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Liposomes as immunostimulant delivery nanosystems

characterization and application in zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*)

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Abstract
(Resum)

The innate immune system is based on the non-specific recognition of conserved elements of the pathogenic metabolism. This recognition is primarily mediated by germ line encoded pattern-recognition receptors (PRRs), present in specialized cells of the innate immune system, that are able to recognize conserved molecular patterns associated to pathogens (PAMPs). This recognition will trigger different signaling pathways that will induce the transcription of pro-inflammatory cytokines and result in local inflammation. Therefore, the innate immune system can be modulated by administration of these PAMPs, simulating a natural pathogen–immune system encounter.

The main hypothesis of this study was that, by encapsulation in the same nanoscaled delivery system, of several PAMPs, also called immunostimulants, we could improve their administration to different fish species. Also, that this delivery system would interact with the cells of the immune system generating its non-specific activation and improving the immune response against different infectious diseases. In this context, a novel immunostimulant delivery nanosystem based on liposomes encapsulating a bacterial lipopolysaccharide from *Escherichia coli*, and a synthetic analog of viral dsRNA, poly (I:C), has been developed. Our data shows that, these biocompatible liposomes were able to be endocytosed *in vitro* by zebrafish (*Danio rerio*) hepatocytes and rainbow trout (*Oncorhynchus mykiss*) macrophages as well as to regulate the expression of immune related genes.

We have also developed a method for *in vivo* imaging of nano-sized liposomes in adult zebrafish, which allowed us to follow the dynamics and the target tissues of the liposomes administered either by intraperitoneal injection or immersion. The biodistribution results showed that the delivery system accumulated mainly in the spleen of zebrafish and in immune relevant cells, such as macrophages, from rainbow trout. Moreover, we showed that these liposomes, administrated by intraperitoneal injection and immersion, could effectively protect zebrafish from bacterial (*Pseudomonas aeruginosa* PAO1) and viral (spring viraemia of carp virus) infections.

In conclusion, these findings suggest that the stimulation of the innate immune system with liposomes encapsulating a bacterial lipopolysaccharide and the synthetic analog of viral dsRNA, poly I:C, could be a good strategy to achieve protection against bacterial and viral infections therefore potentially working as a non-specific prevention tool in fish.

El sistema immunitari innat es basa en el reconeixement no específic d'elements conservats del metabolisme dels patògens. Aquest reconeixement es fa principalment a través de receptors de reconeixement de patrons (PRRs) codificats per la línia germinal, que són presents a cèl·lules especialitzades del sistema immunitari innat, i que són capaços de reconèixer patrons moleculars conservats associats a patògens (PAMPs). Aquest reconeixement iniciarà diferents vies de senyalització que induiran la transcripció de citoquines proinflamatòries per finalment donar lloc a una inflamació local. D'aquesta manera, el sistema immunitari innat pot ser modulat, a través de l'administració d'aquests PAMPs, simulant una trobada natural entre el sistema immunitari i els patògens.

La principal hipòtesi d'aquest estudi va ser que, mitjançant l'encapsulació en un mateix sistema d'administració nanomètric de diversos PAMPs, també anomenats immunoestimulants, es podria millorar la seva administració a diferents espècies de peixos. També, que aquest sistema d'administració podria interaccionar amb les cèl·lules del sistema immunitari generant la seva activació no específica, i millorant la resposta immunitària contra diferents malalties infeccioses. En aquest context, s'ha desenvolupat un nou sistema d'administració d'immunoestimulants basat en liposomes que encapsulen el lipopolisacàrid bacterià d'*Escherichia coli*, i un anàleg sintètic de dsRNA viral, el poli (I:C). Els nostres resultat van mostrar que aquests liposomes eren biocompatibles i capaços de ser endocitats *in vitro* per hepatòcits de peix zebra (*Danio rerio*) i per macròfags de truita irisada (*Oncorhynchus mykiss*). Així mateix, els liposomes van poder modular *in vitro* l'expressió de diversos gens relacionats amb la immunitat.

També s'ha desenvolupat un mètode per a la captació d'imatges *in vivo* dels liposomes nanomètrics en adults de peix zebra. Això ens va permetre seguir la dinàmica i els teixits diana dels liposomes administrats tant per injecció intraperitoneal com per immersió. Els resultats dels estudis de biodistribució van demostrar que els liposomes s'acumulaven principalment a la melsa del peix zebra i en cèl·lules del sistema immunitari com ara macròfags de truita irisada. D'altra banda, hem demostrat que aquests liposomes, administrats mitjançant injecció intraperitoneal i immersió, podrien protegir de manera efectiva el peix zebra tant d'una infecció bacteriana (*Pseudomonas aeruginosa* PAO1) com viral (virèmia primaveral de carpa).

En conclusió, els resultats suggereixen que l'estimulació del sistema immunitari innat amb liposomes que encapsulen un lipopolisacàrid bacterià i l'anàleg sintètic de dsRNA viral, poli(I:C), podria ser una bona estratègia per aconseguir la protecció contra infeccions bacterianes i virals, i que per tant, es podria utilitzar potencialment com una eina no específica per a la prevenció d'infeccions en peix.

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Abbreviations

AF750 Alexa Fluor 750

APC Antigen presentation cell

BCR B cell receptor

CCL4 Chemokine (C-C motif) ligand 4

CD Cluster of differentiation

Chol Cholesterol

CpG ODN Cytosine-guanine oligodeoxynucleotide

CQ Chloroquine

CTL Cytotoxic T lymphocyte

DC Dendritic cell

DLPC 1,2-didodecanoyl-sn-glycero-3-phosphocholine

DOPA 1,2-dioleoyl-sn-glycero-3-phosphoric

dsRNA Double stranded RNA

EIPA 5-(N-Ethyl-N-isopropyl)amiloride

FACS Fluorescence-activated cell sorting

FDA Food and Drug Administration

GIG-2 Grass carp reovirus-induced gene-2

IFN Interferon

Ig Immunoglobulin

IL Interleukin

IRF3 Interferon regulatory factor 3

IS Immunostimulant

LDH Lactate dehydrogenase

LPS Lypopolysaccharide

MβCD Methyl-β-cyclodextrin

MDA-5 Melanoma differentiation-associated gene 5

MD2 Myeloid differentiation factor 2

MFI Mean fluorescence intensity

MHC Major histocompatibility complex

MMP9 Matrix metalloproteinase 9

MPL Monophosphoryl lipid A

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MyD88 Myeloid differentiation factor 88

Mx Myxovirus resistance

NF-κB Nuclear factor-κappa-light chain-enhancer of activated B cells

NK Natural killer

NL_c Nanoliposomes encapsulating the cocktail of immunostimulants (LPS and Poly (I:C))

$NL_{LPS/poly(I:C)}$ Nanoliposomes encapsulating LPS or Poly (I:C)

NL_n Non-encapsulating nanoliposomes

NLR Nod like receptor

NO Nitric oxide

PAMP Pathogen Associated Molecular Patterns

PEG Polyethylene glycol

PGN Peptidoglycan

PGRP Peptidoglycan recognition proteins

PLGA Poly(lactic-co-glycolic acid)

Poly (I:C) Polyinosinic polycytidylic acid

PRR Pattern-recognition receptor

RIG-I Retinoic acid-inducible gene-I

RLR RIG-I-like receptor

RPS Relative percentage of survival

SVCV Spring viraemia of carp virus

TCR T cell receptors

Th T helper

TIR Toll/interleukin-1 receptor

TLR Toll-like receptor

TNFα Tumor necrosis factor-α

TRAM TRIF-related adaptor molecule

TRIF TIR-domain-containing adapter-inducing interferon-β

VHSV Viral hemorrhagic septicemia virus

Introduction

Aquaculture and disease

Aquaculture is the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. Aquaculture supplements the amount of fish obtained by the wildcapture fisheries, offering a solution for the growing protein demand related to the world's growing population. Aquaculture has also an enormous relevance to relieve pressure on wild fish populations as the main stocks of some species are almost fully exploited or very close to their maximum sustainable limits [1–3]. It is important to mention, thought, that the development of this industry has to be both environmentally and socially sustainable in the long term [3].

In the 1960's, aquaculture became a significant commercial practice in Asia, where it had mainly been used as a small-scale means of local community food production for thousands of years. In the last few decades, worldwide aquaculture production has increased significantly. In 1970 aquaculture represented 3.9 % of all fish production, compared to 42.3 % in 2012 [1].

The world aquaculture production in 2012 was 90.43 million tonnes, including food fish, aquatic algae and non-food products [1]. Of those, China alone produced 43.5 million tonnes of food fish and 13.5 million tonnes of aquatic algae that year, whereas some developed countries slightly reduced their aquaculture output in recent years, mainly due to competition from countries with lower production costs. It is estimated that a great diversity of over 600 aquatic species are cultured worldwide normally covering three stages: incubation/hatchery, early rearing and on-growing. 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 [1, 4]. Aquaculture can be carried out in very different methods, for instance, in large recirculating systems or in net cages. In these latter cases, farmed fish are reared in net cages near shore (for marine aquaculture) or in a lake or river (for inland aquaculture) in direct contact with the wild fish reservoire and exposed to pathogens [4, 5].Modern intensive and semi-intensive aquaculture practices are extremely vulnerable to the pollution and disease outbreaks [3]. Infectious disease is a major problem as water-borne pathogens can spread at very fast rates and transmit disease across vast geographic regions. When combined with the crowded conditions of the aquaculture facilities and the warmer temperatures, it provides ideal conditions for disease outbreaks [6–8]. An infectious disease outbreak will mostly lead to severe mortality, requiring also costly decontamination of the facilities and equipment. Therefore, disease has been identified as an important limiting factor to aquaculture production [6]. The most common causative agents of infectious diseases in aquaculture are bacteria (54,9%), followed by virus (22,6%), parasites (19,4%) and fungi (3,1%) [5] (**Figure 1**). This rise in pathology has been accompanied by an increased use of a wide range of chemicals for disease treatment including antimicrobials, which led to bacteria resistance problems [9]. Some examples of the major bacterial diseases are for instance enteric red mouth (ERM) disease or yersiniosis caused by *Yersinia ruckeri*, vibriosis caused by *Listonella anguillarum* and *Vibrio spp.* or furunculosis caused by *Aeromonas salmonicida* [4]. On the other hand, viral diseases have been more difficult to control mostly due to the lack of therapeutics, and some of them are reportable to the World Organization for Animal Health (OIE) [5]. Some of the reportable finfish viral diseases are the koi herpesvirus (KHV) disease caused by the koi herpes virus from the family *Alloherpesviridae*, the viral haemorrhagic septicaemia (VHS) caused by the VHS virus and the spring viraemia of carp (SVC) caused by the SVC virus. both from the family *Rhabdoviridae* [5].

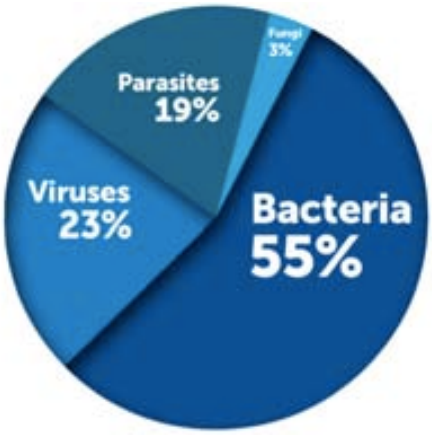

Figure 1. **Schematic of the percentage of total aquaculture diseases caused by each family of pathogens.**

A bacterial or viral outbreak can also have a severe social impact. This was the case, for instance, of the infectious salmon anaemia (ISA) outbreaks that occured in Chile in 2007, probably linked to importation of fertilized salmon eggs from Norway in 1996. Chile experienced exponential growth of the Atlantic salmon industry from 1987 to 2004, but at that time, the production started to show a decline with an increasing mortality due to ''non-identified'' causes. Economic losses due to the outbreak in 2007 were estimated to affect 9% of the total chilean aquaculture industry ($20 million) and approximately, 3.0% reduction in the workforce. In 2009, the outbreak accounted for a 60% drop in Atlantic salmon production, and full recovery was not expected before 2013 with its obvious social impact on the whole chilean economy [5, 10].

This massive increase in aquaculture production and its subsequent related diseases have put greater emphasis on studies of the fish immune system and defence against pathogens. This will eventually lead to a better disease control through prophylactic measures (such as, vaccination, probiotics and immunostimulation), which are far more preferable than treatment measures.

The teleost immune system

The main functions of the immune system are the recognition and elimination of foreign substances and the maintenance of the homeostasis. It can be divided in two major branches: the innate system (non-specific) and the adaptive system (antigen-specific) [11]. The innate system is the first sensor and barrier of pathogenic infections and it is characterized by its rapid appearance, aiming to limit the spread of infection and modulate the consecutive adaptive response. On the other hand, the adaptive system is antigen-specific and is based on receptors generated by somatic recombination of segments of germ line encoded genes affected by the RAG (Recombinase Activating Genes) enzymes.

Evolutionary speaking, the immune system is present in all the metazoan species. This does not mean, though, that the recognition events and the resulting effector reactions are conserved through species [12]: some features may be conserved while some other will be specific to one phylum or even one class. Most of the mechanisms conserved between invertebrates and vertebrates are related to innate immunity [13]. The generation of an adaptive immune system arised in vertebrates, and it seems to have occurred abruptly in the direct ancestors of jawed vertebra-

tes 500 million years ago [12], [14] (**Figure 2**). Thus, cartilaginous fish are the earliest living organisms with a primitive adaptive immune system, as they have immunoglobulins, T cell antigen receptors, major histocompatibility complex (MHC) class I and II molecules, spleen and thymus [15].

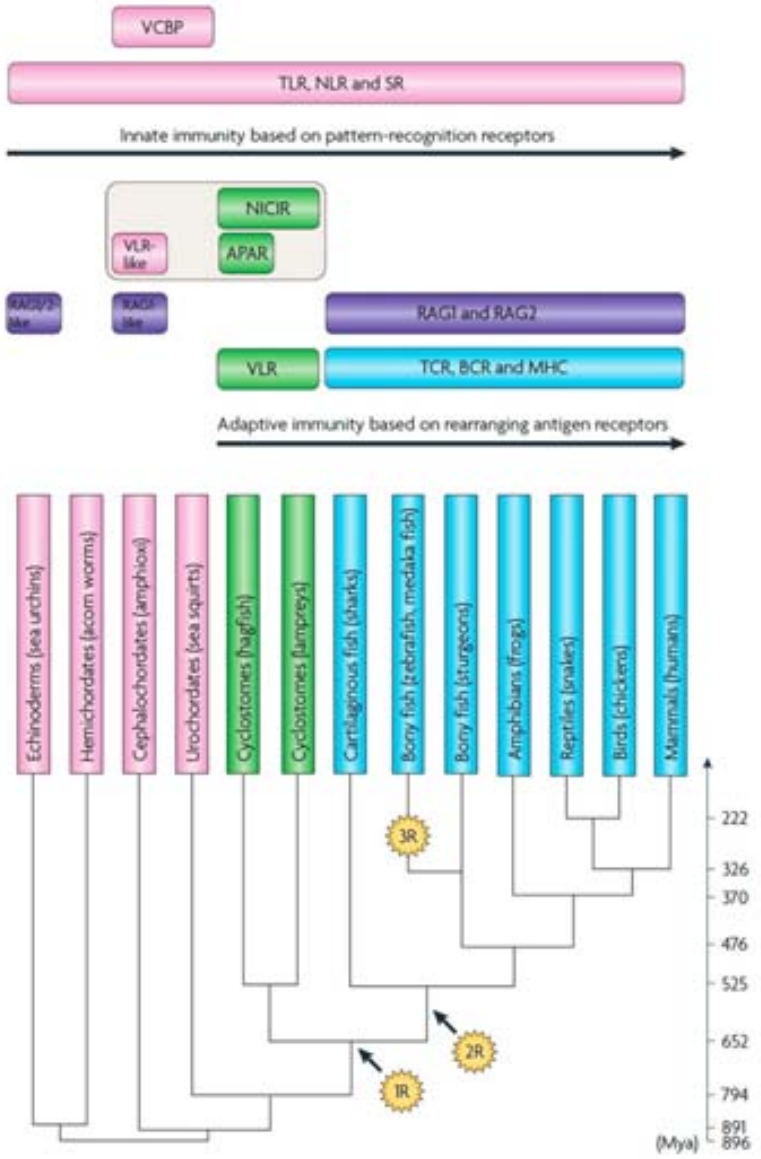

Figure 2. **Overview of the evolution of the immune system in deuterostomes.**

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 also present in the genomes of sea urchins and amphioxi. 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 shown. 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 (from [14]).

The innate immune system is based on the non-specific recognition of conserved elements of the pathogenic metabolism. This recognition is primarly mediated by germ line encoded pattern-recognition receptors (PRRs) that are able to recognize conserved molecular patterns associated to pathogens (Pathogen Associated Molecular Patterns, PAMPs). Specialized cells of the so-called myeloid lineage in vertebrates, such as macrophages and neutrophils, contain a wide spectrum of receptors responsible for the recognition of PAMPs, such as peptidoglycans (PGN) and lypopolysaccharides (LPS), fungal β1,3-glucan, viral double stranded RNA and bacterial DNA. This recognition can induce opsonization and phagocytosis of the pathogen, stimulate natural cytotoxic cells or activate different signalling processes for the attraction of other immune cells to the site of infection, which will result in a local inflammation [11, 16].

In contrast to the large repertoire of rearranged receptors used by the acquired resistance, the **recognition receptors (PRRs) of the innate system** are relatively few and are vertically transmitted [11]. These PRRs share some common features. First, PRRs recognize pathogen components that are essential for the survival of the microorganism and therefore, difficult for the microorganism to alter. Second, PRRs are expressed constitutively in the host and are functional regardless of their life-cycle. And third, PRRs are germline encoded, expressed on a given type of cells and independent of immunologic memory [17, 18]. There are different types of PRRs (**Table 1**), including the Toll-like receptors (TLRs), NOD-like receptors (NLRs), retinoic acid-inducible gene- I (RIG-I)-like receptors (RLRs), membrane-bound C-type lectin receptors (CLR) and peptidoglycan recognition proteins (PGRPs). However, the first to be indentified and the most well characterized PRRs are the TLRs [19].

TLRs are type I transmembrane proteins which comprise an ectodomain with leucine-rich repeats that mediate in the recognition of PAMPs, a transmembrane region, and a cytosolic Toll-

PRR	Location	Recognized PAMPs	Mediated signaling pathway	Adaptors
TLR				
TLR1*	Plasma membrane	Triacyl lipoprotein	Associate with TLR2 mediated signaling pathway	TIRAP; MyD88
TLR2*	Plasma membrane	Lipoproteins; LAM	NF-κB signaling pathway	TIRAP; MyD88
TLR3*	Endosome	dsRNA; poly(I:C)	NF-κB signaling pathway; IRF3 mediated type I IFN production	TRIF
TLR4*	Plasma membrane	LPS	NF-κB signaling pathway	TIRAP; MyD88
	Phagosome	GIPIs; LPS	NF-κB signaling pathway; IRF3 mediated type I IFN production	TRAM; TRIF
TLR5*	Plasma membrane	Flagellin	NF-κB signaling pathway	MyD88
TLR6*	Plasma membrane	Diacyl lipoprotein	Associate with TLR2 mediated signaling pathway	TIRAP; MyD88
TLR7*TLR8*	Endosome	ssRNA	NF-κB signaling pathway; IRF7 mediated type I IFN production	MyD88
TLR9*	Endosome Lysosome-related organelles (LRO)	CpG DNA	NF-κB signaling pathway IRF7 mediated type I IFN production	MyD88 MyD88
TLR10	Endosome	Unknown	Unknown	Unknown
TLR11*	Plasma membrane	Profilin	NF-κB signaling pathway	MyD88
RLR				
RIG-1*	Cytoplasm	dsRNA	NF-κB signaling pathway; IRF3 and IRF7 mediated type I IFN production	MAVS
MDA5*	Cytoplasm	Poly(I:C); dsRNA	NF-κB signaling pathway; IRF3 and IRF7 mediated type I IFN production	MAVS
NLR				
NOD1*	Cytoplasm	iE-DAP dipeptide; Virus	NF-κB signaling pathway; IRF3 mediated IFNβ production	RIP2
NOD2*	Cytoplasm	MDP; ssRNA	NF-κB signaling pathway; MAPK signaling pathway; IRF3 mediated IFNβ production	RIP2; MAVS
NLRC4	Cytoplasm	Flagellin	Inflammasome mediated signaling pathway; IL-1β mediated signaling pathway	-
NLRP1	Cytoplasm	Lethal toxins; MDP	Inflammasome mediated signaling pathway; IL-1β mediated signaling pathway	-
NLRP3	Cytoplasm	Toxins; Intracellular bacteria derived molecules; Viral ssRNA and dsRNA	Inflammasome mediated signaling pathway; IL-1β mediated signaling pathway	-
CLR				
DC-SIGN*	Plasma membrane	High mannose; Fucose	RAF1-p65 acetylation pathway; NF-κB signaling pathway	-
Dectin-1	Plasma membrane	β-1,3-glucan	SYK-CARD9 pathway; NF-κB signaling pathway; IL-1β mediated signaling pathway	-
Dectin-2	Plasma membrane	High mannose	SYK-CARD9 pathway; NF-κB signaling pathway	-
Mincle	Plasma membrane	α-Mannose	SYK-CARD9 pathway; NF-κB signaling pathway	-

Table 1. **Pattern recognition receptors (PRRs).**

* indicate PPRs identified in fish. Abbreviations: GIPIs, Glycoinositolphospholipids; LAM, Lipoarabinomannan; iE-DAP, g-D-glutamyl-meso-diaminopimelic acid; MDP, muramyl dipeptide; MAPK, Mitogen-activated protein kinase; SYK, spleen tyrosine kinase; CARD9, caspase-recruitment domain family member 9. (modified from [94]).

IL-1 receptor (TIR) domains to activate downstream signalling pathways. Expression of TLRs is not static as it can be modulated rapidly in response to pathogens, cytokins or enviromental stresses. To date, 12 members of the TLR family have been identified in mammals [19], each one detecting one kind of PAMP, although some can recognize a diverse collection of ligands. For instance, TLR4 in mammals can either recognize lipopolysaccharide (LPS), the fusion protein of respiratory syncytial virus (RSV), fibronectin and heat-shock proteins. The cellular localization of TLRs can also be related to the pathogen component that they recognize. Thus, TLR1, TLR2, TLR4, TLR5 and TLR6 are mainly localized on the cell surface and they largely recognize microbial membrane components, whilst TLR3, TLR7, TLR8 and TLR9 are mostly expressed within intracellular vesicles and recognize nucleic acids delivered after the uptake of viruses and other pathogens.

As regards to the teleost TLRs, it is hypothesized that the ligand specificity is highly conserved in all vertebrates with clear orthologous relationships. That is the case, for instance, of the TLR5 that recognizes the flagellin protein component of bacterial flagella in mammals and zebrafish [16]. Nevertheless, differences in TLR repertoire exist: while the human genome contains 10 functional TLRs, most fish species present a higher number of TLR genes, most likely related to the early genome duplication of the teleost lineage [20]. Up to 17 TLR types (TLR1, 2, 3, 4, 5, 5S, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, and 23) have been identified in more than a dozen teleost species [21] . Their orthology suggests that they possess the same type of pathogen recognition mechanism as their mammalian counterparts [22], although there are also some remarkable differences. For example, the development of TLR4 mediated endotoxic shock in mammals in contrast to its absence in non-mammalian vertebrates even with the presence of a TLR4 receptor in some species [23]. Most fish lack TLR4 homologues, with the exception of cyprinids (such as zebrafish, rare minnow or carp) and silurids (channel catfish) (see [23] for a review), but different functional studies have proved that these TLR4 homologues do not sense LPS and do not activate the corresponding signalling pathways [24]. Some fish species possess additional TLRs, such as TLR11, 14, 20, 21, 22 and 24, which have not been found in mammals so far, although their existance might not exclude the mammalian-conserved equivalents. For example, both TLR21 and TLR9 can sense CpG-oligodeoxynucleotides, and they cooperate in mediating CpG activity in zebrafish [16].

Stimulation of the TLR by their ligand causes recruitment of the adapter protein MyD88 or other molecules such as MAL, SARM or TRIF. These adaptors are required for activation of MAP kinase family members as well as NF-κB nuclear translocation, which, in turn, activates transcription of pro-inflammatory cytokines [25]. Not only the TLRs themeselves but also the TIR domain-adaptor proteins in their signalling pathways, such as the above mentioned MyD88 and SARM, are mostly conserved among vertebrates [26, 27].

The innate immune system is a collection of physical, humoral and cellular components that have been classified into distinct **modules** [28]. Each module carries out different functions in host defense. Besides, some modules are co-induced by an infection and are usually co-regulated by the same inducing signals, most commonly cytokines. These modules are: (1) mucosal epithelia (which produce mucins and antimicrobial peptides); (2) phagocytes (macrophages, neutrophils and dendritic cells); (3) acute-phase proteins and complement system (for opsonization, recruitment of phagocytes and direct killing of pathogens); (4) inflammasomes (for activation of pro-inflammatory caspases); (5) cytotoxic natural killer cells (for cell apoptosis and cytokine production); (6) cytokines and chemokines (mainly IL-1β, tumor necrosis factor-α, transfor-

ming growth factor-β and type I interferons); and finally (7) eosinophils, basophils and mast cells (mainly for parasite protection) [28].

Teleost have most if not all the features of the innate system present in mammals [15]. And even some components, like the complement, lectins (acute-phase proteins) and natural killer cell receptors, are more diversed than that of mammals [29–31]. This is most likely due to the constraints placed on the adaptive immune response: the limited antibody repertoires, affinity maturation and memory [32]. The mucous surfaces of skin, gills and intestine have an important role in fish as they are aquatic organisms and they are constantly exposed to microbes and stressors. In fact, besides being their first physical barriers, they are also active immunological sites armed with cellular and humoral defenses [33]. Phagocytosis has also great importance in fish as they are poikilothermic animals, and this process is least influenced by temperature [34]. It is also mainly performed by neutrophils (also named acidophilic granulocytes), dendritic cells, monocytes and macrophages [35–37], although lately, it has been demonstrated that B lymphocyte cells from teleosts are also phagocytic and even display the ability to kill internalized bacteria [38]. In fact, theses studies let to the discovery that also B cells from reptilians and even certain mammalian B cell subsets have a significant phagocytic capacity [39]. Macrophages are most commonly associated with the greatest phagocytic capacity, while the role of the acidophilic granulocytes is to actively engulf any potential pathogen at the innfammatory site, and to recruit other immune cells to the site of infection by releasing degradative enzymes, antimicrobial molecules, and toxic metabolites [35]. Dendritic cells, which besides being phagocytic are the main antigen presentation cells (APC) in mammals, have been also described in zebrafish (*Danio rerio*), medaka (*Oryzias latipes*) and rainbow trout (*Oncorhynchus mykiss*) [40–42].

The innate and adaptive immune responses are closely related and the integrated connections between them are highly complex. The innate pathogen recognition will trigger different pathways that will end up in the fast production of proinflammatory cytokines. This, in turn, will mediate the direct defense responses and also stimulate the adaptive immune response [28, 43]. Finally, the adaptive immune system, in a longer term, can also activate innate effector mechanisms in an antigen-specific manner [28] (**Figure 3**).

As previously mentioned, the **adaptive system** is antigen-specific and its receptors generated by somatic recombination are clonally distributed on T and B lymphocytes. This allows clonal selection of pathogen-specific receptors and is the basis for immunological memory: each lymphocyte expresses antigen receptors of a single specificity, and only specific populations of lymphocytes are selected to expand in response to a given pathogen. These receptors can be expressed on B lymphocytes either as antibodies (secretory form) or as B cell receptor (BCRs, membrane form), and on T lymphocytes as T cell receptors (TCRs, membrane form).

In adaptive immunity, two branches of reactions can be distinguished, namely humoral (antibody) and cell-mediated (cytotoxic) responses. When dendritic cells (DCs) and macrophages from the innate immunity encounter a pathogen, they phagocyte it and its protein constituents are processed into antigenic peptides, which are presented at their cell surface by the MHC Class II molecules [44]. On the other hand, the endogenously synthesized proteins are presented on MHC Class I molecules. Importantly, DCs and macrophages can also present exogenous proteins in MHC Class I molecules in a process called "cross-presentation" [44, 45].

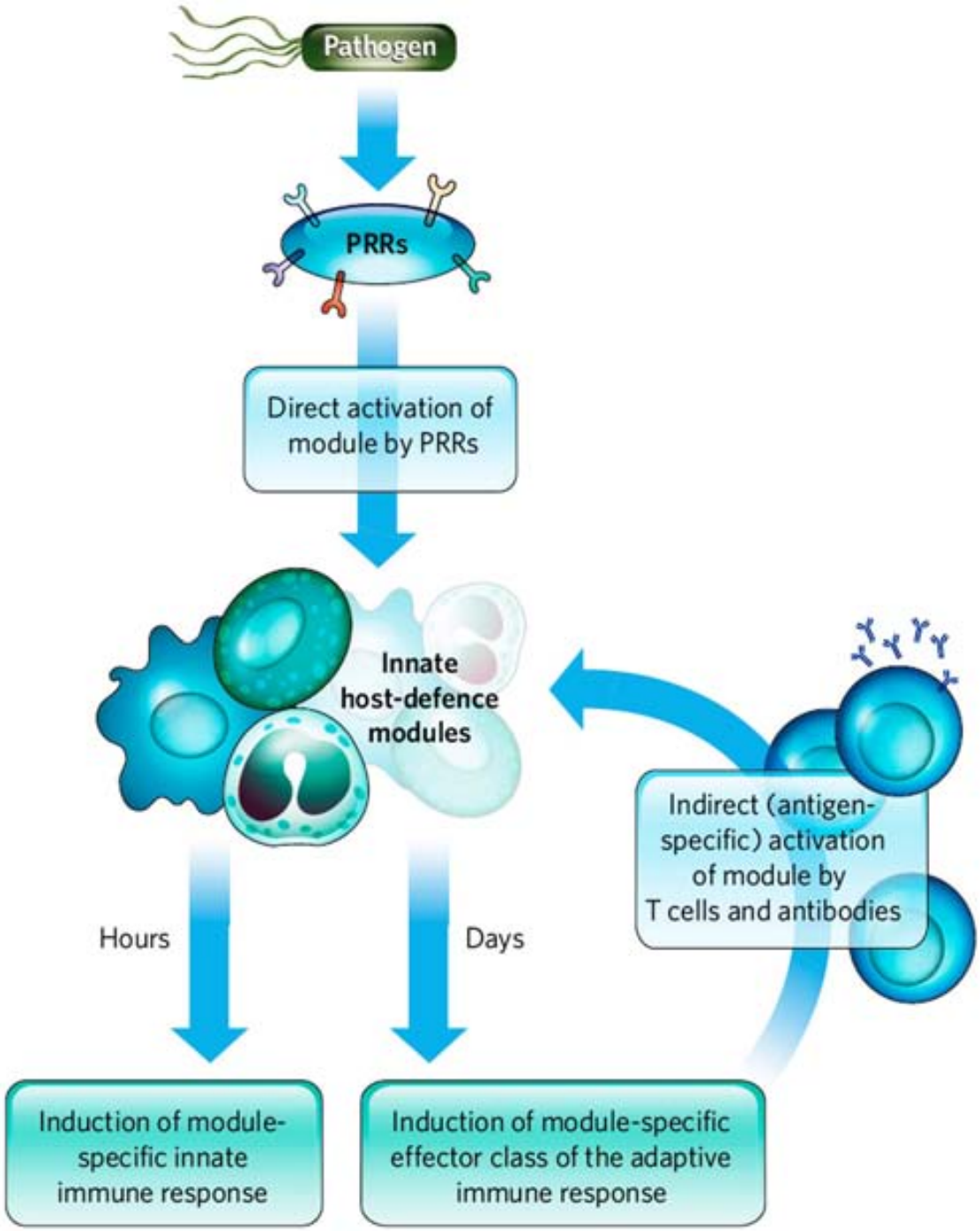

Figure 3. **Activation of host-defence mechanisms.**

Host-defence mechanisms can be induced directly, by engagement of PRRs, or indirectly, by T cells and/or antibodies. After an adaptive immune response has been initiated, it results in antigen specific activation of the same innate immune module that instructed the adaptive immune response (from [28]).

Afterwards, naive T cells are activated by interaction of their TCRs with the presented antigens: after MHC Class I presentation, $CD8^+$ T cells are activated, whereas MHC Class II presentation activates $CD4^+$ T cells. This $CD4^+$ T cells can differentiate into either T helper 1 (Th1) or T helper 2 (Th2) cells, depending on the cytokines that they release [46]. After secretion of these cytokines, T helper cells produce subsequent activation of $CD8^+$ T cells or B lymphocytes. Then,

$CD8^+$ T cells differentiate into Cytotoxic T cells (CTL), producing direct cell-mediated cytotoxic responses [47]. While, B lymphocytes produce antibodies that participate in the humoral response [8], [48] (**Figure 4**).

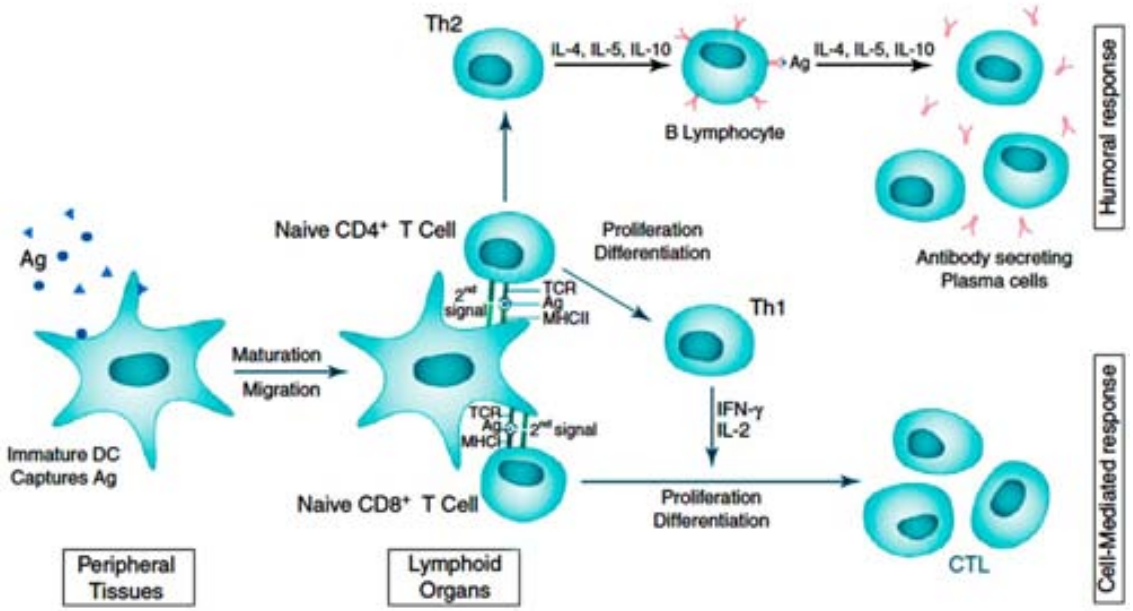

Figure 4. **Schematic overview of antigen presentation and Th priming.**

Upon encounter of DCs with antigen and PAMPs, migration to the lymph nodes and maturation is induced. In the lymphoid organs, Antigen (Ag) presentation via MHC class II and class I molecules leads to activation, proliferation and differentiation of respectively Ag-specific $CD4^+$ and $CD8^+$ T-cells. Activation of $CD4^+$ helper T-cells results in secretion of cytokines and subsequent activation of $CD8^+$ T-cells or B lymphocytes. The humoral branch of the immune response comprises activation of B lymphocytes followed by differentiation into antibody secreting plasma cells. Whereas the cellular response involves activation of $CD8^+$ T-cells followed by proliferation and differentiation into cytotoxic T-cells (modified from [45]).

The teleost adaptive system has some similarities with the mammalian one, although it has been largely hypothesized that it has a lower preeminent role compared to the innate immune system. One of the main differences is in terms of lymphoid tissues since they have spleen and thymus but lack bone marrow and limphatic ganglions/lymph nodes. In replacement of the bone marrow, the anterior part of the kidney (the head-kidney) is the main hematopoietic lymphoid tissue. Another important difference is the lack of class-switch recombination and the number of immunoglobulins (Ig). Indeed, only three classes of Ig have been described: IgM, the most prevalent in plasma; IgD, which role is still in controversy; and IgT which is speciallized in gut mucosal immunity [15, 49] (**Table 2**). Nevertheless some teleost species present unique particularities in their adaptive immune system. For instance, the genome sequence of the Atlantic cod revealed that it had lost genes for the MHC-II and the MHC-II interacting protein CD4, essential for this presentation pathway and T-cell activation. However the lack of these genes was not reflected in an increased susceptibility to disease. The authors suggested that it might be due to an expanded number of MHC-I genes and the unique composition of its TLR families [50]

Comparative developmental vertebrate immunology	Teleosts[a]	Mammals
Physical barriers and interfaces	Skin mucus; scales; gills	Skin; respiratory epithelium of the lungs
Immune effector cell types	Neutrophil/heterophil; Eosinophil; Monocyte/Macrophage; Dendritic cell (only identified in trout gill structures so far); NK cell; T lymphocytes (helper, cytotoxic); B lymphocytes (plasma cells)	Neutrophil; Eosinophil; Basophil; Mast cell Monocyte/ Macrophage; Dendritic cell; NK cell; T lymphocytes (helper, cytotoxic); B lymphocytes (plasma cells)
Major antigen presentation cells	Monocyte/macrophage	Dendritic cell, macrophage/monocyte
Lymphoid tissues	Head kidney; Thymus; Spleen; Gut-associated lymphoid tissue (not well organized); Interbranchial lymphoid tissue	Bone marrow; Thymus; Spleen; Lymph nodes; Gut-associated lymphoid tissue; Germinal centres
Antibody		
Ig Diversity	IgM; IgD; IgT	IgM; IgD; IgA; IgE; IgG
Response to challenge/ rechallenge	Slow and weak memory response (temperature dependent)	Fast and strong memory
Affinity maturation (AM)	Low affinity, and low AM	High affinity and high AM

[a] In particular, salmonids (information restricted to development in environments within physiological optima: 10–15 °C).

Table 2. **Comparative immune system of teleosts and mammals.**

modified from [5].

All together, we could conclude that teleost fish have a strong developed innate immune system, which represents their forefront on immune defense and a crucial factor for their disease resistance. They also have an adaptive immune response, which although being commonly delayed, is essential for its lasting immunity. The presence of both an innate and adaptive immune system has allowed the development of disease preventure measures by manipulation of their immune system through classical vaccinology, probiotics and immunostimulation [8].

Stimulation of the teleost immune system

Profilactic measures based on stimulation of the immune system of the fish have been effective for disease prevention. Importantly, this has allowed for a decrease in the use of antibiotics in aquaculture over the past 20 years, as its excessive use was known to produce resistant bacteria [51]. It is important to mention that proper fish management and limited stress are also key factors in the profilaxis of diseases.

The immunoprophylactic measures involve vaccinology, probiotic treatments and immunostimulation. This Thesis is focused on immunostimulants, although the other prophylactic measures will also be briefly discussed below.

