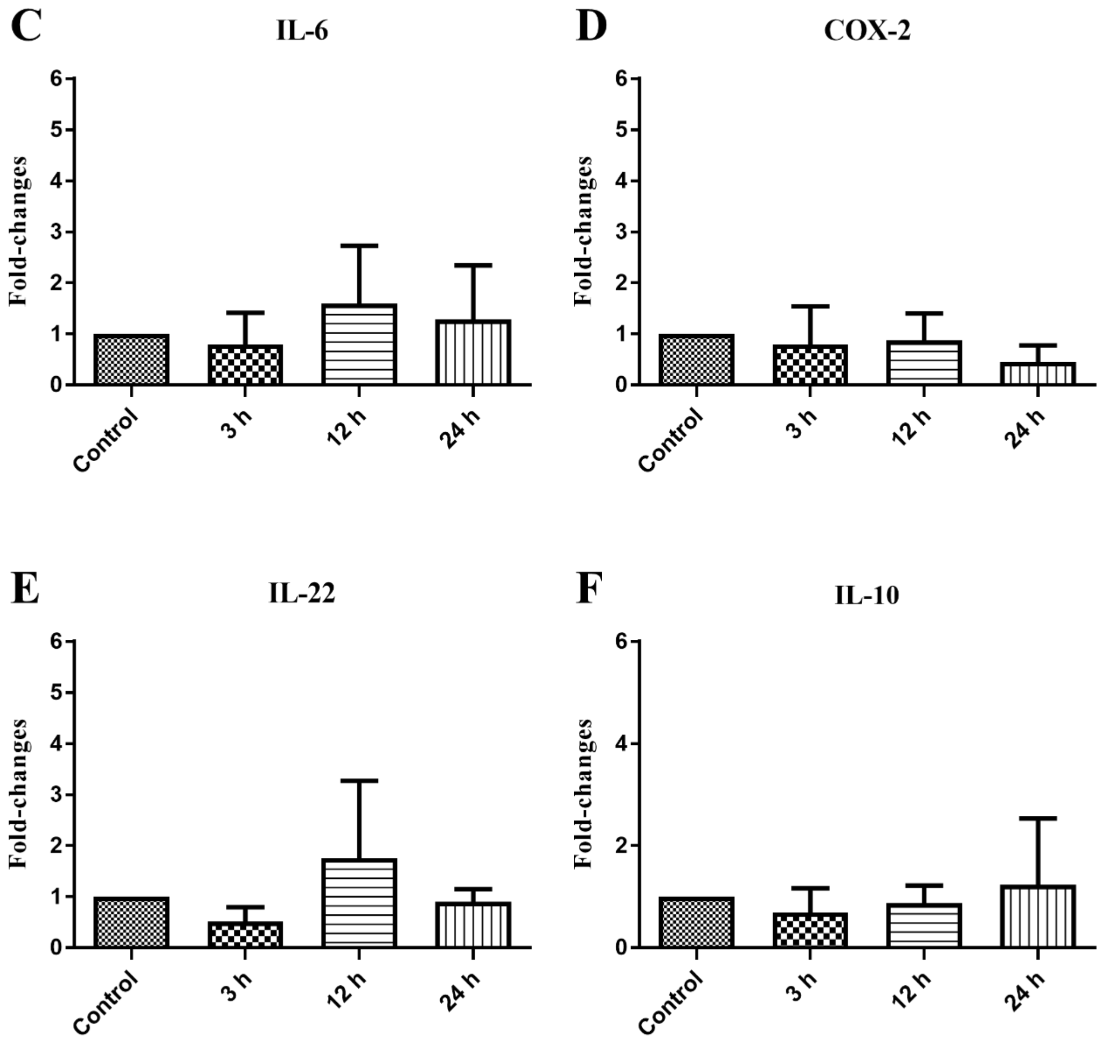
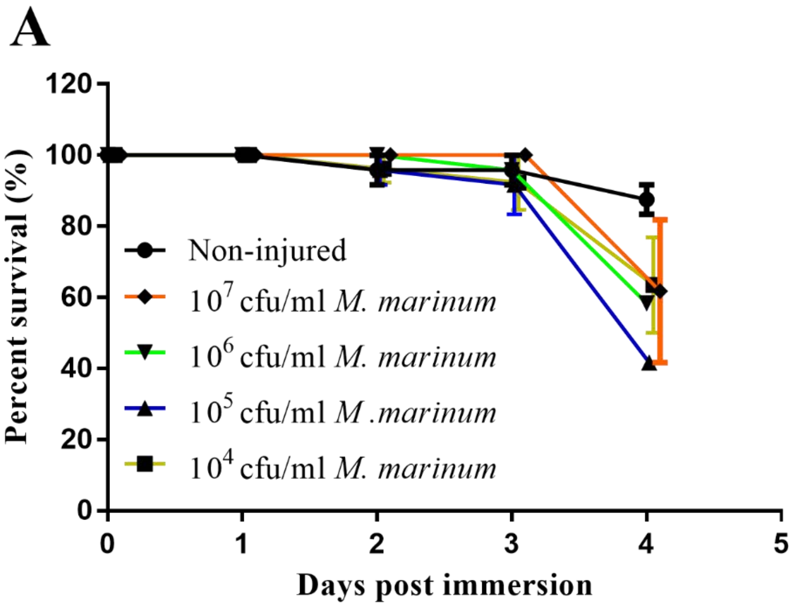


Figure 4. **Gene expression analysis by RT-qPCR.** Zebrafish larvae were immersed with 0.01 mg/ml IBs$^{TNF\alpha}$ for 3 h, 12 h, and 24 h. The gene expression level was showed as fold change using the Livak method ($2^{-\Delta\Delta Ct}$). Statistical significance was determined by one-way ANOVA using Prism software (GraphPad).

