Adjuvants and immunostimulants in fish vaccines: Current knowledge and future perspectives

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Abstract

Vaccination is the most adequate method to control infectious diseases that threaten the aquaculture industry worldwide. Unfortunately, vaccines are usually not able to confer protection on their own; especially those vaccines based on recombinant antigens or inactivated pathogens. Therefore, the use of adjuvants or immunostimulants is often necessary to increase the vaccine efficacy. Traditional adjuvants such as mineral oils are routinely used in different commercial bacterial vaccines available for fish; however, important side effects may occur with this type of adjuvants. A search for alternative molecules or certain combinations of them as adjuvants is desirable in order to increase animal welfare without reducing protection levels. Especially, combinations that may target specific cell responses and thus a specific pathogen, with no or minor side effects, should be explored. Despite this, the oil adjuvants currently used are quite friendlier with respect to side effects compared with the oil adjuvants previously used. The great lack of fish antiviral vaccines also evidences the importance of identifying optimal combinations of a vaccination strategy with the use of a targeting adjuvant, especially for the promising fish antiviral DNA vaccines. In this review, we summarise previous studies performed with both traditional adjuvants as well as the most promising new generation adjuvants such as ligands for Toll receptors or different cytokines, focusing mostly on their protective efficacies, and also on what is known concerning their effects on the fish immune system when delivered \textit{in vivo}. 
Contents

1. Introduction
2. Principles of adjuvant actions
3. Signal 1 adjuvants used in fish vaccinology
   3.1. Oil emulsions
   3.1.1. Freund’s complete adjuvant
   3.1.2. Freund’s incomplete adjuvant
   3.1.3. Montanide
   3.1.4. Other mineral oil adjuvants
3.2. Nano/microparticles as adjuvants
   3.2.1. PLGA particles
   3.2.2. ISCOMs
4. Signal 2 facilitators and TLR ligands as adjuvants or immunostimulants
   4.1. Alum
   4.2. β-glucans – ligands for dectin-1
   4.3. Saponins
   4.4. Poly I:C – toll-like receptor 3 agonist
   4.5. Lipopeptides
   4.6. Flagellin – toll-like receptor 5 agonist
   4.7. CpG – toll-like receptor 9 agonist
   4.8. Cytokines
5. Conclusive remarks and perspectives

Acknowledgments
References
1. **Introduction**

Disease prevention by vaccination is, on economic, environmental and ethical grounds the most appropriate method for pathogen control currently available to the aquaculture sector. Traditionally, vaccines comprise either live-attenuated, replicating pathogens or non-replicating, inactivated pathogens or their subunits. In many countries, live vaccines are not approved for use in aquaculture for safety reasons, while inactivated vaccines based on either killed pathogens or isolated non-replicating pathogen subunits, are in many cases, weakly immunogenic. Thus, adjuvants or immunopotentiators, are highly required for the elicitation of immune responses that may be 100% protective against certain pathogens.

During the past, fish vaccines were made by a trial-and-error approach (conventional vaccine design) including pathogen identification, pathogen cultivation, and vaccine formulation containing whole cell preparation and oils. Through this strategy, vaccines based on whole inactivated extracellular bacterial pathogens were quite efficient; resulting in important reductions in mortalities and antibiotic usage in the aquaculture industry [1]. However, many of the economically disastrous diseases of today are due to intracellular pathogens, and for this type of pathogens the production of effective vaccines has not been an easy task. In this sense, the most promising future vaccines that induce protection against viruses are DNA vaccines. Intramuscular injection of a DNA plasmid encoding an immunogenic antigen has proved very effective in fish, in comparison to the results obtained in other animal models such as mammals [2]. Because the antigen is produced by the fish cells, it is exposed on the cell surface both directly or processed in the context of both major histocompatibility complex (MHC) class I and MHC class II, thus effectively triggering both humoral and
cellular immune responses. Although DNA vaccines offer a number of advantages over conventional vaccines, there are still many aspects that may be optimised with adjuvant help such as alternative routes of immunisation that allow mass-vaccination. Therefore, fish vaccine approaches must be subjected to rational vaccine design wherein a combination of a tailored adjuvant system with the most appropriate antigen is used to create vaccines that may provide a more effective immune response against a specific pathogen with minimal side effects.

On the other hand, many aspects of fish immunology are still unknown and we are far from close to understanding on which immune mechanisms the protection against many of these pathogens resides [3]. Moreover, as we know of today, there are close to 22000 different fish species, and most of them have their “immune peculiarities”. Without a doubt the innate defence system of fish is strongly developed and may cope with many infectious agents, helping the fish to eradicate viruses, bacteria and even parasites. However, many infectious agents resist innate defence mechanisms, and then an adaptive immune response, present for the first time in evolution in teleost fish, must come into play to fight these pathogens, being this adaptive response the basis for vaccinology. The adaptive immune response provides the vertebrate immune system with the ability to recognise and remember specific pathogens, to be able to mount stronger and faster responses each time this pathogen is encountered. In higher vertebrates, adaptive immunity to extracellular pathogens is generally mediated by humoral immune responses (antibodies), while immunity to intracellular pathogens (including viruses) often relies on cellular immune responses (cytotoxic T cells). In fish, and despite the fact that the main elements for an adaptive immune response are present in most species, the regulation of these elements greatly differs from mammalian systems and even among different species. Both immunoglobulin (Ig) or B cell receptor
(BCR) and T cell receptor (TCR) genes are known among all lineages of gnathostomes (jawed vertebrates), but fish Ig are expressed as only as three isotypes (IgM, IgD and IgT) with no isotype switching and low affinity maturation [4]. Interestingly, there is a tight link between the innate and adaptive system that has not been much explored in fish immunology. This link, governed by several innate receptors and signalling molecules such as cytokines and transcription factors, may provide the key for the future rational design of vaccine adjuvants, since recent advances in immunology have shown that the magnitude and specificity of the signals perceived by the innate immune cells following vaccination shape subsequent adaptive immune responses [5].

2. Principles of adjuvant actions

Adjuvants (from Latin *adjuvare* meaning “to help”) have traditionally been defined as helper substances that increase the magnitude of an adaptive response to a vaccine (potency), or ability to prevent infection and death (efficacy). But nowadays scientists have acknowledged that adjuvants may become more important in the way adjuvants guide the type of adaptive response against a specific pathogen. Therefore, adjuvants have been now defined as a group of structurally heterogeneous compounds able to modulate the intrinsic immunogenicity of an antigen [6]. They can be classed according to their chemical nature or physical properties, however since even related compounds can have very different immunomodulating capacities, novel classifications have focused on the immunological events they induce, even though for many of them the exact mechanism of action is unknown. At present, the classification of adjuvants that distinguishes between signal 1 facilitators and signal 2 facilitators has been widely accepted [7]. According to this two-signal model, both the presentation of an antigen
(signal 1) and the additional secondary signals (signal 2) are required for activation of specific T and B lymphocytes, which form the adaptive arm of the immune system [8]. The signal 1 facilitators influence the fate of the vaccine antigen in time, place, and concentration, ultimately improving its immune-availability, while signal 2 facilitators provide the co-stimulation signals during antigen recognition that will provide an adequate environment for the most adequate antigen-specific immune response.