Many experiments have shown how fish surviving an infection are more resistant to a second pathogen encounter. The basic aim of **vaccination** is to imitate that process by activating both innate and immune systems and to achieve a lasting protection due to the appearance of memory cells and specific antibodies. The first report of disease prevention using vaccines was published back in 1938. It showed the immunization of carps with *Aeromonas punctata* [52]. However, it has been in the last 10-20 years when vaccination against the most common diseases became extensive. The large industrial scale vaccination was initially developed for salmonids, but have now been implemented for several species. Vaccines can be made of formalin-inactivated pathogens, live attenuated pathogens, inactivated toxins, recombinant subunits and nucleic acids [53–55]. To date, there are vaccines available for 22 bacterial and 6 viral diseases, but yet no commercial vaccine available for fish parasites [4, 54, 56]. Although the great success of this field, developing fish vaccines is costly and time consuming, and it would probably not be profitable or realistic to develop vaccines against all pathogens identified [54].

One of the main factors to consider is the age of the fish at vaccination, as larvae and fry need to have a fully developed adaptive system when vaccination is attempted [57]. Another critical factor in terms of efficacy, cost and side-effects is the route of vaccination. The administration of vaccines can be performed orally through feed, by immersion in vaccine suspensions or by either intraperitoneal or intramuscular injection. Injection vaccination requires a relatively low amount of vaccine, and it has proven to give the longest protection even though it is more labour intensive, stressful, and has commonly been associated to side-effects at the injection site. Moreover, it is only appropiate for fish over a certain size (>15-20 g.). On the other hand, immersion vaccination can be applied to smaller fish in an easier and less stressful manner, but it requires larger volumes of vaccines and the degree and duration of immune protection is variable. Nevertheless, oral delivery would be the more suitable method for mass administration, as it would not require any change in the fish routine. Unfortunately, it has been found to confer only limited protection, mainly due to destruction of the antigen in the intestinal tract. This difference in the amount of protection between the three methods can also be explained since,

while injection is thought to produce a systemic immune response, immersion and oral delivery mainly induce an integumentary response in the mucous membranes of skin, gills and gut. (**Table 3**) [4, 5].

	Injection vaccination	Immersion vaccination	Oral vaccination
Target	Systemic	Skin and gills	Digestive system
Ease of administration	+(labour intensive)	++	+++ (mass vaccination of fish any size)
Fish weight	>15 g	1–5 g	1–5 g
Stress	Severe (requires anaesthetization & handling); Moderate for automated vaccination	None for baths; Moderate for spray; Severe for dip	None or negligible
Cost	Cost-effective for high value species*	Cost-effective for fish <10 g	Moderate
Efficacy/Potency	+++ (with adjuvants)	++	Weak (inferior)
Side-effects	Severe with oil-adjuvant	None or negligible	None or negligible
Duration of immunity	6–12 months	Shorter	Shortest

* High value species include Atlantic salmon and Rainbow trout.

Table 3. **Comparison of fish vaccination methods.**

modified from [5].

Another approach in the immunoprophylactic control is the use of probiotics and immunostimulants. Both treatments are primarily aimed at enhancing the innate system and therefore, they can be general prevention measures in aquaculture.

Probiotics are definied as live microorganisms which are able to persist in the digestive tract and have beneficial effects for the host. In practice, however, the term is also used for non-viable microbes. Currently, it is known that these microorganisms have an antimicrobial effect either through: (1) modifying the intestinal microbiota; (2) secreting antibacterial substances; (3) competing with pathogens to prevent their adhesion to the intestine; (4) competing for nutrients necessary for pathogen survival; and/or (5) producing an antitoxin effect [58, 59]. Probiotics are also capable of modulating the immune system, enhancing humoral and cellular immune parameters. The use of probiotics in aquaculture is relatively recent but currently, there are several commercial probiotic products prepared from various bacterial species, such as *Bacillus* sp., *Lactobacillus* sp., *Enterococcus* sp., *Carnobacterium* sp., and the yeast *Saccharomyces cerevisiae* among others [8, 58].

The term **immunostimulant**, covers a wide range of substances that activate the immune system through binding to pattern recognition receptors (PRRs), like TLRs. Immunostimulants are highly conserved molecules that can be bacterial, viral, plant or parasitic derivates as well as some synthetic compounds. Some examples are β-glucans, which are carbohydrates that form structural components of different organisms cell walls [60, 61], synthetic cytosine-guanine oligodeoxynucleotides (CpGs) that mimics bacterial or viral DNA [62], and also the synthetic double stranded RNA named Poly (I:C) and the bacterial Lipopolisaccharide (LPS). These two latter will be discussed in further detail below. These substances, or PAMPs (Pathogen Associated Molecular Patterns), have the advantage of directly binding intra- or extracellularly to the innate immune cells [43, 54]. Most of the studied PAMPs activate antigen presenting cells (APCs) together with naive T-cells, and may induce Th1 and Th2 responses with production of signature molecules such as IFN-γ and IL-4, respectively [47] (Figure 3). A Th1 response is more related to the elimination of intracellular pathogens through cell-mediated responses, whereas a Th2 response is more related to an humoral immune response.

The possible routes of administration are the same as the ones used for vaccines, although, immunostimulants are mostly distributed in the feed [8]. In fact, nowadays the use of in-diet immunostimulants has become widely accepted with several commercially available products. For instance, there are diets supplemented with nucleotides, like the AquagenTM (Novartis-Aqua Health Ltd., Charlottetown, Canada), which has proven to provide better protection against diferent infections [63, 64]. They are also supplemented with beta 1,3/1,6 glucans from the yeast *Saccharomyces cerevisiae,* like the MacroGard® (Biotec Pharmacon ASA, Tromsø, Norway), which modulate the immune response in a favorable way and has been used for almost 25 years in animal husbandry and aquaculture [63].

One of the main obstacles in the development of immunostimulants is the poor understanding of the mechanism of action [65]. Therefore, additional research is needed to further investigate their signalling pathways as well as their distribution and interaction with hematopoetic tissues. They have also largely been used as adjuvants in combination with vaccines to enhance their protective effect. Nevertheless, besides being able to induce strong innate responses, they may be decisive for the outcome of acquired responses too [65, 66]. There are also some concerns regarding their use in aquaculture, particularly with the development of the fish larvae immune system and putative tolerance issues [67]. Even so, there are some examples of its use in larviculture in order to protect them before vaccination [57, 67, 68]. Its use has been generally recommended in periods of stress; for instance, during sexual maturation and spawning or transfer to sea cages, or during disease outbreaks.

Lipopolysaccharides (LPS)

Lipopolysaccharides (LPS) are the major component of the outer membrane of Gram-negative bacteria. The negative charge contributed by LPS and their association with divalent cations help to maintain the structural integrity of the outer bacterial membrane, and makes it relatively impermeable to hydrophobic antibiotics or detergents, among others. They are complex amphiphilic molecules composed of three highly immunogenic parts: (1) a negatively charged lipid A group, which anchors LPS molecules to the bacterial membrane; (2) a core oligosaccharide covalently bound to the lipid A group via 3-deoxy-D-manno-octulosonic acid (KDO); and (3) the O- antigen polysaccharide chain (**Figure 5**).

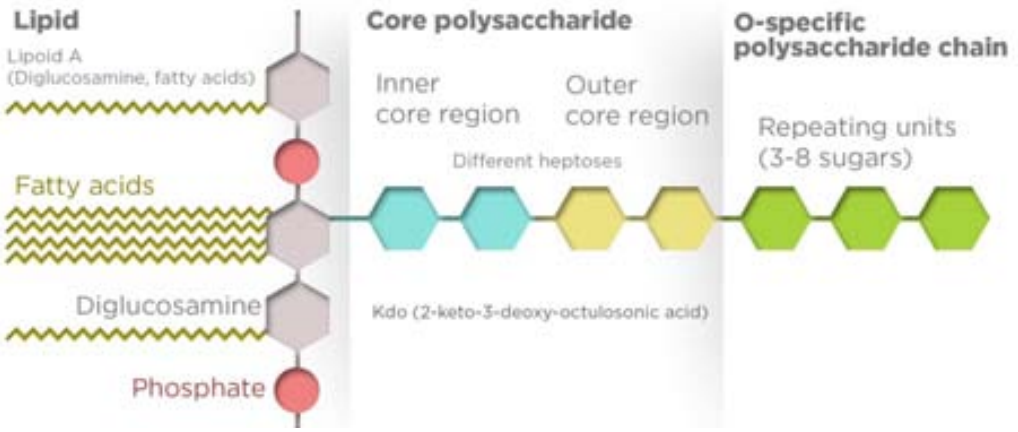

Figure 5. **Lipopolyssaccharide (LPS) complex from Gram-negative bacteria.**

This later can differ in the amount of polysaccharides and extend up to 10 nm outside of the bacterial membrane surface [69, 70]. Different bacteria produce structurally different LPS molecules, varying in their phosphate patterns, number of acyl chains, and fatty acid composition [24]. While the lipid A is highly conserved and is responsible for the endotoxic properties (pyrogenicity, complement activation, B lymphocyte activation, etc.), the O-antigen is highly variable and is responsible for the serological specificity of LPS variants [71, 72] .

The molecular mechanism by which LPS stimulates the immune response has been a focus of attention in mammals. LPS is particularly important since it can elicit septic shock in humans and still remains as one of the most threatening problems in critical-care medicine [73]. Mammalian TLR4 is the central protein in the LPS receptor complex. TLR4, together with MD2 and CD14, can sense up to picograms of LPS and activates two different intracellular pathways: (1) the MyD88-dependent pathway, which leads to the activation of transcription factors such as the Nuclear Factor Kappa B (NF-κB) and Activator Protein 1 (AP-1); and (2) the MyD88-independent pathway based on the TRIF and TRAM effectors, leading to activation of the Interferon Regulating Factor 3 (IRF-3). The Activation of NF-κB, AP-1 or IRF-3 leads to the expression of several inflammatory mediators (cytokines, chemokines or co-stimulatory molecules) (**Figure 6**) [74, 75].

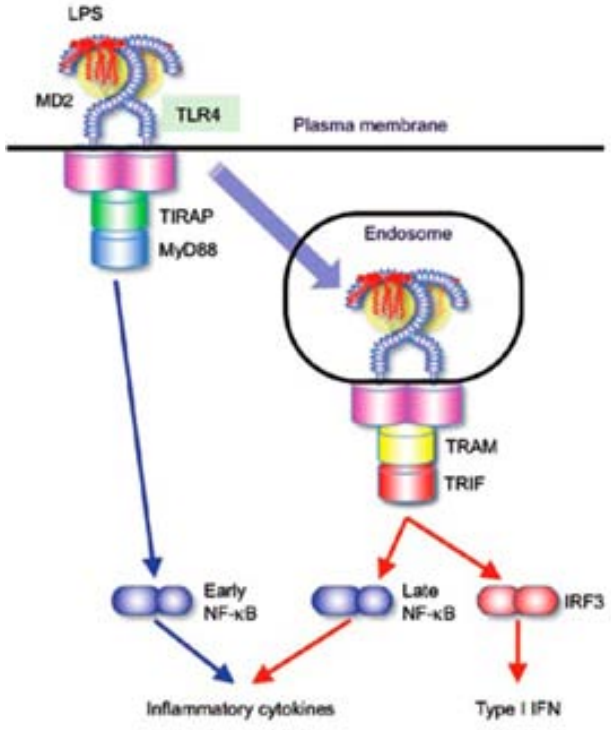

Figure 6. **TLR4 in complex with MD2 engages LPS in mammals.**

Five of the six lipid chains of LPS bind MD2 and the remaining lipid chain associates with TLR4. The formation of a receptor multimer composed of two copies of the TLR4-MD2-LPS complex initially transmits signals for the early-phase activation of NF-κB by recruiting the TIR domain–containing adaptors TIRAP and MyD88 (MyD88-dependent pathway). The TLR4-MD2-LPS complex is then internalized and retained in the endosome, where it triggers signal transduction by recruiting TRAM and TRIF, which leads to the activation of IRF3 and late-phase NF-κB for the induction of type I interferon (TRIF-dependent pathway) (modified from [18]).

In fish, the scenario is quite different: fish can sense LPS but their level of sensitivity is fundamentally different. The concentrations required to stimulate nitric oxide (NO) production in several phylogenetically distant teleost species leukocytes are several-fold higher (microgram range) than the concentrations used for murine and human macrophages (nanogram range) [24, 76]. This could be related to a different LPS sensing mechanism in lower vertebrates. In fact, most fishes lack TLR4 orthologs, although zebrafish and several other cyprinidae family members do have several copies of TLR4 in their genome. Moreover, in the fish species that do have TLR4, different functional studies confirmed that this was not involved in the sensing of LPS [24, 77]. This is an example that sequence homology does not imply a conservation of the ligand specificity. The absence of the genes for the accessory proteins MD2 and CD14 from fish genomes can also help to explain this higher tolerance to LPS [16, 43]. Some authors have speculated that other receptors, such as beta-2 integrins, may play a role in the activation of fish leukocytes by LPS [78], and even that PGN 'contaminating' the crude LPS preparations might be responsible for the cytokine expression stimulation [79].

Even with these differences between mammals and teleost fish, LPS has been widely used for *in vitro* stimulation of fish cultures, and it has demonstrated a high potential for mediating pro-inflammatory cytokines, NO production, acute-phase proteins and macrophage activation/proliferation [80–82]; also observing cytokine expression using ultrapure LPS [24, 83].

The *in vivo* common effects of LPS in fish species have been summarized in a review by Swain et al., [72] and include multiple immunological, physiological and immuno-endocrinological effects. The LPS biodistribution has also been studied and, its accumulation has been found in the main lymphoid organs (spleen and head kidney) as well as in heart, liver and gut [72]. Its effects as immunostimulant and its application in aquaculture have been also largely investigated. When used at a proper dosage, LPS induces beneficial effects to the host like nonspecific activation of macrophages and enhanced protection against disease [57, 84–88]. Moreover, this protective effect against a disease has been proven not only in experiments where LPS was extracted from the same bacteria used for the subsequent challenge [57, 88], but also using LPS from a different source [85–87]. It is important to mention that a good protective effect and enhance immune response have also been found when LPS from a fish non-pathogenic bacteria, such as *E. coli*, was used as immunostimulant [84–87].

Polyinosinic polycytidylic acid (Poly (I:C))

Poly (I:C) is a synthetic double stranded RNA (dsRNA) that mimics a viral infection. It is widely assumed that dsRNA is generated by viral RNA polymerases during genome replication [89]. In fact, the discovery that viral dsRNA was a potent activator of innate immunity was a seminal finding for understanding the host immunity against viral infections [90].

Several synthetic dsRNA analogues are commercially available such as poly (I:C), poly (I:C12U), poly ICLC, poly (A:U) or Poly (I:C) with Poly-lysine. Poly (I:C) is a mismatched dsRNA with one strand being a polymer of inosinic acid and the other, a polymer of cytidylic acid. It was discovered in 1967 by Hilleman's group [91], who also discovered interferon (IFN) induction by dsRNA.

In mammals, dsRNA is sensed mainly by TLR3, but also by two members of RLRs family: the RNA helicases RIG- I and melanoma differentiation-associated gene 5 (MDA-5); and the NLR pyrin domain (NLRP) 3 protein of the NLR family [92]. Both the RIG-I and the MDA-5 have also been found in teleost fish suggesting its conservation through vertebrates [93, 94].

TLR3 can be found both intracellularly and on the cell surface, but it is predominantly located in intracellular vesicles (e.g. endosomes) in most cell types, including dendritic cells and macrophages. Physiologically, TLR3 may encounter viral RNAs in the endosome where viruses enter through the endocytic pathway or by uptake of the apoptotic bodies derived from virally infected cells [92]. Although it is specifically unknown how extracellular dsRNA are delivered to the intracellular vesicles in mammals, some authors have demonstrated that CD14 directly binds to dsRNA and mediates its cellular uptake [95]. Once internalized into the endosome, dsRNA binds to TLR3 and activates the TRIF signaling pathway. This leads to the activation of several transcription factors, including nuclear factor-κB (NF-κB), interferon regulatory factor 3 (IRF3) and activating protein 1 (AP-1) (**Figure 7**). It is important to mention that among the TLR family members, only TLR3 does not use myeloid differentiation factor 88 (MyD88) as a signaling adaptor [17].

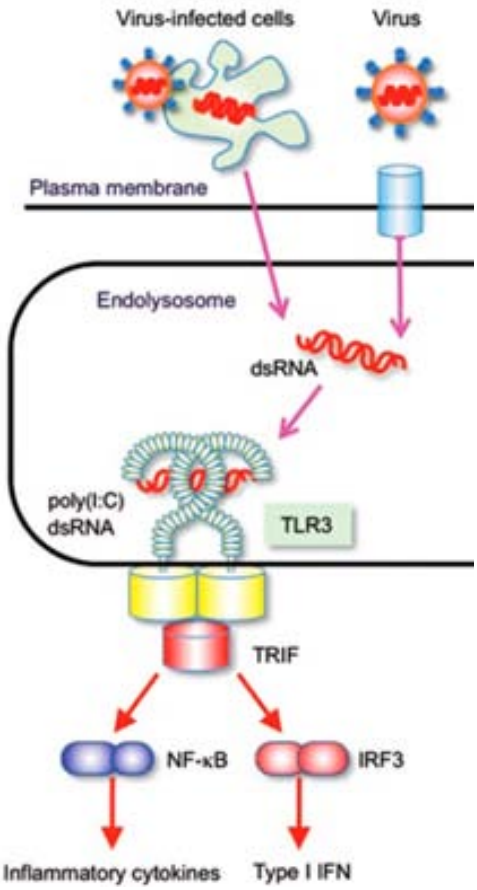

Figure 7. **TLR3 recognizes dsRNA derived from viruses or virus-infected cells.**

dsRNA binds to N- and C-terminal sites on the lateral side of the convex surface of the TLR3 ectodomain, which facilitates the formation of a homodimer via the C-terminal region. TLR3 activates the TRIF-dependent pathway to induce type I interferon and inflammatory cytokines (from [18]).

On the other hand, RLRs are cytoplasmic viral RNA sensors. While RIG-I binds 5′ triphosphate RNA in single- or double- stranded forms or short dsRNA of 300–1000 bp, MDA-5 recognizes long dsRNA of more than 1000 bp in length such as Poly (I:C) [92, 96]. These recognition processes trigger the release of inflammatory cytokines, mainly type I IFNs [97], leading to the the induction of a robust Th1 immunity, and also the Th2 antigen-specific immune response and cytotoxic T lymphocytes (CTLs) activation.

In the case of the dsRNA sensing through NLRs, it has been suggested that the large protein complex termed the inflammasome is activated. This protein platform activates caspase-1. This activation hydrolyzes pro-IL-1β and pro-IL-18 into their mature biologically active forms, which are secreted extracellularly to play a role in immune response [98, 99]. All this immune system activation will eventually help to remove a viral infection.

The induction of antiviral effects by Poly (I:C) have also been largely studied in teleost fish. The dsRNA recognition by the endosomally-located TLR3 has been confirmed in several fish species, such as pufferfish fugu [100], rainbow trout [101, 102] and zebrafish [103]. In the case of the pufferfish fugu, TLR22 has been found to be the cell surface analog sensing the presence of dsRNA outside the cell [100]. Several studies have confirmed the production of IFN [104] and other antiviral proteins induced by IFN, such as Mx protein, after stimulation with synthetic dsRNA analogs like Poly (I:C) *in vitro* and *in vivo*. Therefore, there is substantial evidence that it could be a useful immunostimulant to prevent viral diseases in different fish species [105–109]. Whether if its use is generally applicable in sustainable fish farming remains to be investigated, but it certainly offers advantages as a viral infection profilactic measure. For instance, injected Poly (I:C) does not remain in fish tissues because it is an unstable RNA molecule, which means immunized fish can still be used as a food source [109]. Nevertheless, some adverse effects have also been detected after its use. Lockhart et al. [110] observed pathological changes in the liver of Atlantic salmon after high doses of Poly (I:C) injection resulting from apoptosis and necrosis hepatocytes.

Combined use of Immunostimulants

By using immunostimulants in a combined way, it should be possible to achieve a more broader profilactic effect: the animal might be protected at the same time against different sources of disease. It has also been hypothesized that activation of multiple innate receptors may be more effective than activation of a single pathway [111]. Combinations of TLR agonists can have synergistic effects when used as adjuvants, resulting in greater and more durable responses to antigens, as well as dose sparing [112, 113] . In fact, in vitro studies with defined combinations of TLR ligands support this idea [113, 114]. Nevertheless, when stimulating the immune system with more than one immunostimulant, not only the putative synergistic responses but also the antagonistic ones must be taken into account.

In mammals, it is hypothesised that synergistic effects are mainly seen when MyD88- and TRIF-associated TLRs are used in combination [114–117]. As already mentioned, Poly (I:C) is known to signal mainly through TLR3 (a TRIF- associated TLR), whereas LPS signals through TLR4 in mammals (both a MyD88- and TRIF- associated TLR) and through another sensing mechanism involving other receptors in teleost fish. Indeed, an increased induction of proinflammatory cytokines has been observed specifically after the combination of LPS and Poly (I:C) in some studies [115]–117], and, although the synergia is very mild, no antagonistic effects have been observed.

On the other hand, little is known about these "TLR cooperation" in fish and only some examples of synergia have been studied [118, 119]. Thus, it would be necessary to assess the combined use of LPS and Poly (I:C) in fish species as the different LPS recognition mechanisms might as well involve different synergic behaviour.

In this context, *in vitro* stimulation with LPS and Poly (I:C) was performed with zebrafish hepatocytes (ZFL) and head kidney macrophages from rainbow trout, and RT-PCR quantification of the expression of some immune related genes was performed ("LPS+poly(I:C) control" from

Figure 5; [120]). The obtained values were compared to the results obtained in the group and by others [121, 122] by using each immunostimulant separately. By comparison, we could conclude that the expression levels after the stimulation with LPS and Poly (I:C) together were not lower in any case. For instance, ZFL cells stimulated with Poly (I:C) led to an increase in the GIG-2 expression of 523 ± 23,9 fold-change and an increase in the INFϕ expression of 8,2 ± 4,1 fold-change (Ruyra et al., unpublished results), whereas a combined stimulation of LPS and Poly (I:C) led to an increase in the GIG-2 expression of 2708 ± 62,8 fold-change and an increase in the INFϕ expression of 10,7 ± 3,7 fold-change [120]. After the observation that, in principle, no antagonistic effects were seen on the expression levels of the studied genes, and taking into account the available bibliography, the assumption that no negative effects would arise due to the combined use of LPS and Poly(I:C) was made.

New administration methods for vaccines and immunostimulants

In order to be able to administrate vaccines or immunostimulants to fish avoiding the side effects of the injection administration, new research has focused on exploring the immersion and oral delivery. As mentioned before, both methods are easier to apply but confere only limited protection mainly due to degradation of the compound in the water or in the intestinal tract. Other factors such as the targeted tissues will obviously affect the magnitude of the obtained protection, but an improved delivery of the vaccines/immunostimulants will have a clear impact on the protection that they confere. In this context, the use of delivery 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 act as adjuvants on their own [66, 123]. This has been specially important also in human health, as there has been a swift from whole-cell and live attenuated vaccines towards the safer but less immunogenic subunit vaccines [124–126].

Nanoparticles as drug delivery systems

Drug delivery systems are those materials used for the administration of a pharmaceutical compounds in a controlled manner to achieve a therapeutic effect in humans or animals [127]. These systems are usually used to provide: (1) targeted (cellular o tissular) delivery of actives; (2) improved bioavailability; (3) improved solubility of hydrophobic drugs; (4) sustained release; and also (5) protection of the therapeutic agent from degradation such as enzymatic degradation [128, 129].

The first macrosystems developed for drug delivery appeared during the middle 1960s and evolved to microscopic systems with the development of poly(hydroxy acids) [130]. It was not until the introduction of liposomes, also in the 1960s [131], together with the polymer-drug conjugates, in the mid to late 1970s, that the concept of **nanoengineered drug delivery systems** arised. Nanoencapsulation of drugs involves forming drug loaded particles with diameters ranging from 1 to 1000 nm [132–134]; although other stricter definitions refer only to structures in the 1-100 nm size [135]. This size property enables the nanoscale devices to readily interact with biomolecules, such as enzymes and receptors, both on surfaces and inside cells (**Figure 8**).

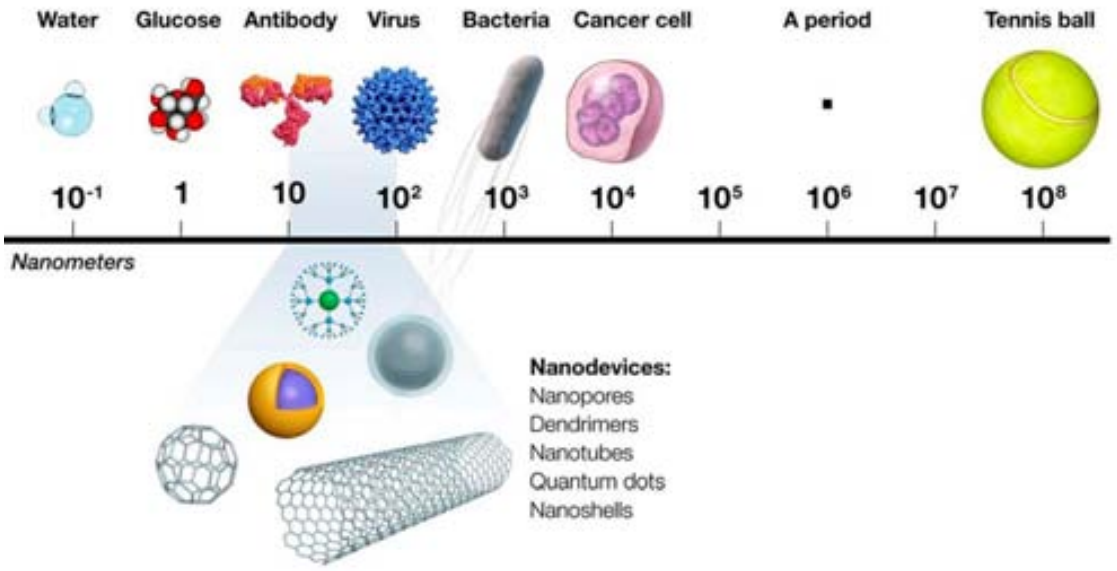

Figure 8. **Length scale showing the size of nanomaterials in comparison to various biological components.**

Subsequently, a variety of other organic and inorganic biomaterials for drug delivery were developed [136] (**Figure 9**). More complex drug delivery systems capable of responding to changes in pH to trigger drug release as well as the first examples of cell specific targeting of liposomes were described in 1980 [137–139]. The first therapeutical nanoparticle approved by the US Food and Drug Administration (FDA) was a mixture of Cyclosporine with Cremophor (a castor oil that solubilized extremely lipophilic drugs through the formation of micelles) back in 1983, whereas the first controlled-release polymer was approved in 1990 [136]. At that time, also the long-circulating liposomes were described. The concept was later named "stealth liposomes" [140], and subsequently, the use of polyethylene glycol (PEG) was shown to increase the circulation time for nanoparticles in the 1990s.

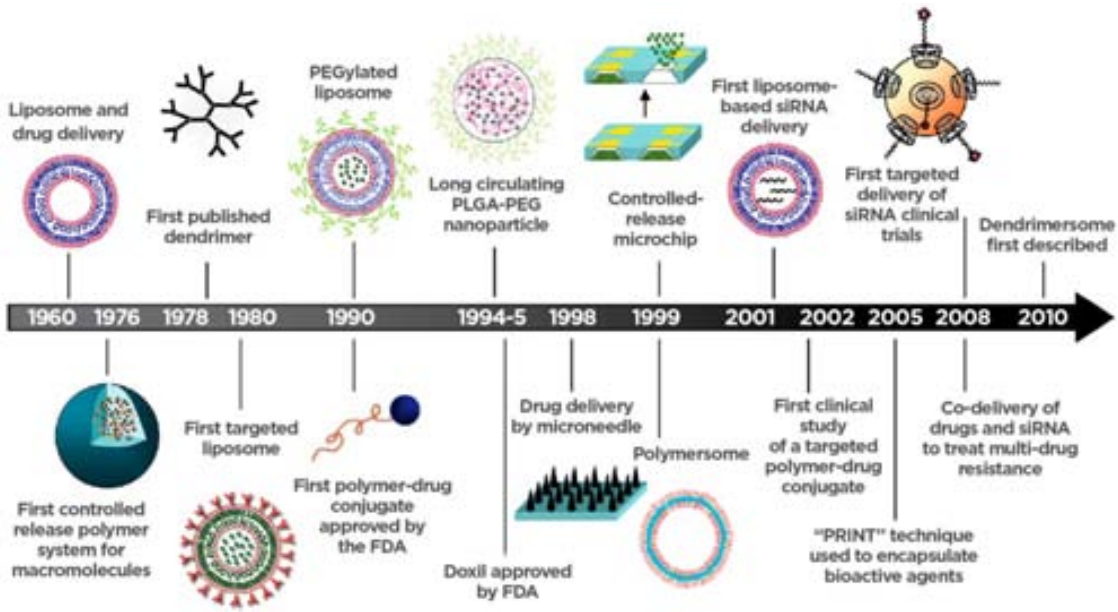

Figure 9. **Timeline of nanotechnology-based drug delivery.**

Modified from [129].

General nanoparticle properties and their influence in cell internalization

The nanosized-encapsulated drugs will generally have completely different properties (e.g., solubility and circulating half-life) compared to the non-encapsulated ones. For this reason, it is very important to understand and control the *in vivo* behaviour of these drugs once encapsulated to predict their efficacy and side effects. Their interaction and possible internalization by cells will also be changed after being encapsulated in a delivery system. The kinetics profile of the nanoparticles will be mainly determined by their **chemical and physical properties**, such as size, charge, and surface chemistry, among others [124, 141] (**Figure 10**).

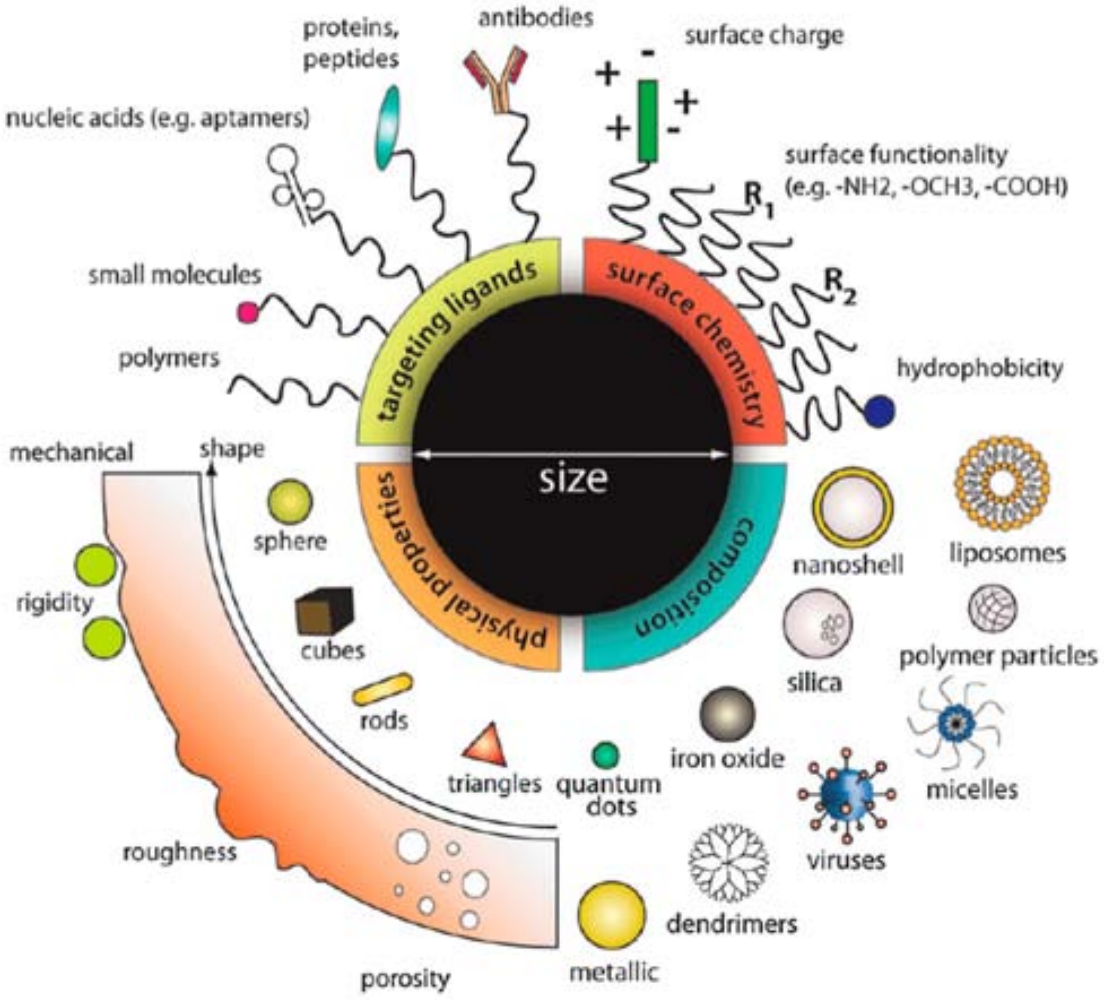

Figure 10. **Nanoparticles can be assembled from different materials with different physical and chemical properties and functionalized with a myriad of ligands for biological targeting.**

From [141].

An important process that will also determine the fate of the nanoparticles is the **opsonization**. Once in the plasma, different proteins, such as immunoglobulins and complement proteins, will adsorp to the nanoparticle surface forming the protein corona in order to "tagg" them and facilitate their recognition by cells of the immune system [142–144]. Although surface modifications (such as the PEGylation) reduce the binding of these biomolecules, some association may still

occur and thus, determine their subsequent cell internalization through specific ligand-receptor interactions [145–147].

Regarding to **the size** of the nanoparticle, it is not only important for the interaction with the biomolecules, but also because it will influence its biodistribution *in vivo*. In mammals, it has been largely studied that particles of less than 5 nm are cleared from the circulation through extravasation or renal clearance, whereas particles from the nanometre range to ~ 15 μm accumulate in the liver, spleen and bone marrow [148, 149]. On the other hand, particle size also influence the mechanism of **cellular internalization** [144, 145, 147]. These mechanisms include phagocytosis, macropinocytosis, caveolar-mediated endocytosis and clathrin-mediated endocytosis among others, and the fate of the internalized material will be different in each case (**Figure 11**).

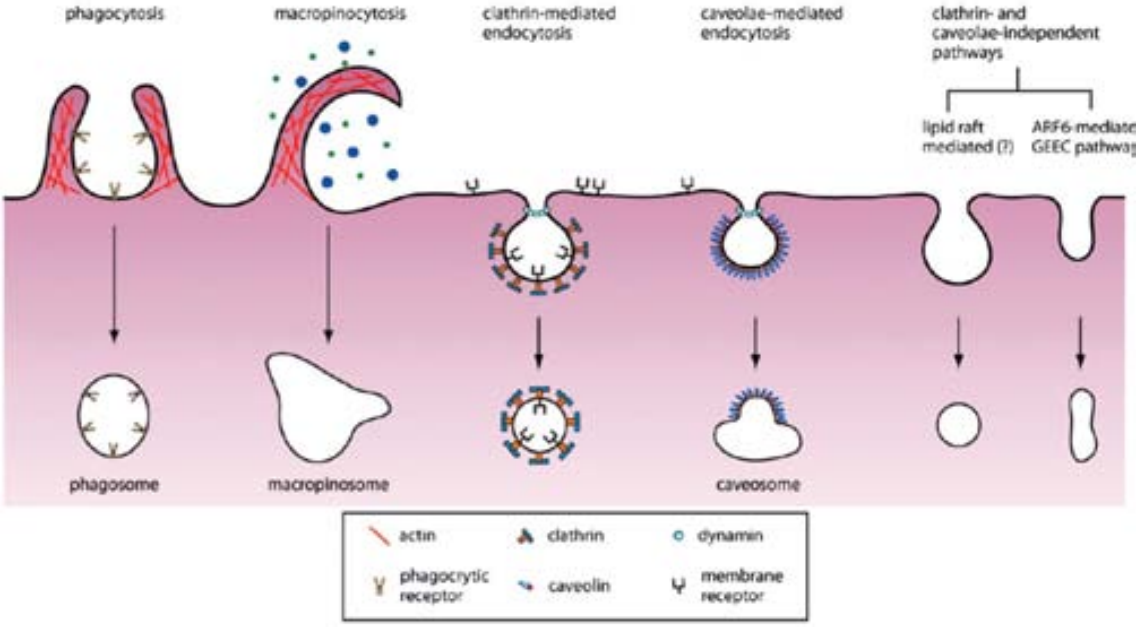

Figure 11. **Pathways of entry into the cell.**

An increasing number of endocytic pathways are being defined. These pathways facilitate cellular signaling and cargo transport. Controlling the route of nanoparticle uptake is important for both mediating their intracellular fate as well as their biological response (from [141]).

Briefly, internalization of large particles by several specialized cells (mainly macrophages and neutrophils) are generally facilitated by phagocytosis while non-specific internalization of smaller particles (<2 μm) can occur through macropinocytosis. The smaller nanoparticles can be internalized through several pathways, including caveolar-mediated (<80 nm) and clathrin-mediated (<300 nm) endocytosis. Nevertheless, nanometre-sized particles, such as polymer nanoparticles or quantum dots, have also been seen to be internalized through phagocytosis [145]. All these mechanisms share some fundamental steps: (1) the binding at the cell surface; (2) invagination of the plasma membrane; (3) formation of the trafficking vesicle; and finally (4) trafficking of the vesicle to a specific subcellular organelle (mostly endosomes). The endocytic pathway is a spatiotemporal succession of different compartments, which continuously interchange their content [145]. The internalized material is transported from one compartment to

another *via* a maturation process; the first step is the early endosome that gradually changes into a late endosome to finally become a lysosome. During this process, the internal pH decreases from ~7.0 to <5.0, while the activity of the proteolytic enzymes increases, therefore favoring the generation of peptides antigens for MHC presentation. Lysosomes contain not only proteolytic enzymes but about 60 soluble and 25 transmembrane degradative enzymes that can hydrolyze nucleic acids, polysaccharides and lipids besides proteins [150]. All of the lysosomal enzymes are acid hydrolases, which are active at the acidic pH but not at the neutral pH characteristic of the rest of the cytoplasm. In the case of the phagocytosis, it has been seen that the phagosomes can skip the early endosomes and directly fuse with lysosomes to accelerate the degradative process [151].

Also, the particle aggregation will obviously have an impact on their particle size and on their endocytosis mechanisms. Therefore, the stability of the nanoparticles is a key factor when trying to predict their interaction with cells and *in vivo* behaviour.

Current findings indicate that particle **shape** and **rigidity** are also key factors for the kinetics of the nanoparticles mainly also due to its effects on the endocytosis. The vast majority of nanoparticles have a spherical shape. However, it has been shown that nanoparticles with similar volumes but different shapes were internalized at different rates [152–154]. This could be explained by the different geometry of the interactions. This effect can be dramatically exemplified with the case of 18 μm-long filamentous micelles, which have been reported to have a circulation half-life of ~5 days [155]. On the other hand, the rigidity also affects the nanoparticle internalization. For instance, macrophages tend to show enhance phagocytosis in the presence of rigid nanoparticles [156].