Zebrafish larvae *M. marinum* infection by immersion

To investigate whether 4 dpf zebrafish larvae can be infected with *M. marinum* by immersion administration, experiments were performed by using five different doses of bacterial suspension. Larvae were divided into two groups, non-injured and injured as described in the materials and methods. Each larvae was immersed with 200 µl of each 10^7,

10^6, 10^5 and 10^4 cfu/ml bacterial suspension in 96 well plate. The survivals were calculated and showed in Figure 5A and B. In non-injured group (Figure 5A), 10^5 cfu/ml bacteria immersed larvae showed highest mortality at 4 days post immersion (around 60%) while at this time point, the rest of doses showed intermediate mortalities (50%). A small mortality was also found in the control (10%). However, the larvae mortalities treated with the same dose of bacteria were variable in different repeats. No significant difference was found between any of the treatment groups and control by using two-way ANOVA. In injured group (Figure 5B), similar mortalities were found in the larvae immersed with 10^7, 10^6, 10^5 and 10^4 cfu/ml bacteria compared to the control. The larvae immersed with all the doses of bacteria suspension did not show significant difference with the control.



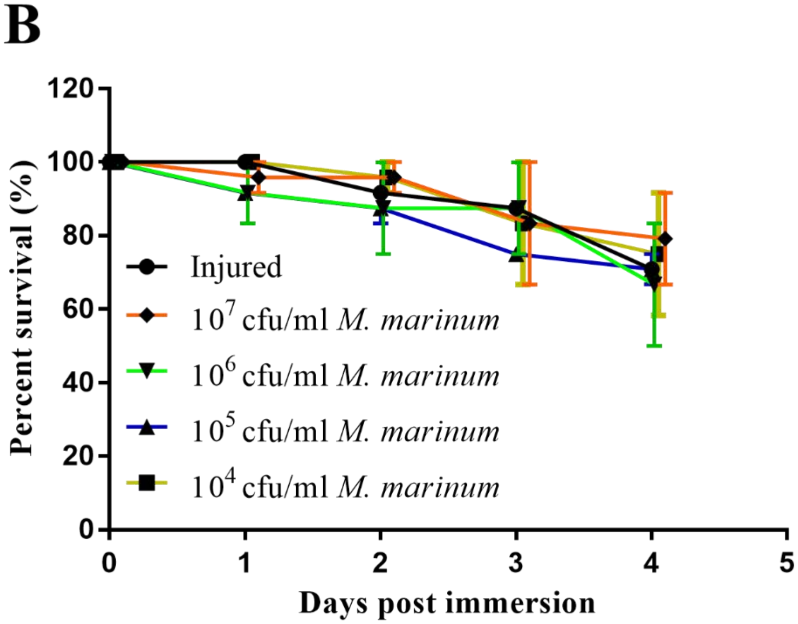


Figure 5. **Survivals of 4 dpf zebrafish larvae infected with *M. marinum* by immersion.** (A) Survivals of non-injured larvae. (B) Survivals of injured larvae

Zebrafish larvae *A. hydrophila* infection model by immersion

To understand whether 5 dpf zebrafish larvae could be infected with *A. hydrophila* by immersion, three different doses of bacterial suspension were used in the experiments. Larvae were divided into non-injured and injured groups as described previously in the Materials and Methods. Each larvae was immersed with 200 µl of each 10^8, 10^6, and 10^4 cfu/ml bacterial suspension in 96 well plate. The survivals were calculated and showed in Figure 6A and B.