Another important aspect of the immune response against many adjuvants is the recognition of microbes through the detection of conserved molecular patterns, designated as pathogen-associated microbial patterns (PAMPs), through pathogen recognition receptors (PRRs) that include Toll-like receptors (TLRs), NOD-like receptors, dectin-1 or RIG-like helicases which are predominantly found on cells of the innate immune system. Nowadays, this recognition is considered critical in signal 2 induction and downstream activation of distinct T helper cell subsets; however, other authors make a distinction and refer to adjuvants that trigger PRRs as signal 0 adjuvants. In fact, recent work on adjuvants has especially focused on different PRR ligands including different PAMPs, and as well as other endogenous TLR ligands (Damage-associated molecular pattern molecules or DAMPs) such as heat-shock proteins (hsp), studying their ability to induce targeted Th responses. Once there is a production and expression of IL-2 (T cell growth factor) and its alpha-subunit of the IL-2 receptors (CD25) during e.g. activation of naïve Th cells into Th0 cells, proliferation of Th cells starts. Th0 cells will differentiate to Th1 or Th2 cells depending on the cytokine environment, wherein IFN-γ drives Th1 cells while IL-4 induces Th2 cell production/differentiation [9]. Additionally, after many cell generations, the Th cells progenitors differentiate to effector Th cells, memory Th cells and regulatory Th cells. Different vaccine adjuvants that are in use in veterinary and human medicine aid
differentiation of Th cells into several T cell lineages – such as Th1, Th2, Th9 and Th17 [10, 11]. In Table 1, we describe known adjuvant actions by both commercial and experimental adjuvants, used mainly in human medicine.

3. Signal 1 adjuvants used in fish vaccinology

3.1. Oil emulsions

To increase the immunogenicity of an antigen, a slow release is often achieved through the introduction of the antigen in the context of an emulsion. An emulsion is defined as a dispersion of a liquid, called the dispersed phase, in a second liquid, called the continuous phase in which the first one is not miscible. In vaccine formulations, these phases are water (antigenic media) and oil. In order to stabilise the emulsions, surfactants are added. A surfactant is a compound containing a polar group that is hydrophilic and a non-polar group that is hydrophobic and often composed of a fatty chain. Surfactants can be defined by their hydrophilic: lipophilic balance (HLB) value that gives information on their relative affinity for both phases. According to the HLB value of the surfactant, different kind of emulsions can be achieved [12]. Those having a low HLB value have a high affinity for oily phases and render W/O emulsions, whereas those with a high HLB value have a high affinity for the aqueous phase and render O/W emulsions, which are well tolerated but induce a shorter term immune response. With certain specific surfactant systems, when the HLB value is intermediate, W/O/W emulsions can be achieved. In this case, the continuous phase is aqueous and the dispersed phase is oil. But inside the oil droplets, an entrapped aqueous phase is found. This type of emulsions has shown to generate long-term immune responses with various antigens.
3.1.1. Freund’s complete adjuvant

The most widely used and most effective adjuvant for experimental purposes has been Freund’s complete adjuvant (FCA). FCA is composed of heat-killed Mycobacteria and a mineral oil with surfactant [13]. Before injection, the antigen in an aqueous solution is mixed with the FCA producing a stable W/O emulsion. Immunisation with FCA and antigens results in strong Th1 and Th17 responses predominantly via the MyD88 pathway. Unfortunately, the use of FCA has been associated with a variety of severe side effects including injection site granuloma; therefore, the use of FCA has been limited to research on animals including fish for establishing an effective immune response. Furthermore, the use of FCA in fish has not always resulted in an increase in immunogenicity or protection.

Pasteurellosis, caused by Pasteurella piscicida, also named Photobacterium damsela subsp. piscicida is one of the major diseases in many species of wild and farmed fish in Asia, USA and Europe. In yellowtail (Seriola quinqueradiata), a susceptible species, vaccination against pasteurellosis has been assayed with a lipopolysaccharide (LPS)-mixed chloroform-killed bacterin which resulted in protection against challenge with the virulent bacterium. In this case, the inclusion of FCA in the vaccine did not significantly enhance the protective effect [14].

Streptococcus iniae is a Gram positive bacterium associated with disease in several commercial species including tilapia (Oreochromis aureus and O. niloticus), yellowtail, hybrid striped bass (Morone saxatilis), turbot (Scophthalmus maximus), and rainbow trout (Oncorhynchus mykiss). Vaccination of rainbow trout with a formalin-killed culture of S. iniae resulted in good protection against experimental challenge that was not significantly potentiated in the presence of FCA [15].
*Aeromonas hydrophila* is a Gram-negative bacterium known to cause motile aeromonas septicemia (MAS) in freshwater fish farming. The major adhesin of *A. hydrophila*, a 43 kDa outer membrane protein, was cloned, expressed and emulsified in FCA to be used in a vaccine for the blue gourami (*Trichogaster trichopterus*) [16]. The vaccine was intraperitoneally (i.p.) injected and after three weeks a booster was given without FCA. Two weeks after the booster, the fish were challenged with two strains of *A. hydrophila*. The recombinant adhesin protected against challenge with both the homologous strain of *A. hydrophila*, and the heterologous strain, providing the same immune protection as the native adhesin [16].

*Aeromonas salmonicida* is the etiological agent for furunculosis. In a study in coho salmon (*Oncorhynchus kisutch*), formalin-killed *A. salmonicida* was i.p. injected in the absence or presence of FCA. In this model, the best protection was found for the FCA adjuvanted vaccine. Interestingly, fish injected with FCA (without antigen) gave some protection even 90 days after challenge [17]. Injection of inactivated *M. bovis* may induce innate defence mechanisms that may result a certain degree of protection to a heterologous pathogen, as shown by Kato *et al.* [18] where Japanese flounder (*Paralichthys olivaceus*) were partially protected against nocardiosis with FCA. In a recent study, Zheng *et al.* [19] compared naturally occurring adjuvants (astragalus polysaccharide and propolis) with FCA used in pentavalent vaccines. In that study, FCA outcompeted the other adjuvants although the natural adjuvants induced some immunostimulant activities.

It has generally been difficult to develop effective vaccines against *A. hydrophila* most probably because of the high degree of antigenic variation [17, 21, 22], this is in contrast to vaccines against Gram-negative pathogens of salmonids like *Aliivibrio salmonicida*, *Vibrio anguillarum*, *Yersinia ruckerii* and *A. salmonicida* –
where vaccines show up to 100% efficiency. Recently, a vaccine against *A. hydrophila* giving protection in rainbow trout was prepared [20]. LaPatra and co-workers developed a new challenge model in rainbow trout with *A. hydrophila* by injection into the dorsal sinus to determine the efficacy of a bacterial lysate. The vaccine was shown to give protection after i.p administration, and this protection could be potentiated in the presence of FCA [20]. Also, fish that survived an *A. hydrophila* challenge were very resistant to re-infection.

*Flavobacterium psychrophilum* is a widespread Gram-negative pathogen in freshwater causing rainbow trout fry syndrome (RTFS) and bacterial cold water disease (BCWD) [23]. In addition to rainbow trout, coho salmon is the most susceptible species together with other non-salmonid species that are also affected. Injection of a low molecular weight fraction emulsified in FCA resulted in an enhanced level of protection for rainbow trout [23].

*Flavobacterium columnare* is a Gram-negative bacterium responsible for columnaris disease. The disease was first described in 1917 in several warm water fish species from the Mississippi river, and since has been isolated from freshwater fish species worldwide [24]. Specific antibodies were found in tilapia plasma and mucus following i.p. injection of formalin-killed sonicated or whole cells of *F. columnare* in FCA within 2 weeks. After a secondary immunisation, the antibody response increased and at 10 weeks post-immunisation the titre remained elevated. Also, antibodies were observed in cutaneous mucus in fish i.p. immunised with formalin-killed sonicated cells (ultrasound disrupted cells) in FCA 6 and 8 weeks post-immunisation [24].