Finally, the nanoparticle **surface charge** critically affects how they will interact with each other and their surrounding, specially regarding the adsorption of opsonins and the interaction with cell membranes. Highly charged particles have proven to fix more complement proteins [157], a process that can only be inhibited by the addition of a hydrophilic coating able to repel opsonins, as already mentioned. The surface electric charge will also determine the nanoparticle interaction with the cell membrane. Briefly, neutral and anionic particles will be less internalized than positively charged ones [145, 146]. Different studies using the same nanoparticles with different surface charges have shown that the ones with cationic groups were internalized more efficiently [158–161]. This was mostly due to its high affinity for the negatively charged proteoglycans expressed in the surface of most cells [162]. Nevertheless, negatively charged particles have also been seen to be slightly more internalized than neutral ones despite the unfavorable interaction between the particles and the negatively charged cell membrane [144, 146, 159, 163]. Their internalization is believed to occur through nonspecific binding and clustering of the particles on relatively scarce cationic sites of the cell membrane [146, 147]. The surface charge can also dictate the specific internalization pathway and subseqüent localization [160]. Perumal et al. [164] demonstrated that anionic dendrimers were internalized through the caveolae mediated pathway, whereas neutral and cationic ones were internalized through non-caveolae and non-clathrin mediated endocytosis. It is imporant to point out, though, that most cationic systems have been reported to be more cytotoxic for the cells, with the obvious consequences on their biocompatibility *in vivo* [145, 165].

Liposomes as delivery systems for the stimulation of the immune system

As above mentioned, the first generation of nanoparticles were mainly based on liposomes and polymer-drug conjugates. Although several other types of nanodelivery systems have been developed (such as dendrimers, carbon nanotubes, virus-like particles, or solid particles made of chitosan or poly(lactic-co-glycolic acid) (PLGA)), (**Figure 12**) this Thesis has focused on the use of liposomes [127, 128].

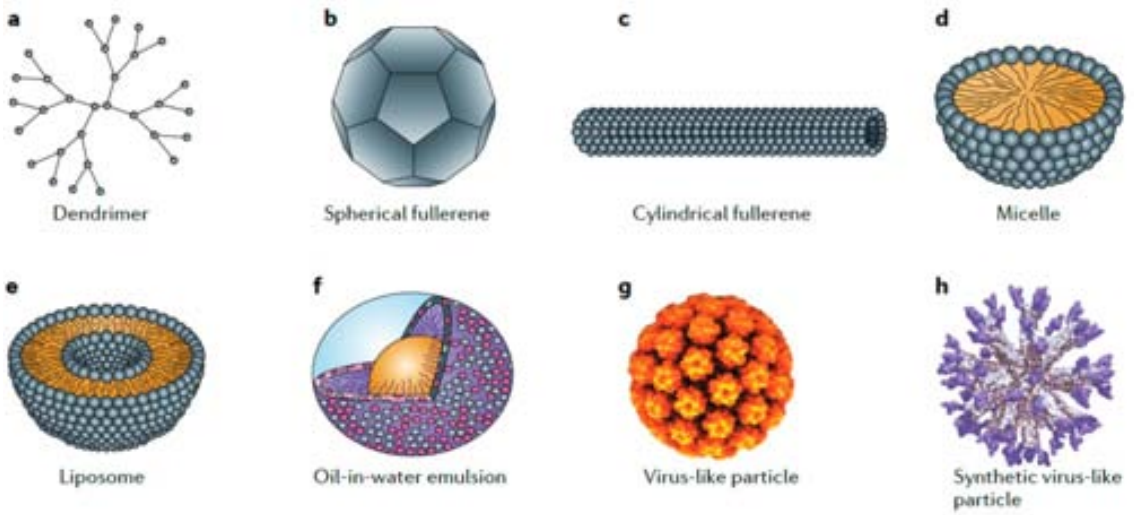

Figure 12. **Examples of different nanodelivery systems.**

Nanodelivery systems include dendrimers (part **a**), carbon molecules known as spherical fullerenes (part **b**) and cylindrical carbon molecules known as cylindrical fullerenes (part **c**). Nanoemulsions incorporate immiscible components such as oil and water that might form amphiphilic molecules such as micelles (part **d**), liposomes with a lipid bilayer (part **e**) and oil-in-water emulsions (part **f**). Virus-like particles are self-assembled structures composed of one or more viral capsid proteins (part **g**), whereas synthetic virus-like particles are self-assembled from chemically synthesized components (part **h**) (from [134]).

Liposomes are spherical, self-closed vesicles made of one or several lipid bilayers and an hydrophobic compartment [166]. Its name derived from the Greek 'lipos' (fat) and 'soma' (body). Liposome formation is highly dependent on the geometry of the lipid monomers, which can be quantified by the critical packing parameter (CPP) of the lipids [167]. Lipids with large head groups and double hydrocarbon chains have a CPP < 1 and therefore, form bilayered structures. Its formation is energy-dependent and post-formation techniques, such as sonication, extrusion or freeze-drying, are needed to avoid the natural heterogenous multilamellar structure.

In this Thesis, liposomes were selected because of some attractive biological properties: (1) they are highly biocompatible; (2) they can encapsulate both hydrophilic and hydrophobic agents; (3) they can deliver actives even inside individual cellular compartments; and (4) their size, charge and surface properties can be easily changed by just adding new components in the lipid mixtu-

re before liposome preparation and/or by variation of the preparation methods. They can also be functionalized on their surface to promote the targeting to specific cells and tissues, and coated with hydrophilic polymers to prolong their circulation half-life. Here, the best example is the long-chain polyethylene glycol (PEG), although other hydrophilic polymers, such as Pluronic F68 and Poloxamer (block copolymer of polyethylene oxide and polypropylene oxide), have been used [142].

The first liposome-based system approved by the FDA was the liposome-encapsulated doxorubicin (Doxil) in 1995 for the treatment of Kaposi's sarcoma. The encapsulation of this cytotoxic drug reduced its overall toxicity and enhanced its deposition in tumours thanks to the enhanced permeability and retention (EPR) effect (macromolecules can escape circulation thanks to the inherent leakiness of the underdeveloped tumour vasculature) [136]. Several other liposome-based systems have been approved for indications as diverse as fungal infections (liposomal amphotericin B) or postsurgical analgesia (liposomal morphine) [127].

Liposomes can interact with cells and be internalized by the endocytosis mechanisms above explained, also in a size-, shape- and charge-dependent manner. Besides, liposomes can also release their contents directly into the cytosol by: (1) fusion with the external cell membrane (fusogenic liposomes); or (2) by destabilizing the endosomal membrane when finding mild acidic conditions (pH 5–6.5) [168–170]. This is highly important when seeking cytoplasmatic or nuclear delivery as otherwise the acidic pH and the enzymes of the lysosomes can degradate the liposome content [171, 172] (**Figure 13**).

Regarding its effect **on the immune system**, liposomes provide adjuvant activity by enhancing the delivery of immunostimulatory compounds to the cells of the immune system, and also by directly potentiating the innate immune responses [134]. In fact, the first report of liposomes as vaccine adjuvants was in 1974, in which negatively charged liposomes combined with a diphteria toxoid was shown to produce an enhanced antibody response [173]. It is widely accepted that the quality of the resulting antibodies and/or cell-mediated immune response and its magnitude depends on the appropiate antigen processing and on the cytokine profile generated.

In this context, liposomes were long ago proven to be effective immunological adjuvants as they are capable of inducing both humoral and cellular immune responses [174]. Briefly, liposomes are phagocyted mainly by macrophages and accumulate in the phagosomes that ultimately become phagolysosomes. After the degradation of the liposomes by lipases, the entrapped antigen is also partially degradated due to the decrease of the pH and to the increase of the proteolytic activity. Then, the resulting peptides are presented to the major histocompatibility class II (MHC-II) complex on the cell surface. This results in the stimulation of T-helper cells and B cells with the subsequent secretion of antibodies. On the other hand, the MHC-I presentation pathway has, for a long time, been considered to be restricted to endogenously synthesized proteins, but the presentation of exogenous proteins on MHC-I molecules has been demonstrated and refered to as "cross-presentation". As mentioned before, the MHC-I pathways eventually lead to an increase of the cytotoxic T-lymphocyte (CTL) responses. These CTL responses are very important for an effective vaccination, specially for vaccines targeting viruses that can not be controlled solely with antibodies [175–177]. Interestingly, some delivery systems, such as liposomes, appear to be specially qualified for using the cross-presentation pathway with their delivered antigens [45]. Therefore liposomes have an enhance effect compared to the soluble antigens and to other traditional adjuvants (such as oil emulsions and aluminium salts) that do not elicit any significant CTL responses [175, 178–180].

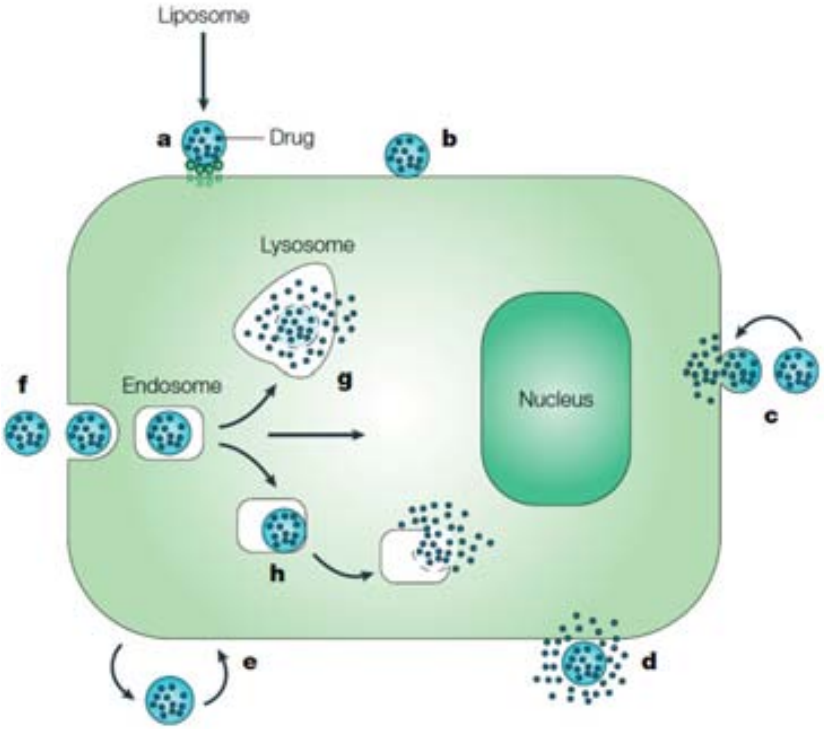

Figure 13. **Liposome-cell interaction.**

Drug-loaded liposomes can specifically (**a**) or nonspecifically (**b**) adsorb onto the cell surface. Liposomes can also fuse with the cell membrane (**c**), and release their contents into the cell cytoplasm, or can be destabilized by certain cell membrane components when adsorbed on the surface (**d**) so that the released drug can enter cell via micropinocytosis. Liposome can undergo the direct or transfer-protein-mediated exchange of lipid components with the cell membrane (**e**) or be subjected to a specific or nonspecific endocytosis (**f**). In the case of endocytosis, a liposome can be delivered by the endosome into the lysosome (**g**) or, en route to the lysosome, the liposome can provoke endosome destabilization (**h**), which results in drug liberation into the cell cytoplasm (from [166]).

Liposomes can also be targeted to enhance its phagocytosis. Dendritic cells and macrophages express a variety of receptors on their membranes for antigen recognition. The coupling of ligands for this receptors on the surface of the liposomes is one strategy to facilitate their uptake. To date, several ligands have been reported; for instance the inclusion of mannosylated phosphatidylethanolamine on the lipidic composition for targeting the mannose membrane receptor facilitated the uptake by monocytes [181]. Another strategy involves the coupling of antibodies in the so-called immunoliposomes, which can also enhance the immune cell recognition and internalization [182].

Among the vast types of liposomal vaccine formulations studied, cationic liposomes appear to be particularly immunogenic, probably due to their high interaction with cells and therefore, with APCs [124, 183]. For instance, the cationic liposome CAF01 composed of dimethyldioctadecylammonium bromide (DDA) and the glycolipid trehalose 6,6′-dibehenate (TDB) has been succesfully used in combination with vaccines against tuberculosis, chlamydia, flu or malaria [173, 184]. In fact, its combination with HIV Type 1 peptides is now on Phase I clinical trials [185]. Cationic liposomes have also been used in experimental vaccines to deliver nucleic acids that have an anionic nature [175]. In addition, different compositions containing DOTAP, DO-

TIM or the comercial Lipofectamine™ have been used in combination with DNA vaccines for Japanese Encephalitis Virus, Influenza A Virus or Tuberculosis [186–188].

Liposomes also offer the possibility of co-delivering antigens and immunostimulatory components to the same cell [45]. In fact, most of the so-called "2nd generation adjuvants" are made of a delivery system combined with one or more immune stimulators to use with the selected antigen. That is because the succesful induction of adaptive immunity depends not only on the direct antigen recognition, but also on stimulation of the innate immune system [172]. There are several examples of improved immunogenicity after liposomal co-delivery of antigens with TLR ligands [189–192]. One of the most studied examples is the AS01 from GlaxoSmithKline formed by DOPC liposomes, the saponin QS21 and monophosphoryl lipid A (MPL). QS21 is a mixture of soluble triterpene glycosides purified from the soap bark tree (*Quillaja saponaria*) and MPL comprises the modificated lipid A portion of *Salmonella minnesota* LPS [193, 194]. Both have been found to be highly immunogenic. Interestingly, AS01 in combination with a malaria antigen is now in Phase III clinical trials [173]. Another exemple is the combination of Poly (I:C) with CpG containing ODNs and a DNA vaccine encapsulated into DOTAP and DOPE liposomes, which have shown to elícit Th1 and therefore CTL-enhanced responses [195].

Overall, one of the principal benefits of using liposomes as delivery systems is their flexibility relating to both physicochemical and immunogenic properties. They are higly versatile and capable of stimulating both $CD4^+$ and $CD8^+$ T cells. There are several studies of the immune response obtained after slight variations on the liposomal composition, as already mentioned [181, 196]. Furthermore, the inclusion of TLR ligands serves not only to stimulate the innate immune system but also to increase the pathogen-like nature of the nanoparticle by mimicking the pathogens themselves [177].

Some examples of LPS and Poly (I:C) encapsulation have already been mentioned [173, 189, 190, 195]. Its use in vaccine applications for human health seems promising as there are some formulations under different development stages. Although they have been encapsulated alone for applications such as inhibition of cancer cell growth [197, 198], they are mostly encapsulated in combination with specific vaccines. Poly (I:C) has been succesfully encapsulated or complexated with cationic liposomes thanks to its polyanionic nature, eliciting strong antigen-specific $CD8^+$ T cells in different animal models [124, 126, 189]. On the other hand, LPS has been encapsulated alone or included in several highly succesfull "adjuvant-platforms" such as the already mentioned AS01, where they use less immunogenic modifications of LPS such as MPL [173, 199].

Delivery systems for aquaculture

Several approaches based on delivery systems have been employed in aquaculture in order to increase the efficacy of vaccines and to obtain alternative routes of immunisation for mass-vaccination [54, 66, 200]. Traditional adjuvants such as mineral oils have been routinely used in different injected commercial fish vaccines to increase their residence time, thanks to a depot effect, but with important side effects at the injection site. The most commonly used was the Freund's Complete Adjuvant (FCA), which is made of a heat-killed *Mycobacteria* and a mineral oil with surfactant. Importantly, due to its significant side effects (e.g., injection site granuloma), its use has been limited to research and it has been replaced by the Freund's Incomplete Adjuvant (FIA), which lacks the mycobacterial component. FIA has been tested succesfully in combination with antigens for different fish pathogenic bacteria, such as *Edwarsiella tarda* or *No-*

cardia seriolae, and fungi oomycetes, such as *Aphanomyces invadans* [201]–203]. Although this adjuvant is still highly effective, it still presents important side effects and therefore, other mineral oil adjuvants such as Montanide have been developed [204, 205]. Several companies have Montanide-containing vaccines available. For instance, MSD Animal Health has the AquaVac FNMPlus, which is an emulsion for injection to Atlantic Salmon that contains inactivated *Aeromonas salmonicida* and that gives a Relative Percentage Survival of ≥ 80 % after vaccination.

Different micro- and nanoparticles have also been used, not only to increase the vaccine residence time in case of injection but also to achieve antigen protection after oral or immersion delivery and to obtain an humoral and cellular response after interaction with the cells of the immune system [206]. For instance, microcapsules made of Poly-(lactide-co-glycolide) (**PLGA**) polymers have been largely studied for oral delivery as their biodegradation rates can be easily changed by alterations in the polymer composition and molecular weights. PLGA nano- and microcapsules have been used to deliver antigens or DNA vaccines to different fish species, such as Japanese flounder, Rainbow trout or Atlantic salmon by oral administration [207, 208] and also by intraperitoenal injection [209, 210] with enhance antibody production and/or higher protection against pathogenic challenges. Another example is the use of **alginate** microcapsules for antigen oral delivery [211]. Alginate is a polysaccharide found naturally in brown algae that has been used also to deliver DNA vaccines with an increase in the confered protection [212, 213]. Nevertheless, these capsules have shown to maintain their stability after its presence in simulate tilapia's gastric conditions, while releasing their content in the foregut [206, 214]. Even with that, there are some examples where their use have not increased the vaccine efficacy [215, 216]. Also, since 1990, the natural biodegradable polysaccharide **chitosan** has been largely studied in aquaculture as its polycationic nature makes it specially suitable for oral DNA vaccination [217–220]. Chitosan also offers the advantage of mucoahesion which can result in improved delivery [200]. Importantly, although great efforts are being made to deliver DNA vaccines (as they have been highly effective against different fish viruses), for the moment, only Canada has allowed their use in fish farming with the development of a commercial prototype against infectious hematopoietic necrosis virus (IHNV), the Apex-IHN® (Aqua Health Ltd., an affiliate of Novartis Aqua Health).

Liposomes have also been extensively studied as potential delivery systems in aquaculture for different types of vaccines. Some bacterial antigens have been encapsulated in liposomes, including an antigen from *Aeromonas salmonicida* or the whole inactivated *A. salmonicida* together with LPS and an inactivated toxin that gave an increase in the protection after immersion administration to Common carp or Rainbow trout, respectively [221, 222]. And even LPS from *A. salmonicida* encapsulated alone stimulated the humoral response compared to free LPS [223]. Related to this, the encapsulation of only LPS from a meningogoccal bacteria in proteoliposomes has been proven enough for increasing the survival in African catfish after their administration with the feed for 15 days [224]. Formalin inactivated koi herpes virus has been encapsulated in liposomes for oral vaccination of Common carp and, subsequent challenge showed an increase in their survival [225]. Cationic liposomes made of DOTAP have also been used for a DNA vaccine encoding for the G protein of the viral hemorrhagic septicemia virus (VHSV). The delivery and transfection of this plasmid vaccine has been succesfully achieved, but it has not given protection against a VHSV infection [226, 227]. In another example, double-stranded RNA has been encapsulated to succesfully inhibit yellow head virus replication in shrimp after injection administration [228]. And DOPS, DOPC and cholesterol liposomes have been coated with a recombinant protein used against the white spot syndrome virus (WSSV) in shrimp, showing

that liposomes improved shrimp survival after challenge with WSSV [229]. It is important to mention that when seeking immersion delivery with liposomes, the charge of the lipidic membrane has to be considered. Fish gills contain a high level of mucin that deprotonates at the pH of the water, facilitating its interaction with cationic liposomes. This could be benefitial but can also have negative effects, such as hypoxia, if the dose is too high [230].

Some advances have been made in immersion and oral delivery but there are still no effective methods commonly available. In the case of the oral delivery, efforts should focus on the understading of the absorption mechanisms of large proteins in the intestine of fish, so that proper delivery systems could be formulated combining protection from proteolysis, improving the permeability of the antigen and controlling the release for a better immune response [231]. In the case of immersion vaccination, not only new delivery systems but also their combination with new methods, such as short pulses of ultrasound are also being studied [227]. Besides, in some studies, the levels of neutralizing antibodies do not correlate with the protection against an infection [215, 216, 232, 233]. This could also be explained because most of the times these antibodies are mesured in the animal serum and do not reflect the mucosal antibodies that might have a key role after oral and immersion vaccinations.

Furthermore, legislation is needed to allow the use of nanoparticles for their administration to fish orally or by immersion, and their safety needs to be extensively addressed both, for the fish themselves and also for human health [206]. Nanotechnology has a wide usage potential in aquaculture and seafood industries. For example for the production of more effective fish feed by protection of delicate fish pellets, but the effects of these applications on environment should be taken into account [234, 235]. In this context, initiatives like the Upstream Oversight Assessment (UOA) of the expected benefits and potential harms of PLGA nanoparticles used to improve vaccines for farmed salmons are highly interesting [236]. This initiative aimed to explore the areas in this field that needed further research, focusing on benefits and issues of hazard like exposure for researchers, workers and consumers.

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Aims & objectives

The fish innate immune system can be modulated by molecules or molecular patterns associated to pathogens (PAMPs) that interact with its pattern-recognition receptors (PRRs). The main hypothesis of this Thesis is whether we could encapsulate several PAMPs or immunostimulants in the same nanoscaled delivery system to improve its administration to different fish species. Also, whether this delivery system could interact with the cells of the immune system generating its non-specific activation and therefore improving the response against a broad spectrum of infectious diseases.

The **overall aim** is ***the development and characterization of a biocompatible delivery system encapsulating a cocktail of immunostimulants that can be administrated to different fish species and enhance their immune system performance***.

Therefore, the **main objectives** of this work are,

(1) The evaluation of the suitability of using an hepatocyte zebrafish cell line as the *in vitro* reference model for cytotoxicity, endocytosis and immune response studies.

(2) The development and characterization of a liposomal delivery system encapsulating two immunostimulants: a bacterial lypopolysaccharide (LPS) and a synthetic analogue of a viral double stranded RNA named Polyinosinic polycytidylic acid (Poly (I:C)).

(3) The evaluation *in vitro* of the cytotoxicity, endocytosis mechanisms and the immuno-modulatory effects of the liposomal delivery system.

(4) The *in vivo* study of the tissue biodistribution as well as the study of macrophages as the immune-specific cellular targets of the liposomal delivery system.

(5) The evaluation of the protection conferred by the liposomes encapsulating the immunostimulants against a model bacterial disease (*Pseudomonas aeruginosa*) and a model viral (*Spring Viremia of Carp Virus*) disease.

(6) The assessment of the protection conferred by the liposomal delivery system to a species of interest for aquaculture (*Onchorhynchus mykiss*) against one of its natural occuring pathogen (*Yersinia ruckeri*).

chapter 1

Zebrafish hepatocytes are able to mount an anti-viral response: **ZFL cells as a model** to study anti-viral responses *in vitro*

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(submitted)

Abstract

The zebrafish (*Danio rerio*) is a widely used model species in biomedical research, which is also starting to be a model for aquaculture research. The ZFL cell line, established from zebrafish liver, has been mostly used in toxicological studies. However, no previous studies have characterised this cell line as a model for immunological studies. The aim of this work was to study the response of the ZFL cell line against different prototypical immune stimuli such as lipopolysaccharide (LPS), peptidoglycan (PGN), zymosan, poly (I:C) and RNA from *Vibrio vulnificus*. Using quantitative real-time PCR, microarrays, confocal microscopy and western blot we have explored the anti-pathogenic response of the ZFL cells. The results showed that the ZFL cells are able to mount an anti-bacterial, anti-fungal and a strong anti-viral response. We can conclude that ZFL would be an excellent *in vitro* model to study the anti-viral response in zebrafish.

Introduction

The zebrafish (*Danio rerio*) has been an important model for developmental studies, vertebrate genetics or ecotoxicology among others because of its small size, rapid generation time, body transparency at early stages and genome conservation compared to mammals [1-3]. It has also been described as a useful model for gene editing using CRISP9 technology [4]. In addition, more recently it has also become a model for those researchers working in fish immunology and vaccinology [5-8]. We [9], and others [7], have previously shown that zebrafish is an excellent *in vivo* laboratory model to study and test the effects of different compounds previously to the tests with commercial freshwater species. Although zebrafish use is growing in many laboratories to replace the use of rodents or commercial fish species, there is a lack of well-characterised zebrafish *in vitro* models. To date, six zebrafish cell lines are commercially available: ZF4, PAC2 and ZEM2 from embryonic origin, AB.9, SJD.1 fibroblast obtained from the zebrafish caudal fin and ZFL obtained from a pool of 10 adult zebrafish livers (ATCC; www.atcc.com). In 2006, He *et al.* characterised in depth, at the genetic and transcriptomic level, the ZF4 and PAC2 cell lines. Their detailed studies indicated that these cell lines were valuable for its use as model cell lines for zebrafish research [10]. As mentioned, ZFL cells were derived by Collodi *et al.* from adult zebrafish livers [11-12]. These cells showed the main characteristics of hepatocytes and can be transfected with conventional plasmids [11]. However, ZFL show differential expression patterns in response to 17alpha-ethynylestradiol when compared with hepatocytes primary cell cultures [13], pointing out the importance of performing further characterization studies on these cells. For the moment, the ZFL cell line has been mainly used for toxicology and ecotoxicology studies [14-15]. For instance, gene transcription and expression or metabolism have been studied following exposure to different metal ions [16-19] and nanoparticles [20-21], as well as brominated flame retardants, pharmaceuticals or 17alpha-ethynylestradiol [22-24]. But there is a lack of information on how these cells respond to immune stimuli and whether they are suitable as a model for immunological studies. In this work we address this issue and we tested different PAMPs (Pathogen Associated Molecular Patterns) to study the ZFL response at the transcriptomic and cellular level, and thus providing basic information for future studies on gene expression and cell signalling. We show that these cells are very useful to dissect the anti-viral but also the anti-bacterial and anti-fungal responses since they can respond to viral, bacterial and fungal stimuli by triggering the expression of genes typically involved in those responses.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and FBS were purchased from PAA Laboratories (Spain). TrypEL and Antibiotic/Antimycotic 100X solution were purchased from GIBCO (Invitrogen, Life Technologies, Spain). Insulin, EGF, LPS (*E. coli* 0111:B4), zymosan, peptidoglycan propidium iodide, Ponceau-S, ethyl 3-aminobenzoate methanesulfonate (MS-222), MTT, Tri-Reagent, ammonium persulfate and TEMED were purchased from Sigma-Aldrich (Tres Cantos, Madrid). Primocin, HMW-poly (I:C) and poly (I:C)-Fluorescein were purchased from Invivogen (Nucliber, Spain). Cell strainers and plasticware were obtained from BD Biosciences (Madrid, Spain). HRP-conjugated goat anti-rabbit IgG was obtained from Jackson Immunoresearch (Soham, UK) and rabbit anti-mouse HRP-conjugated secondary antibody was purchased from Dako (Barcelona, Spain). Bradford protein assay and protein weight marker were obtained from Bio-Rad (Prat del Llobregat, Spain). Supersignal West-Pico chemiluminescent substrate was from Thermo (Rockford, IL, USA).

Methods

Cell culture, PAMP stimulation and pDNA transfection

Zebrafish ZFL cells (CRL-2643, ATCC) were cultured under humidified air atmosphere at 28 ºC, 5% CO2 in DMEM 4.5 g/l glucose, supplemented with 0.01 mg/ml insulin, 50 ng/ml EGF, 5% (v/v) of antibiotic/antimycotic solution, 10% (v/v) heat-inactivated FBS and 0.5% (v/v) heat-inactivated trout serum. Human Embryonic Kidney 293 cells (HEK-293, CRL-1573, ATCC) were cultured at 37 ºC, 5% CO2 in DMEM 4.5 g/l glucose supplemented with antibiotic/antimycotic solution, 10% (v/v) heat-inactivated FBS. Prior to PAMPs stimulation, the ZFL cells were incubated in non-supplemented DMEM for 3 h. Cells were treated with poly (I:C), Lipopolysaccharide (LPS), Peptidoglycan (PGN), Zymosan and RNA extracted from *Vibrio vulnificus* in a range from 1 to 50 μg/ml at the indicated times. For transfection, a DNA construct coding for the TFP protein alone was used [7]. ZFL and HEK-293 cells were seeded in 24-well cell culture plates one day prior to transfection. The plasmid DNA was incubated with the NanoJuice Transfection Kit (1μg pDNA/3μl NanoJuice Transfection Kit, Merck Millipore, Germany) for 30 min in DMEM without FBS and cell culture medium was replaced by the mixture. After incubation at 37ºC for 4 h the medium was removed and supplemented culture medium was added for 20 h. To calculate the transfection efficiencies (%) flow cytometry was performed using a FACS Canto cytometer (Becton Dickinson, USA).

Poly (I:C) uptake analysis

ZFL cells were plated on sterile 6-well plates until 80% confluence was achived. 3 h prior to stimulation the medium was removed and fresh non-supplemented DMEM medium was added. Cells were incubated with 10 μg/ml of fluorescent poly (I:C) for 24 h. Poly(I:C)-Fluorescein was used for the confocal microscopy and flow cytometry analysis. After the incubation, cells were washed with PBS and the nuclei were stained with Hoechst 33342 and the membranes with CellMask Deep Red. The samples were observed using a confocal microscope (Zeiss LSM 700, Germany). For flow cytometry, after the incubation the cells were rapidly cooled down, washed 3x with ice-cold PBS, trypsinized and centrifuged at 200 xg for 5 min. The resulting pellets were resuspended in ice-cold PBS for FACS analysis using a FACSCanto cytometer (Becton Dickinson, USA). Experiments were performed in triplicate and for each sample 10,000 events were

collected. The internalization of the fluorescent marker was calculated as the mean fluorescence intensity (MFI).

Total RNA extraction from *Vibrio vulnificus*

Vibrio vulnificus BT2 SerE strain used in this study was the wild type (R99 – pathogenic and virulent). *V. vulnificus* was grown on Tryptone Soy Agar for 24 h at 30ºC followed by an O/N incubation on Tryptone Soy Broth supplemented with 0.5% (w/v) NaCl medium until saturated concentration of 10^9 cfu was reached. Bacterial suspension was then diluted 1:10 and left to incubate for 3 h. Concentration was then checked and total RNA extracted using RNeasy Mini Kit (Qiagen, Germany) following manufacturer's instructions including a DNase digestion step. RNA quantification was carried out with the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA) and the integrity of the RNA was checked using Bioanalyser-2100 with the RNA 6000 Nano Lab-Chip kit (Agilent Technologies, USA).

Poly (I:C) injection into adult zebrafish

Adult zebrafish (*Danio rerio*) of an average body weight of 1.5–2.5 g were held in tanks with recirculating water under a photoperiod of 12 h light/12 h dark at 28ºC. Twelve individuals for each condition were placed in smaller tanks 24 h before the stimulation. Fish were anaesthetised with ethyl 3-aminobenzoate methanesulfonate (MS-222, 40 ppm) and intraperitoneally injected with 6 mg/kg poly (I:C) or sterile PBS. After 6 h animals were killed by over anesthetization (MS-222, 200 ppm), livers were removed, immediately frozen in liquid nitrogen and stored at –80ºC until use. All experimental procedures were submitted to the Ethical Committee of the Universidad Autonoma de Barcelona (Reference number 1555) and authorized by the "Departament d'Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya", that agree with the International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63).

Gene expression studies

RNA extraction, reverse transcription and qPCR: Total RNA from ZFL cell culture was extracted using TriReagent following manufacturer's instructions whereas RNeasy Micro Kit (Qiagen) was used for the zebrafish liver tissue extraction. RNA quantification was carried out with the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA). The integrity of the RNA was checked using a Bioanalyser-2100 with the RNA 6000 Nano Lab-Chip kit (Agilent Technologies, USA). The cDNA synthesis was performed with 1.5 µg of total RNA using SuperScript III reverse transcriptase (Invitrogen) and oligo-dT15 primer (Promega) according to the manufacturer's instructions. Conventional PCR was carried out to analyze gene expression and 1 µl of cDNA was used as a template with specific primers (**Supplementary Table 1, Annex 1**) Elongation factor 1 (EF1) was used as housekeeping gene for the ZFL cell line. PCR products were resolved on 1% agarose gels stained with GelGreen (Biotium). Quantitative real-time PCR (qPCR) was carried out using SYBR Green I PCR Supermix (Bio-Rad), 500 nM of primers (**Supplementary Table 1, Annex 1**) and 5 µl of cDNA which had been previously diluted (1:25 for target mRNA and 1:500 for reference gene) in a 20 µl final volume reaction. Quantification was done according to Livak method [25]. All samples were run in triplicate. Date were analysed by One-way ANOVA followed by Tukey's post-test.

Microarray analysis: Microarray hybridizations were performed using the *D. rerio* oligonucleotide microarray (ID 0303478005 v.3) 4 x 44K (Agilent). RNA labelling, hybridizations and scanning were performed according to manufacturer's instructions. Briefly, 150 ng of total RNA extracted from ZFL cell culture was amplified and Cy3-labeled with Agilent's One-Color Micro-

array-Based Gene Expression Analysis (Low Input Quick Amp Labelling kit) along with Agilent's One-Color RNA SpikeIn Kit. Each sample (control, 25 μg/ml and 50 μg/ml) in triplicate (1.65 μg) was hybridized at 65°C for 17 h and arrays were scanned with Agilent Scanner G2505B. Spot intensities and other quality control features were extracted with Agilent's Feature Extraction software (10.4.0.0) and microarray data was analyzed using GeneSpring software and DAVID Bioinformatics Resources 6.7 [26].

TNFα secretion.

Adherent trout monocyte/macrophages were isolated as previously described [27] and cultured at 18ºC, 5% CO2 in DMEM 4.5 g/l glucose, supplemented with 10% (v/v) heat-inactivated FBS and 100 μg/ml Primocin. Before treatments, differentiated macrophages were incubated in serum free medium for 3 h. Differentiated macrophages were incubated with poly (I:C) and RNA extracted from *Vibrio vulnificus* in a range from 1 to 20 μg/ml for 24 h. Supernatants were collected, centrifuged to remove cell debris, and precipitated with 25 % trichloroacetic acid (TCA). TNFα secretion from trout macrophages was then assessed by western blot as previously described [28].

Results

ZFL response to different PAMPs stimulation

In order to explore the capacity of the ZFL cells to respond to different immunologically relevant stimuli, we challenged the ZFL cells with different commercial PAMPs (LPS, poly (I:C), PGN and zymosan) at two different concentrations (25 and 50 μg/ml; **Figure 1A**). The ZFL cells were able to respond to bacterial (LPS and PGN), fungal (zymosan) and viral (poly (I:C)) stimuli by up-regulating immune response genes (**Figure 1A**). The most consistent gene expression responses were those involved in antiviral defence such as TLR3, INFγ and INFϕ or Mx genes while TNFα and IL10 showed a more promiscuous expression pattern being up-regulated after LPS, poly (I:C), PGN and Zymosan. PGRP2, one of the canonical peptidoglycan receptors showed up-regulation after peptidoglycan treatment (**Figure 1A**). Surprisingly, we detected a strong and consistent up-regulation of PGRP2 after poly (I:C) treatment (**Figure 1A and 1B**). The expression of PGRPs is usually up-regulated by exposure to bacteria or purified bacterial peptidoglycan, which is an essential cell wall component of virtually all bacteria [29]. This unexpected result led us to better quantify the expression of PGRP2 by qPCR in response to poly (I:C) (**Figure 1B**). We observed a 7.22 ± 2.62 fold change increase in response to 25 μg/ml poly (I:C) and a 5.74 ± 1.71 fold change increase after incubation with 50 μg/ml poly (I:C) in comparison to the expression levels of the viral response gene IFNΦ that were 12.93 ± 2.25 and 11.25 ± 3.97 fold change increased at 25 and 50 μg/ml respectively (**Figure 1B**). Interestingly, no major differences have been observed between the two poly (I:C) doses used, indicating that 25 μg/ml might be sufficient to achieve full activation. To further explore the gene expression pattern after poly (I:C) exposure we carried out a time-course and dose response analysis (**Figure 2A**) indicating again that ZFL cells showed a consistent and a strong anti-viral response. The expression levels of the genes Mxa, INFγ, TLR3 and CCL4 were up-regulated in response to poly (I:C) in a dose- and time-dependent manner. Even low poly (I:C) doses (5 μg/ml) were able to induce expression of antiviral genes (**Figure 2A**). MXa showed a delayed response with a peak at 24 h (**Figure 2**) while INFγ, PGRP2 and CCL4 showed an early response, peaking at 6-12 h. For TLR3 we observed a sustained (6 to 24 h) and dose dependent up-regulation. In addition, we carried out the gene expression analysis in liver of poly (I:C)-injected zebrafish to determine

weather the *in vivo* poly (I:C) injection was also able to induce an antiviral response and compare it to the *in vitro* ZFL response. As shown in **Figure 2B** MXa and GIG2 genes were up-regulated 6 h after poly (I:C) injection in all the individuals while TLR3 showed a constitutive expression in both the treated and non-treated zebrafish.

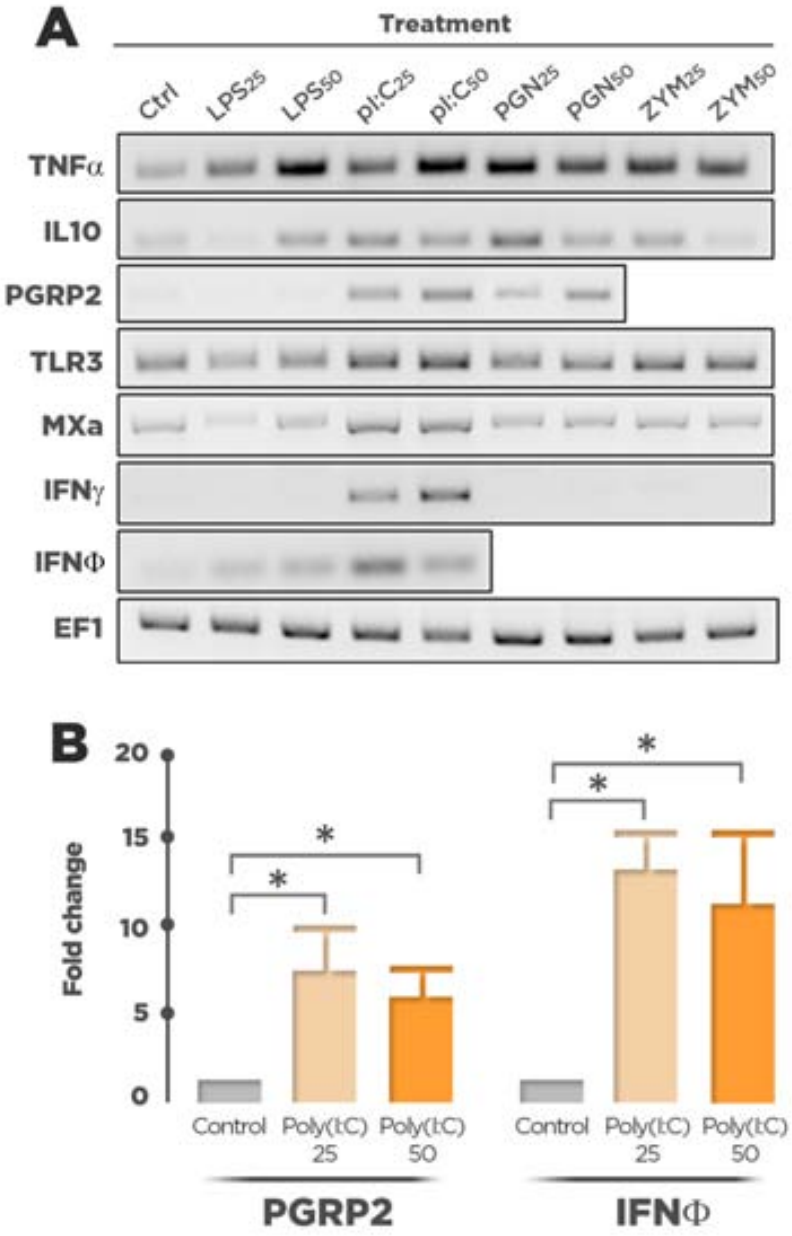


Figure 1. **Analysis of gene expression in ZFL cell culture after PAMPs stimulation.**

(A) ZFL response after 16 h exposure to: 25/50 µg/ml of LPS, 25/50 µg/ml of poly (I:C), 25/50 µg/ml of PGN and 25/50 µg /ml of Zymosan. TNFα, IL10, PGRP2, TLR3, MXa, IFNγ, IFNϕ mRNA abundance was analyzed by conventional PCR. **(B) qPCR analysis of the expression of PGRP2 and IFN ϕ response** after 16 h stimulation with 25/50 µg/ml of poly (I:C). Elongation factor (EF1) was used as reference gene. Data represents means ± SD of three independent experiments for each treatment and differences among groups were analyzed using One-way ANOVA followed by Tukey's post-test. *, $p < 0.05$.