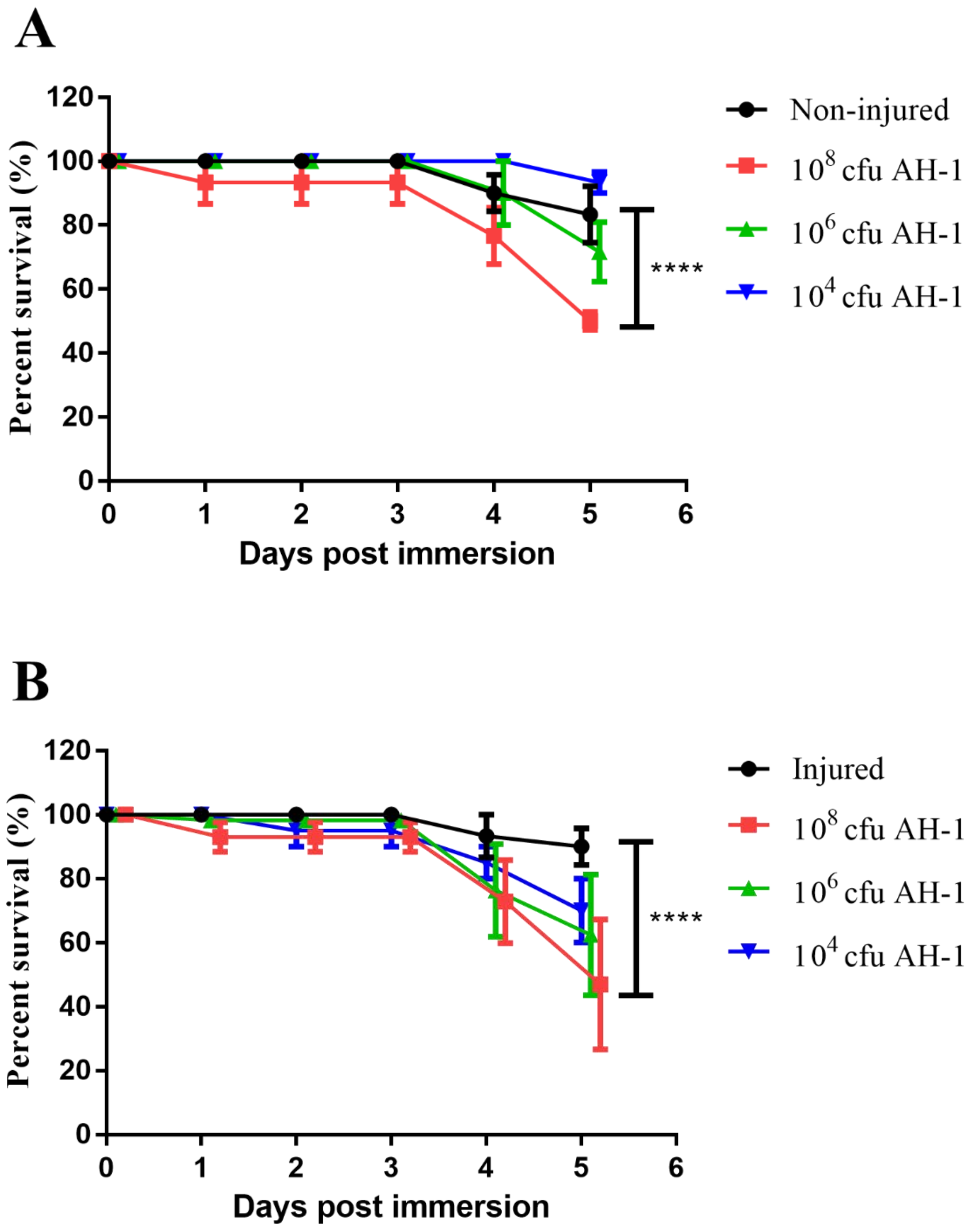


Figure 6. **Survivals of 5 dpf zebrafish larvae infected with *A. hydrophila* by immersion.** A) Survivals of non-injured zebrafish larvae. B) Survivals of injured zebrafish larvae.

IBs$^{TNF\alpha}$ protect zebrafish larvae against *A. hydrophila* lethal challenge

Having confirmed that zebrafish larvae can take up our IBs$^{TNF\alpha}$ at 5 dpf and can be infected with *A. hydrophila* administered by immersion, we then evaluated the IBs$^{TNF\alpha}$ protection of zebrafish larvae against lethal challenge. In this case, zebrafish larvae were immersed with two doses of IBs$^{TNF\alpha}$ at 5 dpf for 5 h and challenged with 10^8 cfu/ml *A. hydrophila* after a 12 h standing time. The survivals were calculated in the following 5 days (Figure 7). However, no significant difference was observed in the entire survival curve.

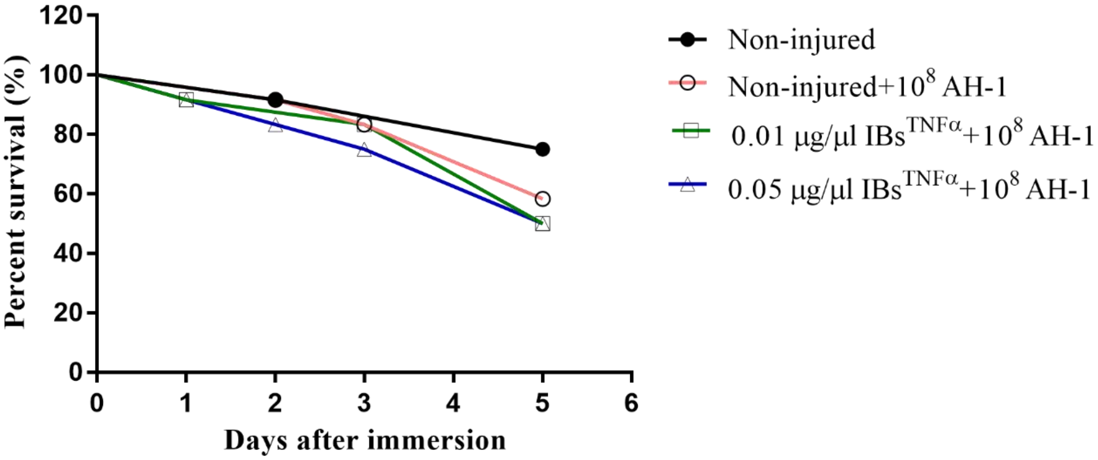


Figure 7. **Survivals of 6 dpf zebrafish larvae challenged with 10^8 cfu/ml *A. hydrophila.*** The zebrafish larvae were immersed with two doses of IBs$^{TNF\alpha}$ at 5 dpf for 5 h and challenged with 10^8 cfu/ml *A. hydrophila* after a 12 h standing time. The survivals were calculated in the following 5 days.

Discussion

Millions of microorganisms are present in the aquatic environment and are especially threatening to fish during the early developmental stage when the immune system is still immature. Although a mixed passive immunity exists during vitellogenesis and oogenesis (59) (60), fish larvae depend fundamentally on their innate defense mechanisms, since adaptive immunity develops later. Most of the innate immune genes are already expressed at hatching, including those coding for innate immune receptors (TLR5 and TLR9), pro-inflammatory (IL-1β, TNFα and COX2) and anti-inflammatory (TGF-β1) molecules, antiviral (MX, IRF1, IRF-9, IFI-30 and PRT-1) and antibacterial (C3, LYZ, HAMP and LBP/BPI) molecules, and phagocyte markers (M-CSFR and NCF4) (61). Thus, it is possible that the immune defenses against pathogens could be enhanced by stimulating the innate immune system of fish larvae. Zebrafish larvae have become a convenient animal model due to the low costs, nearly transparent body, easy mass breeding and fully sequenced genome (62) (63). One of the main aims of this study was to establish infection models useful for testing prophylactic approaches for larvae stages focusing on immersion administration and subsequent infection using natural routes. In this study, zebrafish larvae were established as a model to test the biodistribution *in vivo* and the immune regulating ability of two potential adjuvants, NL_c liposome and $IBs^{TNF\alpha}$. We further attempted to establish two bacterial infection models in zebrafish larvae and tested the immune protection evoked by those adjuvants using these models.