### 3.1.2. Freund’s incomplete adjuvant
Because of its high toxicity, the use of FCA has been widely replaced by Freund’s incomplete adjuvant (FIA) that lacks the mycobacterial components of the emulsion, being therefore just a W/O emulsion. This adjuvant is still highly effective in vaccination with a significant reduction of toxicity, however, some important side effects are still present, effects very well detailed for Atlantic cod (Gadus morhua) in a very recent paper [25].

_Edwardsiella tarda_ is a Gram negative intracellular bacterium that can infect both marine and freshwater fish, including Japanese flounder. In order to develop effective vaccines against this pathogen, fish were i.p. injected with a vaccine containing a major antigenic protein of _E. tarda_ in the absence or presence of FIA [26]. Protection against experimental challenge achieved by the vaccine without adjuvant resulted in a relative per cent survival (RPS) of 34% that was increased to 81% in the presence of FIA. Moreover, vaccination with the oil-adjuvanted antigen stimulated the expression of a series of genes like complement component 3 (C3), MHC class I and MHC class II, CD8α, CD40, Mx, interferon γ (IFN-γ), tumour necrosis factor α (TNF-α) and interleukin 6 (IL-6), whereas vaccination with the antigen alone resulted in increased expression of just IgM, MHC class I and class II, and Mx [26].

_Nocardia seriolae_ is a Gram-positive acid-fast bacterium that causes nocardiosis in cultured marine and freshwater fish in Taiwan, Japan and China. Although the disease results in considerable economic loss, no commercial vaccines are available. Very recently, an oil-adjuvanted vaccine was developed and tested on protection against challenge with a virulent strain [27]. Formalin-inactivated whole cell antigen was used as a vaccine with or without FIA, however, and even though antibody levels increased, no protective effects were found.
Another Gram-positive bacterium that causes disease (lactococcosis) and mortality in rainbow trout is *Lactococcus garvieae*. Recently a vaccine was prepared based on formalin inactivated bacterin or bacterin together with FIA. Fish were given i.p. injections and challenged by exposure to virulent bacteria 30, 75, and 125 days after vaccination [28]. A hundred and twenty five days after vaccination the RPS in fish vaccinated with bacterin only was 54% and whereas it was 85% in fish vaccinated with bacterin together with FIA.

*Tenacibaculum maritimum* is a marine bacterium that causes flexibacteriosis worldwide. In Australia (Tasmania), Atlantic salmon (*Salmo salar*) and rainbow trout are the most heavily affected species, and due to the lack of vaccines, so far the disease has been treated with trimethoprim and oxytetracycline with the subsequent negative impact on the environment [29]. Salmon injected with formalin inactivated bacteria mixed with FIA provided protection against challenge with *T. maritimum* while the vaccine without the adjuvant could not provide sufficient protection against a moderate challenge of *T. maritimum*.

Infection with fungi oomycetes such as *Aphanomyces invadans* may cause heavy mortalities of fresh water and estuarine fish species as a result of granulomatous inflammation. In catla (*Catla catla* Hamilton), fungal extract combined with FIA showed to increase both the survival rate during experimental challenge with *A. invadans* and the antibody response compared to non-adjuvanted vaccines [30].

3.1.3. Montanide

Mineral oil adjuvants registered under the trademark of Montanide have been optimised in order to improve efficacy and stability of vaccine formulations and to
reduce side effects. These adjuvants are based on either mineral oil, non-mineral oil or a mixture of both, as well as those made from specific surfactant chemistry using mannitol oleate and may be used to manufacture different type of emulsions, W/O, O/W or W/O/W, for use in both mammals and fish [31, 32].

*Philasterides dicentrarchi* is a scuticociliate parasite that causes mortalities and significant economic losses in cultured turbot [33]. An important attempt to optimise a vaccine against this parasite was performed on the basis of antigenic dose, concentration of inactivating agent (formalin) and proportion of the adjuvant Montanide ISA763A (W/O, non-mineral oil) in the emulsion. The results of the study showed that a high concentration of antigen, 0.2 % formalin and 50 % adjuvant generated the longest time of survival after challenge 30 days after the second injection, and the highest levels of antibodies in the vaccinated fish [33].

*Pseudomonas plecoglossicida* is a bacterium causing bacterial hemorrhagic ascites of cultured ayu (*Plecoglossus altivelis*). To develop a vaccine against the disease, formalin-killed *P. plecoglossicida* bacterin was emulsified with Montanide and injected i.p. The fish were challenged with an i.p injection of virulent *P. plecoglossicida* 22 and 52 days after vaccination [34]. The RPS of vaccinated fish was 17-58% without adjuvant, 57-92% with Montanide ISA711 and 65-86% with Montanide ISA763A. Another study on the same disease and adjuvant (Montanide ISA 763A) concluded that there is a good correlation between antibody levels and protection against disease in a challenge test [35].

To study the efficacy of different adjuvants in Atlantic halibut (*Hippoglossus hippoglossus*), fish were injected i.p. with a model vaccine of human gamma globulin with either FCA or Montanide ISA711 as adjuvants [36]. Antibody responses and
intraperitoneal adhesions were examined every month for up to 12 months. FCA produced the highest and fastest antibody response, since in the group injected with the Montanide adjuvant only 4 of 47 fish reached a titre of 1:1000 (month 6) compared to 27 of 48 fish in the FCA group (after 2 months), however, FCA also induced the fastest intraperitoneal adhesions [36].

In a very recent study in carp (Cyprinus carpio), a recombinant S-layer protein of *A. hydrophila* was used to assess the ability to protect fish against six virulent isolates of *A. hydrophila*. The recombinant S-layer protein of *A. hydrophila* was produced, diluted in phosphate buffered saline and mixed with a Montanide adjuvant at a ratio of 30:70. Common carp were i.p. injected with the emulsion, and after 35 days the fish were challenged with six different isolates of *A. hydrophila* [37]. The RPS values varied between the different challenge isolates (40-75%), but they suggested that the S-layer protein together with Montanide adjuvant is a good candidate for an efficacious vaccine.

Furthermore, Montanide ISA-763 has also been used as an adjuvant in experimental bivalent vaccine for *L. garvieae* and *A. hydrophila* with high degree of efficacy in rainbow trout [38].

### 3.1.4. Other mineral oil adjuvants

*Moritella viscosa* is the causative agent of winter ulcers in farmed fish like Atlantic salmon and Atlantic cod. Vaccination of Atlantic salmon against *M. viscosa* is performed with oil-adjuvanted polyvalent injection vaccines based on formalin-inactivated bacterial cultures, using an AJ-oil (Alpha Ject 5200) used in some vaccines commercialised by Pharmaq [39]. However, a multivalent commercial salmon vaccine containing *M. viscosa* as one of five bacteria mixed in a mineral oil adjuvant (Alpha Ject
5200) did not protect turbot against challenge [40], whereas moderate intra-abdominal adhesions were detected in vaccinated fish.

Other commercial oil-adjuvanted vaccines have been shown to give protection in Atlantic salmon against bacterial diseases like vibriosis, coldwater vibriosis and furunculosis for a long time. However, side effects and retardation in growth have been clearly demonstrated [41, 42]. Mutoloki and co-workers investigated the intraperitoneal lesions induced by an oil-adjuvanted vaccine against infection with *A. salmonicida* and *M. viscosa* in Atlantic salmon [43]. The cellular composition was typical of granulomas containing large macrophages, eosinophilic granular cells, lymphocytes and multinucleated cells.