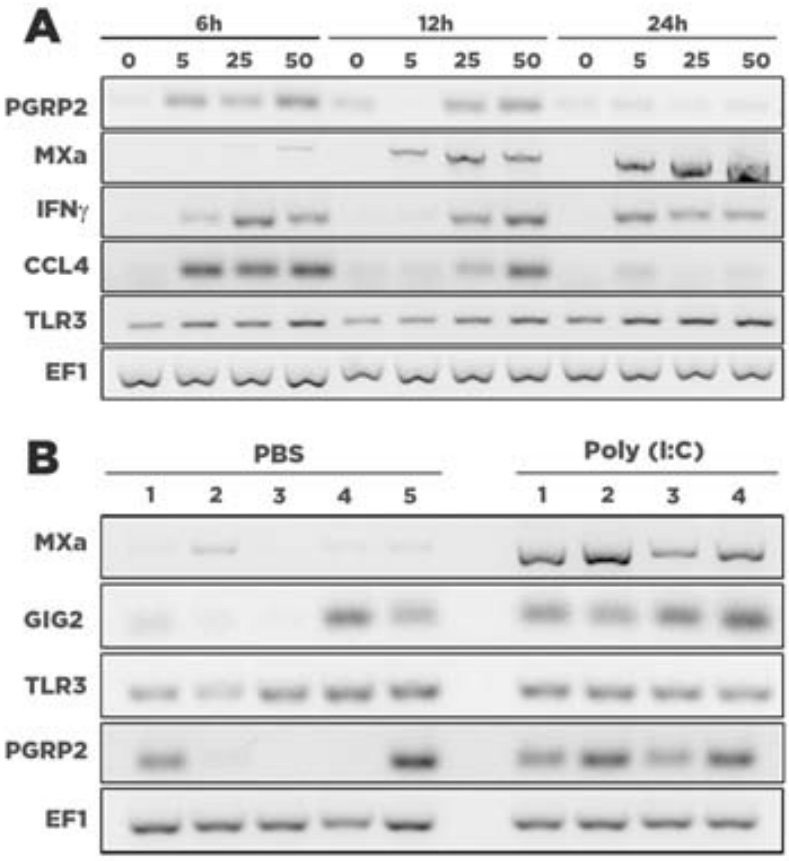


Figure 2. **Analysis of gene expression after poly (I:C) administration.**

(A) ZFL time course response to poly (I:C) ZFL transcript expression after exposure to: 5 - 50 μg/ml of poly (I:C) at different times (6 to 24 h). PGRP2, MXa, IFNγ, CCL4, TLR3, mRNA abundance was analyzed by conventional PCR. **(B) Analysis of transcript expression after adult zebrafish *in vivo* i.p injection of poly (I:C).** Gene expression at 6 h after injection of 6 mg/kg of poly (I:C) or PBS (controls). MXa, GIG2, TLR3, mRNA abundance was analyzed by conventional PCR. Elongation factor (EF1) was used as reference gene.

RNA isolated from *Vibrio vulnificus* stimulates the immune response in ZFL cells

In addition to commercially available PAMPs we wanted to evaluate the response against a non-commercial molecule isolated from a natural fish pathogen (*Vibrio vulnificus*). We purified total RNA from *Vibrio vulnificus* (vvRNA) in order to test weather bacterial RNA could also induce an immune response in ZFL cells. Dose response experiments with vvRNA at 1, 5 and 10 μg/ml were performed in parallel to poly (I:C) at 1, 5, 10 and 20 μg/ml (**Figure 3A**). Results showed that vvRNA was able to induce the expression of CCL4 and TLR3 at 10 μg/ml but not at the lowest concentrations. On the other hand, MX, INFΦ, TNFα and PGRP6 expression was induced even at 5 μg/ml while no GIG2 induction was observed at any vvRNA concentration (**Figure 3A**). The total RNA concentrations are in the range of concentrations previously published. For example, Erbele *et al.*, used 1 and 10μg/ml to stimulate PBLs [30] and Kanneganti *et al.*, used 2 and 4 μg/ml to stimulate peritoneal macrophages [31]. We could also see that the expression of most of the studied genes was different compared to the one obtained after the stimulation with poly (I:C). The levels of expression of CCL4, TLR3, GIG2, MX and TNFα were clearly higher after poly (I:C) stimulation.

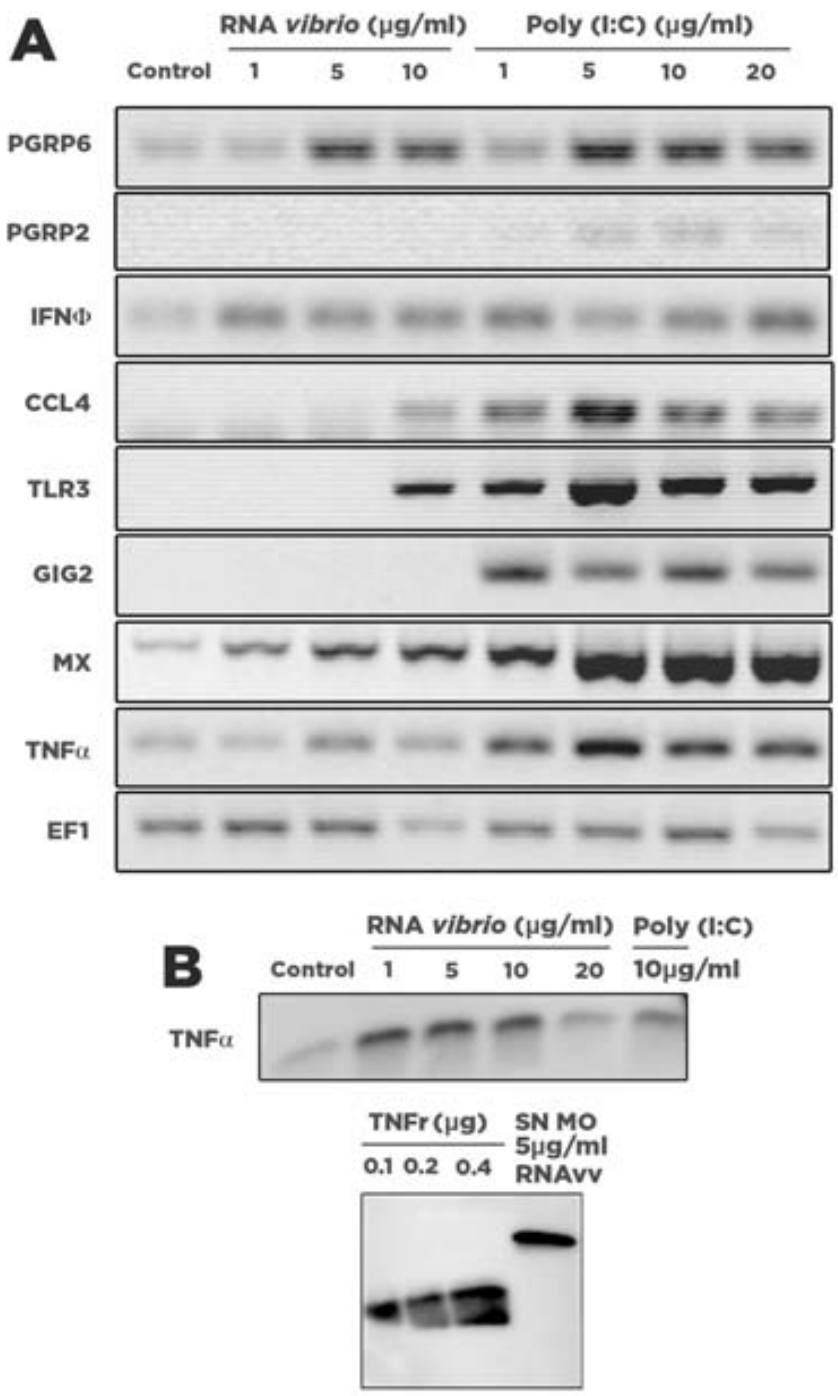


Figure 3. **Analysis of gene expression after *Vibrio vulnificus* RNA stimulation.**

(A) ZFL response after 16 h exposure to 1 -10 µg/ml of total RNA extracted from *Vibrio vulnificus* and 1 - 20 µg/ml of poly (I:C). PGRP6, PGRP2, IFNϕ, CCL4, TLR3, GIG2, MXa, TNFα mRNA abundance was analyzed by conventional PCR. Elongation factor (EF1) was used as reference gene. One representative of 3 individuals is shown. **(B) TNFα secretion from trout macrophages stimulated 24 h** with 10 µg/ml of poly (I:C) and 1 - 20 µg/ml of total RNA extracted from *Vibrio vulnificus* was assessed by western blot (upper panel). Western blot control (lower panel) with different amounts of recombinant trout TNF α and the supernatant from macrophages stimulated with 5 µg/ml of vvRNA. A representative western blot is shown.

The ability of vvRNA to induce pro-inflammatory cytokines was also evaluated in trout macrophage cultures. TNFα secretion was observed after treating trout macrophages with vvRNA (**Figure 3B, upper panel**). We compared the amount of secreted TNFα after vvRNA stimulation with a standard curve of recombinant TNFα from rainbow trout (trTNFα) and we observed high levels of TNFα secreted to the cell medium after stimulation with 5 μg/ml vvRNA (**Figure 3B, lower panel**). The viability of ZFL cells was also evaluated using MTT assay and it was not compromised by the vvRNA treatment (data not shown).

Gene expression analysis of poly (I:C) stimulated-ZFL cells

We were mainly interested to study the anti-viral response of ZFL cells and to better understand its anti-viral response profile we performed a high throughput expression analysis with zebrafish commercial microarrays using poly (I:C) at two different concentrations (25 and 50 μg/ml, 24 h). As seen before by conventional PCR in the dose response and time course experiments, no major differences were observed in the expression between the two poly (I:C) doses (**Figure 4A**). Only 14 genes (1% total regulated genes) were differently expressed (**Figure 4A**) and p-values and fold changes were very close to the chosen limit indicating that 25 μg/ml was a suitable dose to fully activate the antiviral response. As a general view we found 364 down-regulated genes (36%) mainly related with functional categories like DNA replication and metabolism, cell cycle or biosynthesis of fatty acids; and 646 up-regulated genes (64%). A list of selected differentially up-regulated genes is shown in **Figure 4B** and we can observe the activation of the antiviral programme in ZFL cells. These cells increase the expression of multiple genes related to antiviral response such as GIG2, interferons, TLR3, IFIH1 (MDA5) or IFIT5 among others (**Figure 4B**). A KEGG pathway analysis showed that we were able to detect a strong enrichment in the RIG-I-like receptor signaling pathway (p value < 1.7E-5) and in the Toll-like receptor signalling pathway (p value < 2.4E-4).

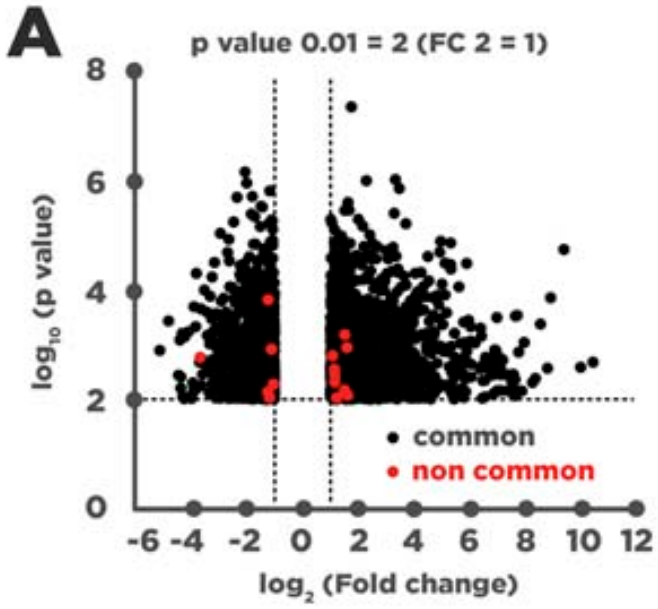
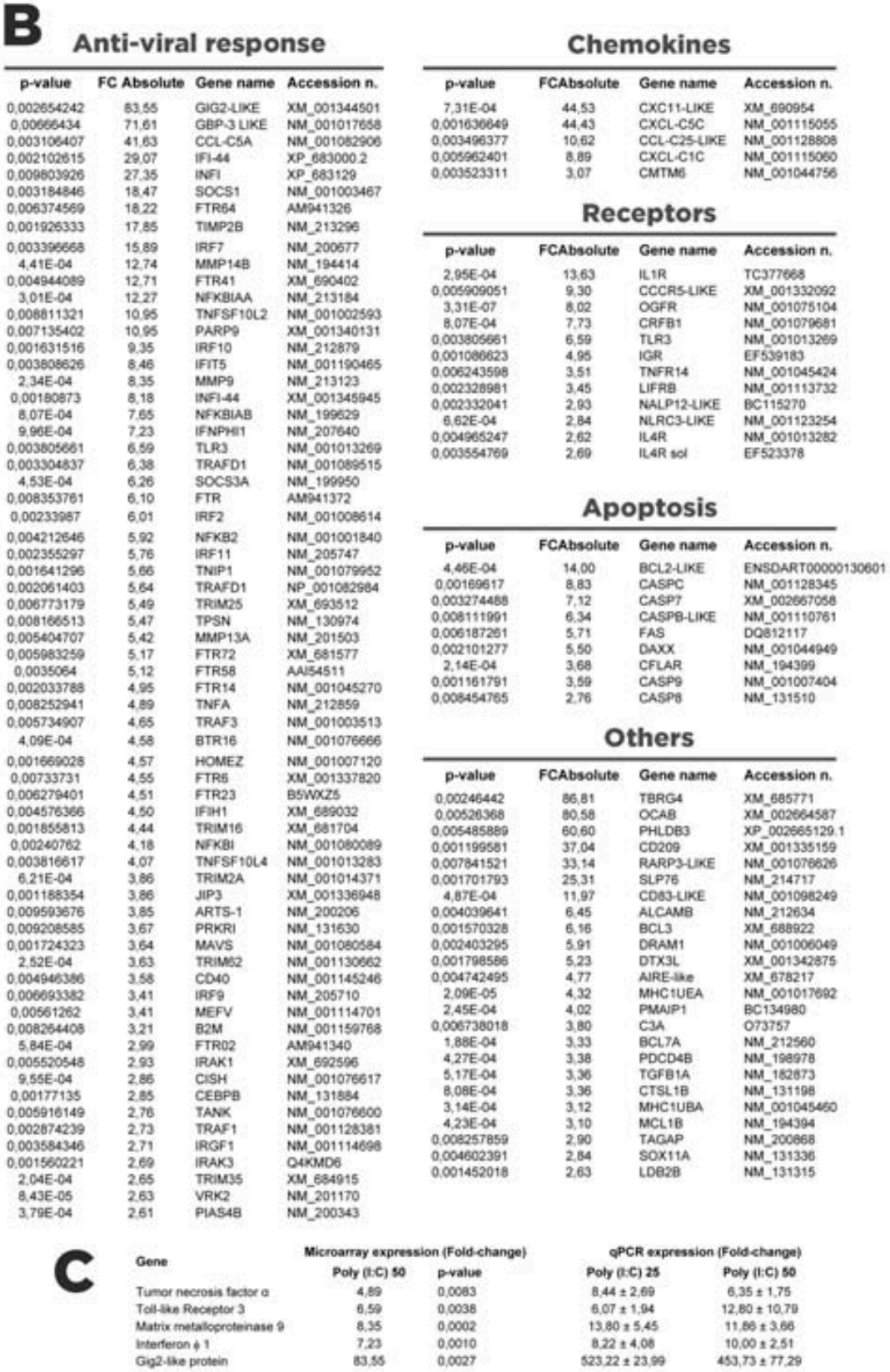


Figure 4. **Microarray and QPCR analysis of specific mRNA transcript expression in ZFL after poly (I:C) stimulation.**

(A) Vulcano plot of the regulated genes at 25 and 50 μg/ml poly (I:C).



B

Anti-viral response

p-value	FC Absolute	Gene name	Accession n.
0,002654242	83,55	GIG2-LIKE	XM_001344501
0,00666434	71,61	GBP-3 LIKE	NM_001017658
0,003106407	41,63	CCL-C5A	NM_001082906
0,002102615	29,07	IFI-44	XP_683000.2
0,009803926	27,35	INFI	XP_683129
0,003184846	18,47	SOCS1	NM_001003467
0,006374569	18,22	FTR64	AM941326
0,001926333	17,85	TIMP2B	NM_213296
0,003396668	15,89	IRF7	NM_200677
4,41E-04	12,74	MMP14B	NM_194414
0,004944089	12,71	FTR41	XM_690402
3,01E-04	12,27	NFKBIAA	NM_213184
0,008811321	10,95	TNFSF10L2	NM_001002593
0,007135402	10,95	PARP9	XM_001340131
0,001631516	9,35	IRF10	NM_212879
0,003808626	8,46	IFIT5	NM_001190465
2,34E-04	8,35	MMP9	NM_213123
0,00180873	8,18	INFI-44	XM_001345945
8,07E-04	7,65	NFKBIAB	NM_199629
9,96E-04	7,23	IFNPHI1	NM_207640
0,003805661	6,59	TLR3	NM_001013269
0,003304837	6,38	TRAFD1	NM_001089515
4,53E-04	6,26	SOCS3A	NM_199950
0,008353761	6,10	FTR	AM941372
0,00233987	6,01	IRF2	NM_001008614
0,004212646	5,92	NFKB2	NM_001001840
0,002355297	5,76	IRF11	NM_205747
0,001641296	5,66	TNIP1	NM_001079952
0,002061403	5,64	TRAFD1	NP_001082984
0,006773179	5,49	TRIM25	XM_693512
0,008166513	5,47	TPSN	NM_130974
0,005404707	5,42	MMP13A	NM_201503
0,005983259	5,17	FTR72	XM_681577
0,0035064	5,12	FTR58	AAI54511
0,002033788	4,95	FTR14	NM_001045270
0,008252941	4,89	TNFA	NM_212859
0,005734907	4,65	TRAF3	NM_001003513
4,09E-04	4,58	BTR16	NM_001076666
0,001669028	4,57	HOMEZ	NM_001007120
0,00733731	4,55	FTR6	XM_001337820
0,006279401	4,51	FTR23	B5WXZ5
0,004576366	4,50	IFIH1	XM_689032
0,001855813	4,44	TRIM16	XM_681704
0,00240762	4,18	NFKBI	NM_001080089
0,003816617	4,07	TNFSF10L4	NM_001013283
6,21E-04	3,86	TRIM2A	NM_001014371
0,001188354	3,86	JIP3	XM_001336948
0,009593676	3,85	ARTS-1	NM_200206
0,009208585	3,67	PRKRI	NM_131630
0,001724323	3,64	MAVS	NM_001080584
2,52E-04	3,63	TRIM62	NM_001130662
0,004946386	3,58	CD40	NM_001145246
0,006693382	3,41	IRF9	NM_205710
0,00561262	3,41	MEFV	NM_001114701
0,008264408	3,21	B2M	NM_001159768
5,84E-04	2,99	FTR02	AM941340
0,005520548	2,93	IRAK1	XM_692596
9,55E-04	2,86	CISH	NM_001076617
0,00177135	2,85	CEBPB	NM_131884
0,005916149	2,76	TANK	NM_001076600
0,002874239	2,73	TRAF1	NM_001128381
0,003584346	2,71	IRGF1	NM_001114698
0,001560221	2,69	IRAK3	Q4KMD6
2,04E-04	2,65	TRIM35	XM_684915
8,43E-05	2,63	VRK2	NM_201170
3,79E-04	2,61	PIAS4B	NM_200343

Chemokines

p-value	FCAbsolute	Gene name	Accession n.
7,31E-04	44,53	CXC11-LIKE	XM_690954
0,001636649	44,43	CXCL-C5C	NM_001115055
0,003496377	10,62	CCL-C25-LIKE	NM_001128808
0,005962401	8,89	CXCL-C1C	NM_001115060
0,003523311	3,07	CMTM6	NM_001044756

Receptors

p-value	FCAbsolute	Gene name	Accession n.
2,95E-04	13,63	IL1R	TC377668
0,005909051	9,30	CCCR5-LIKE	XM_001332092
3,31E-07	8,02	OGFR	NM_001075104
8,07E-04	7,73	CRFB1	NM_001079681
0,003805661	6,59	TLR3	NM_001013269
0,001086623	4,95	IGR	EF539183
0,006243598	3,51	TNFR14	NM_001045424
0,002328981	3,45	LIFRB	NM_001113732
0,002332041	2,93	NALP12-LIKE	BC115270
6,62E-04	2,84	NLRC3-LIKE	NM_001123254
0,004965247	2,62	IL4R	NM_001013282
0,003554769	2,69	IL4R sol	EF523378

Apoptosis

p-value	FCAbsolute	Gene name	Accession n.
4,46E-04	14,00	BCL2-LIKE	ENSDART00000130601
0,00169617	8,83	CASPC	NM_001128345
0,003274488	7,12	CASP7	XM_002667058
0,008111991	6,34	CASPB-LIKE	NM_001110761
0,006187261	5,71	FAS	DQ812117
0,002101277	5,50	DAXX	NM_001044949
2,14E-04	3,68	CFLAR	NM_194399
0,001161791	3,59	CASP9	NM_001007404
0,008454765	2,76	CASP8	NM_131510

Others

p-value	FCAbsolute	Gene name	Accession n.
0,00246442	86,81	TBRG4	XM_685771
0,00526368	80,58	OCAB	XM_002664587
0,005485889	60,60	PHLDB3	XP_002665129.1
0,001199581	37,04	CD209	XM_001335159
0,007841521	33,14	RARP3-LIKE	NM_001076626
0,001701793	25,31	SLP76	NM_214717
4,87E-04	11,97	CD83-LIKE	NM_001098249
0,004039641	6,45	ALCAMB	NM_212634
0,001570328	6,16	BCL3	XM_688922
0,002403295	5,91	DRAM1	NM_001006049
0,001798586	5,23	DTX3L	XM_001342875
0,004742495	4,77	AIRE-like	XM_678217
2,09E-05	4,32	MHC1UEA	NM_001017692
2,45E-04	4,02	PMAIP1	BC134980
0,006738018	3,80	C3A	O73757
1,88E-04	3,33	BCL7A	NM_212560
4,27E-04	3,38	PDCD4B	NM_198978
5,17E-04	3,36	TGFB1A	NM_182873
8,08E-04	3,36	CTSL1B	NM_131198
3,14E-04	3,12	MHC1UBA	NM_001045460
4,23E-04	3,10	MCL1B	NM_194394
0,008257859	2,90	TAGAP	NM_200868
0,004602391	2,84	SOX11A	NM_131336
0,001452018	2,63	LDB2B	NM_131315

C

Gene	Microarray expression (Fold-change)		qPCR expression (Fold-change)	
	Poly (I:C) 50	p-value	Poly (I:C) 25	Poly (I:C) 50
Tumor necrosis factor α	4,89	0,0083	8,44 ± 2,69	6,35 ± 1,75
Toll-like Receptor 3	6,59	0,0038	6,07 ± 1,94	12,80 ± 10,79
Matrix metalloproteinase 9	8,35	0,0002	13,80 ± 5,45	11,86 ± 3,66
Interferon ϕ 1	7,23	0,0010	8,22 ± 4,08	10,00 ± 2,51
Gig2-like protein	83,55	0,0027	523,22 ± 23,99	453,73 ± 77,29

Figure 4 (continuation). **Microarray and QPCR analysis of specific mRNA transcript expression in ZFL after poly (I:C) stimulation.**

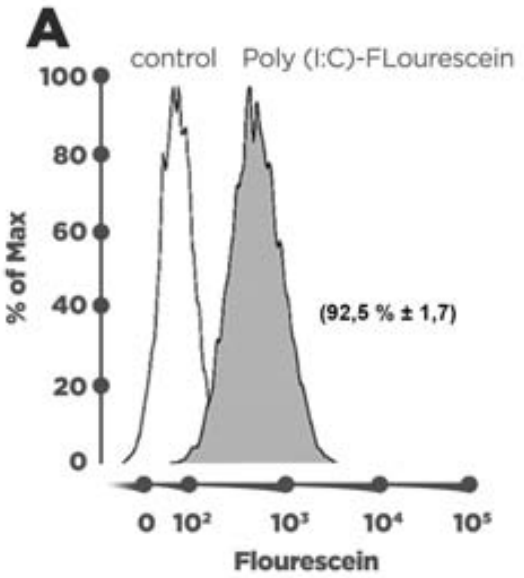
(B) Table of the significant (p-value <0.01) up-regulated genes of the ZFL cells microarray. **(C) Comparative table showing the expression (Fold-change)** obtained by microarray analysis and qPCR of representative genes after stimulation with poly (I:C).

The pathways included the main intracellular receptors involved in virus detection (TLR3, RIG-I and MDA5) together with several components of their signalling pathways (MAVS, TRAF, IRAK or SOCS) (**Supplementary Table 1, Annex 1**). Also a KEGG pathway involved in apoptosis was significantly enriched with genes like Fas, CASP8 or 9 (data not shown). Interestingly a functional category named Zinc finger-RING type included a set of finTRIM genes (finTRIM 2, 25, 35, 39, 58, 62, 64 and 72) (data not shown). The finTrim genes has also been involved in mammals in innate immune response against virus and have been described in several fish species [32]. Validation of the microarray results using representative genes (TNFα, TLR3, GIG2, MMP9 and INFϕ) is shown in **Figure 4C**. An excellent correlation between microarray and qPCR fold changes was observed and we also observed the qPCR data the saturation of the expression values between the 25 and 50 μg/ml poly (I:C) doses.

Uptake of Poly (I:C) labelled with fluorescein and transfection of ZFL cells

To further demonstrate that ZFL were able to respond to Poly (I:C) treatment we performed confocal microscopy and flow cytometry analysis using Poly (I:C) labelled with fluorescein. The ZFL cells treated with Poly (I:C)-Fluorescein were analysed by flow cytometry and 92.3 ± 1.7% of the treated cells were positive for fluorescein and therefore Poly (I:C) uptake (**Figure 5A**). Confocal microscopy imaging also showed that Poly (I:C)-Fluorescein (10 μg/ml) was efficiently endocytosed by ZFL cells (**Figure 5B**). The 3D reconstruction analysis demonstrated its full internalization forming cytosolic agglomerates compatibles with endosomal localization (**Figure 5 i, ii, iii**). This would indicate that poly (I: C) might be able to reach endosomal compartments for TLR3 binding.

Finally, we performed a set of experiments to evaluate the suitability of ZFL for transfection experiments and the results are shown in Table 1. ZFL cells cannot be transfected using PEI based systems (0,2% mean transfection efficiency) but can be transfected with moderate efficiency with cationic lipid-based system (GeneJuice). We achieved around 20% efficiencies using 1 μg of GFP plasmid at 24 h after transfection. When we increase the transfection time we did not observe an increase in the transfection efficiency (data not shown).



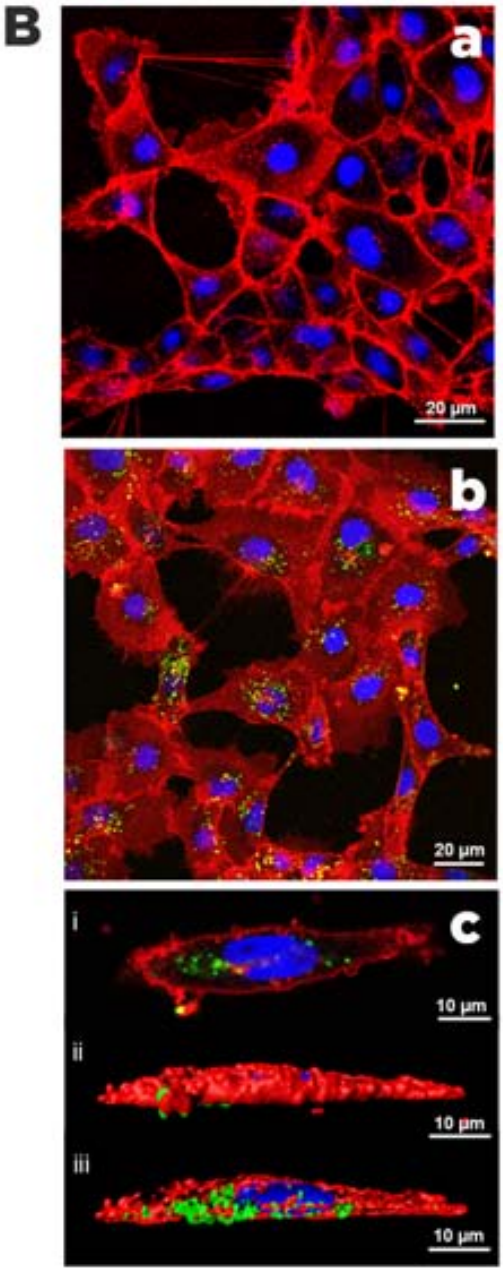


Figure 5. **Endocytosis of poly (I:C) by ZFL cells.**

(A) Representative flow cytometry plot shift after Poly (I:C) endocytosis by ZFL cells (dark grey). Cells were incubated 24 h with 10 µg/ml of Poly (I:C)-Fluorescein and the percentage of positive cells is shown.

(B) Representative confocal microscopy image of **(a) ZFL control cells** and **(b) ZFL cells treated with 10 µg/ml of poly (I:C)-Fluorescein for 24 h incubation**. Cell membranes were stained with CellMask Deep Red (red) and nuclei were stained with Hoechst 33342 (blue). **(c) (i) View of a single cell**, **(ii) 3D reconstruction of the whole cell** and **(iii) longitudinal stack of the cell showing the endocytosis of the poly (I:C).**

Discussion

In this study we characterised the immune response of the ZFL cell line under different relevant immune stimuli and we showed that ZFL cells were able to respond to bacterial (lipopolysaccharide, peptidoglycan, RNA), viral (poly (I:C)) and fungal (zymosan) stimuli by up-regulating canonical genes related to pathogenic defence [33, 34]. Many groups have been using zebrafish as a model to study fish viral response but to date no virus naturally infecting zebrafish has been described [35]. However the zebrafish embryo and adult are able to counteract model viral infections, such as spring viremia of carp virus [36, 37], snakehead rhabdovirus [38] and infectious hematopoietic necrosis virus [39]. Thus, we were interested on characterizing in depth, at the cellular and transcriptomic level, whether ZFL cells had a typical anti-viral response and could be used as a model. As emerged from the microarray and qPCR data the main antiviral pathways involving TLR3, MDA5 (IFIH1) and IFIT5 are strongly up-regulated. These cytosolic receptors have overlapping binding properties with viral PAMPs and share similar signalling pathways both leading to the activation of the interferon system [40]. We also found up-regulated genes acting downstream of these receptors like MAVS (*Mitochondrial Antiviral Signaling protein*) that coordinates pathways leading to the activation of NF-kappa-B, IRF3 and IRF7, and to the subsequent production of antiviral cytokines such as IFN and CCL5 [41, 42]. The highest expression values obtained in the microarray were the ones for the GIG2-like and GBP-3-like genes (83.55 and 71.61-fold change respectively). GIG2 (*grass carp reovirus-induced gene 2*) is an interferon inducible gene specific of non-amniotes organisms with no significant homology to any known genes in mammalian genomes [43] and GBP3 is a guanylate binding protein belonging to the family of large GTPases [44]. Both genes have been described as interferon inducible genes. Also MXa is an interferon-induced GTP-binding protein with direct antiviral activity and well described to assess viral response due to its high and consistent expression levels [45-47]. By comparing the expression of MXa and GIG2 in ZFL cells (PCR and microarray data) to adult zebrafish livers (PCR) we found a good correlation between the *in vitro* and *in vivo* data. Thus, the ZFL cells anti-viral response would be a consistent model also to study the anti-viral zebrafish liver response *in vivo*.

Surprisingly, poly (I:C) treatment was also able to stimulate the expression of PGRP2 and PGRP6 genes in ZFL cells and adult zebrafish liver too. These genes have been involved in the response to peptidoglycan both in fish and mammals [29, 48, 49]. Recently, Hua Li *et al.* showed that grass carp PGRP6 was up-regulated after poly (I:C) treatment [50] and Chang *et al.* showed that silencing of PGRP5 in zebrafish embryos provoked a strong upregulation of TLR3 [51]. All these data lead us to hypothesised that there might be a crosstalk between poly (I:C) and peptidoglycan responses in fish that needs to be explored.

In this study, we have showed that total RNA from *V. vulnificus* was also acting as a PAMP triggering an inflammatory response in ZFL cells. An emerging area of research indicates that bacterial RNA acts as an antigen and, indeed some authors postulate that bacterial mRNA is a special type of PAMP that allows the immune system to detect bacterial viability activating a robust immune response [52]. On the other hand, Kannegani *et al.* described Nalp3 (cryopyrin) as the bacterial RNA cytosolic sensor leading to the activation of inflammasome [31], while Kawashima *et al.* proposed TLR3 as the main RNA sensor of self-microbiota in the gut [53] and Eberle *et al.* proposed the TLR7 present in human dendritic cells as the bacterial RNA receptor [30]. The RNA doses used by different authors [30, 31, 52, 54] to stimulate expression or release of cyto-

kines are similar to those used in ZFL cells in this work (1-10 μg/ml). Here, we provide preliminary evidence that bacterial RNA is also a PAMP in zebrafish cells and may be sensed through TLR3 although further experiments would be necessary to characterise this response.

The study of fish innate immune system is of great importance since it sheds light on the pathogenic mechanisms and therefore, helps on the design of adjuvants and vaccination strategies for fish. Viral infections in particular, are devastating diseases in fish farms and there is a clear need for the design of new vaccines, adjuvants and delivery systems to improve its efficacy [55]. A promising strategy in fish vaccination against viral infections is the use of DNA vaccines [56] and references herein). For instance, a DNA vaccine against infectious hematopoietic necrosis virus (IHNV) disease was licensed in Canada with good protection results [56]. DNA vaccines can be administrated naked or using delivery systems based on cationic liposomal formulations [57]. In this context, the ZFL cell line can be used to study and model the cellular response to different DNA vaccine delivery systems that are usually evaluated *in vitro* using lipofectamine-like products. The ZFL cells can also be transfected using lipofectamine-like methods with transfection percentages (11,9 ± 3,9%) similar to the transfection levels obtained in ZF4 cells (15-20,0% [19], and in ZFB1 cells, a non-commercial zebrafish cell line (22,0%, [58]. Although these percentages are quite low when we compared them to HEK-293 cells transfected with a lipofectamine-like compound or PEI (71,5 ± 4,6% and 65,0 ± 10,0 respectively), they are still in the range of transfection levels described for other fish cell lines using cationic lipid-based systems described for other fish cell lines [10, 58, 59] (Table 1). Is worth to mention that when using a nucleofector system the transfection efficiencies of ZFL increased until 70% [10, 60]. In summary, our analysis showed that ZFL cell line is a valuable tool for its use as model cell line in fish immunology research, specially to address the study of anti-viral responses and the putative DNA vaccination strategies using delivery systems.

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chapter 2

A Novel **Liposome-Based Nanocarrier** Loaded with an **LPS-dsRNA** Cocktail for Fish **Innate Immune** System **Stimulation**

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Abstract

Development of novel systems of vaccine delivery is a growing demand of the aquaculture industry. Nano- and micro-encapsulation systems are promising tools to achieve efficient vaccines against orphan vaccine fish diseases. In this context, the use of liposomal based-nanocarriers has been poorly explored in fish; although liposomal nanocarriers have successfully been used in other species. Here, we report a new ~125 nm-in-diameter unilamellar liposome-encapsulated immunostimulant cocktail containing crude lipopolysaccharide (LPS) from *E. coli* and polyinosinic:polycytidylic acid [poly (I:C)], a synthetic analog of dsRNA virus, aiming to be used as a non-specific vaccine nanocarrier in different fish species. This liposomal carrier showed high encapsulation efficiencies and low toxicity not only *in vitro* using three different cellular models but also *in vivo* using zebrafish embryos and larvae. We showed that such liposomal LPS-dsRNA cocktail is able to enter into contact with zebrafish hepatocytes (ZFL cell line) and trout macrophage plasma membranes, being preferentially internalized through caveolae-dependent endocytosis, although clathrin-mediated endocytosis in ZFL cells and macropinocytocis in macrophages also contribute to liposome uptake. Importantly, we also demonstrated that this liposomal LPS-dsRNA cocktail elicits a specific pro-inflammatory and anti-viral response in both zebrafish hepatocytes and trout macrophages. The design of a unique delivery system with the ability to stimulate two potent innate immunity pathways virtually present in all fish species represents a completely new approach in fish health.

Introduction

The development of sustainable aquaculture, a strategic sector to feed the ever-increasing human population [1], relies on disease prevention through the implementation of preventive immunostimulation and effective vaccination strategies [2]. With the advent of liposomal vaccines, one can begin to conceive new non-invasive, non-stressful and easy-to-manage methods for administering immunostimulants and vaccines to a large number of cultured fish at any time of their life cycle. Liposomes are hollow spherical, safe and well-tolerated assemblies formed by a single lipid bilayer or multiple concentric bilayers that can be tailored (via selecting their composition, size, charge, etc.) to efficiently entrap a wide variety of immunostimulants and vaccines [3]. This encapsulation provides the obvious potential advantages of increasing their stability and protection, thus enhancing their immune response and disease protection, and opening up the possibility to design more efficient immunostimulant-vaccine cocktails. In addition, liposomes have been proven to act as adjuvants to potentiate immune responses alone and to be rapidly cleared from sites of administration, being preferentially distributed among macrophages [4]. Taking into account these excellent properties and since liposomes can be stable in solution or be dried [5], new opportunities will be available to aquaculture to study such systems as new immunostimulant vehicles, which could be administered either dissolved in water (immersion bath), by injection, or orally via coated-food.