After the extensive characterization of the NL_c liposome and $IBs^{TNF\alpha}$ in adult zebrafish, we wanted to detect the biodistribution of these two nanoparticles in zebrafish larvae. To this end, the fluorescently-labeled NL_c liposome and $IBs^{TNF\alpha}$ were prepared and the experiments were performed by immersion administration. Immersion administration seems to be the only convenient and suitable method for immunization in fish larvae to date due to the small size and frangibility when handled. Moreover, absorption of antigens by the intestine could be very important to develop a successful mucosal immunity and to develop a strong mucosal response against a later infection through natural routes.

The NL_c liposome was found in the pharynx and intestine of 4 dpf larvae after a 48 h immersion. We could not find NL_c liposome in 3 dpf larvae after a 24 h immersion. This is probably because the zebrafish mouth is not widely open before 3 dpf during development (58). Probably the main route that zebrafish larvae take up the NL_c liposome is through the oral-intestinal pathway. This implies that exposure time and larvae age are important factors to consider when designing an immersion experiment. Similar to NL_c liposome uptake, the $IBs^{TNF\alpha}$ filled the intestine of 5 dpf larvae after 1 h, 3 h, 5 h, and 7 h after immersion. Not surprisingly, $IBs^{TNF\alpha}$ was also found in the pharynx in 5 dpf larvae at all the time points. These results indicate that zebrafish larvae may take up antigens or vaccines rapidly after 3 dpf when the mouth is opened widely and the intestine may be the main organ responsible for the uptake. Differences observed between NL_c and $IBs^{TNF\alpha}$ uptaken times may be due to the different composition of these two delivery systems. While IBs is a new biomaterial made with protein, the NL_c liposomes are made with phospholipids and cholesterol. The transit and absorption times through the gastrointestinal system of lipids and proteins could be different. For instance, the rate of lipid digestion and absorption in fish is slower than in mammals (64). *In vivo* experiments with IBs$^{TNF\alpha}$ in adult zebrafish (Chapter 2), have shown fast absorption rates in the intestine. On the other hand experiments with NL$_c$ liposomes have shown a peak of absorption at 24 h post-administration (28) showing a delayed uptake compared to proteic nanoparticles. In addition, transparent zebrafish larvae could be generated by PTU treatment (55), it benefits to the in vivo tracking of fluorescent vaccines or adjuvants.

The innate immune factors of the immature zebrafish larvae are functional from 1 dpf (65). Mature neutrophils and primitive macrophages are present from 24–30 hpf (66), which are the primary actors of the larval innate immune system (51). In this study, the effect of two potential adjuvants, NL$_c$ liposome and IBs^{TNFa}, was evaluated by testing the differential expression of some selected immune genes in zebrafish larvae. The selection of genes was made considering different functions in the innate immune system. TNFα, IL-1β, IL-6, and IL-22, were selected as markers of proinflammatory and antibacterial activity (40). IL-10 is an inhibitory factor for the production of Th1 cytokines (67). COX2 is an inflammatory related enzyme expressed after the stimulation of proinflammatory agents (68). INOS is an early feature of inflammation. TLR9 is a protein receptor and can be stimulated by bacterial and viral CpG-deoxynucleotides containing DNA (CpG-DNA) (69). IRF1 is a marker of immune system response to virus infection (70). The up-regulations of TNFα, IL-1β, INOS, COX2, TLR9, and IRF1α indicate a stimulation of the zebrafish larvae immune system by NL$_c$ via immersion. The gene expression of TNFα and IL-1β provided results similar to the previous study in our lab (71). Analysis of genes expression in IBs$^{TNF\alpha}$ immersed larvae showed TNFα and IL-1β were slightly up-regulated at 12 h, indicating a stimulation of the zebrafish immune system. The gene expression analysis by RT-qPCR showed that zebrafish larvae could be developed as a model to test the immune regulating effects of adjuvants.

To test whether the NL$_c$ liposomes and IBs$^{TNF\alpha}$ could protect zebrafish larvae against bacterial infection, we tried to develop two models using both gram-positive bacteria *M. marinum* and Gram-negative bacteria *A. hydrophila* by immersion administration. The zebrafish larvae model infected with *M. marinum* had already been successfully developed using microinjection (72). However, this technique requires specialized equipment and a high level of operator expertise, so might not practical in general. Zebrafish larvae infected with *A. hydrophila* had been established both by administrating via micro-injection and bath immersion (57). The infection model by bath administration resembles the natural conditions of infection. In the *M. marinum* immersion experiments, we could not observe significantly different mortalities of the larvae between treatments and control. Therefore, *M. marinum* could not lethally infect zebrafish larvae by immersion or the mortality may occur in a longer monitoring time. This result is in agreement with the previous reports that *M. marinum* usually results in a long-term chronic infections (73) (74). Interestingly, zebrafish larvae infected with 10^8 cfu/ml *A. hydrophila* showed significantly different mortality rates from control at 5 days post immersion in both injured and non-injured groups. We selected the non-injured infection method because it is much easier to perform and the deviation of the mortality is smaller than the injured group. However, $\text{IBs}^{\text{TNF}\alpha}$ did not show protection to the zebrafish larvae after a challenge with *A. hydrophila*. This negative result also matches the gene expression data, that no significant up-regulation of immune genes was observed in the previous study.

Thus, it could be concluded that the most appropriate time for testing the uptake of adjuvants or vaccines is 3 dpf. In addition, most of the innate immune-related genes are detectable in this developmental stage. So far, the bacterial pathogens that were reported to be capable of infecting zebrafish larvae by immersion administration are *A. hydrophila* (injured larvae) (57), *E. tarda* (75) and *F. columnare* (76). More efforts need to be made to develop a bacterial infection model for larvae. In this vein, zebrafish larvae could be considered as a model system to study the *in vivo* uptake of immunostimulants, innate immune regulation by immunostimulants and the protection of immunostimulants to larvae against pathogen infection.