Oil-adjuvanted vaccines are also used to control sea bass (*Dicentrarchus labrax*) against bacterial diseases like vibriosis and pasteurellosis. Sea bass is one of the most explored fish species in the Mediterranean area, and suffers from infection by *V.anguillarum* and *Photobacterium damsela* subsp. *piscicida*. Oil-adjuvanted vaccines against these diseases have been prepared and injected i.p., but despite their effectiveness, granulomatous peritonitis was also recognised [44].

The major bacterial disease of farmed Atlantic cod is classical vibriosis [45]. Cod vaccinated by injection with mineral oil adjuvanted vaccines against both *V.anguillarum* and atypical *A. salmonicida* were very well protected against homologous challenges [46]. In this model, even without adjuvant the fish were protected against *V.anguillarum*, but not against atypical *A. salmonicida* challenge.
3.2. **Nano/microparticles as adjuvants**

Microparticles offer a promising option to oil emulsions, and their beneficial use as carriers for vaccine delivery has been widely discussed [47]. An association or/and encapsulation of antigen(s) with/in microparticles can be achieved by covalent linkage or physical entrapment. Compared to the latter technique, where the antigen is non-covalently and physically incorporated in the interior of the microparticle, covalent coupling offers distinct advantages: fewer amount of antigen is required; processing and presentation by antigen-presenting cells is more efficient; a higher stability during storage is obtained and any excess of (valuable) material can easily be regained. With the use of microparticles, a very low dose of antigen can give rise to an optimal humoral response.

The structure and the properties of microparticles may change markedly with slight alterations in production conditions, but nanoparticles can be prepared in a physico-chemically reproducible manner within narrow size limits. For this reason, adjuvants on the basis of these submicron polymeric particles were developed and have also been suggested for use as potent adjuvants in mammalian systems [48].

3.2.1. **PLGA particles**

Encapsulation of vaccines in biocompatible and biodegradable Poly-(lactide-co-glycolide) (PLGA) polymers has been studied for over twenty years. Antigen is released from the microspheres by diffusion through matrix pores and by matrix degradation. Biodegradation rates can be regulated by alterations in polymer composition and
molecular weights. In addition, there is often instant release of surface associated antigens that may be beneficial to aid a rapid response.

So far, a few studies have been carried out on fish with regard to uptake and degradation of PLGA particles and the immune response obtained. For the most part, these studies have been on oral administration [49-53]. A recent article appeared on parenteral immunisation of Indian major carp, rohu (*Labeo rohita*) with PLGA encapsulated antigen [54]. Outer membrane proteins (OMP) of *A. hydrophila* were encapsulated in PLGA microparticles and mixed with FIA in an emulsion or administered alone by i.p. injection in rohu. Twenty-one and 42 days after immunisation, the antibody titres were significantly higher in the PLGA-encapsulated antigen group containing FIA [54].

A dose-dependent transient increase of antibody response following i.p injection of PLGA particles containing human gamma globulin (HGG) has been shown by Fredriksen and Grip [55] where it was shown that microparticle carriers were superior compared to nanoparticles. Furthermore, when the formulation of PLGA entrapped HGG was performed with β-glucan or oil, it resulted in a continuous increase of antibodies over time (over 120 days).

Oral vaccines encapsulated in PLGA have been also used in Japanese flounder [51, 53] and salmonids like rainbow trout [50, 52, 56] or Atlantic salmon [49]. In the case of Japanese flounder, a plasmid encoding the major capsid protein of lymphocystis disease virus (LCDV) was constructed and encapsulated in PLGA. Controls were naked plasmid vaccine and blank PLGA particles [53]. The fish were orally intubated, and 28 days post vaccination the fish were challenged by intramuscular injection with LCDV. Vaccine-effects were evaluated by observing the presence of lymphocystis nodules. The
cumulative percentage of Japanese flounder with nodules after challenge was greatly
reduced in the group receiving the plasmid coding for the LCDV protein in PLGA
particles in the period of 15 to 120 days post-immunisation [53]. In addition, the levels
of antibody in sera of fish vaccinated with PLGA microcapsules increased for up to nine
weeks; although from this point it started to decrease [51].

In rainbow trout, oral vaccination (as a feed additive) against lactococcosis was
attempted with antigens encapsulated in PLGA particles [52]. RPS of the PLGA-
vaccine amounted to 63 % and booster vaccination with oral administration of the
PLGA-vaccine gave a RPS of more than 60 % 120 days after the first vaccination. Also
in rainbow trout, human gamma globulin (HGG) was microencapsulated in PLGA [50].
Specific antibodies were detected in the intestinal mucus of fish that were administered
with the microencapsulated antigen after boosting with soluble HGG, but not in fish that
were primed with the soluble antigen. The fate of orally administered HGG in Atlantic
salmon was determined, demonstrating that fifteen minutes after administration, the
HGG-PLGA was found in the intestine resembling the observation for free HGG [49].
The results from this study indicate that orally delivered HGG-PLGA had higher levels
and greater persistence of HGG systemically than free HGG.

Finally, feeding of rainbow trout with feed containing plasmid DNA encoding
IHNV G protein induced slightly higher amount of neutralising antibodies against
IHNV but no increased survival after experimental challenge with IHNV [56].

3.2.2. ISCOMs

Immune-stimulating complexes (ISCOMs) were conceived to co-formulate antigen
and adjuvant in a particle [57]. ISCOMs represent an interesting approach to stimulation
of the humoral and cell-mediated immune response towards amphipathic antigens. They are relatively stable but non-covalently-bound complex of approximately 40 nm diameter of saponin adjuvant Quil-A (saponin extracted from the cortex of the South American tree *Quillaja saponaria molina*), cholesterol and amphipathic antigen in a molar ratio of approximately 1:1:1. ISCOMs produced through the patented Matrix™ technology by Isconova have been widely studied in combination with different veterinary vaccines, and are currently incorporated in a number of commercialized animal vaccines. At this moment, Pharmaq is studying the introduction of these adjuvants in commercialised fish vaccines.

4. **Signal 2 facilitators and TLR ligands as adjuvants or immunostimulants**

A large number of adjuvants that have been investigated do not directly affect the concentration and distribution of antigen between injection site and presentation site (this has not been established in fish yet). This category of vaccine adjuvant has dominated the literature on vaccine research in the last decade, and comprises the category of signal 2 facilitators, which include stranger and danger molecules, as well as inflammatory cytokines.

A number of so-called toll-like receptors (TLR) ligands (agonists) may induce strong innate responses that may be decisive for the outcome of acquired responses. Teleost fish species may possess close to twice the number of different TLR compared to mammalian species presumably due to an ancient genome duplication event. Many similarities between mammalian and fish with respect to intracellular and downstream signaling events exist, but there are dissimilarities that warrant focus. In this issue, a
A detailed review authored by Aoki and Robertsen has been included, giving an excellent overview of the current knowledge on fish TLR (technical editor: Check whether this review is included in the special issue, and give reference). Another up-to-date review on immune relevant genes including TLR-like receptors in fish is also authored by Zhu et al. [58]. In general, those TLRs that, after ligand binding, induce the production of IL-12 favour a Th1 response (TLR 3, 4, 5, 7, 8, 9 and 11) and in addition, the activation of these TLRs may induce cross-presentation of antigens facilitating a cytotoxic T cell response under certain conditions [59]. It should be mentioned that ligand binding to TLRs 3 and 4, 7 and 9 may also induce type I IFN responses via interferon regulating factors. Within this group of signal 2 facilitators, we have also included alum, as it has been recently discovered that this adjuvant directly interacts with dendritic cells in a similar way to that of danger signals [60].