Herein, we describe a novel liposomal immunostimulant cocktail (hereafter called liposomal IS-cocktail) composed of two immunostimulants: the bacterial lipopolysaccharide (LPS) and the synthetic analog of dsRNA viruses, poly (I:C). Both bacterial and viral compounds were chosen to stimulate two potent innate immune pathways (TLR3 and TLR4 pathways) virtually present in all fish species [6]. The molecular basis of the immunostimulant action lies in the stimulation

of innate immunity through the binding and activation of innate pathogen recognition receptors (PRRs) located on antigen-presenting cells (APCs) [7]. The principal APCs in fish are macrophages, neutrophils, dendritic cells and B cells [8, 9, 10]. Upon immunization, APCs release a variety of cytokines and chemokines regulating both innate and adaptive immunity [11]. Triggering combinations of PRRs on APCs with natural or synthetic ligands can induce synergistic activation and production of cytokines [12, 13]. Indeed, LPS is present in the cell wall of G negative bacteria and signals through TLR4 in mammals. The synthetic analog poly (I:C) (dsRNA) mimics RNA viruses and signals through TLR3 located on endosomal membranes and through RIG-I and MDA5 located in the cytosol [11]. Teleost fish can respond to dsRNA through TLR3, RIG-I and MDA5 [14] and to crude LPS preparations probably through a sensing mechanism not involving TLR4 [15–17], but involving peptidoglycan recognition proteins and other intracellular receptors like Nod-like receptor 3 [18]. LPS would be an excellent candidate for immunostimulation purposes, but it has been scarcely used due to its high endotoxic potential in mammals. Fish are much less sensitive to LPS toxic effects [17] and, by encapsulating LPS, we have assayed a simple way to stimulate fish innate immune system. On the other hand, the addition of dsRNA to the nanocarrier would also target anti-viral response pathways [13].

Prior to this study, some advances have been made on the encapsulation of vaccines for fish vaccination and immunostimulation. Some of these studies have suggested that microencapsulated vaccines significantly enhance the protection and immune response in various fish species [19–22]. Thus far, however, no one has demonstrated the ability to simultaneously control the encapsulation of several immunostimulants in unilamellar, bio-compatible liposomes. Such capabilities would allow one to construct much more sophisticated and efficient liposomal immunostimulants for aquaculture. The approach used herein relies on the ability of using the surface charge of liposomes, which can be tailored by properly selecting the lipid head-groups, to optimize the encapsulation of both negatively charged LPS and dsRNA. In such design, PEGylated lipids have also been used in liposomal immunostimulant formulations to control the unilamellarity of liposomes and to prolong the plasma half-life of the immunostimulants [23, 24]. This study provides evidence that the optimized multifunctional liposomal IS-cocktail induces a concurrent anti-viral and pro-inflammatory state in zebrafish hepatocytes and trout macrophages. Moreover, insights into the mechanisms controlling the cell interaction and metabolism of the liposomes have demonstrated the possibility to target plasmatic membrane and intracellular compartments essential to achieve an optimum immune response. Our findings have also shown that the designed liposome formulations are safe at therapeutic doses and could be used in future fish health applications.

Materials and Methods

Materials

1,2-didodecanoyl-sn-glycero-3-phosphocholine (DLPC), 1,2- dioleoyl-sn-glycero-3-phosphoric acid monosodium salt (DOPA), Cholesterol (Chol), 3β-N-(di-methyl-amino-ethyl)carbamate hydrochloride (Cholesteryl), Cholesterol-PEG_{600} (Chol-PEG), lipopolysaccharides (LPS) from *E. coli* 0111:B4, TriReagent, insulin, EGF, chloroquine and all endocytosis inhibitors were purchased from Sigma-Aldrich. MarinaBlue-DHPE, fluorescein-DHPE, LPS-AlexaFluor594, antibiotic/antimycotic solution, TrypLE Express, Cell Mask Deep Red, Hoechst 33342 and Superscript III reverse transcriptase were purchased from Invitrogen. Poly (I:C) High Molecular Weight, poly (I:C)-Fluorescein and Primocin were purchased from InvivoGen, whereas ZFL

cells were purchased from ATCC. Oligo-dT15, GelGreen and SYBR Green I were purchased from Promega, Biotium and Bio-Rad, respectively.

Ethics statement

All experimental procedures involving rainbow trout (*Onchorynchus mykiss*) and 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).

Preparation and characterization of liposomes of immunoliposomal formulations

Liposomal formulations were prepared by the thin film hydratation method [25] with some modifications. Briefly, DOPA, DLPC, Chol, Cholesteryl and Chol-PEG_{600} were dissolved in chloroform solutions (100 mg/ml) and mixed at the desired molar ratios (Table 1). The organic solvent was then evaporated by rotary evaporation to obtain a lipid film. Later, the film was hydrated with 2 ml of PBS at 0.5 mg/ml poly (I:C) or 1.5 mg/ml LPS. The encapsulation of poly (I:C) or LPS was done with an immunostimulant:lipid ratio of 1:30 and 1:10, respectively. For the preparation of the liposomes that contained a cocktail of immunostimulants (hereafter called liposomal IS-cocktail), 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 110000 *xg* for 30 min at 10uC. Liposome integrity was checked by DLS and Cryo-TEM. 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). The morphology was examined using Cryo-Transmission electron microscopy (Cryo-TEM) in a JEOL-JEM 1400 microscope (JEOL Ltd., Japan). Liposome stability was followed (48 h at 28ºC) by turbidity measurement in a Turbiscan Lab Expert (Formulaction, France).

Encapsulation efficiency (EE)

Encapsulation efficiencies (EE) were calculated according to the equation $EE(\%) = [(C_{IS,total}-C_{IS,out})/C_{IS,total}] \times 100$, where $C_{IS,total}$ is the initial immunostimulant concentration and $C_{IS,out}$ is the concentration of non-encapsulated immunostimulant. To measure the $C_{IS,out}$, all liposome suspensions were centrifuged at 110000 *xg* for 30 min at 10ºC. Supernatant aliquots were taken to quantify the concentration of non-encapsulated poly (I:C) and LPS by UV-Vis spectroscopy using a Nanodrop ND-1000 (Thermo Scientific, USA). Poly (I:C) was linearly detected in a range from 2.5 μg/ml to 1 mg/ml (Abs at 250 nm, $r^2 = 0.999$), whereas LPS was linearly detected in a range from 4.0 μg/ml to 1 mg/ml (Abs at 269 nm, $r^2 = 0.999$). Liposomes that did not contain any encapsulated immunostimulant were also ultracentrifuged and their supernatant quantified (Abs at 220 nm) to verify that liposomes were properly precipitated. To calculate the EE of the liposomal IS-cocktail, the putative non-encapsulated immunostimulants in the supernatant were separated by aqueous Gel Permeation Chromatography (GPC, Ultrahydrogel 120, Waters, USA) and quantified by UV-Vis spectroscopy, where poly (I:C) and LPS were linearly detected. All experiments were done in triplicate.

Localization of liposome-encapsulated immunostimulants

Evaluation of the distribution of encapsulated immunostimulants in liposomes was done by confocal microscopy. The liposome bilayer was labeled with MarinaBlue-DHPE (0.005 molar ratio). Fluorescent LPS-AlexaFluor594 and poly (I:C)-Fluorescein were individually or simultaneously encapsulated in liposomes and the resulting liposomal formulations were examined using a Leica TCS SP5 confocal microscope (Leica Microsystems, Germany).

Cell culture

Zebrafish ZFL cells (CRL-2643, ATCC) were cultured at 28ºC, 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) 4.5 g/l glucose, supplemented with 0.01 mg/ml insulin, 50 ng/ml EGF, 5% (v/v) of antibiotic/antimycotic solution, 10% (v/v) heat-inactivated FBS and 0.5% (v/v) heat-inactivated trout serum (TS). HepG2 cells were grown at 37ºC, 5% CO2 in DMEM 4.5 g/l glucose, supplemented with 5% (v/v) of antibiotic/antimycotic solution and 10% (v/v) heat-inactivated FBS. Adherent trout monocyte/macrophages were isolated as previously described [8]. Before treatments, cells were incubated 3 h in serum free medium.

Cytotoxicity assays

Two different cell viability assays (MTT and LDH) were simultaneously performed using three cell lines (ZFL, HepG2 and primary trout macrophages). Cells were seeded at $2.5x10^5$ cells/ well. The medium was removed and fresh non-supplemented medium containing the liposome formulation at indicated concentration was added, incubating the cells for 24 h. Lactate dehydrogenase (Cytotoxicity Detection Kit LDH, Roche) activity in the medium and MTT assay on the cells were performed. Cell viability was expressed as a percentage of the control. All the measurements were done in triplicate in 3 independent experiments. Dose-response curves were fitted using a sigmoidal dose-response curve model provided in the GraphPad Prism 5.0 (GraphPad software, USA). EC_{50} value was derived from these fitted curves for single experiments. Differences among data were analyzed using One-way ANOVA followed by Tukey's post test $p<0.001$.

Endocytosis analysis using flow cytometry

To visualize liposome endocytosis, DHPE-fluorescein was incorporated at a 0.05 molar ratio into the liposomal IS-cocktail. Labeled liposomal IS-cocktail was added to either ZFL or trout macrophages to a final concentration of 750 μg/ml liposomal IS-cocktail (containing 25 μg/ml poly (I:C) and 12.5 μg/ml LPS) and incubated for selected times. After treatment, cells were cooled down, washed 36 with ice-cold PBS, trypsinized and centrifuged at 200 *xg* for 5 min. Pellets were resuspended in ice-cold PBS for FACS analysis using a BD FACSCanto cytometer (Becton Dickinson, USA). Experiments were performed in triplicate (10,000 events for each sample). The internalization of fluorescence was calculated as the mean fluorescence intensity (MFI). To compare membrane-bound versus endocyted liposomes, the medium was removed at different times (5, 15, 30 and 60 min), and the cells were washed either with ice-cold PBS (pH = 7.4) or with an ice-cold PBS-acetic acid (pH = 4.2) to remove the liposomes attached to the membrane. The remaining (internal) fluorescence of the cells was then analyzed using the PBS washed cells as a total uptake. The uptake of liposomes at long incubation times was also studied. When needed, cells were pretreated for 1 h with 100 μM chloroquine. Then, fluorescent liposomes were added and left to incubate 15 min for the ZFL cells and 30 min for the trout macrophages. After 3x PBS washes, liposome-free medium was added and cells were incubated for 1, 6 or 16 h in the presence of chloroquine, when required. Finally, cells were routinely treated for flow cytometry analysis. To determine the liposome endocytosis pathways, the following

inhibitors were used: methyl-β-cyclodextrin (MβCD, 5 mM), 5-(N-Ethyl-N-isopropyl)amiloride (EIPA, 50 mM), sucrose (300 mM for ZFL, 150 mM for trout macrophages) and wortmannin (W, 100 nM). The inhibitor's toxicity was assessed (**Figure S4 in Annex 2**) and working concentrations were selected. Cells were pretreated for 1 h with each inhibitor, and liposomes were added for 15 min (ZFL cells) or 30 min (trout macrophages). Finally, 1 h after adding the liposomes, cells were analysed by flow cytometry.

Endocytosis analysis using confocal microscopy

Cells were seeded one day before the endocytosis experiments. For short incubation times (from 30 min to 1.5 h), liposomal IS-cocktail was added at 750 μg/ml liposomal IS-cocktail (containing 25 μg/ml poly (I:C) and 12.5 μg/ml LPS). For the 16 h incubation time, liposomal IS-cocktail was added at 375 μg/ml liposomal IS-cocktail (containing 12.5 μg/ml poly (I:C) and 6.25 μg/ml LPS). After 3x PBS washes, cells were stained with CellMask and Hoechst and viewed under a Leica TCS SP5 confocal microscope (Leica Microsystems, Germany). Image analysis was performed using Imaris software and z-stacks were analyzed to visualize the particle contact sites and location.

Gene expression studies

Cells were stimulated for 16 h with 750 μg/ml of liposomal IS-cocktail containing 25 μg/ml poly (I:C) and 12.5 μg/ml LPS and 375 μg/ml of liposomal IS-cocktail containing 12.5 μg/ml poly (I:C) and 6.25 μg/ml LPS. Non-loaded liposomes and non-encapsulated IS were used as controls. Total RNA from the ZFL and trout macrophages cell cultures was extracted using TriReagent following manufacturer's instructions. The RNA quality and concentration was assessed and cDNA was synthesized with 1.0 μg and 0.5 μg of total RNA for ZFL cells and macrophages, respectively, using SuperScript III reverse transcriptase and oligo-dT15 primer. PCR was carried out with 1 ml of cDNA as a template with specific primers (Table S1 in File S1) and qPCR was carried out using SYBR Green I mix, 500 nM of primers and 5 μl of cDNA. Samples from 3 independent experiments were run in triplicate, and quantification was done according to Livak method [26].

TNFα secretion

Trout macrophages were incubated for 16 h with 375 μg/ml of liposomal IS-cocktail (with 12.5 μg/ml poly (I:C) and 6.25 μg/ml LPS). Non-loaded liposomes and free LPS were used as controls. Supernatants were collected, centrifuged and precipitated with 25% trichloroacetic acid. TNFα secretion was assessed by Western blot as previously described [16].

in vivo toxicological assays

Adult AB zebrafish (*Danio rerio*) were held in tanks with recirculating water under a photoperiod of 14 h light/10 h dark at 28ºC. Embryos were obtained from random pair-wise mating collected, rinsed and kept in E3 medium at 28ºC. Viable embryos and post-hatching larvae were plated in 96-well plates. Liposomal IS-cocktail (liposome concentrations from 0.75 to 6 mg/ml) were added to the wells (200 μl), and incubated for 120 h. The plate evaporation rate was minimized as previously described [27]. Non-loaded liposomes and non-encapsulated immunostimulants were used as controls, and 24 individuals for each condition were used. Hatching rate, cumulative mortality and malformations of the embryos were recorded every 24 h, and survival curves were plotted using the Kaplan-Meier method and analysed using the log-rank test. Larvae were also frozen at 280ºC and total RNA was isolated as indicated before for gene expression evaluation.

Results

Preparation and characterization of liposomal formulations

Series of liposomal formulations with different lipid membrane composition and net surface charges were prepared to determine the optimal liposomal formulation to achieve the maximum encapsulation efficiency of LPS and poly (I:C). Three lipid mixtures were studied, $NL_{1,n}$ and $NL_{2,n}$, formed by the cationic lipid mixture of DLPC-Cholesteryl-Chol-PEG, $NL_{3,n}$, constituted by the neutral mixture DLPC-Chol, and $NL_{4,n}$ and $NL_{5,n}$, formed by the anionic lipid mixture DLPC-DOPA-Chol-PEG (**Table 1**).

Name	Liposome composition		ζ' potential (mV)	Size (nm)
$NL_{1,n}$	DLPC 50% - Cholesteryl 35% - Cholesterol 10% - PEG5%	++	23.5±0.4	197.3±54.7
$NL_{2,n}$	DLPC 50% - Cholesteryl 10% - Cholesterol 35% - PEG5%	+	10.4±1.8	182.7±8.4
$NL_{3,n}$	DLPC 50% - Cholesteryl 45% - PEG5%		−5.4±1.7	204.5±21.6
$NL_{4,n}$	DLPC 40% - DOPA 10% - Cholesterol 45% - PEG5%	−	−19.0±0.5	185.1±9.5
$NL_{5,n}$	DLPC 15% - DOPA 35% - Cholesterol 45% - PEG5%	− −	−30.9±2.5	161.1±12.6

Table 1. **Composition and characterization of non-loaded liposomal formulations.**

In all formulations, small unilamellar vesicles (**Figure 1A**) were obtained with a mean size ranging from 161.1±12.6 nm to 204.5±21.6 nm. In all cases, a 5% of Chol-PEG_{600} was included to achieve uniform samples. Encapsulation efficiencies of LPS or poly (I:C) in the different $NL_{1,n}$ to $NL_{5,n}$ formulations were studied, showing that a positively charged liposome surface, like in $NL_{1,n}$ (+23.47±0.40 mV) and $NL_{2,n}$ (+10.43±1.77 mV), favors the encapsulation of LPS and poly (I:C). In contrast, the encapsulation efficiency of both LPS and poly (I:C) in liposomes decreased as the surface charge became more negative like in $NL_{5,n}$ (-30.87±2.53 mV), as previously described by Balazs *et al.* and Nakhla *et al.* [28,29]. It has been suggested that the attractive interaction between the negative charge of the immunostimulants and the positive charge of the liposome surface results in near-perfect conditions to achieve the highest encapsulation efficiencies [30]. For example, the influence of these interactions to the encapsulation of both LPS and poly (I:C) was further confirmed by a decrease of the positive f9 potential value down to -4.34±0,41 and 4.5±1.1 for both $NL_{2,LPS}$ and $NL_{2,poly\ (I:C)}$, respectively. The maximum loading efficiencies for LPS were 49.6±5.9% and 66.0±0.1% for $NL_{2,LPS}$ and $NL_{1,LPS}$, respectively. Interestingly, loading efficiencies achieved for poly (I:C) were always higher, with values of 95.0±1.4% and 91.2±0.1% for $NL_{2,\ poly\ (I:C)}$ and $NL_{1,\ poly\ (I:C)}$, respectively (**Table 2**). To further characterize the physico chemical structure of such cationic liposomal formulations, we encapsulated AlexaFluor594-labeled LPS (**Figure 1C**) and fluorescein-labeled poly (I:C) (**Figure 1D**) into Marina Blue-labeled liposomes (**Figure 1B**). Confocal microscope images of non-extruded liposomes demonstrated that both LPS and poly (I:C) were incorporated into their lipidic bilayer. **Figures 1C–D** show the spatial superimposition between fluorescence intensities of Alexa-Fluor594-LPS and Marina Blue-liposomes (**Figure 1C**) as well as of fluorescein-poly (I:C) and Marina Blue-liposomes (**Figure 1D**), further confirming that both immunostimulants are localized in the lipidic bilayer of cationic liposomes. Next, we investigated the cytotoxicity of cationic liposomes without encapsulated immunostimulants of both, $NL_{1,n}$ and $NL_{2,n}$ formulations, showing the maximum loading efficiencies (**Figure S1 in Annex 2**). Both types of liposomes were *in vitro* assayed on ZFL and HepG2 cell lines using MTT and LDH assays.

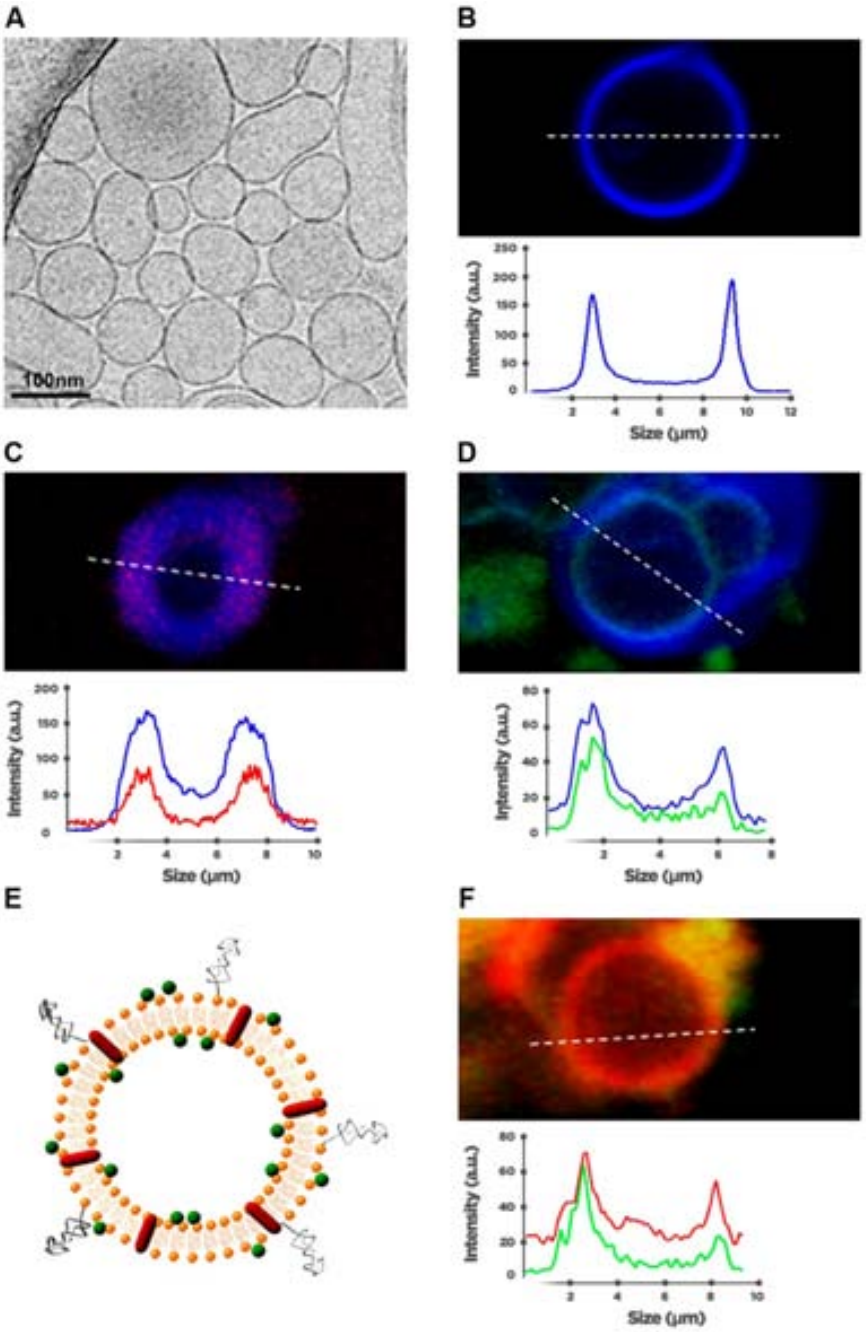

Figure 1. **Characterization of liposomal formulations.**

(A) Representative Cryo-TEM image of DLPC/Chol/Cholesteryl/PEG$_{600}$-Chol (5:3.5:1:0.5) liposomes extruded through a 200 nm pore size membrane. **(B) Confocal fluorescence image of a single liposome** tagged on its lipid bilayer with Marina Blue-DHPE **(blue)** and its corresponding fluorescence intensity profile. **(C) Confocal fluorescence image of a single Marina Blue-labeled liposome** containing AlexaFluor594-labeled LPS **(red)** and their corresponding fluorescence intensity profiles. **(D) Confocal fluorescence image of a single Marina Blue-labeled** liposome containing fluorescein-labeled poly (I:C) and their corresponding fluorescence intensity profiles. **(E) Schematic representation of the liposomal IS-cocktail (NL$_c$)** showing the presence of both encapsulated LPS (red) and poly (I:C) **(green)** in the lipidic bilayer of liposomes. **(F) Confocal fluorescence image of a single liposome containing** both fluorescein-labeled poly (I:C) **(green)** and AlexaFluor594-labeled LPS **(red)** and their corresponding fluorescence intensity profiles,

Name	EE LPS (%)	EE poly (I:C) (%)
$NL_{1,LPS}$	66.0±0.1	
$NL_{1,\ poly\ (I:C)}$		91.2±5.9
$NL_{2,LPS}$	49.6±5.9	
$NL_{2,\ poly\ (I:C)}$		95.0±1.4
$NL_{3,LPS}$	6.9±0.4	
$NL_{3,\ poly\ (I:C)}$		25.8±7.6
$NL_{4,LPS}$	5.9±3.2	
$NL_{4,\ poly\ (I:C)}$		38.0±4.5
$NL_{5,LPS}$	2.0±1.3	
$NL_{5,\ poly\ (I:C)}$		12.9±4.3

Table 2**. Efficiencies for the encapsulation of LPS and poly (I:C)**.

Encapsulation efficiencies (EE) for separately encapsulating an initial concentration of 1.5 mg/ml of LPS and 0.5 mg/ml of poly (I:C) into 15 mg/ml of the liposomal (NL_{1-5}) formulation.

Interestingly, $NL_{1,n}$ and $NL_{2,n}$ liposomes showed similar cytotoxicity activity in HepG2 cells (**Figure S2 in Annex 2**). However, the more cationic liposomes ($NL_{1,n}$) clearly showed higher toxicity on ZFL cells (EC50 = 0.166 mg/ml) than the less cationic one ($NL_{2,n}$). Because of their similar loading efficiencies but different cytotoxicity, the less toxic $NL_{2,n}$ formulation (DLPC 50%-Cholesteryl 10%-Chol 35%-Chol-PEG 5%) was finally chosen as the ideal liposomal composition to co-encapsulate LPS and poly (I:C) (**Figure 1E**).

Using these conditions, the resulting liposomal IS-cocktail (hereafter referred to as NL_c formulation) was composed of 125.8±6.6 nm-in-diameter liposomes that entrapped both LPS and poly (I:C) with loading efficiencies of 22.3±2.1% and 99.6±0.1%, respectively. Therefore, the NL_c formulation was composed of a mixture of 15 mg/ml of liposomes containing 250 μg/ml and 500 μg/ml of LPS and poly I:C, respectively. Importantly, after co-encapsulating LPS and poly (I:C), such liposomes exhibited a slightly positive surface charge (1.37±3.58 mV), which was attributed to electrostatic interactions between their positively charged lipidic bilayer and the negatively charged immunostimulants. The occurrence of these attractive interactions was corroborated by co-encapsulating AlexaFluor594- labeled LPS and fluorescein-labeled poly (I:C) into cationic liposomes, from which the localization of both immunostimulants in the lipidic bilayer was observed (**Figure 1F**).

Evaluation of cell toxicity of liposomal $NL_{2,LPS}$, $NL_{2,poly\ (I:C)}$ and NL_c formulations on zebrafish hepatocytes and trout macrophages primary cultures

To fully characterize the safety of our formulations, we carried out *in vitro* cytotoxic studies (**Figure 2** and **Figures S2, S3 in Annex2**). The therapeutic immunostimulant doses were chosen according to our previous results on LPS and poly (I:C) responses in different fish species [16,31]. Based on these results, dose-response experiments were conducted with $NL_{2,n}$, $NL_{2,LPS}$, $NL_{2,poly\ (I:C)}$ and NL_c in ZFL cells at the indicated concentrations (**Figure 2**). None of the encapsulating formulations showed toxicity at potential therapeutic doses in these cells. Moreover, free LPS toxicity (50 mg/ml LPS, 51.8%±17.9 viability and 25 μg/ml LPS, 62.0%±6.01 viability) was avoided by nanoencapsulation. Also, poly (I:C) treatment prompted a slight decrease in viability (50 μg/ml poly I:C, 80.32%±7.01 viability) that was fully reverted when this molecule was encapsulated (**Figure 2B**).

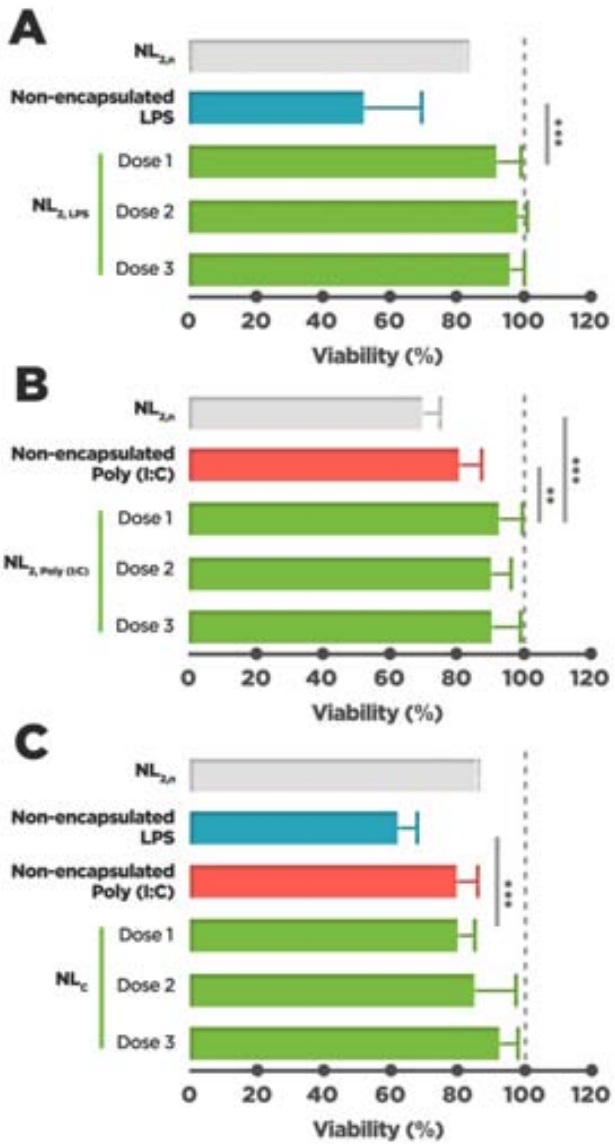

Figure 2. **Cytotoxicity of $NL_{2,\ LPS}$, $NL_{2,\ poly\ (I:C)}$, and NL_c formulations in ZFL cells by MTT-based assay.**

(A) Viability of ZFL after 24 h incubation with liposome-encapsulated LPS ($NL_{2,\ LPS}$, green bars) at Dose 1 = 1 mg/ml liposome with 50 µg/ml LPS, Dose 2 = 0.5 mg/ml liposome with 25 µg/ml LPS and Dose 3 = 0.20 mg/ml liposome with 10 µg/ml LPS. The white bar is the empty liposome control ($NL_{2,n}$, 1 mg/ml liposome) and the blue bar is the free LPS control (50 µg/ml). **(B) Viability of ZFL after 24 h incubation the liposome-encapsulated poly (I:C)** ($NL_{2,\ poly\ (I:C)}$, green bars) at Dose 1 = 1.5 mg/ml liposome with 50 µg/ml poly (I:C), Dose 2 = 0.75 mg/ml liposome with 25 µg/ml poly (I:C) and Dose 3 = 0.375 mg/ml liposome with 10 µg/ml poly (I:C). The white bar is the empty liposome control treatment ($NL_{2,n}$, 1.5 mg/ml liposome) and the red bar is the non-encapsulated poly (I:C) control (50 µg/ml). **(C) Viability of ZFL cells after 24 h incubation with liposomal LPS-poly (I:C) cocktail** (NL_c, green bars) at Dose 1 = 1.5 mg/ml liposome with 50 µg/ml poly (I:C) and 25 µg/ml LPS, Dose 2 = 0.75 mg/ml liposome with 25 µg/ml poly (I:C) and 12.5 µg/ml LPS and Dose 3 = 0.375 mg/ml liposome with 12.5 µg/ml poly (I:C) and 6.25 µg/ml LPS. The white bar is the empty liposome control treatment ($NL_{2,n}$, 1.5 mg/ml liposome), the blue bar indicates the free LPS (25 µg/ml) and the red bar is the free (I:C) control (50 µg/ml). Non-treated cells were used as 100% viability control (dotted line). Data represent means ± SD of three independent experiments. Differences were analyzed using One-way ANOVA followed by Tukey's post test. **, $p<0.01$; ***, $p<0.001$.

Further, empty $NL_{2,n}$ showed low toxicity but higher than NL_c in all cases, which can be attributed to changes suffered by the liposomes after the encapsulation of LPS and poly (I:C) that further improve its biocompatibility.

The same results were obtained by using the LDH assay (**Figure S2 in Annex 2**). Finally, toxicity studies were also carried out using trout primary cell cultured APCs (**Figure S3 in Annex 2**). In this cells, we observed low toxicity levels of NL_c formulations (20% over basal mortality), but did not observe a LPS/poly (I:C) mediated toxicity at the indicated doses.

Endocytosis of NL_c formulation by ZFL cells and trout macrophages primary cultures

Since hepatocytes play a major role in physiological detoxification processes and APCs are the key targets of our liposomes, we next evaluated the liposome uptake in both systems using flow cytometry and confocal microscopy. In ZFL cells, we observed a rapid (5 min) and efficient liposome uptake (**Figure 3A**) that reached a maximum in 1 h, and then started to decrease during the next 16 h, indicating that NL_c were probably metabolized by the endosomal/lysosomal system. Different studies have shown the ability of cationic liposomes to deliver different compounds to endosomal compartments [12, 32]. To further explore this process, we assayed the NLc endocytosis in the presence of chloroquine (CQ), an inhibitor of lysosomal acidification, and we observed a significant increase of fluorescence in the presence of CQ (**Figure S5A in Annex 2**). This observation confirmed the occurrence of NL_c in the endosomal/lysosomal compartment (55.53±0.83% CQ-dependant endocytosis inhibition at 16 h).

To discriminate between membrane-bounded and endocytosed NLc, we measured the total versus endocytosed fluorescence at different times, observing that around 80% of total fluorescence signal corre sponded to endocytosed liposomes (**Figure 3A**) that accumulated intracellularly forming cytosolic agglomerates of 1.13±0.42 mm mean size (**Figure 3C**). To distinguish between the various mechanisms of endocytosis, a series of FITC-labelled NL_c liposome uptake assays were performed in the presence of inhibitors (methyl-β-cyclodextrin, MβCD, sucrose, wortmannin and EIPA) known to block a particular endocytosis pathway (**Figure 3B**). Treatment of cells with MβCD, a caveolae-mediated endocytosis inhibitor, led to a 60±5.9% ($p<0.001$) decrease in liposome uptake, whereas treatment with the macropinocytosis inhibitors wortmannin and EIPA provided contradictory results. While wortmannin inhibited uptake (19±4%; $p<0.01$), EIPA, a more specific macropinocytosis inhibitor, did not. The PI3K inhibitors (e.g wortmannin) have been described to have pleiotropic effects on endocytosis as they can block the internalization of ligands of the clathrin- and caveolae- mediated pathways [33, 34]. Thus, in ZFL cells, wortmannin could affect caveolae-mediated endocytosis instead of macropinocytosis. Finally, treatment with hypertonic medium (sucrose) led to a 15±6% ($p<0.05$) inhibition, indicating that clathrin-mediated endocytosis may also contribute to NL_c uptake. All these data suggested that NL_c could be endocytosed by ZFL cells mainly through the caveolae-dependent endocytosis pathway, but clathrin-mediated endocytosis could also be involved in liposome uptake.

The uptake in differentiated trout macrophages was also evaluated. As shown in Figure 4, these cells were able to efficiently endocyte NL_c. We measured total versus intracellular fluorescence by flow cytometry, and similarly to ZFL cells, macrophages were able to internalize around 80% of fluorescent liposomes in 1 h (**Figure 4A**).

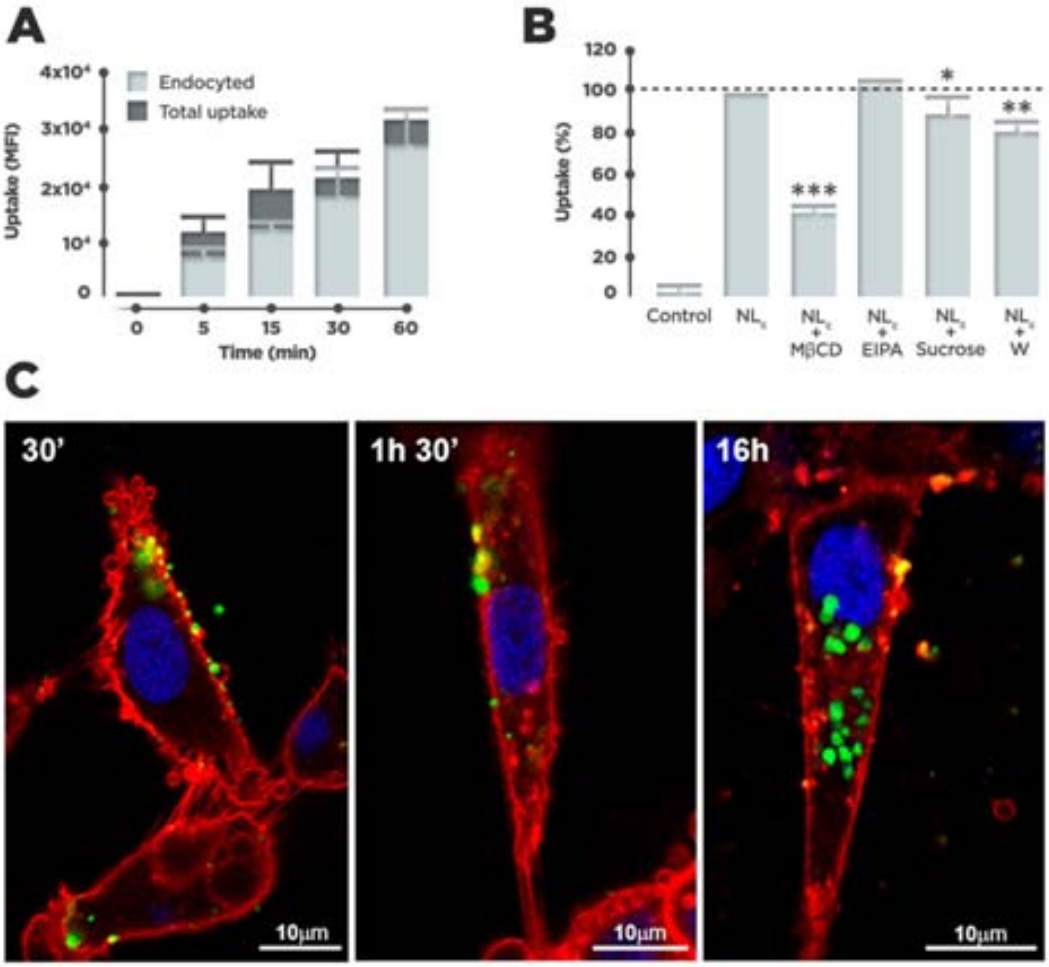

Figure 3. **Endocytosis of NL_c formulation by ZFL cells.**

(A) Flow cytometry time-course comparison of the membrane-bound (dark grey bar) versus the endocyted liposomes (light grey bar) after incubation with NL_c (750 µg/ml liposome, 25 µg/ml poly (I:C) and 12.5 µg/ml LPS) at the indicated times. Data represent means ± SD of three independent experiments. **(B) Effect of chemical inhibitors on the endocytosis of the NL_c** (750 µg/ml liposome, 25 µg/ml poly (I:C) and 12.5 µg/ml LPS). Inhibitors were used at the following concentrations: MβCD at 5 mM, EIPA at 50 µM, sucrose at 300 mM and W at 100 nM. The uptake of cells without inhibitors (NL_c bar) was used as 100% uptake control and non-treated cells were used as control (control bar). Data represent means ± SD of three independent experiments. Differences were analyzed using One-way ANOVA followed by Tukey's post test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). **(C) Confocal microscopy images of fluorescent liposomes** (NL_c) endocyted by ZFL cells. Cells were incubated for 30 min, 1.5 h and 16 h with NL_c containing DHPE-Fluorescein (green) at a 0.05 molar ratio. Cell membranes were stained with CellMask **(red)** and the nucleus was stained with Hoechst **(blue)**.