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General discussion

The aim of this thesis was systematically study the immunity in low vertebrates, especially in teleost fish, from the fundamental theory to practical application in aquaculture. This includes several aspects.

The first one was to study the toll like receptors in a fish-like invertebrate, the Mediterranean amphioxus (*B. lanceolatum*), from a functional and evolutionary point of view. Amphioxus is an ancient chordate lineage which shares key anatomical and developmental features with vertebrates. The amphioxus is a good model for understanding the evolution of vertebrates (1) (2) because of its phylogenetic position at the base of the chordate and genomic simplicity. By studying the whole TLRs gene family, we tried to annotate the TLRs from the *B. lanceolatum* genome using phylogenetic analysis and protein domain comparison with known vertebrates TLRs. By studying one particular TLR, we deciphered its full-length sequences, its expression profile in response to LPS and Poly (I:C) by RT-QPCR analysis, its heterologous expression in mammalian cells, and its ligand specificity *in vitro*. We believe that through this study we can contribute with a piece of information to the evolutionary puzzle of TLRs by understanding the size of the TLRs family in amphioxus, their annotations and the prediction of their ligands.

TLRs play crucial roles in the innate immune system by recognizing PAMPs and in adaptive immune system by activating antigen-presenting cells. In addition to immunity, TLRs have multiple functions ranging from developmental signaling to cell adhesion. The study of TLRs may help in understanding the role of TLR-mediated responses which could increase our range of weapons to treat infectious diseases and manipulate immune responses by drug intervention (3). TLRs are conserved across invertebrates to vertebrate, and the successful study of mammalian TLRs broads our tools in the study of non-vertebrates and other vertebrate TLRs. Numbers of TLRs in different phylum of deuterostome are variable. In echinodermata, *Strongylocentrotus purpuratus* and *Lytechinus variegatus*, the genomes of these two sea urchin species contain 253 and 68 TLRs or related genes even through it is still remaining unknown whether most of the genes are functional (4). In cephalochordate, genome research showed *Branchiostoma floridae* has 48 TLRs (5). In our study, between 22 and 37 TLRs were found in three different genomes databases of three *Branchiostoma* species. To include a sequence as a TLR in our database it must include at least one LRR domain, one transmembrane domain and a TIR domain (from N-terminal to C-terminal). The final number of TLRs in the three *Branchiostoma* species was: 22, 28 and 37. It has been published that *B. floridae* has 48 TLRs but in our opinion only 28 sequences are true TLRs.

In urochordates subphylum, *C. intestinalis* only possesses two functional TLRs (6). In vertebrates, 20 different TLRs have been reported in teleost species (7) and 20 TLR genes are known in the South African clawed frog, *Xenopus laevis* (8). 10-12 TLRs were found in mammals. This phenomenon may be explained by two evolutionary scenarios of TLRs according to the review with some different presumptions (9). First, only a few TLRs existed in a common deuterostome ancestor. Sea urchins expanded their TLRs or related gene paralogs during their divergence. Next, the gene loss happened in the amphioxus TLRs (from 253 to 48) and sea squirt suffered longer gene loss therefore less TLRs were left (only 2 TLRs). However, the two rounds of whole-genome duplication (WGD) and one bony fish lineage-specific WGD significantly increased the TLRs family in vertebrates (10-12 in mammals and 20 in fish). Alternatively, a common deuterostome antecedent might have numerous and complex TLR family genes. The gene loss happened alone with the evolution as seen the numbers of TLRs decreased from sea urchin and amphioxus to sea squirt. Again, the whole-genome duplication (WGD) significantly increased the TLRs family in vertebrates. However, recent genome data is revealing an unexpected perspective of gene loss that is a pervasive source of genetic change (10) which may be more favor to the second evolutionary presumptions. If so, vertebrate and probably most of amphioxus TLRs might share their specific PAMP recognition and intracellular localization during their evolution. In addition, different numbers of TLRs in deuterostome may in concert with the variation of their lifetimes (months or years), feed habits (vegetarian or omnivore), or environments (land or marine). Therefore, characterization of PAMPs and intracellular localization of amphioxus TLRs is expected to contribute not only to elucidation of their biological roles but also to investigation of molecular and functional divergence of the deuterostome TLR family.

Phylogenetic analysis of all TLRs in *Branchiostoma* and representative vertebrates showed that in *Branchiostoma* species appears a distinct cluster of TLRs which could not be grouped within any of the vertebrate TLRs. This distinct cluster may be due to the inclusion of incomplete protein sequences from the genomic databases used in the phylogenetic analysis. On the other hand, this cluster may be a real new TLR family which has been lost later in vertebrates during evolution. Interestingly, the phylogenetic analysis of *B. floridae* TIR domain and vertebrate TLRs has indicated that 33 variable-type TLRs show a paraphyletic relationship with vertebrate TLR11 lineage (5). However, this cluster shows a paraphyletic relationship with vertebrate TLR1 lineage in our case.