4.1. Aluminium containing adjuvants

The adjuvant property of aluminium salts was discovered in 1926 [61]. Aluminium compounds, especially aluminium phosphate and aluminium hydroxide, are some of the few adjuvants that have been allowed and considered safe to use in human vaccines. Aluminium adjuvants have been shown to induce Th2 responses almost exclusively [26], thus they have been used as adjuvants with great success, being particularly effective at promoting protective humoral immunity. However, alum is not optimally effective for diseases where cell-mediated immunity is required for protection. It was believed that alum activates NLRP3 inflammasome and induces necrotic cell deaths that release the danger signal uric acid [62]. However, very recently, it has been discovered that being a crystal, alum binds dendritic cell plasma membrane lipids with substantial force, independent of inflammasome and membrane proteins.
The subsequent lipid sorting activates an abortive phagocytic response that leads to antigen uptake. Such activated dendritic cells, without further association with alum, show high affinity and stable binding with CD4\(^+\) T cells via the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen-1 (LFA-1). Only a few studies have been performed with aluminium adjuvants in the optimization of fish vaccines.

Fifteen years ago a vaccine against *A. salmonicida* mixed with potassium aluminium sulphate (alum) as an adjuvant was tested in Atlantic salmon [63]. Alum appeared to enhance the protection against challenge, but not at a statistically significant level. In another study, an *Escherichia coli* mutant was used for vaccination against *Edwardsiella ictaluri*-induced enteric septicaemia of catfish (*Ictalurus punctatus*). Killed *E. coli* bacteria with or without alum were administered i.p to catfish and the fish were challenged with virulent *E. ictaluri* bacteria [64]. Fish given *E. coli* in alum showed an enhanced survival (92 %) compared with the fish for which *E. coli* was administered alone (54%) or fish given saline (56%).

Recently, an aluminium hydroxide adjuvanted *E. tarda* vaccine was prepared and injected i.p in Japanese flounder. The RPS was found to be 69 % [26] while immunisation with the antigen alone followed by an experimental challenge gave a RPS of 34, however, the FIA coupled vaccine showed a RPS of 81%.

Another experiment has been recently carried out by Fan *et al.* [65], in which formalin-inactivated reddish body iridovirus (TRBIV) were mixed with alum and either injected or bath administered twice in turbot. The resulting RPS calculated was 83.3% and 90.5%, respectively.
4.2. β-glucans – ligands for dectin-1

β-glucans are known to stimulate the non-specific immune response of both mammals and fish where dectin-1 may be involved [66, 67]. To obtain protective effects against diseases the glucan is injected i.p., and there seems to be a dosage-dependent and short-lived protection. In addition, there are some reports on the adjuvant effect of β-glucans [41, 42, 68-75].

DeBaulney and co-workers prepared an oral vaccine against vibriosis for use in turbot, and after feeding the vaccine for 5 days the fish were challenged 28 days thereafter. Fish given the vaccine alone resulted in a RPS of 52 %, while a combination of the vaccine and the β-glucan gave a RPS on 61 %, higher protection levels but not statistically different from the vaccine alone [71]. In 1998, an attempt to establish immunisation protocols to obtain the highest immune response against V. damsela was performed in Spain [72]. In this study they i.p. injected the O-antigen of V. damsela in combination with β-glucan. As a correlate to vaccine efficacy, the phagocytic index of head kidney macrophages was evaluated. The obtained results were as follows: the enhancement of the phagocytic index lasted longer in fish injected with β-glucan at the same time or after being injected with the antigen when compared with fish injected with β-glucan before the antigen. Similar results were obtained with regard to antibody titres [72].

Yeast glucan (mainly a β-1,3-D glucan) was included in a furunculosis vaccine that consisted in a formalin-killed culture of A. salmonicida and V. salmonicida [70]. The vaccine, either with or without β-glucan, was injected i.p. and salmon challenged 3-46 weeks after vaccination. Vaccines supplemented with β-glucan induced significantly higher protection against furunculosis than vaccines without this adjuvant [70], but β-glucan alone did not result in protection after 11 weeks. In another study, β-glucan-
adjuvanted vaccines against furunculosis seemed to give protection at an early time-point after vaccination (6 weeks), but no protection was seen after 3 and 6 months [41]. As a side effect, the average weight of the β-glucan-adjuvanted group was significantly lower compared to the controls, but the weight of fish given oil-adjuvant was also significantly lower than the β-glucan-adjuvanted group [42]. In a further study performed in coho salmon, Nikl et al. evaluated the potentiating effect of seven substances on the protection after vaccination with formalin-treated A. salmonicida bacterin [68]. Statistically significant improvement in survival over the group receiving bacterin alone was noted in fish groups that also received β-glucans like Vitastim-Taito and lentinan. However, agglutinin levels were significantly elevated in all cases where the bacterin was injected, and no significant elevation in agglutinin titer occurred as a result of combining an immunostimulant with the bacterin [68].

Catla is one of the major Indian carp species often affected with A. hydrophila, thus a formalin-inactivated A. hydrophila vaccine was developed and protection was studied in the absence and presence of a β-glucan adjuvant [74]. A reduction in mortality was found in the presence of β-glucan compared to the vaccine itself, although the differences were not statistically significant (RPS of 67.7 % and 58.0% with and without the adjuvant, respectively). In carp, a vaccine against A. hydrophila showed a higher antibody titer when β-glucan was i.p. injected prior to vaccination, while bath and oral administration of β-glucan before vaccination did not result in enhanced antibody response [75]. In a further study by Selvaraj and coworkers, carp were vaccinated against A. hydrophila with LPS from a virulent strain of the bacterium in the presence of different concentrations of β-glucan and administered through various routes such as i.p, oral or bath [76]. The RPS was significantly higher in i.p. injected groups even at the lowest concentration of β-glucan and fish given a mixture of LPS and
β-glucan orally obtained a higher RPS compared to controls. The administration of the LPS-glucan by bath did not result in increased survival, and antibodies were never detected in fish vaccinated either orally or by bath. However, no possible analysis of the contribution of β-glucan in the vaccine efficacy could be established because an obvious control group in this study was missing, namely the protective effect of LPS without adjuvant [76].

In another study, the i.p. injection of β-glucan on days 1 and 3 followed by two i.p. immunisations of *E. ictaluri* on days 7 and 14 performed in channel catfish resulted in higher serum antibody levels relative to catfish receiving PBS instead of β-glucan before administration of *E. ictaluri* [69]. Serum antibody levels were determined on day 7 (day 21) after the last immunisation, reaching with β-glucan antibody titers that were typically two-fold higher than those of fish without β-glucan.

In order to investigate possible treatments against *A. hydrophila* in blue gourami, laminaran, a β-1,3-D-glucan, was injected i.p. in the absence and presence of formalin-killed *A. hydrophila* bacteria [77]. A single i.p. injection of 20 mg kg⁻¹ laminaran alone was sufficient to protect the fish against infection by a virulent strain of *A. hydrophila* up until 29 days after injection in correlation with an increased phagocytic activity of head kidney phagocytes. Despite this, the addition of 20 mg kg⁻¹ laminaran to a formalin-killed *A. hydrophila* did not significantly improve the protection [77].