In contrast to ZFL cells, however, macrophages did not metabolize liposomes in the endosomal/lysosomal compartment since we could detect the same fluorescence levels even 24 h later (**Figure S5 in Annex 2**). Note that the intracellular liposomes, as in ZFL cells, were present primarily in the cytosol as agglomerates (1.09±0.37 mm), with no fluorescence in the nuclei (**Figure 4C**). Again, we performed a series of liposome uptake assays in the presence of inhibitors, and we observed that in macrophages both MβCD and EIPA were able to inhibit the endocytosis by 31.09±14.52% ($p<0.01$) and 15.57±1.72% ($p<0.05$), respectively (**Figure 4B**). These results indicated that caveolae-mediated endocytosis and macropinocytosis/phagocytosis are the main endocytic pathways for liposome internalization in trout macrophages.

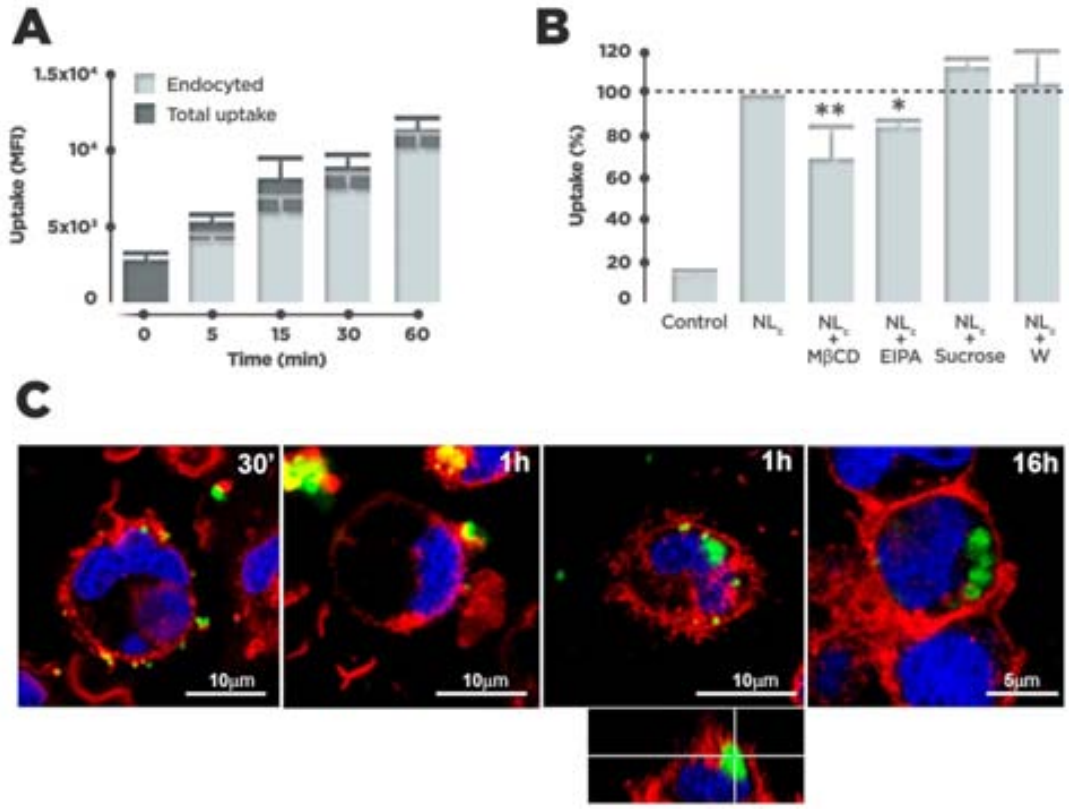

Figure 4. **Endocytosis of NL_c formulation by trout macrophages.**

(A) Flow cytometry time-course comparison of the membrane-bound (dark grey bar) versus the endocyted liposomes (light grey bar) after incubation with 750 µg/ml liposome-encapsulated 25 µg/ml poly (I:C) and 12.5 µg/ml LPS at the indicated times. Data represent means ± SD of three independent experiments. **(B) Effect of chemical inhibitors on the endocytosis of the NL_c** (750 µg/ml liposome-encapsulated 25 µg/ml poly (I:C) and 12.5 µg/ml LPS) macrophages uptake. Inhibitors were used at the following concentrations: MβCD at 5 mM, EIPA at 50 µM, sucrose at 150 mM and W at 100 nM. The uptake of cells not treated with inhibitors (NL_c bar) was used as 100% uptake control and non-treated cells were used as control (control bar). Data represent means ± SD of 3 independent experiments. Differences were analyzed using One-way ANOVA followed by Newman-Keuls post-test. *, $p<0.05$; **, $p<0.01$). **(C) Confocal microscopy images of fluorescent liposomes** (NL_c) endocyted by macrophages. Cells incubated 30 min, 1 h and 16 h with NL_c containing DHPE-Fluorescein **(green)** at a 0.05 molar ratio. Cell membranes were stained with CellMask (red) and nucleus with Hoechst **(blue)**.

The immunostimulatory effects of NL_c formulation on ZFL cells and trout macrophages

We examined the gene expression patterns in response to NL_c treatment in ZFL cells and trout macrophages (**Figure 5A and 5B**) by evaluating the expression of marker genes of proinflammatory (TNFα and IL-6) and anti-viral responses (IFNΦ and α, GIG2 and CCL4). **Figure 5A** shows that IFNΦ and GIG2 gene expression was significantly induced by the NL_c formulation at both doses, but we did not observe significant differences between Dose 1 and 2. Importantly, IFNΦ (NL_c Dose 1: 11±2 - fold change; $p<0.01$) and GIG2 (NL_c Dose 1: 2250±49 -fold change; $p<0.01$) had higher expression levels in NL_c formulation than in non-loaded liposomes ($NL_{2,n}$: 564 -fold change and 17±1.5 -fold change, respectively). The chemokine CCL4, a chemotactic cytokine that is induced in fish after viral infection35], was also efficiently induced after NLc treatment (**Figure 5A**). We also observed that non-loaded liposomes ($NL_{2,n}$) were still able to induce low levels of gene expression (**Figure 5A and 5B**). Several groups have indeed described that cationic liposomes have an immunological adjuvant effect and that they are able to regulate the transcription of several chemokines and cytokines [36].

We also assessed the IFNα, IL-6 and TNFα expression levels in trout macrophages (**Figure 5B**) to further evaluate the stimulatory ability of NLc formulation. The IFNα expression was significantly induced after NL_c Dose 1 and 2 treatment (68±5 and 50±10.5 - fold change; $p<0.001$) as compared to non-loaded liposomes ($NL_{2,n}$; 9.2±3.8 -fold change; $p<0.001$) and to the free LPS/poly (I:C) mixture (1264 -fold change; $p<0.001$). The pro-inflammatory cytokines IL-6 and TNFα showed a slightly different pattern, achieving good stimulation levels after NL_c treatment with respect to non-loaded liposomes, but similar or lower levels when compared to the free-LPS/poly (I:C) mixture (**Figure 5B**). Consistent with gene expression results, TNFα protein secretion was strongly induced by NL_c formulation, and most importantly, it was undetectable after stimulation with non-loaded liposomes $NL_{2,n}$ (**Figure 5C**). TNFα is one of the pivotal early response cytokines that are secreted by macrophages and enters the circulation to exert its systemic action [37].

In vivo biocompatibility of the NL_c formulation

We conducted different dose-response survival experiments with the NL_c formulation and non-loaded liposomes $NL_{2,n}$ in pre- and post-hatching larvae (**Figure 6 and Figure S6 in Annex 2**). A NL_c concentration range from an extremely high dose (NL_c Dose 4 = 6 mg/ml) to a putative therapeutic dose (NL_c Dose 1 = 0.75 mg/ml) was chosen. We did not observe significant differences in survival curves obtained with pre-hatched larvae incubated with NL_c formulation at different doses (**Figure 6A**), and only very high doses (NL_c Dose 4) caused a significant increase in mortality (100% at 72 h, $p<0,0001$). In contrast, high LPS toxicity with free-LPS treatment both in pre- and post-hatching larvae was observed (**Figure S6A and S6B in Annex 2**). A moderate poly (I:C) toxicity in pre-hatching larvae (62.5% mortality at 120 hpf; $p<0.0001$) versus control (36.12% mortality at 120 hpf; $p<0.0001$) was also recorded. Therefore, and in accordance with our previous *in vitro* toxicity studies (**Figure 2**), the encapsulation of both immunostimulants avoided the embryo/ larvae mortality induced by free LPS and poly (I:C) (**Figure 6A and Figure S6 in Annex 2**). Importantly, the embryos incubated with NL_c formulations were able to hatch and develop normally until 120 h with no morphological defects. The survival curves in post-hatching larvae incubated with these liposomal formulations were substantially different (**Figure 6B**).

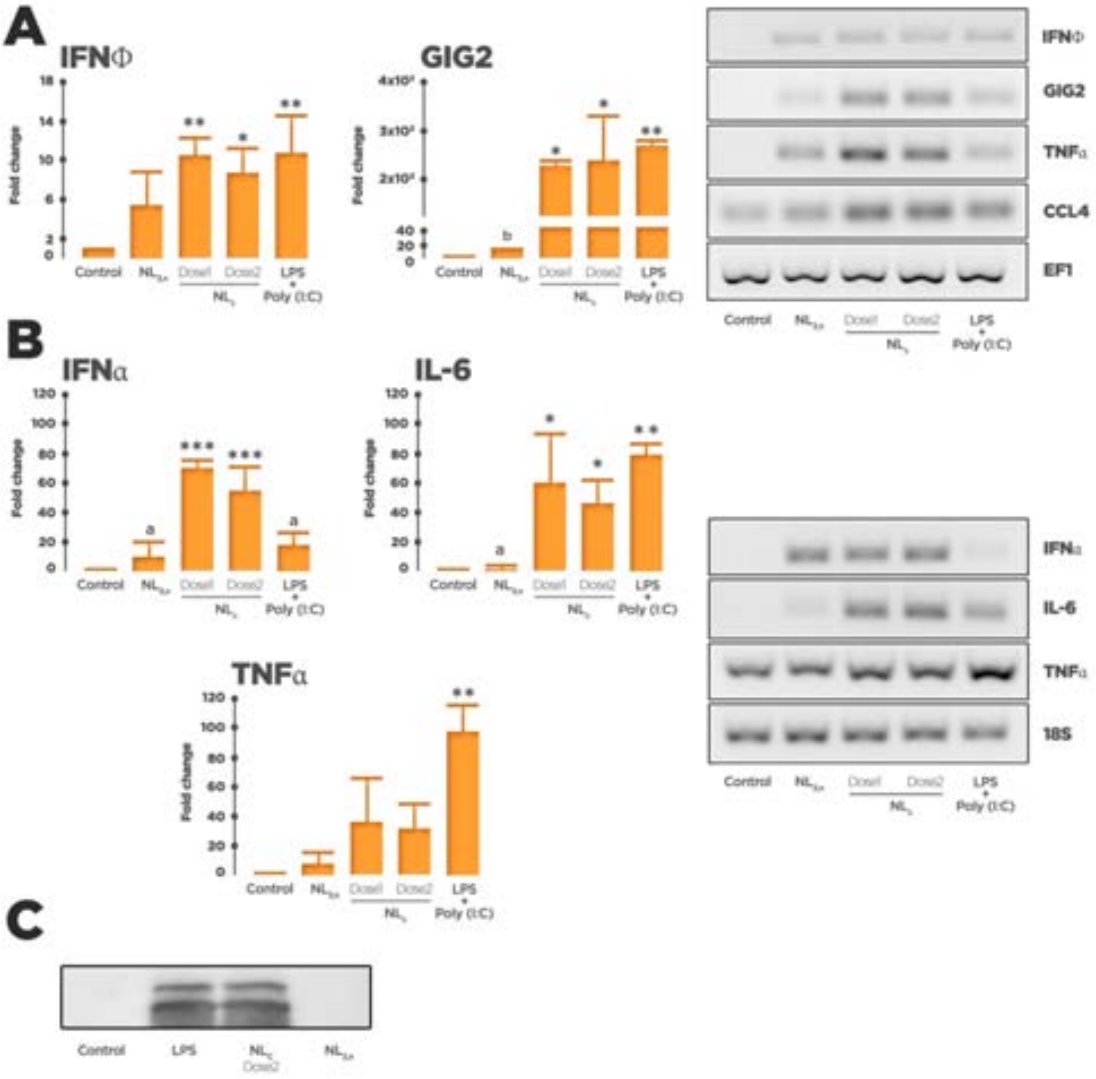

Figure 5. **Analysis of gene expression in ZFL cell culture (A) and trout macrophage primary cell culture (B) after 16 h exposure to liposomes.**

$NL_{2,n}$ = liposomes without immunostimulants (750 µg/ml), NL_c Dose 1 = liposomes (750 µg/ml) containing 25 µg/ml poly (I:C) and 12.5 µg/ml LPS, NL_c Dose 2 = liposomes (375 µg/ml) containing 12.5 µg/ml poly (I:C) and 6.25 µg/ml LPS, and LPS+poly (I:C) = stimulation control (25 µg/ml poly (I:C), 12.5 µg/ml LPS). Elongation factor (EF1) was used as reference gene for ZFL cells and 18S for trout macrophages. IFN (φ for ZFL and α for macrophages), GIG2, CCL4, IL-6 and TNFα abundance was analyzed by Q-PCR (left panel) and conventional PCR (right panel). Data represent means ± SD of 3 independent experiments. Values with asterisk are statistically significant relative to the control (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) and values with letters ($_{a,b}$) are statistically significant relative to NL_c Dose 1 ($_a$, $p<0.001$, $_b$, $p<0.05$). Differences were analyzed using One-way ANOVA and Tukey's post test. **(C) TNFα secretion from trout macrophages stimulated with liposomes** for 16 h was assessed by Western blot. NL_c Dose 2 = 375 µg/ml liposomes, 12.5 µg/ml poly (I:C), 6.25 µg/ml LPS, $NL_{2,n}$ = empty liposomes (375 µg/ml) and LPS = stimulation control (6.25 µg/ml). A representative Western Blot is shown.

In this case, non-loaded liposomes ($NL_{2,n}$ Dose 2, 1.5 mg/ml) showed less toxicity than that of the corresponding liposomal IS-cocktail (NL_c Dose 2, 1.5 mg/ml liposomes, 50 µg/ml poly (I:C), 25 µg/ml LPS). In addition, a dose-dependent toxicity for the NL_c formulation after 48 h incubation was observed (**Figure 6B**). Analysis of gene expression in NL_c challenged larvae at 24, 48 and 72 h showed expression of marker genes of pro-inflammatory (TNFα and iNOS) and antiviral responses (TLR3 and GIG2) (**Figure S7 in Annex 2**), indicating a stimulation of the zebrafish larvae immune system. Finally, DLS measurements done using NL_c and $NL_{2,n}$ formulations after 5 days incubation in E3 medium indicated a good stability after the *in vivo* challenge. We also characterized the NL_c stability in *in vivo* experimental conditions by Turbiscan, and we found that the NL_c stability index was not significantly changed after incubation in aquarium water or in E3 medium at 28°C for 2 days (stability indexes of 6.16 and 3.8, respectively). These data further confirm that this liposomal IS-cocktail might be used for future *in vivo* immunization in aquatic species.

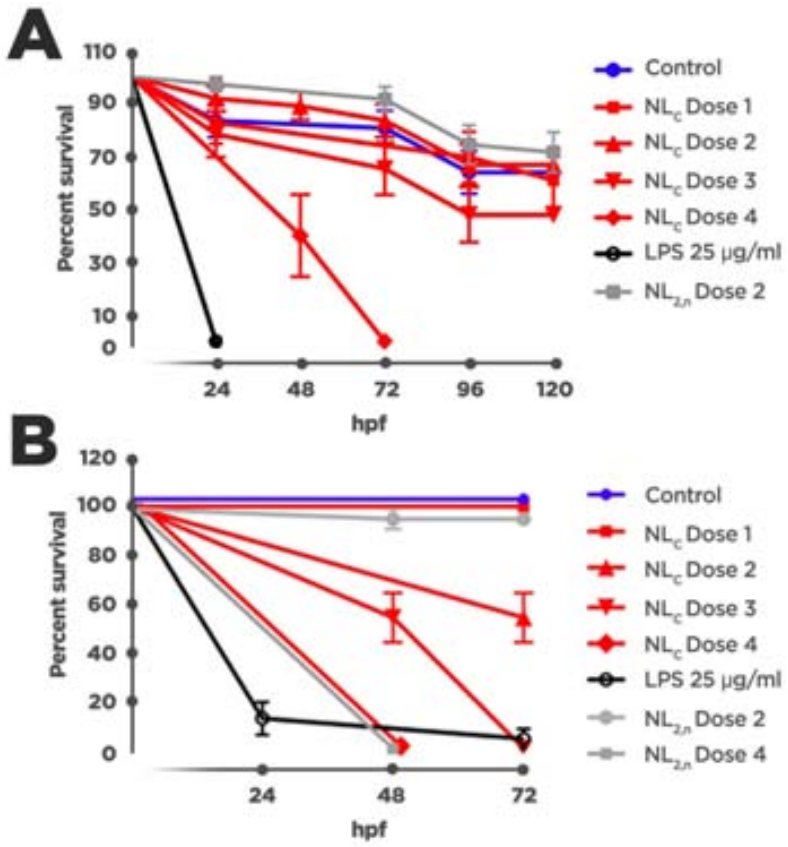

Figure 6. ***In vivo* NL$_c$ formulation toxicities.**

Survival of zebrafish embryos was recorded every 24(hpf) **(A)** and 72 h post-hatching (hph) **(B)** after exposure to four concentrations of liposomal IS cocktail (red, NL_c Dose 1 = 750 µg/ml liposomes, 25 µg/ml poly (I:C) and 12.5 µg/ml LPS; NL_c Dose 2 = 1.5 mg/ml liposomes, 50 µg/ml poly (I:C) and 25 µg/ml LPS; NL_c Dose 3 = 3 mg/ml liposomes, 100 µg/ml poly (I:C) and 50 µg/ml LPS; NL_c Dose 4 = 6 mg/ml liposomes, 200 µg/ml poly (I:C) and 100 µg/ml LPS). Liposomes without encapsulated immunostimulants (grey, $NL_{2,n}$ Dose 2 = 1.5 mg/ml, $NL_{2,n}$ Dose 4 = 6 mg/ml) and non-treated embryos (blue) were used as controls. Non-encapsulated LPS (black, 25 µg/ml and 100 µg/ml) was used as mortality control. Differences were analyzed using log rank test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Discussion

Vaccination and preventive immunostimulation has become the principal prophylactic tool for disease control in aquaculture. Some conventional vaccines made with inactivated bacteria (e.g. *Listonella anguillarum* causing vibriosis) have achieved good protection levels against different fish infections [38]. However, most diseases have no prevention tools, causing massive mortalities in fish farms and generating important economic losses. It is still unclear whether teleost fish have immunological memory but the secondary humoral responses are by far less prominent than in mammals [9,38]. Thus, the activation of the innate immune system seems the most effective way for the initiation of an efficient immune response in fish. The binding of antigens to the innate pathogen receptors (PRRs) located on antigen-presenting cells (APCs) is critical for developing an effective immune response. Fish have a powerful innate immune system with a high molecular diversity and complexity [39], being APCs (especially the macrophages and dendritic cells) the main players of the innate immune response and responsibles for the activation of adaptive immunity [40]. With these specific premises, we have designed a nanosized and non-toxic unilamellar liposomal formulation loaded with TLR ligands (LPS and poly (I:C)) which was able to induce a potent anti-viral and pro-inflammatory response *in vitro* and *in vivo* in fish. As far as we know, this study is the first attempt to co-encapsulate two model immunostimulants specifically designed to target fish APCs in nanosized liposomes. To date, the unique attempt to vaccinate fish using liposomes was done by Irie *et al.*, who explored the use of microsized liposomes containing *A. salmonicida* total extracts in carp [22]. Recently, Fredriksen *et al.* have also shown that a combination of poly (lactic-*co*-glycolic acid) microparticles loaded with β-glucan and human γ-globulins were able to target head kidney macrophages inducing an adaptive *in vivo* immune response in salmon [41]. The LPS would be an excellent candidate for immunostimulation purposes, but it has been scarcely used due to its high endotoxic potential in mammals. Fish are less sensitive to LPS toxic effects [17], and by encapsulating LPS we have assayed a simple way to stimulate fish innate immune system. On the other hand, we also target antiviral response pathways by adding dsRNA to the nanocarrier [13]. We have achieved high co-encapsulation efficiencies by using liposomes with positive charge that can easily incorporate LPS and poly (I:C) into the lipid bilayer and become neutral liposomes. Although liposomes are in principle highly biocompatible, *in vitro* toxicity of cationic liposomes has been reported by several groups [42, 43]. Thus, the observed charge neutralization has been an advantage, making our formulation highly biocompatible. Another advantage of this encapsulation system has been the elimination of the free LPS associated toxicity observed in cells and larvae (**Figures 2 and 6**). The LPS toxicity *in vitro* and *in vivo* has been well documented in different vertebrates [15], and it has also been demonstrated that encapsulation of LPS into liposomes decreased its toxicity compared to the free form [29]. Our system minimizes the detrimental effects of LPS while maintaining the immune system activation potency.

By developing an *in vitro* endocytosis assay with fish cells, we have also demonstrated that NLc liposomes contact with plasma membranes and they are efficiently internalized by fish macrophages and zebrafish hepatocytes. Different studies in rodents and humans have shown the ability of liposomes to deliver different compounds to endosomal compartments [12,32]. The liposomes developed in this study are 125 nm in size and its endocytosis is inhibited mainly by MβCD and sucrose, which indicates that they likely utilize the caveolae-mediated and the clathrin-mediated endocytosis pathways to reach intracellular compartments. The fact that the NLc

liposomes accumulate in endosomal-lysosomal compartments is a potential advantage since TLR3 is located in endosomal membranes, and antigen processing for MHCII presentation takes place in this compartment [3]. In addition, this simple and active formulation designed for virtually all fish species vaccination could be upgraded with specific pathogenic antigens of any particular fish species.

In recent years, health and environmental safety of nanoparticle-based therapeutics is a major concern for nanotechnology that has to be carefully addressed [44]. The zebrafish embryos and larvae have become a reference model for *in vivo* toxicological studies due to its sensitivity and logistic convenience [45–47]. Zebrafish embryos are protected from the environment with the chorion, a rigid but permeable membrane, which embryos lose after 48 h (hatching) to become free-swimming larvae [48,49]. We have taken advantage of the zebrafish model to demonstrate the biocompatibility of our formulation at therapeutic doses and also the ability of NL_c to target innate immune system. The activation of the innate immune system in trout macrophages and in zebrafish larvae can be assessed by following the expression of key cytokines [16,50]. Our study demonstrates that NL_c formulation stimulates the expression of several cytokines involved in anti-viral and bacterial response, and in some cases, the treatment with empty NL formulations also stimulates cytokine gene expression. Importantly, TNFα secretion by trout macrophages is potently and specifically stimulated by the liposomal IS-cocktail and not by the non-loaded liposomes. However, several groups have indeed described that cationic liposomes had an immunological adjuvant effect and that they were able to regulate the transcription of different chemokines and cytokines [36]. The induction of specific immune responses with liposomal immunostimulant formulations should be a promising strategy to improve disease control in fish farms.

Acknowledgments

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chapter 3

Targeting and **stimulation** of the zebrafish (*Danio rerio*) **innate immune system** with **LPS/dsRNA**-loaded **nanoliposomes**

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Abstract

Herein we report the use of immunostimulant-loaded nanoliposomes (called *NL$_c$* liposomes) as a strategy to protect fish against bacterial and/or viral infections. This work entailed developing a method for *in vivo* tracking of the liposomes administered to adult zebrafish that enables evaluation of their *in vivo* dynamics and characterisation of their tissue distribution. The NL$_c$ liposomes, which co-encapsulate poly (I:C) and LPS, accumulate in immune tissues and in immunologically relevant cells such as macrophages, as has been assessed in trout primary cell cultures. They protect zebrafish against otherwise lethal bacterial (*Pseudomonas aeruginosa PAO1*) and viral (*Spring Viraemia of Carp Virus*) infections regardless of whether they are administered by injection or by immersion, as demonstrated in a series of *in vivo* infection experiments with adult zebrafish. Importantly, protection was not achieved in fish that had been treated with empty liposomes or with a mixture of the free immunostimulants. Our findings indicate that stimulation of the innate immune system with co-encapsulated immunostimulants in nano-liposomes is a promising strategy to simultaneously improve the levels of protection against bacterial and viral infections in fish.

Introduction

The immune system of vertebrates encompasses adaptive immunity and innate immunity, the former of which involves immunological memory. Fish posses a highly diverse, strong innate immune system and were the first vertebrates to develop an adaptive immune system. Interestingly, fish lack IgG and class switch-recombination machinery [1], but have IgM, IgT and IgD generated by somatic rearrangement, somatic mutation and gene conversion [2]. Another important distinctive feature of teleosts is that they have phagocytic B lymphocytes. It has been reported the presence of phagocytic B lymphocytes in trout, catfish, cod and Atlantic salmon ([1] and references herein) but not in zebrafish [3]. Nevertheless, farm-raised fish respond well to vaccination. Recently, the concept of "innate memory" has been proposed [4, 5] and has also inspired the design of vaccination approaches focused on the stimulation of innate immunity.

Several fish vaccines against viral or bacterial diseases, most of which comprise inactivated pathogens are now available [6]. However, researchers are working intensively to enhance vaccine efficiency by developing new vaccines, containing adjuvants and immunostimulants [7], and new formulations based on encapsulation [8-12]. Encapsulating vaccines makes them more stable to the environment and to low pH and/or enzymatic reactions inside the treated organism [12, 13]. Among the various encapsulation systems available, liposomes are especially attractive, as they are biocompatible and highly tuneable [14]; can actually enhance the efficacy of the vaccine, as has been reported in fish [15, 16]; and can be used as labels to enable *in vitro* or *in vivo* tracking of the vaccine. Another factor that researchers are endeavouring to improve in fish vaccines is administration, which is typically done by injection in adults. Research efforts are focused on creating non-stressful, easy to manage and low-cost vaccination protocols to improve large-scale procedures based on immersion rather than on injection [6] and [17].

Our group recently developed nanoliposomes (called *NL$_c$liposomes*) for simultaneous wide-spectrum anti-bacterial and anti-viral protection of farm-raised fish. First, we co-encapsulate two general immunostimulants: bacterial lipopolysaccharide (LPS) and poly (I:C), a synthetic

analogue of dsRNA viruses. Then, we demonstrated that the NL_c liposomes were taken up *in vitro* by macrophages and that they regulated the expression of immunologically relevant genes (likely, by triggering innate immune signalling pathways) [18].

In the work reported here, we studied the biodistribution and immunological efficacy of NL_c liposomes in zebrafish *in vivo*. We chose zebrafish as the model organism for the *in vivo* assays for multiple reasons: they have been widely used to study the pathogenicity of different fish and human pathogens; they have innate and adaptive immune systems; and they are easy to breed and handle [19]. We adapted a non-invasive imaging method widely used in mammalian models [20, 21], and then used it to track the nanoliposomes in adult zebrafish *in vivo*. To the best of our knowledge, this is the first report of this method being applied to live zebrafish. In addition, we studied which cells were preferentially targeted by the NL_c liposomes in rainbow trout (*Oncorhynchus mykiss*), by performing *ex vivo* analysis of the main immune relevant tissues. We also developed a new model for infection of adult zebrafish by the bacterium *Pseudomonas aeruginosa*, an opportunistic pathogen in fish and in humans [22, 23]. Although most of the zebrafish infection models employ larvae [24], the maturity of larval immune systems remains poorly understood. We believe that the development of infection models in adult zebrafish might ultimately prove valuable for designing new therapeutic approaches and for elucidating the functions of the teleost immune system.

Materials and Methods

Preparation and lyophilisation of NL_c liposomes

The NL_c (*NanoLiposome cocktail*) liposomes were prepared as previously described in Ruyra et al. [18]. Liposomal formulations were prepared by the thin film hydratation method [25] with some modifications. Briefly, DLPC, cholesterol, cholesteryl and chol-PEG600 were dissolved in chloroform solutions (100 mg/ml) and mixed at the desired molar ratios (0.5:0.35:0.10:0.05). The organic solvent was then evaporated by rotary evaporation to obtain a dry lipid film. For the preparation of the liposomes that contained a cocktail of immunostimulants 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 were homogenised 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 and Cryo-TEM. The final NL_c liposomes comprised 125.8 ± 6.6 nm liposomes containing both poly (I:C) and LPS (1 mg/ml liposome encapsulates 33.3 μg/ml poly (I:C) and 16.6 μg/ml LPS) and had a neutral surface charge (1.37 ± 3.58 mV). The co-encapsulation efficiencies (EE) were of 22.3 ± 2.1% for LPS and of 99.6 ± 0.1% for poly (I:C). 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 re-suspended in PBS and the morphology of the reconstituted NL_c liposomes was assessed by Cryo-TEM (JEOL-JEM 1400, Japan). To quantify the amount of immunostimulants leaked after lyophilisation, liposomes encapsulating either poly (I:C) or LPS

were prepared lyophilised and finally, stored at RT. At 0 h and 4 months, the dried liposomal cakes were resuspended with PBS and the free poly (I:C) or LPS was separately quantified as described in Ruyra et al. [18].

Animals

Adult wild type (wt) zebrafish were held in tanks with recirculating water under 14 h light/10 h dark at 28 °C. Adult rainbow trout (*O. mykiss*) were held in tanks under 12 h light/12 h dark at 15 °C. All the experimental protocols with animals were reviewed and approved by the Ethics and Animal Welfare Committee and Biosecurity Committee of the Universitat Autònoma de Barcelona and Universidad Miguel Hernandez. All of these protocols followed the International Guiding Principles for Research Involving Animals.

Fluorescent labelling of NL_c liposomes

Alexa Fluor 750 (AF750) succinimidyl ester and DOPE-NH_2 were conjugated as previously described [25]. Only conjugated Alexa Fluor 750 was detected by TLC (Rf = 0.6), indicating that conjugation was complete. The fluorescently labelled AF750-NL_c liposomes were prepared by incorporating AF750-DOPE into the lipid mixture (0.01 molar ratio). Similarly, fluorescently labelled FITC-NL_c liposomes were prepared by incorporating Fluorescein-DHPE (Molecular Probes, Life Technologies Corp., USA) into the lipid mixture (0.01 molar ratio).

Biodistribution of the NL_c liposomes in zebrafish

The *in vivo* biodistribution of the NL_c liposomes in adult zebrafish (0.39 ± 0.04 g weight) was studied using the AF750-NL_c liposomes. The liposomes were administered by intraperitoneal (i.p.) injection or by immersion. Administration by i.p. injection: adult zebrafish (n = 4 per condition) were anaesthetised (MS-222, 40 ppm) and given 10 µl of AF750-NL_c liposomes (380 mg/kg liposome containing 12.6 mg/kg of poly (I:C) and 6.3 mg/kg of LPS). At 24, 48 and 72 h post-injection, the fish were anaesthetised (160 ppm) and imaged in the IVIS Spectrum platform (excitation: 745 nm; emission: 800/820/840 nm, Calliper, PerkinElmer, USA). For the *ex vivo* imaging, the zebrafish were killed by over-anaesthetisation (200 ppm) and their organs were extracted and then, imaged in the IVIS Spectrum platform. Administration by immersion: adult zebrafish (n = 4 per condition) were immersed in a tank containing AF750-NL_c liposomes (500 µg/ml liposome containing 16.6 µg/ml of poly (I:C) and 8.3 µg/ml of LPS) for 30 min, and then placed back into a tank of clean water. At 0 and 12 h post-immersion, the fish were anaesthetised and imaged in the IVIS Spectrum platform (as described above). For the *ex vivo* imaging analyses, the zebrafish were killed by over-anaesthetisation (200 ppm), and their organs were extracted and then, imaged in the IVIS Spectrum platform. The images were analysed using Caliper Living Image 4.1 software (PerkinElmer). For the *ex vivo* analysis, the Region of Interest (ROI) was measured and the data were represented as the Radiance Efficiency (RE) divided by the mean area of each organ.

Targeting of cells by the NL_c liposomes in rainbow trout

FITC-NL_c liposomes were used to study the cells targeted by the NL_c liposomes in rainbow trout. Animals (n = 4, ~125 g weight) were anaesthetised and i.p. injected with 200 µl of FITC-NL_c liposomes (96.0 mg/kg liposome containing 3.18 mg/kg of poly (I:C) and 1.59 mg/kg of LPS) or 200 µl PBS (controls). After 24 h, the fish were sacrificed for head kidney and spleen dissection. Adherent trout monocyte/macrophages were isolated as previously described [26]. Every 24 h, cells were studied by flow cytometry analysis (FACSCanto cytometer, Becton Dickinson, USA) or by confocal microscopy imaging (Zeiss LSM 700, Germany).

P. aeruginosa bacterial challenge in zebrafish after NL_c liposome administration

Adult zebrafish (0.61 ± 0.12 g weight) were transferred to an isolated system and acclimated for 1 day before each experiment. *P. aeruginosa* (PAO1, sub-line MPAO1; obtained from Seattle PAO1 transposon mutant library, University of Washington) was grown at 37 °C in blood agar plates (BioMérieux, France), collected directly from the plates and then, dispersed in sterile PBS. The LD_{50} for PAO1 infection was calculated in fish infected by i.p. injection with 20 µl of PAO1 suspension at concentrations ranging from 3.2×10^7 to 2.5×10^8 cfu. The fish were observed daily for signs of disease and mortality, and the dead fish were assessed for bacterial presence and identification (data not shown). For the survival experiments, the fish were i.p. injected with either 10 µl of NL_c liposome (246 mg/kg liposomes containing 8.2 mg/kg poly (I:C) and 4.1 mg/kg LPS), 10 µl of empty liposomes (246 mg/kg), 10 µl of a mixture of the free immunostimulants (8.2 mg/kg poly (I:C) and 4.1 mg/kg LPS) or 10 µl of PBS (control). At 1, 7 or 30 days post-injection (dpi), the fish were challenged with *P. aeruginosa* ($1.5 \times LD_{50}$) and their survival was assessed for 5 days. All experiments were done in triplicate and 12 individuals were used for each condition and experiment. A total number of 36 fish were used for each condition. Survival curves were analysed using the Kaplan–Meier method and the statistic differences were evaluated using the log-rank test (GraphPad, USA). Relative percentage of survival (RPS) was calculated according to RPS (%) = [(1 − mortality treated group)/mortality control] × 100.

Cell cultures and *Spring Viraemia of Carp Virus* (SVCV) preparation

The fish-cell line ZF4 [27] used in this work was purchased from the American Type Culture Collection (ATCC number CRL-2050). ZF4 cells were maintained at 28 °C in a 5% CO_2. The 56/70 isolate of SVCV isolated from carp [28] was propagated in ZF4 cells at 22 °C. Supernatants from SVCV-infected cell monolayers were clarified by centrifugation at $4000 \times g$ for 30 min and stored in aliquots at −70 °C. The clarified supernatants were used for *in vivo* infection assays.

Spring Viraemia of Carp Virus (SVCV) challenge in zebrafish after liposome administration

Zebrafish were given NL_c liposomes, empty liposomes or a mixture of the free immunostimulants by either i.p. injection or immersion, as described below. I.p. injection: the fish were injected with either 10 µl of NL_c liposomes (246 mg/kg liposome containing 8.2 mg/kg poly (I:C) and 4.1 mg/kg LPS), 10 µl of empty liposomes (246 mg/kg), 10 µl of the mixture of free immunostimulants (8.2 mg/kg poly (I:C) and 4.1 mg/kg LPS) or 10 µl of PBS (control). Immersion: the NL_c liposomes (500 µg/ml liposomes containing 16.6 µg/ml poly (I:C) and 8.3 µg/ml LPS), empty liposomes (500 µg/ml) and a mixture of the free immunostimulants (16.6 µg/ml poly (I:C) and 8.3 µg/ml LPS) were each administrated for 30 min, including a handling control. At 7 dpi, the zebrafish (n = 15/each condition) were infected by immersion with SVCV ($7.1 \pm 2 \times 10^7$ pfu/ml) according to previously described infection protocols [29, 30]. Fish were assessed for survival, abdominal distension, exophthalmia, impaired swimming and skin/fin base haemorrhages for 15 days. Survival curves were analysed using the Kaplan–Meier method and the differences were evaluated using the log-rank test (GraphPad). Relative percentage of survival (RPS) was calculated according to RPS (%) = [(1 − mortality treated group)/mortality control] × 100. At 5 dpi, two surviving fish from each group were randomly sampled for virus recovery [30].

Results

Biodistribution of NL_c liposomes in zebrafish after administration by i.p. injection

The biodistribution of the NL_c liposomes in adult zebrafish was studied following i.p. injection of the fish with fluorescently labelled liposomes (AF750-NL_c liposomes). Whole-animal images revealed a fluorescence signal in the peritoneal cavity of all the individuals up to 72 h with no detectable fluorescence signal in any other part of the fish (**Figure 1A**). Quantification of this signal confirmed a sustained presence of the liposomal formulation. A slight decrease was observed at 72 h: from 3.76×10^9 Radiant Efficiency (RE) at 0 h to 2.16×10^9 RE at 72 h (**Figure 1B**). Organ *ex vivo* analysis was performed at 0, 24, 48 and 72 h post-injection, and the corresponding signal intensities were quantified (**Figure 1C**).