Besides of the view in whole TLRs family, we studied one particular TLR in in *B. lanceolatum*. Firstly, we systematically analyzed the bioinformatics of its protein sequence and compared with vertebrate TLRs. Bl_TLRj has a similar protein structure like most of the vertebrate sccTLRs (11). The full-length protein showed the highest 30.43% identities with TLR22 of *Squaliobarbus curriculus.* According to the ectodomain architecture analysis and the LRRfinder (12) (13), Bl_TLRj is most similar to vertebrate TLR5 since both have 21 LRR and a single domain architecture. N-linked glycosylation is involved in trafficking, binding activities and PAMP recognition (14) (15) (16). Bl_TLRj has 10 N-linked glycosylation sites which is closest to TLR4 family (mean number of 9.1). Next, the phylogenetic analysis of Bl_TLRj protein sequence with representative vertebrates TLRs revealed that Bl_TLRj is grouped with the TLR11 family of vertebrates. The value of probability of the divergence between Bl_TLRj and TLR11 family is 86%. The Bl_TLRj has different characters that are similar with different vertebrate TLRs. We could not classify the Bl_TLRj to any known TLR. Therefore, the *in vivo* experiments were performed by treating amphioxus with LPS and Poly I:C. No significant up- or down-regulation of Bl_TLRj gene expression was observed. This observation might be explained that the doses are not enough to induce the Bl_TLRj expression or the immersion method is not suitable when purified PAMPs are used. It has been described in *B. belcheri* TLR1 receptor showed an expression induction *in vivo* in response to the injection of LPS and *V. vulnificus* (17).

In order to further analyze the function of Bl_TLRj protein and its ligand specificity, Bl_TLRj was cloned into a mammalian expression vector and expressed in HEK293 cells. Since amphioxus does not have a transfectable cell line, human cell line seems the best choice for us. It is noticed that establishment of cultured cells derived from each organism will strongly enhance various functional studies of TLR. The advantages of using HEK293 cell are that these cells do not normally express any of the TLRs and are amenable to systematic manipulation. Cytometry and western blot confirmed the correct protein expression in this cells thus facilitating the intracellular localization, and the signaling pathways activated in response to different PAMPs. Immunofluorescence and confocal microscopy further help to understand the localization of this protein at subcellular level. From the experience of vertebrate TLRs, The subcellular location affects the ligand recognition. TLRs located on the plasma membrane recognize microbial pathogenic components of cell wall, while the others located in endosomes, Golgi or endoplasmic reticulum recognize nucleic acids and their derivatives (18). Therefore, the Bl_TLRj is located in the cytoplasm and may recognize nucleic acid-like ligands. One of the most important character of vertebrate TLRs is that they could transactivate the transcriptional factor NF-κB in response to their specific ligands. To shed light on the role of Bl_TLRj in PAMPs recognition, 12 mammalian TLR ligands were tested in HEK293 cells that were transiently transfected with Bl_TLRj. None of the ligands could activate the NFκB luciferase reporter in the HEK293 cells, indicating that Bl_TLRj might not directly utilize other components (like adapter proteins) of this pathway in HEK293T cells or Bl_TLRj could not directly bind any PAMPs. For example, the functional Tolls in *D. melanogaster* need the assistant of the cytokine-like molecule Spätzle (20) and mammalian TLR4 cannot recognize LPS without the assistance of MD2 and CD14 proteins (71). However, the Bl_TLRj itself could induce the NFκB luciferase reporter. Furthermore, we constructed a new protein by fusing the Bl_TLRj extracellular and transmembrane domain with the Human TLR2 intercellular TIR domain. We tested to see whether this construct could respond to ligand stimulation or not when stably transfected in HEK293 cells. Indeed, the chimeric Bl_TLRj in HEK293 cells activated NF-κB reporter in response to Poly I:C (LMW) and Poly I:C (HMW). Poly I:C is specific ligand for mammalian TLR3, ligand for TLR13 of Miiuy croaker (43), and ligand for TLR22 of Japanese flounder (48) and pufferfish (50). Taken together, we suggest that Bl_TLRj could be classified as TLR13 subfamily, and it may be an ancestor of the vertebrate TLR13 subfamily.

The next aim of this thesis is to evaluate the potential capability of two nanoparticles, NL_c liposomes and $IBs^{TNF\alpha}$, in the role of adjuvants *in vivo*. Firstly, NL_c liposomes was developed previously in our lab as nanodelivery vehicle which consists of two immunostimulants, LPS and poly I:C encapsulated in nanosized liposomes. The *in vitro* experiments showed that NL_c liposomes could significantly stimulate immune genes expression in both zebrafish hepatocytes and trout macrophages (21). Furthermore, by the injection of fluorescence-labelled NL_c liposomes to zebrafish and rainbow trout, we assessed that NLc liposomes could target immune tissues and macrophages *in vivo*. At last, NL_c liposomes was able to significantly increase the survival of the zebrafish when challenged with *Pseudomonas aeruginosa* bacteria and spring viraemia of carp virus (SVCV) (22). However, NL_c liposomes were tested using a *P. aeruginosa* infection model this microorganism is not a relevant pathogen in fish but is an opportunistic pathogen of human (23). For this reason, we developed *M. marinum* infection model in adult zebrafish *M. marinum* is the mycobacterial species most closely related to the *M. tuberculosis* complex and causes TB-like infections in fish and human (opportunistic infection) (24). Diseases caused by *M. marinum* usually result in chronic infections and systemic formation of granulomas which is still incurable except a long term treatment of antibiotic (25). In addition, we chose zebrafish as our animal model because it has been widely used as a teleost model to study infectious diseases and also it is starting to be a relevant model in aquaculture. Zebrafish has a complete set of genes required for the establishment of a fully functional innate and adaptive immune system (26). Adult zebrafish *M. marinum* infection model had been set up successfully and described in the protocol (27). However, we have modified different steps that made the infection more easily and efficiently performed. For example, we used the frozen bacterial aliquots to perform the experiments instead of using living bacteria in order to reduce the error between repeats. We found that zebrafish mortality reached 100% within two weeks by injecting around 10^6 cfu/fish *M. marinum*. We selected this dose for further experiments due to the less time and labor costs. Prouty et al. 2003 found that zebrafish infected with *M. marinum* ATCC 927 progresses in a dose-dependent manner and intraperitoneal injection of $>10^6$ cfu/fish leads to death of the majority of infected fish within 10 weeks (28). This difference may be due to the different strain we used in the experiments. NL_c liposomes increased the survival of the zebrafish when challenged around 10^6 cfu/fish *M. marinum* both 7 days and 40 days after the liposome injection. Besides, either the empty liposome or non-encapsulated LPS and Poly (I:C) mixture, failed to protect the zebrafish against any of the studied challenges. These good results confirmed the adjuvant capability of liposomes in protection of immunostimulants. Interestingly, the long term protection by the stimulation of innate immunity might be explain by the novel concept in immunity called innate immune memory or trained immunity. Trained immunity is expressed as protection against reinfection by the same or different pathogens in organisms lacking adaptive immune responses such as plants (29) or invertebrates (30) (31). In addition, trained immunity is also an important feature of innate immunity in vertebrates, in which it provides protection in parallel with the existence of classical T/ B cell dependent adaptive responses (32). In zebrafish, the innate immune memory was proved by using rag1(-/-) mutant zebrafish which lacks functional B and T lymphocytes of acquired immune system, but includes Natural Killer (NK)-like cells and Non-specific cytotoxic cells (NCC) of innate immune system. The results showed that the survival of mutant zebrafish increased significantly when re-exposed to the same bacteria (33).