4.3. **Saponins**

Saponins are natural glycosides of steroid or triterpene which have been widely explored as adjuvants in different mammalian systems due to their capacity to stimulate both Th1 and Th2 responses [78]. The most widely used saponins are Quil A
(component of ISCOMs) and their derivatives, however, due to their high cytotoxicity and instability in aqueous phase, the use of different kinds of saponins is being explored.

In Japanese flounder, formalin-killed *E. tarda* cells were administered to fish by feeding in the absence or presence of curdlan or curdlan together with Quil A saponin. Although the incorporation of curdlan gave higher survival rates, only the group in which the vaccine was administered with both curdlan and Quil A showed significantly better survival [73].

4.4. Poly I:C – toll-like receptor 3 agonist

Polyinosinic polycytidylic acid (poly I:C) is a double stranded polyribonucleotide, that mimics a viral infection and therefore has been widely used to induce a type I IFN in many species including fish [79-81]. IFNs are cytokines with a major role in the early defence against viral infections, thus Poly I:C induces a non-specific antiviral state after its binding to TLR3 and the subsequent activation of intracellular signalling events. This non-specific antiviral activity of Poly I:C has been recently tested in rainbow trout infected with infectious hematopoietic necrosis virus (IHNV) [82]. Fish pre-injected with Poly I:C were protected against IHNV challenge 2 days later and IHNV-specific antibodies were detected in survivors. The survivors showed a 100% survival rate following re-challenge with IHNV both 21 and 49 days after the primary IHNV challenge [82], demonstrating that the fact that fish were at an antiviral state during the encounter of a virus, gave them an important advantage for posterior specific antibody production. A similar study was performed in the sevenband grouper *Epinephelus septemfasciatus* in which fish were immunised against the
nodavirus red-spotted grouper nervous necrosis virus (RGNNV) [83]. Fish injected with
50 mg Poly I:C fish$^{-1}$ or more intramuscularly (i.m.) and challenged i.m. with RGNNV
2 days post-injection showed more than 90% survival rate. When surviving fish were re-
challenged with RGNNV 3 weeks after the primary challenge, no mortalities were
detected in the group that had been previously exposed to Poly I:C, since upon RGNNV
challenge the antibodies against RGNNV were higher in these fish. All survivors that
were re-challenged with RGNNV showed even higher levels of specific antibodies. In
addition, the RGNNV titres in brain tissues of the survivors in the Poly I:C-RGNNV-
RGNNV group were all under the detection limit [83]. Following up this work, this
research group conducted a field trial exploring the vaccine efficacy of a RGNNV
vaccine followed by Poly I:C injection. The Poly I:C-adjuvanted vaccine showed
reasonable efficacy, but a one-shot Poly I:C injection in sevenband grouper 20 days
after a natural RGNNV outbreak also induced a high survival rate (93.7%) compared to
non-treated fish (9.8%) [84].

A prophylactic strategy using poly I:C was also used by Takami and co-workers
in Japanese flounder experimentally infected with viral haemorrhagic septicaemia virus
(VHSV) [85]. The survival rate in Japanese flounder after VHSV challenge following
Poly I:C administration was 100%, while all untreated fish died within 9 days. Survival
rates of the fish given a secondary challenge VHSV were 100% in the Poly I:C-VHSV
group (Poly I:C-VHSV-VHSV group), while non-immunized fish showed a 0%
survival.
4.5. Lipopeptides

Lipoproteins and lipopeptides have been found in a large number of microorganisms, the most prominent being mycobacteria and mycoplasms. These molecules have been found to exhibit both a strong innate (inflammatory) response and a long-lasting adaptive immune response in mammals. Very few studies have been performed on lipopeptides in fish. The adjuvant effect of polar glycopeptidolipids in experimental vaccines against *A. salmonicida* was investigated [86]. Polar glycopeptidolipids (pGPL-Mc) were extracted from *Mycobacterium chelonae*, which is one of three mycobacteria species that are fish-pathogenic. At 12 weeks post vaccination, the antibody response of fish given 0.25 mg kg\(^{-1}\) pGPL-Mc in combination with an *A. salmonicida* bacterin was significantly higher than that induced by a non-adjuvanted bacterin. Increased doses of pGPL-Mc suppressed the antibody response, while no significant side effects were observed in the peritoneal cavity after use of this adjuvant [86].

4.6. Flagellin – toll-like receptor 5 agonist

The structural protein of Gram-negative flagella is called flagellin. Flagellin is a potent activator of a broad range of cell types within the innate and adaptive immune system. Several studies have demonstrated the ability of flagellin as an adjuvant, promoting cytokine production [87]. Flagellin is known to induce immune responses via the TLR5 signalling resulting in a mixed Th1 and Th2 response, although it has also been reported that inflammasomes containing NLRC4/IPAF may bind cytosolically located flagellin [62]. During the last decade, the adjuvant effect of flagellin has widely been studied in vertebrates and during the last couple of years also in fish [88-90]
Piscirickettsiosis is a severe disease reported in salmonids that has caused especially great problems for the Chilean aquaculture industry. In 1989, the bacterium *Piscirickettsia salmonis* was isolated from a moribund coho salmon and was found to be the etiological agent of this disease. The pathogen is a Gram-negative obligate intracellular bacterium. The disease has also been reported to affect Atlantic salmon, rainbow trout and other farmed salmonid species [88]. A recombinant subunit vaccine was developed in order to control the disease due to poor responses after treatment by antibiotics. Three experimental formulations were prepared containing two or three recombinant proteins of the bacterium, and the formulations were emulsified with one volume of FIA [88]. The highest protective response was obtained with a vaccine formulation containing the subunit of the flagellum and chaperonins Hsp60 and Hsp70 of *P. salmonis*, suggesting that the use of more than one recombinant protein antigen is needed to obtain a good protective effect against this disease.

Jiao and co-workers have been studying different vaccine concepts against *E. tarda* in the Japanese flounder to obtain effective protective formulations, based on both recombinant proteins and DNA vaccine constructs [89, 90]. The most promising vaccine concept was the one consisting in a chimeric DNA vaccine coding for the *E. tarda* proteins Eta6 fused in-frame to FliC, the flagellin for *E. tarda*. Although they found that *E. tarda* FliC induced low protective immunity by itself, it could function as a molecular adjuvant and potentiate the specific immune response induced by the *E. tarda* antigen Eta6. Fish immunised with pEta6 and FliC produced specific serum antibodies and exhibited enhanced expression of genes that are involved in both innate and adaptive immune responses (IL-1β, IFN, Mx, CD8α, MHC-Iα, MHC-IIα, IgM) [89, 90]. Such up regulation following immunisation with flagellin has also been described by Hynes *et al.* [91], where TNF-α, IL-6, IL-8 and IL-1β were significantly
up regulated compared to non-adjuvanted controls. In this study, however, there was no
induction of specific antibody response against flagellin or the model antigen \textit{Limulus}
\textit{polyphemus} hemolymph (LPH) in the Atlantic salmon.

4.7. \textit{CpG – toll-like receptor 9 agonist}

Bacterial DNA and synthetic oligodeoxynucleotides (ODNs) expressing
unmethylated CpG motifs trigger an immunostimulatory cascade that culminates in the
maturation, differentiation and proliferation of multiple immune cells, including B and
T lymphocytes, NK cells, monocytes, macrophages and dendritic cells. CpG motifs are
approximately 20 times less common in mammalian than microbial DNA, and therefore
are recognised as a danger signal by cells that express TLR9. In mammals, it has been
widely demonstrated that CpG ODNs function as adjuvants when co-administered with
vaccines, being able to both accelerate and magnify the immune response [92]. In fish,
although many studies have been carried out on the immunomodulatory effects of CpGs
[93-96], only few studies have focused on the adjuvant effect of these molecules.