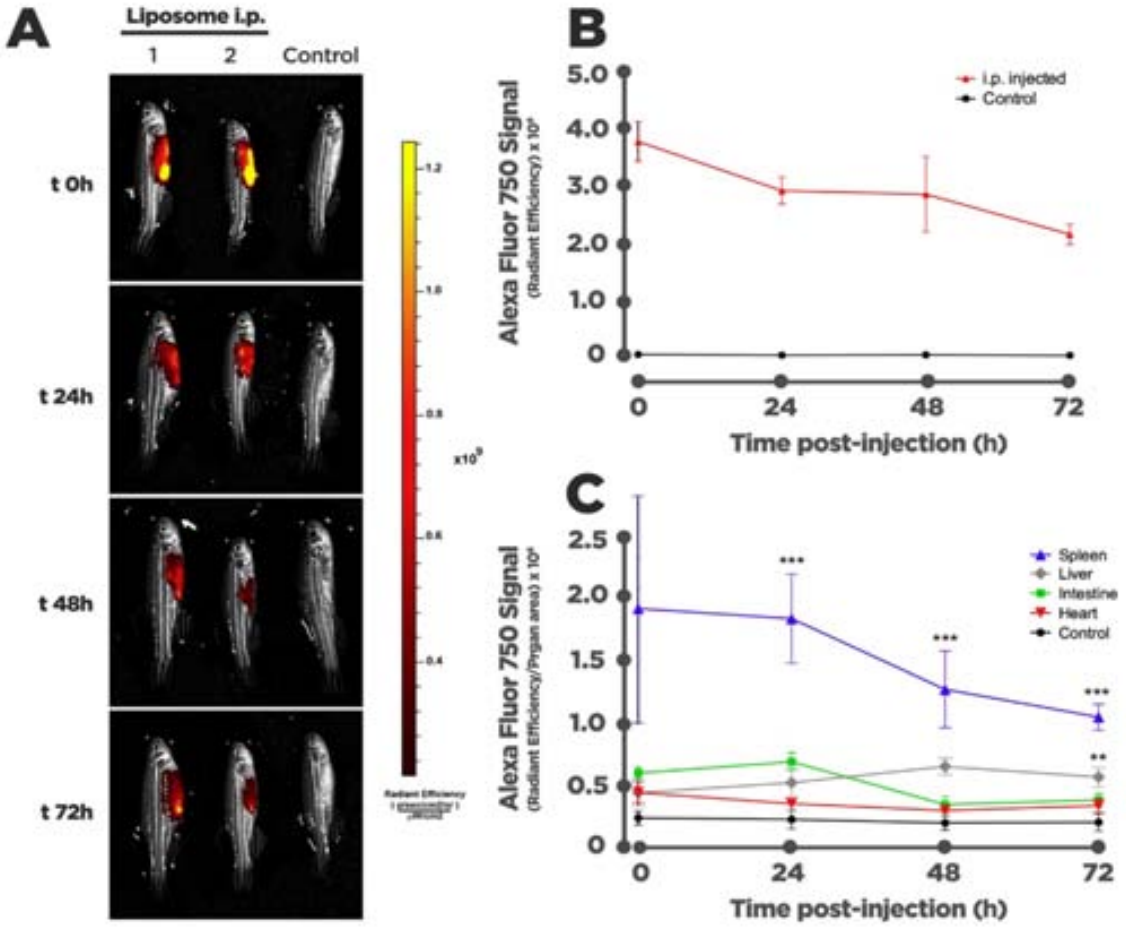

Figure 1. **Biodistribution time-course of the NL_c liposomes after i.p. injection.**

(A) Representative IVIS Spectrum image of adult anaesthetised zebrafish at 0, 24, 48 and 72 h after being i.p. injected with 10 µl of AF750-NL_c liposome formulation. Untreated fish were used as control for background subtraction. Image intensity is represented as Radiant Efficiency. **(B) AF750-NL_c liposome fluorescent signal quantification from 0 to 72 h.** Untreated fish were used as control (black dots). Image intensity is represented as mean ± SD (n = 4) Radiant Efficiency. **(C) Organs including spleen, liver, intestine and heart** were removed at 0–72 h and imaged to reveal accumulation of the AF750-NL_c liposomes. The fluorescence intensity of the different organs was measured using the Caliper Living Image software. Data represent the mean ± SD (n = 4) Radiant Efficiency/organ area. Differences were analysed using One-way ANOVA followed by Tukey's post-test. $^{**}p < 0.01$; $^{***}p < 0.001$.

Significant accumulation of the NL_c liposomes was observed in the spleen from 0 to 72 h (from 1.92×10^6 RE/organ area at 0 h to 1.05×10^6 RE/organ area at 72 h), and in the liver at 72 h (5.71×10^5 RE/organ area). These values are consistent with those from previous studies using radioactive labelling, which had shown that large unilamellar liposomes injected into fish had localised mainly in the spleen [13].

Cells targeted by NL_c liposomes in rainbow trout

To identify the cells targeted by the NL_c liposomes *in vivo*, we worked with adult rainbow trout instead of zebrafish, as the larger size of the former enabled us to isolate mononuclear phagocytes from the main immunologically related organs (spleen and head kidney) for subsequent characterisation by flow cytometry and by confocal microscopy. In a typical experiment, fluorescent NL_c liposomes were injected into trout ($n = 4$), and at 24 h post-injection the spleen and the head kidney were dissected for primary cell culture. The NL_c liposomes were tracked by flow cytometry and by confocal microscopy at 24, 48 and 72 h. Fluorescence signals were significantly detected by flow cytometry (**Figure 2A**) in spleen-derived cells at 24, 48 and 72 h. NL_c liposomes were also found in head kidney-derived cells, although in far lower levels than in the spleen. For example, at 72 h, the percentage of total positive cells in the spleen was 30.3 ± 12.6%, compared to 2.9 ± 1.2% for the head kidney. Interestingly, fluorescent cells were detected even up to 6 days post-injection, indicating that the NL_c liposomes can persist for at least 1 week (data not shown). For the confocal microscopy analysis, the cell membranes and nuclei were stained with either CellMask or Hoechst, respectively. The monocytes/macrophages were easily distinguishable by the kidney-shaped nuclei and the rugosity of their plasma membranes (**Figures 2B and C**) [31, 32]. The presence of NL_c liposomes in macrophage-like cells from the spleen was confirmed at 24, 48 and 72 h (**Figure 2B**). Fluorescent NL_c liposomes were also found in macrophage-like cells isolated from head kidney (**Figure 2C**). The membrane-staining and the z-stack images enabled visualisation of the exact location of the liposomes, and the images demonstrated that the liposomes had been completely taken up by the cells; no fluorescent NL_c liposomes attached to the plasma membrane were detected (**Figures 2B and C (iii, iv)**).

NL_c injected liposomes protect zebrafish against *P. aeruginosa* lethal challenge

In previous work, we showed that NL_c liposomes induced the expression of immunologically relevant genes *in vitro* [18]. Having determined, in the present work, that these liposomes target macrophage-like cells *in vivo*, we next studied the protective effect of the system against *P. aeruginosa* infection. Before the immunisation experiments, the PAO1 infection model in adult zebrafish was fully characterised by determining the $LD_{50} = 5.3 \times 10^7$ cfu (**Annex 3 Figure 1**), and then recovering and subsequently identifying the PAO1 strain by 16S rRNA sequencing (data not shown). The zebrafish were immunised with the NL_c liposomes, and then challenged with the PAO1 bacteria at 1 day, 1 week or 1 month post-immunisation. Their survival rates were assessed and the results were used to compare the different immunisation protocols (**Figure 3 and Annex 3 Figure 2 and Table 1**). Neither the empty liposomes nor the mixture of free immunostimulants (poly (I:C) and LPS) protected the zebrafish against PAO1 infection when injected 1 day (**Annex 3 Figure 2**) or 1 week (**Figure 3A**) before the challenge. In contrast, the fish that had received NL_c liposomes exhibited significantly higher survival rates than the control group, regardless of the date of administration (RPS of 33.2% at 1 day; 47.1% at 1 week; and 36.3% at 1 month (**Figure 3, Annex 3 Figure 2 and Table 1**).

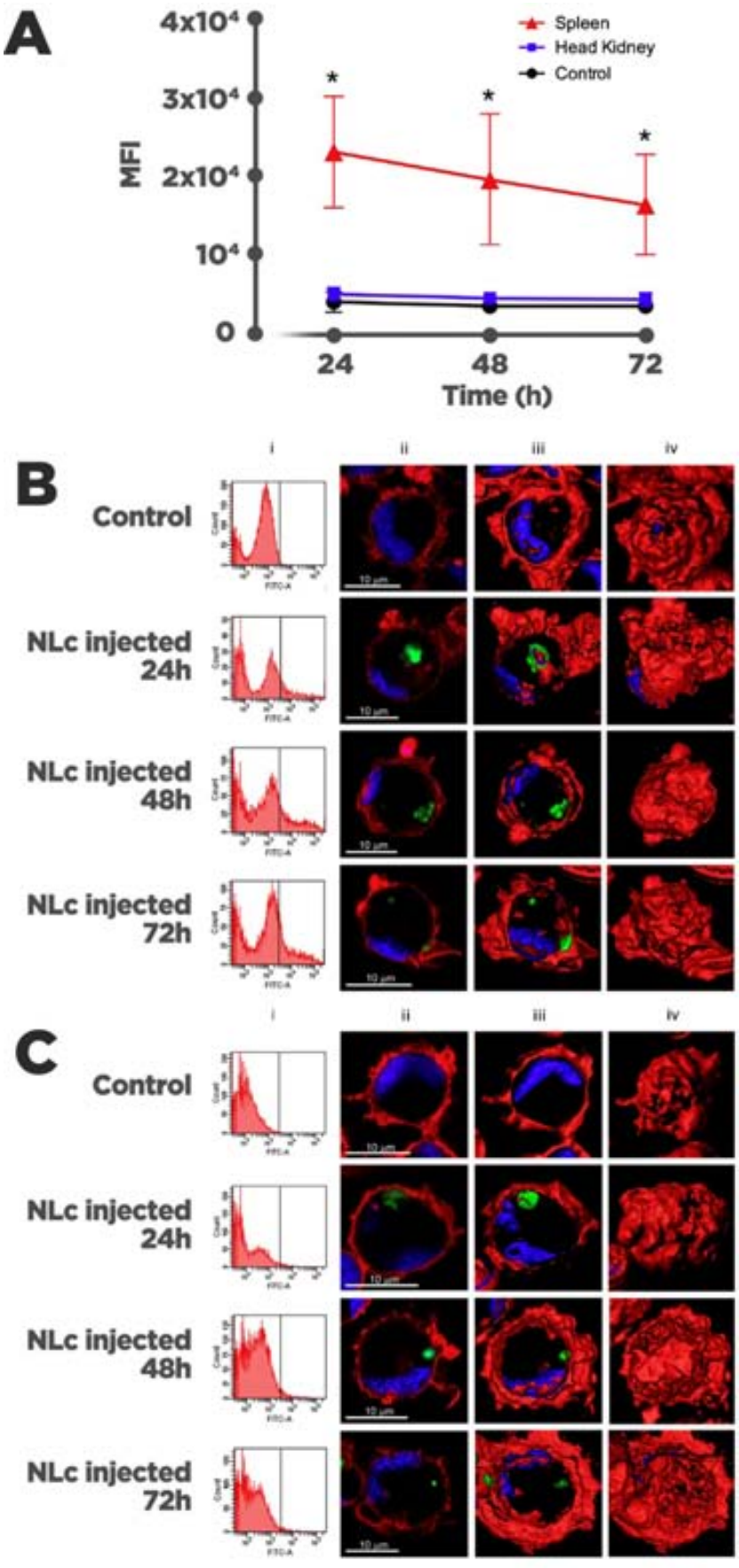

Figure 2. **Uptake of NL_c liposomes by phagocytes from trout spleen and head kidney.**

(A) Quantification of NL_c liposomes uptake in spleen phagocytes and head kidney phagocytes at 24, 48 and 72 h. FITC-NL_c liposomes were i.p. injected into adult rainbow trout. Cells from untreated individuals were used as control (black dots). Data represent mean ± SD (n = 4) fluorescence intensity (MFI) and differences were analysed using One-way ANOVA followed by Tukey's post-test. *$p < 0.05$. Time-course of FITC-NL_c liposome uptake in **spleen phagocytes (B)** and in **head kidney phagocytes (C). Representative flow-cytometry plot** of FITC positive cells **(i)** and corresponding confocal images of FITC-NL_c liposomes (green) internalised in macrophage-like cells **(ii).** Cell membranes are shown in red and nuclei, in blue. Z-stack **(iii)** and whole-membrane **(iv)** digitalised image of the same cells.

To determine the feasibility of using a storable version of the NL_c liposomes (**Annex 3 Figure 3**), we also evaluated the efficacy of lyophilised NL_c liposomes against *P. aeruginosa* infection. Thus, adult zebrafish were treated with rehydrated lyophilised NL_c liposomes or with freshly prepared NL_c liposomes, and then infected at 1 week post-injection (**Figure 3A**). Interestingly, the lyophilised liposomes were as effective as the freshly prepared ones (58.3% survival *vs.* 50% survival, respectively; **Figure 3A**). This result confirmed that lyophilised liposomes are amenable to use after long-term storage.

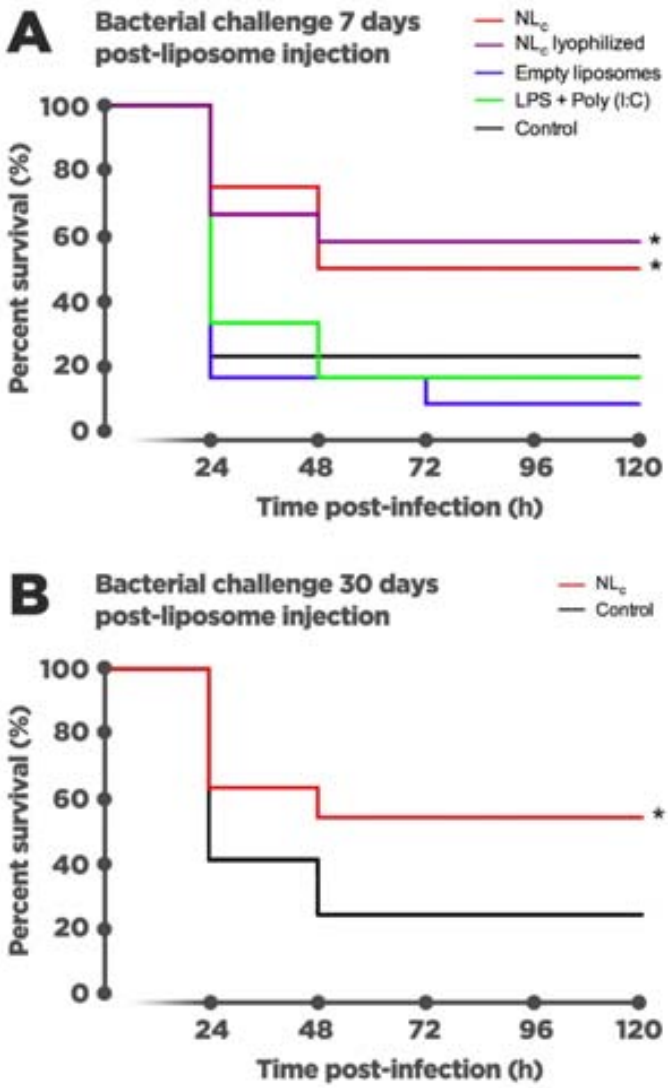

Figure 3. **Survival of adult zebrafish after i.p. injection of NL_c liposomes and challenge with *P. aeruginosa* (PAO1).**

(A) Fish were i.p. immunostimulated with NL_c liposomes, empty liposomes or free Poly (I:C)/LPS 7 days before being challenged with PAO1 at the LD_{50}. NL_c liposomes lyophilised for 4 months were also used. **(B) Fish i.p injected with NL_c liposomes were also challenged with PAO1** one month after immunostimulation. Untreated zebrafish that had been infected with PAO1 at the LD_{50} were used as mortality control. Differences were analysed using log rank test. $^*p < 0.05$.

NL_c liposomes administered by i.p. injection protect zebrafish against *Spring Viraemia of Carp Virus* (SVCV) lethal challenge

The protective efficacy of NL_c liposomes *against Spring Viraemia of Carp Virus* (SVCV) administered by i.p. injection was assessed in adult zebrafish. The fish were treated with NL_c liposomes, empty liposomes, the mixture of free immunostimulants (poly (I:C) and LPS) or PBS. At 7 days post-injection, all the fish were subjected to an immersion challenge with SVCV (**Figure 4**). Similarly to the bacterial challenge neither the empty liposomes nor the mixture of free immunostimulants offered any significant protection relative to the control fish, as measured at 15 days (RPS of empty liposomes: 0%; free immunostimulants: 7.7%). Only the fish that had received NL_c liposomes showed a significantly higher survival rate (RPS of 42.3% after 15 days) (**Figure 4 and Annex 3 Table 1**). This difference was evident throughout the entire experiment.

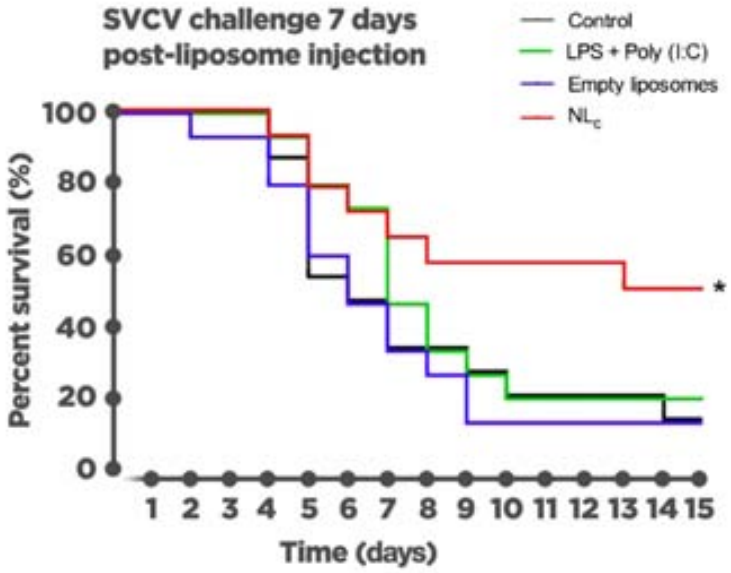

Figure 4. **Survival of adult zebrafish after i.p. injection of NL_c liposomes and immersion challenge with *Spring Viraemia of Carp Virus* (SVCV).**

Fish were immunised with NL_c liposomes, empty liposomes or free Poly (I:C)/LPS by i.p. injection 7 days before being challenged with SVCV by immersion. Untreated zebrafish that had been infected with SVCV were used as mortality control. Differences were analysed using log rank test. *$p < 0.05$.

Biodistribution of NL_c liposomes in zebrafish after administration by immersion

We also evaluated the biodistribution of fluorescently labelled NL_c liposomes (AF750-NL_c liposomes) in zebrafish following administration by immersion. The zebrafish were treated by placing them into water tanks containing AF750-NL_c liposomes. At 0 h, fluorescence was detected in the gills of all fish and by 12 h post-immersion, fluorescence was still detected in the gills but was also detected in the abdominal region of most of the fish (83.3%) (**Figure 5A**). To accurately gauge the organ distribution of the NL_c liposomes, *ex vivo* imaging was performed at 12 h post-immersion (**Figure 5B**). Fluorescence was observed in the gills of all fish (100%), and in the intestine and the liver of some fish (83.3% and 50% of fish, respectively). Thus, the results suggest that the NL_c liposomes had attached to the gill surface, and that they had reached the liver and the intestine. We cannot discard that NL_c liposomes also reached the intestine by the fish having swallowed water during immersion [33].

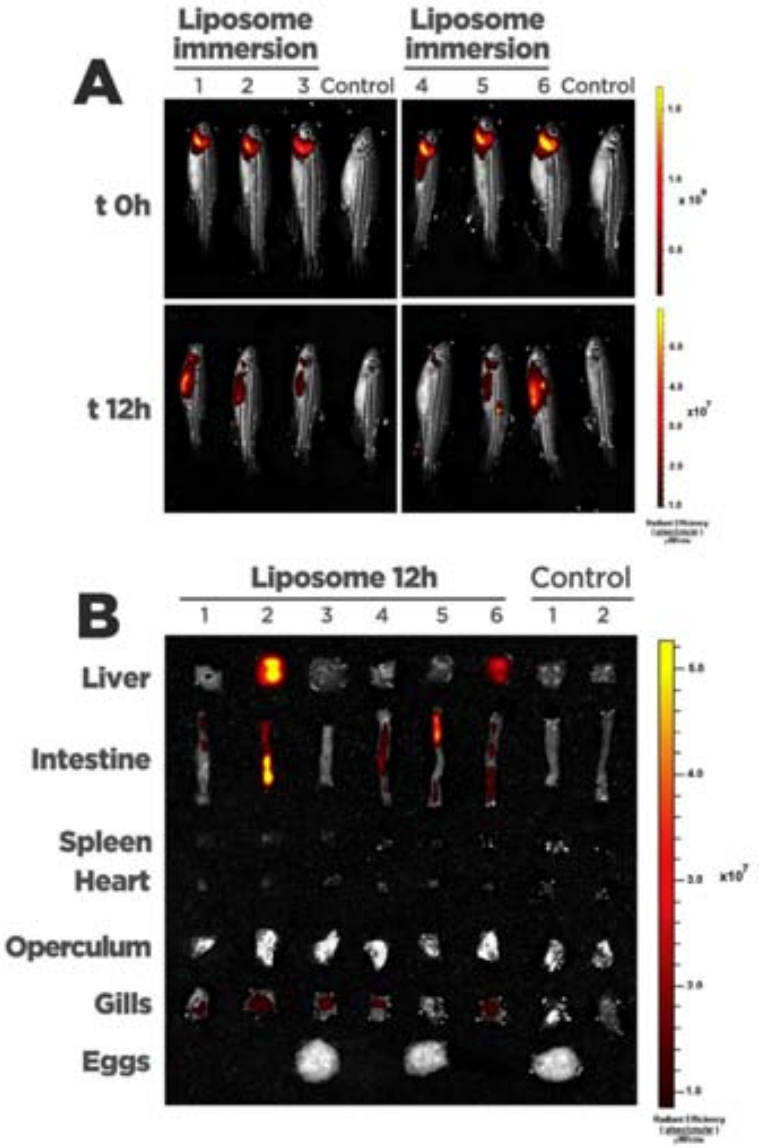

Figure 5. **Biodistribution of the NL_c liposomes after immersion.**

(A) IVIS Spectrum images of adult anaesthetised zebrafish, (n = 6) at 0 and 12 h, after 30 min immersion in water containing AF750-NL_c liposomes. **(B)** Organs (including liver, intestine, spleen, heart, operculum and gills) and eggs were removed at 12 h and imaged to reveal accumulation of AF750-NL_c liposomes. Image intensity is represented as Radiant Efficiency. Non-immersed fish were used as control for background subtraction

NL_c liposomes administered by immersion protect zebrafish against *Spring Viraemia of Carp Virus* (SVCV) lethal challenge

Having confirmed that these liposomes can be administered by immersion, we then evaluated their efficacy by the latter route against SVCV immersion challenge. In this case, the empty liposomes and the mixture of free immunostimulants gave a slight increase in the survival at 13 days: RPS was 20.0% with empty liposomes, 21.4% with free poly (I:C)/LPS (**Figure 6 and Annex 3 Table 1**). However, the only statistically significant difference in the entire survival curve was observed in the NL_c liposome-treated fish, whose mortality was clearly delayed throughout the experiment (RPS value of 33.3%) (**Figure 6 and Annex 3 Table 1**).

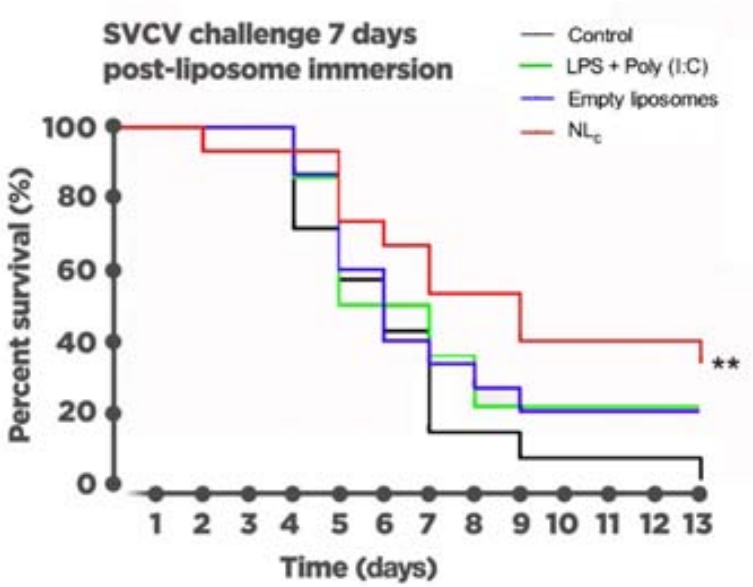

Figure 6. **Survival of adult zebrafish after bath immersion with NL_c liposomes and immersion challenge with *Spring Viraemia of Carp Virus* (SVCV).**

Fish were immunostimulated by immersion in water containing NL_c liposomesempty liposomes or free poly(I:C)/LPS 7 days before challenge. Untreated zebrafish infected with SVCV were used as mortality control. Differences were analysed using log rank test. $^{**}p < 0.01$.

Discussion

Our experiments on NL_c liposomes administered to adult zebrafish by i.p. injection clearly indicated that the spleen was the main organ in which the liposomes had accumulated. This finding is consistent with the fact that the spleen is amongst the most important organs for filtering out foreign agents [34] and is the main organ for antigen presentation in teleost fish [31]. Furthermore, this result is in agreement with those of previous studies, in which the uptake and retention of injected bacteria, vaccine antigens and liposomes were demonstrated in the spleen and the head kidney [35, 36]. However, we did not detect any fluorescent signal in zebrafish head kidney *in vivo*, although this was probably related to the detection limit of the method. Nevertheless, our experiments on NL_c liposomes administered to adult rainbow trout by i.p. injection demonstrated that the liposomes had accumulated in macrophage-like cells extracted from the spleen and, to a lesser extent, from the head kidney. These cells were identified as macrophages by their size, phagosome-rich cytoplasm, characteristic kidney-shaped nuclei and membrane rugosity [31, 32].

The NL_c uptake mechanisms *in vivo* probably would be different depending on the tissue. *In vitro* trout macrophages internalised the NL_c liposomes mainly through caveolae-mediated endocytosis and phagocytosis, while zebrafish hepatocytes (ZFL cells) internalised the NL_c liposomes through caveolae-dependent and clathrin-mediated endocytosis [18].

The difference in the amount of NL_c liposomes found in spleen and head-kidney macrophages could be explained by the fact that the majority of the circulating monocyte/macrophages would migrate to the spleen after mobilisation to the inflammatory site [37]. Another possible explanation might be that macrophages isolated from different tissues exhibited different phagocytic

responses [38]. Macrophages help regulate the immune response by producing cytokines and interferons and by presenting antigens to lymphocytes [39]. Therefore, targeting the delivery systems to these cells should be an excellent strategy to achieve optimal protection levels.

To test whether the NL_c liposomes could protect fish against bacterial infection, we developed a new model using *P. aeruginosa*. Despite the current lack of models in adult zebrafish, researchers have developed several models of bacterial (*e.g. Streptococcus iniae* or *Mycobacterium marinum*) or viral (*e.g.* VHSV) infection in zebrafish larvae over the past few years [24, 40]. However, the maturity of larval immune systems remains poorly understood. We chose *P. aeruginosa* because it is an opportunistic pathogen in fish [22] and in humans [23], is easy to handle, and is available in multiple virulence mutants. We would like to highlight that animal models of bacterial infection such as the one we developed in this work might also prove valuable in therapeutic research for humans, especially given the fact that immunosuppressed patients (*e.g.* cystic fibrosis patients) are highly susceptible to *P. aeruginosa* infection.

The level of protection against infection by *P. aeruginosa* or by SVCV that we observed in the fish treated with NL_c liposomes, regardless of the administration route, suggests the potential utility of these liposomes as a broad-spectrum tool for immunological protection of fish. Furthermore, the fact that the mixture of free immunostimulants did not offer protection in any of the infection models underscores the importance of encapsulating in liposomes to ensure optimal activation of the immune system. Although i.p. injection remains the most widely used route to administer vaccines, it suffers some disadvantages, such as stress and side-effects at the injection site [41]. On the other hand, immersion and oral administration would be the preferable methods as they involve less handling costs and stress. However, the suitability in terms of cost-effectiveness of each vaccination method will have to be studied for each particular disease/case. In regard to this, we also evaluated the use of immersion to deliver the liposomes, as this method – in addition to being less time- and cost-dependent – offers another major advantage: the vaccine generates mucosal immunity at the site on the organism's body at which it is most likely to encounter the pathogen [42]. Thus, liposomes not only protect encapsulated actives, they also enhance the immune response by increasing mucosal adhesion [12, 43].

In the present work, we found that the NL_c liposomes had accumulated in the gills, where they most likely attached to the epithelial cells and underlying phagocytes [33], and in the intestine, another reported route of antigen entry in bath-immunised fish [33, 44]. The presence of NL_c liposomes in the liver following administration by immersion might be down to this organ's role in detoxification and lipid-processing [34]. This observation is consistent with previous studies in which encapsulated LPS was found in the liver after oral administration, indicating that they undergone intestinal absorption [45]. Although there have been reports of failed attempts at using immersion to administer vaccines [46], this failure might be related to the vaccine composition or because the use of the same route for vaccination and experimental challenge is probably very important [9, 11]. Accordingly, we used an immersion infection model, observing a significant increase in the survival and a delay in the mortality. Thus, given the promising results we have obtained with NL_c liposomes and the fact these liposomes, once lyophilised, can be easily stored for long periods of time without losing their efficacy, we are confident that this approach will ultimately prove fruitful for use in diverse therapeutic contexts.

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general
Discussion & Conclusions

General discussion

The overall aim of this Thesis was the development and characterization of a biocompatible formulation containing two immunostimulants encapsulated into liposomes, that could be administrated to different fish species and enhance their immune system performance.

This involved several specific objectives. The first one was to evaluate the suitability of using an hepatocyte zebrafish cell line (ZFL) as the *in vitro* model for performing the cytotoxicity, endocytosis and immune response studies. By characterization of this cell line, we have demonstrated that indeed it can be used not only for the toxicological studies but also to assess the anti-viral response as well as the response to other stimuli such as anti-bacterial and anti-fungal response. In such context, we believe that this cell model will be very important because it will increase the available tools for those researchers working in fish immunology and vaccinology.

Teleost fish have a highly developed innate immune system and also an adaptive immune response, which is essential for its lasting immunity. This has allowed the development of disease preventive measures by manipulating their immune system. Teleost species live in aquatic environments that can contain a high concentration of pathogens [1]. Given the fact that these species mostly relay on their strong innate immune system to fight against these pathogens, the specific modulation of their innate immune system seemed the best approach to improve their ability to deal with infections. The fish innate immune system can be modulated and enhanced by molecular patterns associated to pathogens (PAMPs) that interact with its pattern-recognition receptors (PRRs), triggering different pathways [2]. It is for this reason that we decided to work with these molecules, also named immunostimulants.

Another key aspect of our approach was the use of PAMPs from different biological sources, expecting that the animals would be able to cope with infections of different origin. In an heterologous approach, the lypopolysaccharides (LPS) from a gram negative bacteria (*Escherichia coli*) and the synthetic viral double stranded RNA, Poly (I:C), were selected because bacteria and virus are the most common causative agents of infectious diseases in aquaculture (54,9% and 22,6%, respectively) [3]. It is important to keep in mind though, that crude LPS preparations, like the one we have used, could potentially include contaminants (e.g. peptidoglycans (PGNs), nucleic acids or lipoproteins), which could be responsible for part of the proinflammatory activity [4]. Other factors, such as the availability and the physicochemical properties of the immunostimulants, were also considered; for instance, the fungal polysaccharide Zymosan was taken into consideration, but finally discarded because of its size above the seeked nano range.

More importantly, the focus of this Thesis was to improve the administration of these PAMPs with the use of a biocompatible delivery system. By using nanosized delivery systems, we were searching for a different interaction with the cells of the immune system, an improved bioavailability, a sustained release, and a better protection of the therapeutic agents from degradation.

That entailed, in a first stage, the encapsulation of both types of immunostimulants in nanosized liposomes and a comprehensive characterization of the process. Parameters, such as the size, charge and stability, are extremely important for their future *in vivo* behaviour. First of all, extensive characterization was performed with five empty liposomal preparations with positive, neutral and negative surface charges. All these liposome compositions contained not only a phospholipid and cholesterol, but also a small percentage of polyethylene glycol (PEG). Not because we wanted to increase the circulation time of the liposomes (as our target were the im-

mune system cells), but because the unilamelarity and homogeneity of the samples were increased after its addition. Then, each immunostimulant was encapsulated separately in the five liposome formulations. Both immunostimulants are negatively charged and therefore, the obtained encapsulation efficiencies were markedly higher with the positively charged liposomes, due to the expected electrostatic interactions between the liposomes and both immunostimulants. For example, in the case of the Poly (I:C), the encapsulation percentages obtained with both cationic formulations were extremely high, with values ranging from 91.3 % to 95.0 %. Indeed, the presence of an electrostatic interaction between cationic lipidic membranes and nucleic acids has been extensively reported [5–8]. Choosing those formulations with higher encapsulation efficiencies, cytotoxicity studies were performed with the two empty positively charged liposomes. A clear difference was observed between the two compositions and, as reported, the cytotoxicity was increased as the positive charge increased too. That allowed for the selection of the more suitable lipidic composition to encapsulate both immunostimulants, separately and/or together, without compromising the cell viability.

It is important to mention that the co-encapsulation of the two immunostimulants together led to a decrease in the encapsulation efficiency of the LPS, probably as a result of a weaker electrostatic interaction with the lipidic membrane. Still, it was preferable for us to have both molecules encapsulated in the same delivery system, as administration of LPS and Poly (I:C) in separate liposomes would have meant an increase in the overall liposome concentration and therefore, an increase in its related cytotoxicity. More interestingly, it has already been stated that it is important to administrate the active molecules in the same delivery system to improve their immunogenicity [9, 10]. For instance, one of the most recent succesful "2nd adjuvant platforms", the AS01, is made of liposomes encapsulating together the saponin QS21 and the monophosphoryl lipid A (MPL) [11]. Nevertheless, the liposomes containing both immunostimulants have proven to be highly biocompatible at the concentrations required for *in vitro* immune stimulation.

Another valuable aspect of the characterization was the assessment of the immunostimulant location in the liposomes. The confocal images, performed on liposomes encapsulating each one of the immunostimulants, confirmed that they were placed in the lipidic membrane (as expected due to the abovely mentioned electrostatic interactions). More interestingly, it also allowed us to see that, after their co-encapsulation, they were both still attached to the liposome membrane. In the case of the LPS, we could hypothesize that its affinity for the positively charged liposome membranes might come from the negatively charged lipid A part, which in fact, also anchors LPS molecules to the bacterial outer membrane made of phospholipids too. Due to technical limitations, we were not able to specify the exact orientation of the molecules on the lipidic membranes, but we would expect a random attachment of both LPS and Poly (I:C) on the internal and external faces of the membrane. If that was the case, one could speculate that the antigen-presenting cells (APCs) would have ready access to the immunostimulants placed outside the membrane, and that might even increase the liposome tagging and/or phagocytosis [12]. In fact, the incorporation of immunoactive compounds in the surfaces of nanoparticles has been proposed to better mimic interactions between "pathogen-like" particles and the immune cells in an strategy called "biomimetic vaccine carriers" [13].

The endocytosis assays were necessary to assess the liposome interaction with the cells. Most clinical nanomaterials are in the range of 10–300 nm in diameter and typically accumulate in organs containing high numbers of tissue macrophages, such as the liver or spleen [14]. As al-

ready mentioned, the target of our developed liposomes were the APCs or "professional phagocytes", but it was necessary to study their interaction with other non-professional phagocyting cells where they might also accumulate. The role of hepatocytes in the hepatic clearance of small sized liposomes (< 80 nm) have been demonstrated before [15]. In this context, the zebrafish hepatocyte cell line (ZFL) was used in addition to the trout macrophages. Indeed, some differences were observed in the kinetics, intensity and endocytosis mechanisms between the two cell types. Both cells efficiently internalized the liposomes after contact with the plasma membrane, as observed by confocal microscopy. The uptake mechanisms were studied using different chemical inhibitors, and the caveolae-mediated endocytosis seemed to be the most prominent pathway used by both cells types. The hepatocytes also used the clathrin-mediated pathway, whereas trout macrophages used phagocytosis too. The caveolae-mediated pathway has been related to the endocytosis of smaller nanoparticles (< 80 nm), while the clathrin-mediated pathway is associated to nanoparticles slightly bigger (< 300 nm) [16, 17]. Based on that, our developed liposomes would have to be preferentially internalized through clathrin-mediated mechanisms, as their size is 125.8 ± 6.6 nm. However, other factors, such as the surface charge, rigidity and/or the putative presence of toll-like receptor (TLR) ligands on the liposome surface, might also have an impact on their endocytosis mechanisms. Nevertheless, the chemical inhibitors used for these studies could be slightly unselective and therefore, disturb more than one endocytosis pathway [18, 19]. However, in order to better identify and dissect the intracellular trafficking of the liposomes, colocalization assays with known markers of early endosomes, lysosomes, or even with biomarkers of caveosomes or clathrin-coated vesicles, would have been a better approach [20, 21].

Confocal microscopy and flow cytometry results suggested that, after endocytosis by the zebrafish hepatocyes, liposomes accumulated in the endosomal/lysosomal system and started to be metabolized. This liposome degradation was inhibited by the addition of chloroquine, an inhibitor of the lysosomal acidification, which, in turn, confirmed the liposome location into endosomes. It is important to mention that TLR3, the known dsRNA sensing receptor for some teleost fish species, is located in the endosomes [22–24]. Regarding to the trout macrophages, accumulation in endosome-like vesicles (phagosomes) after long incubation times was seen by confocal microscopy. On the other hand, no apparent degradation of the liposomes was observed by flow cytometry. This low degradation could be due to the different "activation states" of the fish macrophages, which have an impact on their resulting effector functions [25], or even to the kinetics of the phagosomal/lysosomal system. Internalization of particles by professional phagocytes triggers signalling cascades that result into the fusion of phagosomes with lysosomes, leading to the formation of phagolysosomes [26]. It has been stated that the kinetics of the phagosome maturation are dependent, for instance, on what they are engulfing [27, 28]. Another explanation could be related to the different roles that the macrophages and dendritic cells would have in mammals and in teleost fish. Dendritic cells are the main APCs in mammals, and therefore, they are able to avoid a rapid lysosome acidification that would otherwise lead to an excesive antigen degradation. By recruiting active NADPH oxidase complexes to the phagosome, mammalian dendritic cells are able to temper the acidification to effectively process, and subsequently, present phagosome-derived peptides on their surface [29]. In teleost fish, dendritic cells have been described in some species, but their role in antigen-presentation is still unknown [30–32]. In fact, high expression of MHC-II molecules has been found, not only in macrophages [33], 34] but in other immune cells, such as in seabream acidophilic granulocytes (functionally analogous to mammalian neutrophils), in zebrafish eosinophils and mast cells [34, 35]. Also, B lym-

phocytes have been proven to be at least phagocytic [36]. Therefore, macrophages, together with other cells, might be having a more prominent role in antigen presentation. In that case, one could speculate that the phagosomal acidification, maturation and processing of antigens could also be different between the mammalian and teleost macrophages.

One way to assess the activation of the innate immune system is by following the expression and release of immune cytokines [37]. The *in vitro* expression of some of these cytokines was increased with the liposomes containing the immunostimulants in comparable levels to the ones obtained by the free immunostimulants. More importantly, TNFα protein secretion was also strongly induced by the liposomes containing the immunostimulants, whereas the empty liposomes generally failed to stimulate the cytokine gene expression or the TNFα protein secretion. This reinforces the idea that the encapsulated immunostimulants are able to reach their cell receptors and activate their signalling pathways, and that this cytokine increase is not a consequence of the empty liposomes themselves.

Another important thing to note is that, by encapsulation of the LPS in liposomes, its related toxicity decreased significantly in the zebrafish hepatocyte cell model and also in the case of the unhatched zebrafish embryos. This could be probably related to its different interaction with the cells and a different bioavailability related to a putative sustained release of the LPS.

Nevertheless, after an extensive characterization of the liposomes and their *in vitro* behaviour, we further wanted to assess their tissue and cell biodistribution in a living system. For that, injection of fluorescently tagged liposomes to zebrafish (*Danio rerio*) was performed. The *ex vivo* organ imaging demonstrated accumulation of the liposomes in the spleen, an organ with a high population of resident tissue macrophages [33]. Besides, after liposome injection to adult rainbow trout, liposomes were found internalized in macrophages from the spleen and head kidney. Still, many questions were kept unsolved. For example **(1)** ***do liposomes accumulate only in macrophages or also in other phagocytic cells such as dendritic cells or B lymphocytes?***; or **(2)** ***does liposome uptake occur at the injection site or is the uptake happening in the tissue-resident macrophages?*** Whether cellular liposomal uptake occurs directly in tissue macrophages or in monocytes, which subsequently accumulate at the site of inflammation and migrate to antigen presentation organs, has not been examined widely due to technical constrictions. But what we can confirm is that liposomes were detected in the spleen of zebrafish right after injection, indicating either a fast liposomal migration to this organ or a fast monocyte uptake of the liposomes and subsequent migration to this organ.