Secondly, a recombinant cytokine protein, $IBs^{TNF\alpha}$, was developed in our lab and showed good performance as adjuvant like biocompatibility, high stability, and immunostimulatory ability. In previous work, oral administration of $IBs^{TNF\alpha}$ were clearly taken up and located in the villi base and submucosa within the pyloric caeca section and, in the villi apex and lamina propria within the midgut of rainbow trout (34). When intraperitoneally injected, it provide *in vivo* excellent protection levels against *P. aeruginosa* lethal infection (34). However, a deep understanding of $IBs^{TNF\alpha}$ as mucosal adjuvant needed to be achieved. Similarly, the recombinant TNFα from European sea bass (*D. labrax L.*) also used as oral vaccine adjuvant and it significantly extended the protection of fish against *Vibrio anguillarum* challenge when orally immunized with vaccine (35). But the author did not focus on the explanation of mucosal vaccination mechanisms, such as whether be diluted in mucosal secretions, be captured in mucus gels, be attacked by proteases and nucleases, and be excluded by epithelial barriers (36). One of the bottlenecks for developing intestinal mucosal adjuvants is how to be resistant to the harsh environment of fish stomach (37). The IBs$^{TNF\alpha}$ were produced as aggregated proteins in the form of inclusion bodies (IBs) in *E. coli*. This form significantly increased the stability in extreme temperature and pH, even lyophilization (38). For this reason, we believe IBs$^{TNF\alpha}$ might be a good candidate as mucosal adjuvant and requires study in detail. We showed that IBs$^{TNF\alpha}$ was taken up in the zebrafish intestine when orally administrated. It crosses the intestinal mucosal epithelial barriers, pass through the lamina propria, and reach muscle cell layer. The uptake is probably because IBs$^{TNF\alpha}$ have a complex compositions (LPS, lipids, PGN and nucleic acids) that can be recognized by intestinal mucosal surfaces as microbial pathogens. LPS or PGN are patterns associated to pathogens (PAMPs) that interact with its pattern recognition receptors (PRRs) on the surfaces of immune cells, triggering fish innate immune system (39). In addition, IBs$^{TNF\alpha}$ were able to induce the local immune responses in the zebrafish intestine. Some cytokine genes (IL-6 and IL-1β) and inflammation-related genes (COX2 and MMP9) were significantly upregulated in a short time frame. The efficient immune stimulation meets with one of the mucosal adjuvant principle. Although the expression levels of immune genes are not so high, we may improve this by increasing the dose or mixing with vaccines. However, the expression of IgT, IgM and MHC-II did not show significant difference between IBs$^{TNF\alpha}$ treated fish and control fish. This may be due to the short monitoring period of time after immunization. Finally, we wanted to study whether the survival of the animals against an infection increased after IBs$^{TNF\alpha}$ administration. We tried to infect zebrafish with *M. marinum* by oral intubation. However, we could not observe any mortality of zebrafish by orally intubating *M. marinum* even with a very high dose (3.5×10^{8} cfu/fish) in a one month monitoring period. Therefore, the injection model that had been settled previously was used to test the IBs$^{TNF\alpha}$ by injection administration. The IBs$^{TNF\alpha}$ was able to efficiently protect zebrafish against a *M. marinum* lethal infection by increasing the survivals at both 7 days and 40 days post IBs$^{TNF\alpha}$ administration. Cytokines as IL-1β, IL-8 and TNFα from different fish species have showed their role as adjuvants and protect fish against challenges when co-injected with specific vaccine (40) (41) (42) (43) (35). However, using cytokine adjuvant alone protects fish against challenge has not been reported except that IL-1β showed resistance to VSHV challenge (44). The protection conferred by the IBs$^{TNF\alpha}$ is probably the result of their nanostructure, the sequence of the building protein, combined with their compositional complexity (LPS, lipids, PGN and nucleic acids) as described previously (34).