Chinook salmon (\textit{O. tshawytscha}) reared in the Pacific Northwest of the United
States often suffers from infection with \textit{Renibacterium salmoninarum}, the causative
agent of bacterial kidney disease (BKD). The conclusion from a study in which whole
cell vaccines with or without CpG adjuvants were used, was that either the vaccine
alone or that with CpG provided protection against i.p. challenge with \textit{R. salmoninarum}
[93]. However, a combination of a commercial \textit{R. salmoninarum} vaccine (Renogen)
with a CpG adjuvant significantly reduced the level of bacterial antigens in the kidney
of naturally infected fish [93].
In a study in rainbow trout, four groups were i.m. injected with a commercially available, non-adjuvanted aqueous vaccine against furunculosis containing inactivated cultures of *A. salmonicida* (Aquavac Furovac 5) alone, or together with CpG ODN 1982, CpG ODNs 2133 or ODN2143. The fish were challenged with i.p. injection of a pathogenic strain of *A. salmonicida* 7 weeks after injection and the only group that showed a significantly lower mortality compared to those injected with Furovac alone (mortality of 52%) was the group injected with Furovac and the CpG ODN 2143 in which only a 21% of the fish died [94].

The protective effect of CpG motifs was also studied by Liu and co-workers in turbot and Japanese flounder [95, 96]. Sixteen CpG ODNs were synthesized and examined for the ability to inhibit bacterial dissemination in Japanese flounder blood. Four ODNs with the strongest inhibitory effects were selected and a plasmid pCN6 was constructed containing the sequences of the 4 selected ODNs. Japanese flounder were injected i.m. with plasmids pCN6 and pCN3 (control) and PBS. Four weeks post-vaccination the fish were challenged with *A. hydrophila* and mortality was monitored over a period of 20 days. Accumulated mortalities were 30%, 66.7% and 63.3% in pCN6-, pCN3, and PBS-immunised flounder, respectively [96]. Fish were also vaccinated as above and challenged with *E. tarda* 4 weeks after vaccination and the mortalities were 53.3, 90%, and 93.3% respectively. Therefore, the pCN6 plasmid provided a nonspecific protection against both *A. hydrophila* and *E. tarda* infections. This nonspecific protective effects have also been observed in fish parasitic infections since certain CpGs (e.g. CpG-ODN 1668 and CpG-ODN 2359) have also proved to have effects protecting fish against *Miamiensis avidus* [97]. Following on, a salmonid alphavirus (SAV) vaccine containing antigen plus CpG and Poly I:C as adjuvants induced a significant production of neutralizing antibodies and conferred some level of
protection – as evaluated by percentage of SAV positive fish compared to controls [98].

The authors reported that the adjuvanted vaccines induced prominent IFN type I expression – that is crucial in antiviral response.

To analyse the adjuvant effect of CpGs in turbot, fish were vaccinated with a *Vibrio harveyi* recombinant subunit vaccine, DegQ, in combination with a CpG that had been shown to provide anti-bacterial effects in the host species after injection. Fish were vaccinated by i.p. injection including all the appropriate controls and twenty-eight days after vaccination, the fish were challenged by a virulent strain of *V. harvey*, and accumulated mortalities were recorded [95]. The only vaccine formulation that induced a significant protection was DegQ in combination with this pCN5 CpG. The duration of the adjuvant effect was found to last at least 50 days.

One of the unique features of DNA vaccines is the ability to stimulate both cellular and humoral immune responses through the administration of a bacterial plasmid coding for a protective antigen [99]. Thus, these DNA vaccines possess intrinsic immunostimulatory capacity due to the presence of CpG motifs in the bacterial plasmid backbone. Therefore, the inclusion of additional CpG motifs in the vaccine plasmid would provide us with an intrinsic adjuvant within the same construct, being an easy method to increase the immunogenicity. In this sense, a recent work by Martinez-Alonso *et al.* [100] explored the possibility of increasing the immunogenicity of a VHSV DNA vaccine though the introduction of several copies (either two or four) of a fragment containing multiple CpG sequences of known immunostimulatory effects into the DNA vaccine. The addition of these CpG motifs significantly increased the titre of neutralising antibodies in serum and increased the levels of transcription of several immune genes such as Mx or MHC-I, demonstrating for the first time that additional CpG motifs may also be used to increase the immunogenicity of these DNA vaccines.
4.8. Cytokines

In the past years, a great number of cytokine genes have been identified in many fish species, however, and despite the fact that the use of cytokines as adjuvants has been widely explored in mammals, not many studies have focused on the possible use of cytokine genes as vaccine adjuvants in fish. This may be due to the fact that for the majority of these molecules, many details concerning their immunological role are still lacking, and until we know what immune processes they are regulating, their use would be a mere trial and error process. In any case, some attempts to explore their potential have been made in some fish species.

Interferon regulatory factors (IRFs) form a large family of transcription factors. IRF-1 has been shown to have a role in cytokine signalling and host defence against pathogens. For example, IRF-1 is up-regulated in response to virus infection in fish cells, inducing an antiviral state [101]. In a recent study, the potential use of IRF-1 as a vaccine adjuvant was investigated in Japanese flounder. The co-injection of IRF-1 with a DNA vaccine encoding the major capsid protein (MCP) gene of red sea bream iridovirus (RSIV) resulted in elevated serum neutralisation antibodies but was not significantly different from that in the fish vaccinated with the DNA vaccine alone [102]. Despite the moderate effect in protection, in this study, IRF-1 was responsible for the up-regulation of antiviral substances like nitric oxide (NO), interferon ß (IFN ß) and interferon inducible genes such as Mx.

Interleukin 8 (IL-8) is a CXC chemokine produced by many cell types in mammals like macrophages, monocytes, epithelial cells, neutrophils and fibroblasts upon infection, or stimulated by cytokines like IL-1ß and tumor necrosis factor a (TNF-
In mammals, chemokines have been widely used as adjuvants in vaccines against viral infections, since not only they attract more cells to the site of inflammation, but they also regulate the immune functions of the recruited cells. In fish, IL-8 has been characterised in rainbow trout among other species, and its chemo attractant properties established [103]. In this species, a vaccine plasmid coding for the glycoprotein gene of VHSV was co-injected with a plasmid coding for rainbow trout IL-8 to explore its potential adjuvant effect [104, 105]. When the plasmid coding of IL-8 (pIL-8+) was administered together with the VHSV vaccine, an increase of IL-1β in the spleen was found together with a greater cellular infiltration at the site of inoculation. Furthermore, fish injected with pIL-8+ alone showed a significantly higher expression of TNF-α, IL-11, TGF-β and IL-18 in the spleen [104]. In a further study, the transcription of different inducible CC chemokines were studied in rainbow trout in response to both the VHSV DNA vaccine and/or pIL8+, demonstrating that when IL-8 is used as an adjuvant, the expression of other chemokines such as CK5A, CK6, CK7 and CK5B is also modulated [105]. All these results showed that IL-8 was able to modulate the early immune response and could be a potent vaccine adjuvant in fish against viral infections.

Administration of IL-1β-derived peptides to rainbow trout by i.p. injection induced reduced mortality of fish when exposed to VHSV after 2 days [106]. The peptides also induced leukocyte migration into the peritoneal cavity 1-3 days post-injection, however its possible use as adjuvant was not further explored. The role of IL-1β as an adjuvant was investigated in carp after i.p. injection of killed *A. hydrophila* in the absence and presence of recombinant C-terminal peptide of carp IL-1β. It was found that the agglutinating antibody titre was significantly higher in the fish injected with killed bacteria plus recombinant IL-1β peptide compared with killed bacteria alone 3 weeks after vaccination [107].
Conclusive remarks and perspectives

The development of effective vaccines should be approached by combining the search for protective antigens together with the application of specific, and targeting, adjuvants that maximise the immunogenicity with a desired immune response. These vaccine-specific adjuvants may be able to trigger specific immunological processes, without producing a generalised response with strong side effects. However, an obvious consequence for the lack of detailed knowledge on vaccine potency and efficacy using novel adjuvants such as the TLR ligands or cytokines - is that the vaccine producers may use oil-adjuvants instead for simplicity reason. The oil adjuvants, being able to induce very strong and durable immune responses may “overshadow” significant protective mechanisms that have been overlooked up till now. Thus, the search for real molecular correlates of protection should be pursued with strong efforts. In future vaccine research, the immunostimulatory potential of a given substance followed by vaccine potency and efficacy studies should be unequivocally established in the context of vaccination. Only then, we will be able to convince the pharmaceutical industry to move from traditional adjuvants to more sophisticated adjuvants that specifically trigger adequate immune responses that may be optimised for a specific pathogen.

Acknowledgements

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References

[9] Steinman L. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. Nat Med 2007; 13: 139-145.


Ninomiya K, Yamamoto M. Efficacy of oil-adjuvanted vaccines for bacterial hemorrhagic ascites in ayu Plecoglossus altivelis. Fish Patology 2001; 36: 183-185.


[81] Plant KP, Harbottle H, Thune RL. Poly I:C induces an antiviral state against Ictalurid Herpesvirus 1 and Mx1 transcription in the channel catfish (Ictalurus punctatus). Dev Comp Immunol 2005; 29: 627-635.


[104] Jimenez N, Coll J, Salguero FJ, Tafalla C. Co-injection of interleukin 8 with the glycoprotein gene from viral haemorrhagic septicemia virus (VHSV) modulates the cytokine response in rainbow trout (Oncorhynchus mykiss). Vaccine 2006; 24: 5615-5626.
Table 1. Adjuvants, central components, receptors/process and principal immunological responses elicited by licensed and experimental adjuvants mainly for human medicine.
Adapted from Coffman et al. [57].

<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>Central immunostimulatory component(s)</th>
<th>PPR/Process</th>
<th>Principal immune response elicited</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alum MF59 and AS03</td>
<td>Aluminum salts, Squalene in water emulsions</td>
<td>NLRP3 (?)</td>
<td>Ab, Th2 (+Th1 in humans)</td>
</tr>
<tr>
<td>AS04</td>
<td>MPL + Alum</td>
<td>TLR4 and NLRP3 (?)</td>
<td>Ab and Th1</td>
</tr>
<tr>
<td><strong>Adjuvants in experimental use or in late stage clinical development</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly I:C</td>
<td>Synthetic dsRNA</td>
<td></td>
<td>Ab, Th1, CTL</td>
</tr>
<tr>
<td>MPL, and in diff. formulations</td>
<td></td>
<td></td>
<td>Ab, Th1</td>
</tr>
<tr>
<td>Flagellin, flagellin-Ag fusion proteins</td>
<td>Recombinant flagellin from bacteria</td>
<td>TLR5</td>
<td>Ab, Th1 + Th2</td>
</tr>
<tr>
<td>Imiquimods</td>
<td>Imidazoquinoline derivatives</td>
<td>TLR7, TLR8 and both</td>
<td>Ab, Th1, CTL (when conjugated)</td>
</tr>
<tr>
<td>CpG, and in different formulations</td>
<td>Synthetic phosphorthioate-linked DNA oligonucleotides with optimized CpG motifs</td>
<td>TLR9</td>
<td>Ab, Th1, CTL (when conjugated)</td>
</tr>
<tr>
<td>ISCOMS</td>
<td>Saponins</td>
<td>Not defined</td>
<td>Ab, Th1 + Th2, CTL</td>
</tr>
<tr>
<td>IFA (and montanide formulations)</td>
<td>Mineral or paraffin oil + surfactant</td>
<td>Not defined</td>
<td>Ab, Th1 + Th2</td>
</tr>
<tr>
<td>CFA</td>
<td>IFA + peptidoglycan, trehalose dimycolate</td>
<td>NLR, TLR?</td>
<td>Ab, Th1, Th17</td>
</tr>
</tbody>
</table>

Abbreviations and descriptions: MF59 (Novartis proprietary adjuvant MF59 containing squalene, polyoxyethylene sorbitan monooleate and sorbitan trioleate), AS03 (GlaxoSmithKline) contains squalene, DL-α-tocopherol, polysorbate), AS04 (Aluminum hydroxide and monophosphoryl lipid A (MPL), ISCOMs (immune stimulating complex; nanostructure of cholesterol, phospholipids and Quil-A saponis), IFA (incomplete Freund’s adjuvants). Ab: antibodies.
Table 2. Adjuvants currently used in fish vaccines commercialised by the main fish vaccine manufacturers.

<table>
<thead>
<tr>
<th>Company</th>
<th>Vaccine name</th>
<th>Pathogen</th>
<th>Adjuvant</th>
<th>Immunization route</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PHARMAQ</strong></td>
<td>Alpha Ject and Alpha Marine vaccines</td>
<td>Different bacterial and viral pathogens</td>
<td>Mineral oil</td>
<td>i.p.</td>
</tr>
<tr>
<td></td>
<td>Alpha Dip</td>
<td></td>
<td>No adjuvant</td>
<td>Immersion</td>
</tr>
<tr>
<td><strong>MSD Animal Health</strong></td>
<td>AquaVac</td>
<td>A. salmonicida, Y. ruckeri, Vibrio</td>
<td>No adjuvant</td>
<td>i.p., immersion</td>
</tr>
<tr>
<td></td>
<td>AquaVac FNMPlus</td>
<td>A. salmonicida</td>
<td>Montanide ISA711</td>
<td>i.p.</td>
</tr>
<tr>
<td></td>
<td>Norvax Compact PD</td>
<td>Salmonid alphavirus (SAV1 and SAV3)</td>
<td>Montanide ISA763A</td>
<td>i.p.</td>
</tr>
<tr>
<td></td>
<td>AquaVac ERM Oral</td>
<td>Y. ruckeri, Vibrio</td>
<td>No adjuvant</td>
<td>oral</td>
</tr>
<tr>
<td><strong>Novartis</strong></td>
<td>Birnagen Forte As</td>
<td>A. salmonicida and infectious pancreatic necrosis virus (IPNV)</td>
<td>Mineral oil (Drakeol 6VR)</td>
<td>i.p.</td>
</tr>
<tr>
<td></td>
<td>Ermogen</td>
<td>Y. ruckeri</td>
<td>No adjuvant</td>
<td>Immersion</td>
</tr>
<tr>
<td></td>
<td>Apex®-IHN</td>
<td>IHNV</td>
<td>No adjuvant</td>
<td>i.m.</td>
</tr>
</tbody>
</table>