The third aim of this thesis was to investigate the *in vivo* uptake and innate immune regulation of our two potential adjuvants, NL_c liposome and $IBs^{TNF\alpha}$ in fish larvae. It is still a challenging problem that most fishes in aquaculture suffer high and unpredictable mortality during early development stages. Except egg quality, nutrition and physiochemical conditions which could be fairly well controlled, pathogen infections are one of the most important reasons resulting in high mortality. For example, turbot larvae showed a relatively high survival rate in the absence of culturable bacteria or with the addition of the antibiotic rifampicin (45) (46). We used zebrafish larvae as model due to the low costs, nearly transparent body, easy large breeding and fully sequenced genome. The first key aspect of our approach is to determine when, where, and how larvae take up the adjuvants. Interestingly, 4 dpf larvae showed a clear uptake of NL_c in the intestine and pharynx after 48 h immersion but not at 3 dpf after a 24 h immersion. Zebrafish development reveals that the mouth is widely open at 3 dpf (47). In such context, we believe that the uptake of NL_c might be through oral-intestinal pathway. Similar result was also observed in the uptake of $IBs^{TNF\alpha}$ at 5 dpf larvae after a very short immersion. This efficient uptake strongly supports the oral-intestinal hypothesis since the development of mouth may help the uptake. It further suggests that 3 dpf zebrafish larvae might be considered as a good time point for immersion. Next, the immune stimulation of zebrafish larvae by immersion in NL_c and $IBs^{TNF\alpha}$ were studied. This part was divided into two sections, expression of immune genes and protection post bacterial lethal challenge. The up-regulation of the innate immune genes suggested the immune stimulation ability of NL_c in zebrafish larvae. The components LPS and Poly I:C encapsulated in NL_c might be responsible for the significant up-regulation of IL-1β and IRF-1, respectively. IL-1β is a marker for proinflammatory and antibacterial activity while IRF-1 is a marker of antivirus response (48) (49). However, no significant up-regulation was found in the gene expression analysis of $IBs^{TNF\alpha}$. This difference may be explained by the different compositions of NL_c and $IBs^{TNF\alpha}$ or maybe due to the fact that higher amount of $IBs^{TNF\alpha}$ are needed to trigger the innate immune response. On the other hand, one gram-positive bacteria *Mycobacterium marinum* and one gram-negative bacteria *A. hydrophila* were studied and used to set up the infection model. However, infection model of *M. marinum* could not set up by immersion administration which probably is because of the long-term chronic infection character of this bacteria (50). We successfully developed the *A. hydrophila* infection model by immersion and significant mortality was observed at 5 days post immersion in both injured and non-injured groups. We selected the non-injured infection method because it is much easier to manipulate and the deviation of the mortality is smaller than the injured group. However, $\text{IBs}^{\text{TNF}\alpha}$ could not significantly increase the survival of zebrafish larvae after challenged with *A. hydrophila* comparing to the control. Higher doses of IBs^{TNFa} to trigger the innate immune responses and thus improve the survival against bacteria challenge maybe necessary. Taken all this into account, further experiments with higher doses of IBs^{TNFa} have to be taken into account.

In conclusion, with the wild fish population too depleted to harvest, aquaculture offers enough animal protein for human consumption. However, infectious diseases seriously challenge the health of famed fish species. Vaccination strategy has been proved to play an important role in the prevention of fish diseases but commercial available vaccines are still lacking. New studies or approaches for development of vaccines must be sought. These include adjuvant which is tailored and combined with the most appropriate antigen to create vaccines that may provide a more effective immune response against a specific pathogen (51). Nevertheless, the successful development of vaccines could not do without the study of fundamental research in immunology, such as the study of toll like receptor in this thesis. The reason why we designed this thesis is that not only insight into different organisms leading to a greater understanding of the complex immune system but also by merging the acquired knowledge to improve the fish health in practice.

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General conclusions

1) 28 new TLR genes of *Branchiostoma lanceolatum* were identified from genomic data via RT-PCR. Phylogenetic analysis showed that the repertoire of the *B. lanceolatum* TLRs consists in both non-vertebrate- and vertebrate-like TLRs.
2) A complete coding sequence of Bl_TLRj was obtained in *B. lanceolatum* and was fully characterized. Protein sequence and structure analysis showed that it is a sccTLRs. Phylogenetic analysis showed that it clusters with the vertebrate TLR11 family.
3) The Bl_TLRj expressed in HEK293 cells showed that it localized in the cytoplasm, but it did not recognize most of the common mammalian ligands for TLRs.
4) The ectodomain of the Bl_TLRj fused to the TIR domain of human TLR2 expressed in HEK293 could induce NFκB transactivation in response to both poly I:C (LMW) and poly I:C (HMW) treatments.
5) The recombinant cytokine TNFα inclusion bodies (IBs$^{\mathrm{TNF\alpha}}$) were able to cross the intestinal mucosal epithelial barriers, passed through the lamina propria, and reached the muscle layer in adult zebrafish intestine by oral intubation. In addition, IBs$^{\mathrm{TNF\alpha}}$ could stimulate the local immune response at the level of gene expression in the intestine.
6) A zebrafish infection model was established by i.p. injection of *Mycobacteria marinum*, a natural fish pathogen. IBs$^{\mathrm{TNF\alpha}}$ administrated by i.p. injection could protect zebrafish against *M. marinum* lethal infection. Our previously developed nanoliposomes encapsulating LPS and poly I:C (NL$_c$) could protect zebrafish against *M. marinum* infection by i.p. injection.
7) A zebrafish larvae infection model was established by bath immersion of *A. hydrophila*, a Gram-negative fish pathogen. In contrast, our results showed that *M. marinum* could not infect zebrafish larvae at the level of mortality in a 4 days' monitoring period.
8) The IBs$^{\mathrm{TNF\alpha}}$ could be taken up and accumulated in the pharynx and intestine at 5 dpf in zebrafish larvae. The NL$_c$ could also be taken up and accumulated in the pharynx and intestine at 3 dpf zebrafish larvae.

9) The IBs$^{TNF\alpha}$ could not significantly alter the expression of the immune-related genes in 5 dpf larvae. The expression of immune-related genes such as IL-1β and IRF1α was significantly up-regulated after 48 h treatment with NLc in 2 dpf larvae.
10) IBs$^{TNF\alpha}$ administered by immersion could not protect zebrafish larvae against *A. hydrophila* lethal infection.

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