Another question might be the role of the migration of monocytes/macrophages to the spleen (and in a lesser amount to the head-kidney) after endocytosis of liposomes. Authors have suggested that the melanomacrophage centers found in the spleen and the head kidney of teleost fish may serve as sites for antigen presentation [38]. A study from Iliev *et al.* [33] in salmon demonstrated that there was a specific population of cells expressing MHC-II molecules on their surface, which were able to uptake the model antigen ovalbumin (OVA) and CpGs in the periphery and accumulate predominately in the head-kidney and spleen in the first 24 hours post-administration. These cells had a macrophage-like morphology. Our *in vitro* studies demonstrated that liposomes were able to enhance different proinflammatory cytokines gene expression and also the TNFα secretion. So, one would expect a similar behaviour by the monocyte/macrophages *in vivo* after interaction with the liposomes. Nevertheless, after assuming a general activation of the macrophages, their possible role in antigen presentation of the LPS

and/or Poly (I:C) (although not being antigenic proteins) in the spleen or head kidney would also have to be studied.

Finally, we wanted to study whether the survival of the animals against an infection increased after administration of our designed delivery system. Zebrafish has been extensively used as a model animal in infection and immunity experiments, based on its immune system similarity to the mammalian one. Also, researchers working on fish immunology and aquaculture are starting to use zebrafish as a valuable *in vivo* model [39–43]. For instance, it has been used as a model with several virus infections, such as infectious hematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), nervous necrosis virus (NNV), spring viraemia of carp virus (SVCV) and viral haemorrhagic septicaemia virus (VHSV) [44–48]. Also, a successful infection of zebrafish has been demonstrated using a variety of bacterial pathogens. They include *Mycobacterium marinum*, *Edwardsiella tarda*, *Streptococcus iniae*, *Aeromonas salmonicida* and *Staphylococcus aureus* [49–54]. In this context, two adult zebrafish infection models were used to test the liposome efficacy: (1) an injection bacterial infection model based on *Pseudomonas aeruginosa*, which was developed in the laboratory; and (2) an immersion viral infection model based on the spring viraemia of carp virus (SVCV). Interestingly, our formulation was able to significantly increase the survival of the zebrafish when challenged with either of the tested infections, confirming that the same delivery system, containing LPS from *E. coli* and dsRNA, can confer protection against two distinct pathogens. Besides, the non-encapsulated LPS and Poly (I:C) mixture, which gave similar levels of proinflammatory cytokine expression *in vitro*, failed to protect the zebrafish *in vivo* against any of the studied challenges.

Furthermore, liposomes significantly increased the survival of the zebrafish even when challenged one month after the liposome injection. This might be a bit surprising as liposomes are, in principle, stimulating the innate immune system which is characterized by a rapid appearance. The good results after one month could be related: **(1)** ***to the duration of the innate immune stimulation;*** **(2)** ***to an indirect stimulation of the adaptive immune response***; or could be also related **(3)** ***to an adaptive component of the innate immunity***. As a matter of fact, several authors have stated that there might be an adaptive component in the innate immunity responsible for this lasting state of enhanced innate immunity or "innate memory" [55]. In mammals, evidence of adaptive responses of prototypic innate immune cells, such as natural killer (NK) cells and monocytes/macrophages has been demonstrated and viewed as a paradigm shift in immunity [56–58]. In the case of the monocytes/macrophages, stimulation with different molecules has been seen to result in changes in expression of specific receptors that can confer or enhance physiologically important properties, and possibly become refractory to further stimulation [58]. On the other hand, NK cells, an innate lymphocyte population with the capacity to directly recognize and kill tumor target cells and virus-infected cells without any prior induction period, have been proven to mount antigen-specific immunological memory [59, 60]. In this regard, the T and B cell deficient $rag1^{-/-}$ zebrafish mutant has been used to investigate immune protection in response to infections in the absence of an acquired immune system [61]. Results showed that these mutant zebrafish were able to develop and maintain through the time a protective immunity following a primary vaccination exposure. The same authors suggested that the NK cells would most likely be the cells mediating the protective immunity in these $rag1^{-/-}$ mutant zebrafish [61]. Taken all this into account, further experiments challenging the animals at longer post-liposome administration times would be of great relevance. It would also be very interesting to distinguish the contribution of each type of immunity: the classical innate immun-

ity, which is expected to be the main part of the global immune response, and the putative contribution of the adaptive response and/or the possible role of the "innate memory".

The fact that the spring viraemia of carp virus (SVCV) challenge was performed by immersion allowed us to assess the performance of the liposomes administrated by bath immersion too. Before, it was also necessary to assess the targeting of the liposomes to the fish mucosae, the main portals of entry of pathogens [1, 62]. The liposomes were administrated by immersion, and tissue biodistribution results demonstrated mucosal adherence to the zebrafish gills and intestine. No cellular target studies were performed. However, based on previous studies, we could speculate that liposomes might be attached to epithelial cells and the underlying phagocytes [63, 64]. Interestingly, bath immersion administration of the liposomes also resulted in zebrafish increased survival when challenged with the SVCV, even though a lower infection dose would had been more suitable in the assessment of an immunostimulant protection effect.

Results obtained in the group further proved that liposomes administrated to zebrafish larvae by immersion had preeminent intestinal accumulation (unpublished data). This finding opens the door to future studies performed by immersion to larvae and juveniles in order to confer protection during development or growth stages, where they might be more susceptible to disease because of their not fully-competent immune system [65].

Besides its application in model species like zebrafish, its possible applicability to a real aquaculture species has also been taken into account. For that, ongoing work is being performed to study the rainbow trout survival after liposome administration and subsequent lethal challenge with its natural infecting pathogen *Yersinia ruckeri*, a gram-negative bacteria from the *Enterobacteriaceae* family. This microorganism causes the enteric redmouth disease (ERM) or yersiniosis, a serious infectious disease affecting salmonids and other fishes cultured worldwide [66, 67]. Outbreaks of yersiniosis are often associated with poor water quality, excessive stocking densities and the occurrence of environmental stressors, although the severity of yersiniosis is dependent mainly on the virulence of the strain.

Importantly, preliminary results seem very promising as our formulation administrated by injection have been able to delay and decrease the mortality compared to controls (PBS, empty liposome and free LPS and Poly (I:C) mixture groups) (**Figure 1**). Nevertheless, the cumulative mortality in the PBS control group reached almost 100 %, and it was higher than desired in the assessment of any immunostimulant molecule protective effect. Therefore, further trials will be performed with lower infective doses.

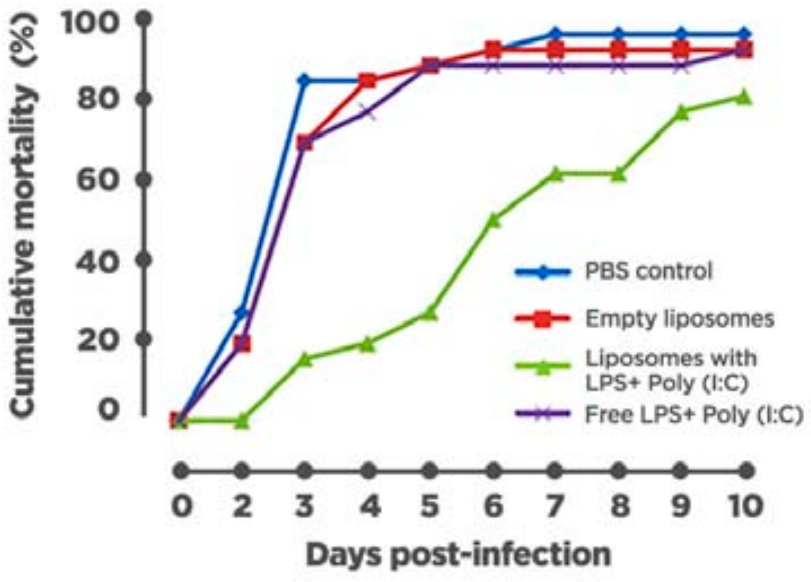

Figure 1. **Mean cumulative mortalities (%) obtained in rainbow trout after *Yersinia ruckeri* challenge.**

The assessment of the expression of immune related genes, both from the innate and adaptive immunity, in the head kidney and spleen of the experimental animals will also be performed in order to better understand the processes by which trouts injected with liposomes containing LPS and Poly (I:C) respond more favorably to an infection. A good result in the challenges carried out with rainbow trout would allow us to extend the range of action of our designed delivery system. Besides, survival assays with more pathogens could also be performed, as well as protection and subsequent infection assays at the larval and juvenile developmental stage.

As the capture fishing industry has declined, the aquaculture has become an important source of seafood. Nowadays, the members of the cyprinid family (*e.g.* carps and barbels) are the most predominant in fish aquaculture. However, these species have relatively low value compared to salmon and trout, which are produced mainly by companies based in Northern Europe, Chile, Canada and the USA [3]. Vaccines have been basically produced over the last years for these most valuable species, just because the value of a healthy population justified the price paid for the vaccines. Nevertheless, it is estimated that the fish farming will continue growing and become more industrialized with increasing investment [3]. With this in mind, the onset of new tools that could be administrated, for instance, in stressful or disease-related seasons, and that could improve the animal survival against a wide range of pathogens, might be very useful. Obviously, further work would also have to be performed to assess the suitable effective lowest doses and the liposome administration times, together with extensive economic cost and benefit studies.

General conclusions

The conclusions of this Thesis are summarized below:

(1) A liposomal delivery system composed of DLPC, Cholesterol, PEG and the positively charged cholesteryl, encapsulating two immunostimulants has been developed and characterized. Liposomes were at the nanometric scale (125 nm) and neutrally charged after coencapsulation of the lipopolysaccharide from *E. coli,* and the synthetic analogue of a dsRNA virus, Poly (I:C). The encapsulation efficiency of the Poly (I:C) was almost 100% while the encapsulation efficiency of the lipopolysaccharide was markedly lower. Both immunostimulants were found to be preferentially located in the liposome lipidic membrane.

(2) The liposomal carrier showed low cytotoxicity in vitro, in a zebrafish hepatocyte cell model and a trout macrophage primary cell model, as well as low in vivo toxicity using zebrafish embryos and larvae. Liposomes incubated with the zebrafish hepatocytes and the trout macrophages were able to elicit a specific pro-inflammatory and anti-viral response. More interestingly, liposomes encapsulating both immunostimulants, induced the TNFα protein secretion in vitro while the empty liposomes did not.

(3) Liposomes were internalized in vitro by: **(1)** zebrafish hepatocytes, mainly through caveolae-dependent endocytosis and clathrin-mediated endocytosis; **(2)** and by trout macrophages, through caveolae-dependent endocytosis and phagocytosis.

(4) An imaging method mostly used with rodents, has been adapted for in vivo tracking of fluorescent nanoliposomes in adult zebrafish. That, has enabled the evaluation of the in vivo dynamics and tissue distribution of the liposomal delivery system.

(5) After intraperitoneal injection, liposomes were found to accumulate in immune-related tissues of the zebrafish (spleen) and in immunologically relevant cells such as head-kidney and spleen macrophages from rainbow trout. After administration by bath immersion, the liposomes were able to be attached to the gills and intestine, which are mucosal tissues and the main portal of entry of pathogens.

(6) Liposomes protected zebrafish against otherwise lethal bacterial (*Pseudomonas aeruginosa* PAO1) and viral (spring viraemia of carp virus) infections regardless of whether they were administered by intraperitoneal injection or immersion. Importantly, protection was not achieved in fish treated with empty liposomes or a mixture of the free immunostimulants.

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Supplementary data

Annex 1

supplementary data

Zebrafish hepatocytes are able to mount an anti-viral response: **ZFL cells as a model** to study anti-viral responses *in vitro*

Chapter 1

Supplementary data

Zebrafish hepatocytes are able to mount an anti-viral response: ZFL cells as a model to study anti-viral responses in vitro

Ruyra A., Torrealba D., Morera, D., Tort, L., MacKenzie, S., Roher N.

(submitted)

Gene	Primer name	Sequence	Accession nº
Peptidoglycan recognition protein 2	zfPGRP2	For_ TGCCCAGGAAATGCCCTTTA Rev_ CTGCTGTGGGGACCCCTAGT	NM_001045166.1
Myxovirus resistance a	zfMXa	For_TTGACCTCCCTGGCATTGCA Rev_GCACTGGGCCAGTTGGATGA	NM_182942.4
Tumor necrosis factor a	zfTNFα	For_TGCTTCACGCTCCATAAGACC Rev_CAAGCCACCTGAAGAAAAGG	NM_212859.2
Interleukin 10	zfIL10	For_TCACGTCATGAACGAGATCC Rev_CACCATATCCCGCTTGAGTT	NM_001020785.2
Toll-like Receptor 3	zfTLR3	For_TGGTCCGGTGCTGTTTCTGA Rev_GGACAGTGGCACACCAAACG	AY616582.1
Interferon γ 1	zfINFγ1	For_GCGCATACAGATTTCGACGG Rev_TTTTTCTGTGGAGGCCCGAT	NM_001020793.1
Interferon ϕ 1	zfINFϕ_1	For_AAGTTTTTAGTCCTGACATTGGATCA Rev_TCCCAGTTCACCGAGTTCATG	NM_207640.1
Matrix metalloproteinase 9	zfMMP9	For_CACGCTTATCCTCCAGGTGAAG Rev- CCGTAGCGGGTTTGAATGG	NM_213123.1
Gig2-like protein	zfGIG2	For_AGGGTACGACACTGCCTGGT Rev_AGGGTCACCAAAGCCACAAT	NM_001245991.1
Elongation factor 1α	zfEF1	For_CTTCTCAGGCTGACTGTGC Rev_CCGCTAGCATTACCCTCC	AY422992

Table 1. **Zebrafish (*Danio rerio*) specific primers for PCR and qPCR.**

Annex 2

supplementary data

A Novel **Liposome-Based Nanocarrier** Loaded with an **LPS-dsRNA** Cocktail for Fish **Innate Immune** System **Stimulation**

Chapter 2

Supplementary data

A Novel Liposome-Based Nanocarrier Loaded with an LPS-dsRNA Cocktail for Fish Innate Immune System Stimulation

Ruyra A., Cano-Sarabia M., MacKenzie S.A., Maspoch D., Roher N.
(2013) PLoS ONE 8(10): e76338 doi:10.1371/journal.pone.0076338

Danio rerio

Gene	Primer name	Sequence	Accession n°
Interferon ϕ 1	INFϕ_1	For_AAGTTTTTAGTCCTGACATTGGATCA Rev_TCCCAGTTCACCGAGTTCATG	NM_207640.1
Gig2-like protein	GIG2	For_AGGGTACGACACTGCCTGGT Rev_AGGGTCACCAAAGCCACAAT	NM_001245991.1
Chemokine CCL4-like	CCL4	For_TAAAATGAGCACCTCTCGCTTTG Rev_GACTGAGGCTTACGGCACACA	XM_002663010.1
Toll-like Receptor 3	TLR3	For_TGGTCCGGTGCTGTTTCTGA Rev_GGACAGTGGCACACCAAACG	AY616582.1
Tumor necrosis factor α	TNFα	For_TGCTTCACGCTCCATAAGACC Rev_CAAGCCACCTGAAGAAAAGG	NM_212859.2
Inducible Nitric oxid synthase	iNOS	For_ GAGCAGGCCCAATGCATTT Rev_TGCGCTGCTGCCAGAAAC	NM_001104937.1
Elongation factor 1α	EF1	For_CTTCTCAGGCTGACTGTGC Rev_CCGCTAGCATTACCCTCC	AY422992

Oncorhynchus mykiss

Gene	Primer name	Sequence	Accession n°
Interferon α	INFα	For_GCCCCAGTCCTTTTCCCAAC Rev_CCTCTCAGGTTTCATGGCAGGT	NM_001124531.1
Tumor necrosis factor α	TNFα	For_CGCTGACACAGTGCAGTGGA Rev_TCCCCGATGGAGTCCGAATA	NM_001124374.1
Interleukin 6	IL-6	For_TTTCAGAAGCCCGTGGAAGAGA Rev_TCTTTGACCAGCCCTATCAGCA	NM_001124657.1
Ribosomal 18S	18S	For_CGAGCAATAACAGGTCTGTG Rev_GGGCAGGGCATTAATCAA	AF243428.2

Rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) specific primers for PCR and Q-PCR.

Table S1. **Rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) specific primers for PCR and Q-PCR.**

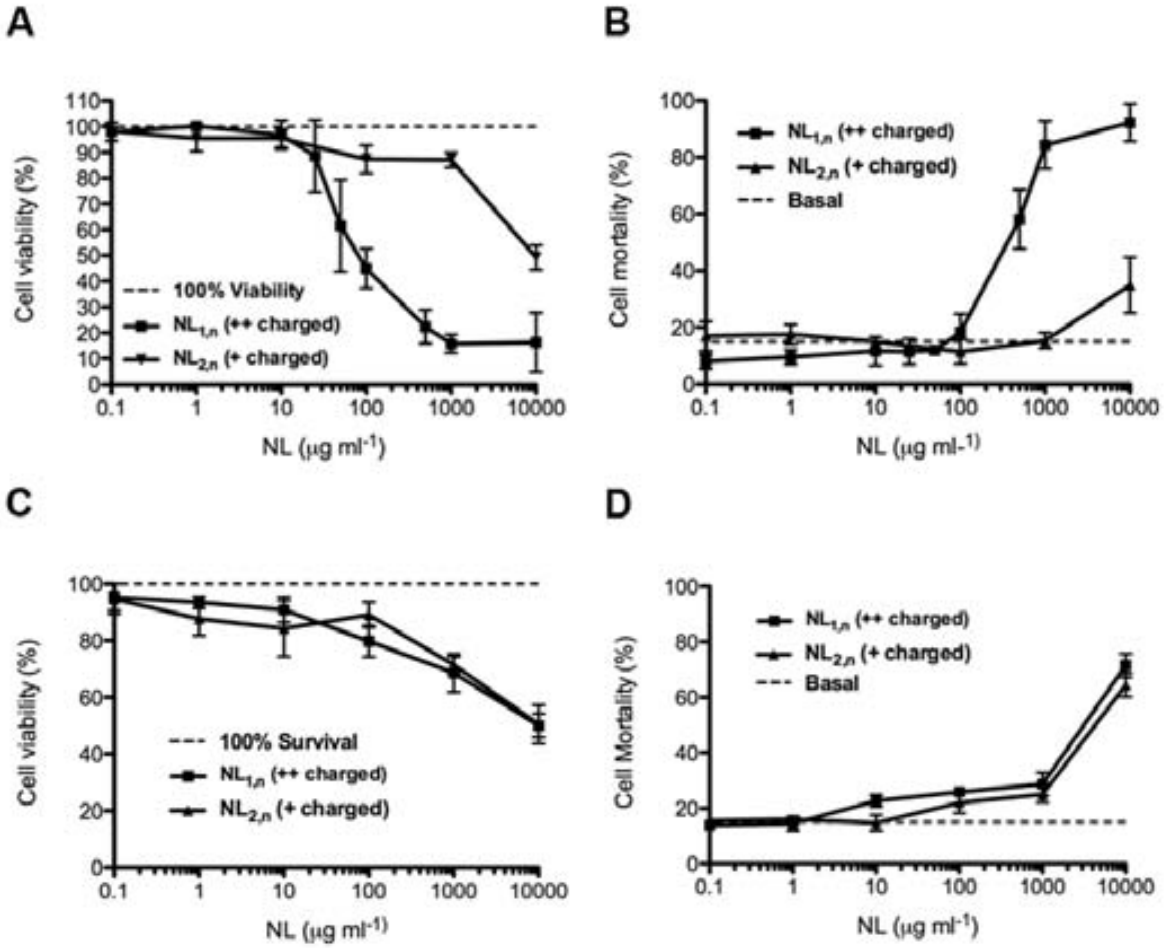

Figure S1. **Evaluation of toxicity of cationic liposomes without encapsulated immunostimulants ($NL_{1,n}$ and $NL_{2,n}$).**

Viability of ZFL cell line was assessed with the MTT assay **(A)** or LDH assay **(B)** after a dose response (0.1 μg/ml-10 mg/ml) with the two liposomal formulations ($NL_{1,n}$ and $NL_{2,n}$). Viability of HepG2 cell line was determined with the MTT assay **(C)** and with the LDH assay **(D)** after a dose response (0.1 μg/ml-10 mg/ml) with the two liposomal formulations ($NL_{1,n}$ and $NL_{2,n}$). Non-treated cells were used as 100% viability control (dotted line) in the MTT assays and non-treated cells were used as control of the basal death (dotted line) in the LDH assays. Data represent means ± SD of three independent experiments. Differences were analyzed using One-way ANOVA followed by Tukey's post-test. *, $p < 0.05$; **, $p < 0.01$;
***, $p < 0.001$.

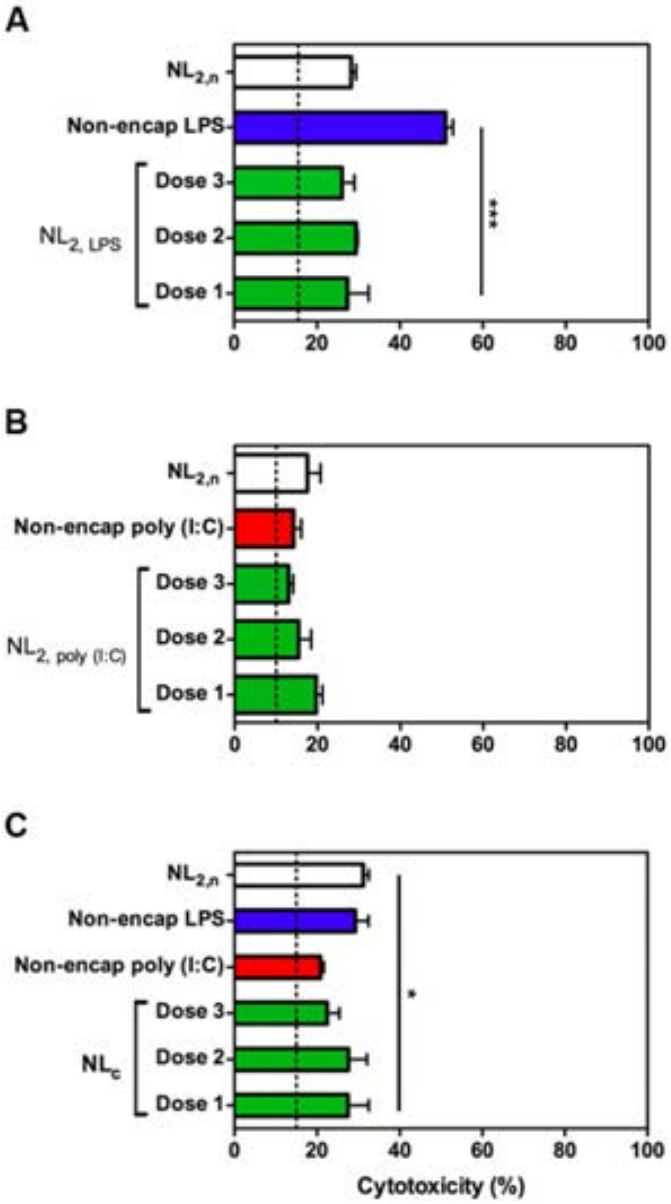

Figure S2. **Cytotoxicity of NL_c formulation in ZFL cells by LDH assay.**

(A) Viability of ZFL after 24 h incubation with the liposome-encapsulated LPS ($NL_{2, LPS}$, green bars) at Dose 1 = 1 mg/ml liposome with 50 µg/ml LPS, Dose 2 = 0.5 mg/ml liposome with 25 µg/ml LPS and Dose 3 = 0.20 mg/ml liposome with 10 µg/ml LPS. The white bar is the control treatment with liposomes without encapsulated immunostimulants ($NL_{2,n}$, 1 mg/ml liposome) and the blue bar is the non-encapsulated LPS control (50 µg/ml). **(B)** Viability of ZFL after 24 h incubation with the liposome-encapsulated poly (I:C) ($NL_{2, poly (I:C)}$,green bars) at Dose 1 = 1.5 mg/ml liposome with 50 µg/ml poly (I:C), Dose 2 = 0.75 mg/ml liposome with 25 µg/ml poly (I:C) and Dose 3 = 0.375 mg/ml liposome with 10 µg/ml poly (I:C). The white bar is the control treatment with empty liposomes ($NL_{2,n}$, 1.5 mg/ml liposome) and the red bar is the non-encapsulated poly (I:C) control (50 µg/ml). **(C)** Viability of ZFL cells after 24 h with liposomal LPS-poly (I:C) cocktail (NL_c, green bars) at Dose 1 = 1.5 mg/ml liposome with 50 µg/ml poly (I:C) and 25 µg/ml LPS, Dose 2 = 0.75 mg/ml liposome with 25 µg/ml poly (I:C) and 12.5 µg/ml LPS and Dose 3 = 0.375 mg/ml liposome with 12.5 µg/ml poly (I:C) and 6.25 µg/ml LPS. The white bar is the control treatment with empty liposomes ($NL_{2,n}$, 1.5 mg/ml liposome), the blue bar is the non-encapsulated LPS (25 µg/ml) and the red bar represents the non-encapsulated poly (I:C) control (50 µg/ml). Non-treated cells were used as 100% viability control (dotted line). Data represent means ± SD of three independent experiments. Differences were analyzed using One-way ANOVA followed by Tukey's post- test. *, $p < 0.05$; ***, $p < 0.001$.

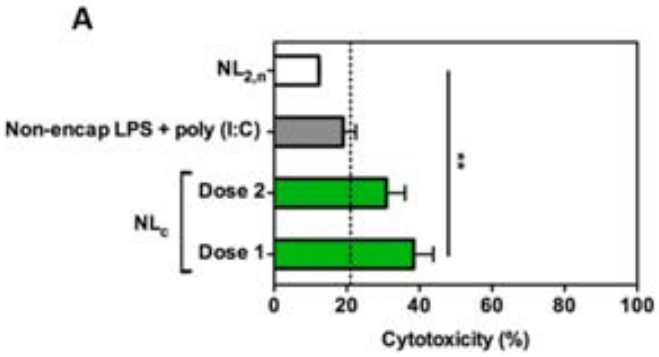

Figure S3. ***In vitro*** **cytotoxicity of NL_c formulation in trout macrophages.**

(A) The cytotoxicity of NL_c was assessed by the LDH assay. Viability of the trout macrophage primary cell culture after 24 h incubation with NL_c encapsulating both poly (I:C) and LPS (green bars) at Dose 1 = 0.75 mg/ml liposome with 25 µg/ml poly (I:C) and 12.5 µg/ml LPS and Dose 2 = 0.375 mg/ml liposome with 12.5 µg/ml poly (I:C) and 6.25 µg/ml LPS. The white bar is the control treatment with non-encapsulating liposomes ($NL_{2,n}$, 0.75 mg/ml liposome) and the grey bar is the non-encapsulated poly (I:C) and LPS control (25 µg/ml and 12.5 µg/ml, respectively). Basal dead cells of the non-treated cells were used as control (dotted line). Data represent means ± SD of 3 independent experiments. Differences were analyzed using One-way ANOVA followed by Tukey's post-test **, $p < 0.01$.

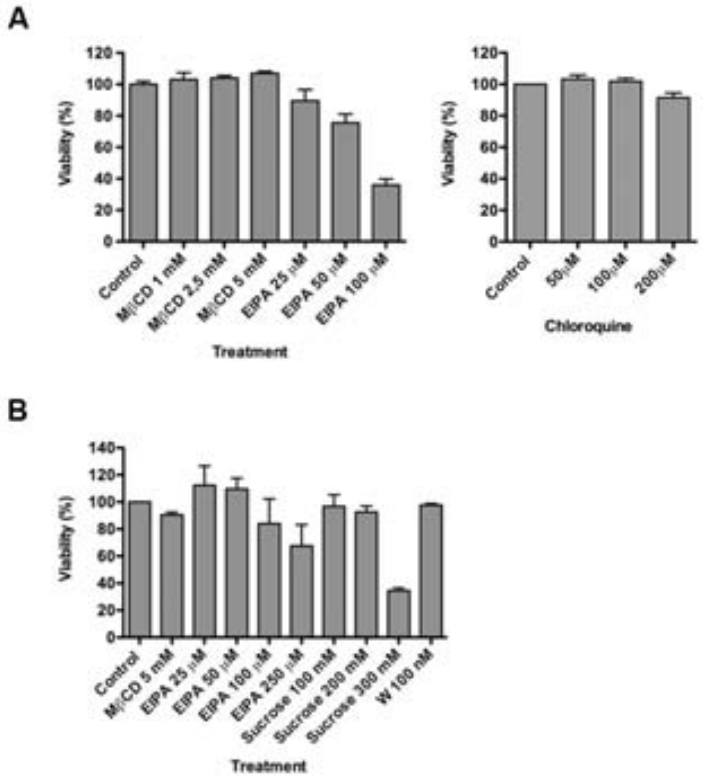

Figure S4. ***In vitro*** **cytotoxicity of endocytosis inhibitors.**

(A) Viability of ZFL cells after 1 h exposure (16 h in the case of the chloroquine) to different endocytosis inhibitors, assessed by the MTT assay. **(B)** Viability of trout macrophages after 1 h exposure to different endocytosis inhibitors, assessed by the MTT assay. Non-treated cells were used as a 100% viability control (Control bar).

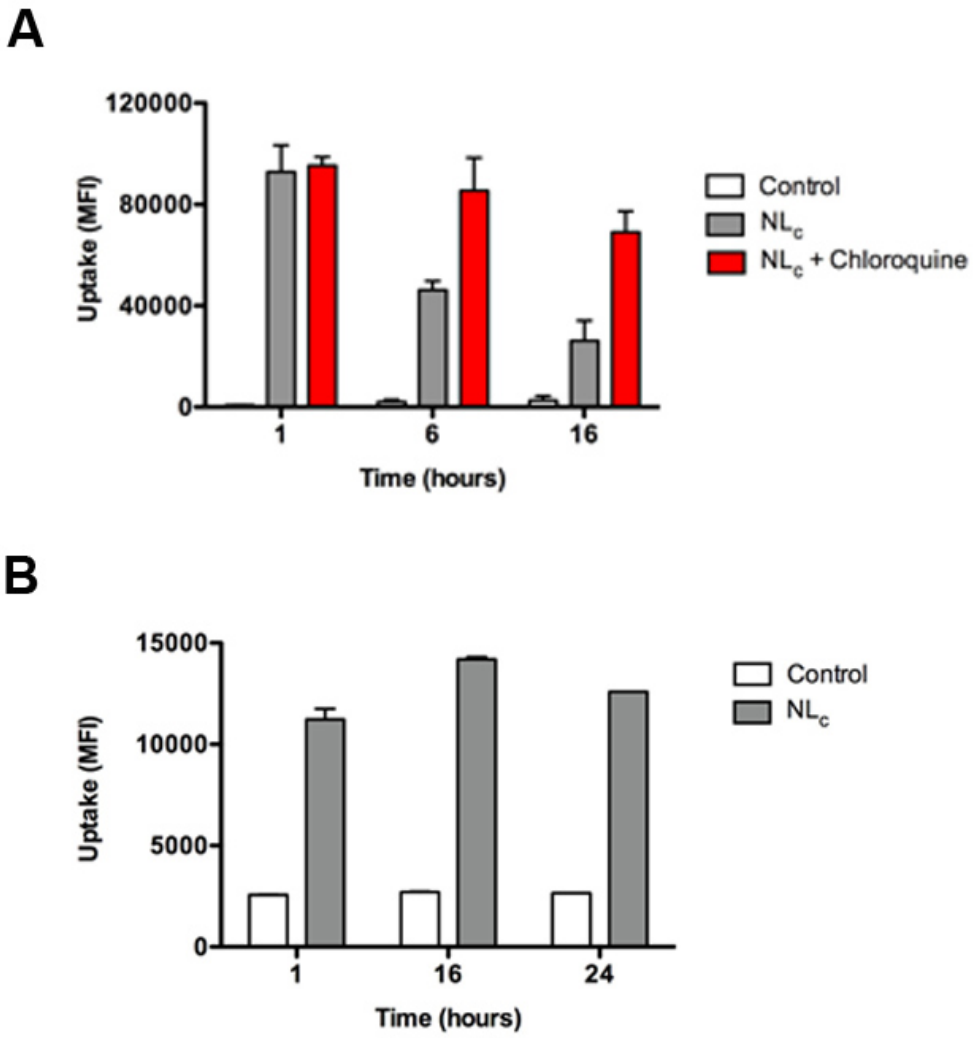

Figure S5. **Time-course of NL_c uptake in vitro.**

(A) Flow cytometry time course of NL_c uptake (grey bars, liposomes at 750 μg/ml containing 25 μg/ml poly (I:C) and 12.5 μg/ml LPS) by ZFL cells. To study the metabolization of NL_c, ZFL cells were also pretreated for 1 h with chloroquine at 100 μM (red bars). Then, liposomes were added (750 μg/ml liposome containing 25 μg/ml poly (I:C) and 12.5 μg/ml LPS), and left to incubate in the constant presence of chloroquine. **(B)** Flow cytometry time course of NL_c uptake (grey bars, liposomes at 750 μg/ml containing 25 μg/ml poly (I:C) and 12.5 μg/ml LPS) by trout macrophages. Cells not exposed to NL_c were used as controls (white bars). Data represent means ± SD of triplicates of three independent experiments.

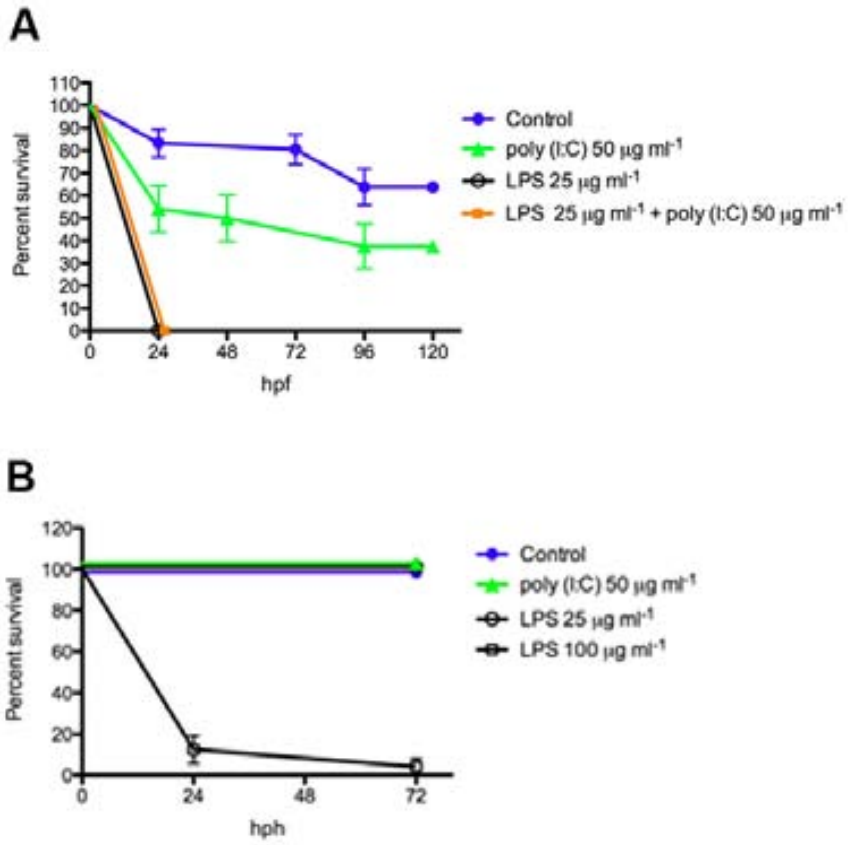

Figure S6. ***In vivo*** **NL_c toxicity assay controls.**

Survival of zebrafish embryos was recorded every 24 h at 120 h post fertilization (hpf) **(A)** and 72 h post hatching (hph) **(B)** after exposure to non-encapsulated LPS (black, 25 µg/ml and 100 µg/ml), non-encapsulated poly (I:C) (green, 50 µg/ml) and non-encapsulated LPS (25 µg/ml) and poly (I:C) (50 µg/ml) in combination (orange). Non-treated embryos (blue) were used as controls. Survival curves were analyzed using the log rank test (n=24 individual)

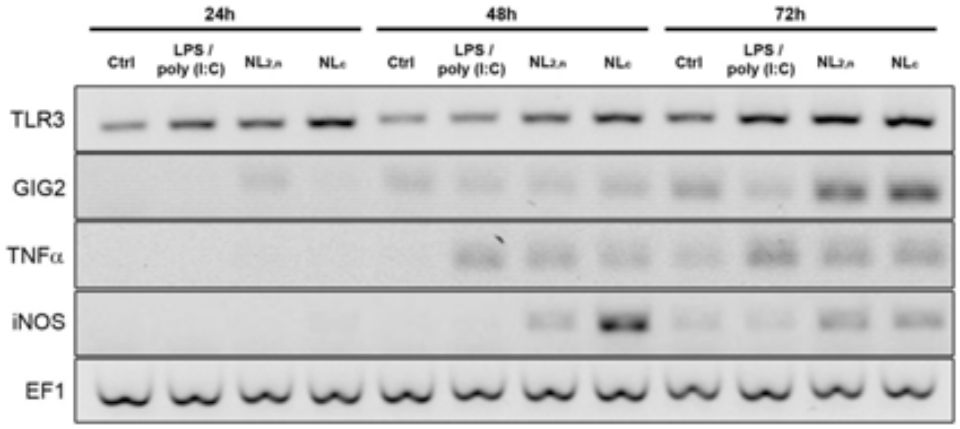

Figure S7. **Analysis of gene expression in zebrafish larvae after time-course exposure to liposome preparation.**

$NL_{2,n}$ = liposomes without encapsulated immunostimulants (1.5 mg/ml), NL_c = liposomes (1.5 mg/ml) with 50 µg/ml poly (I:C) and 25 µg/ml LPS and LPS + poly (I:C) = stimulation control (50 µg/ml poly (I:C), 25 µg/ml LPS). Non-treated embryos were used as control (Ctrl). Elongation factor (EF1) was the reference gene and TLR3, GIG2, TNFα and iNOS mRNA abundance was analyzed by conventional PCR (right panel). Representative images of three independent experiments are shown.

Annex 3

supplementary data

Targeting and **stimulation** of the zebrafish (*Danio rerio*) **innate immune system** with **LPS/dsRNA**-loaded **nanoliposomes**

Chapter 3

Supplementary data

Targeting and stimulation of the zebrafish (*Danio rerio*) innate immune system with LPS/dsRNA-loaded nanoliposomes

Ruyra A., Cano-Sarabia M., García-Valtanen, P., Yero, D., Gobert, I., MacKenzie S.A., Estepa, A., Maspoch D., Roher N.
(2014) Vaccine 32(31): 3955-3962 doi:10.1016/j.vaccine.2014.05.010

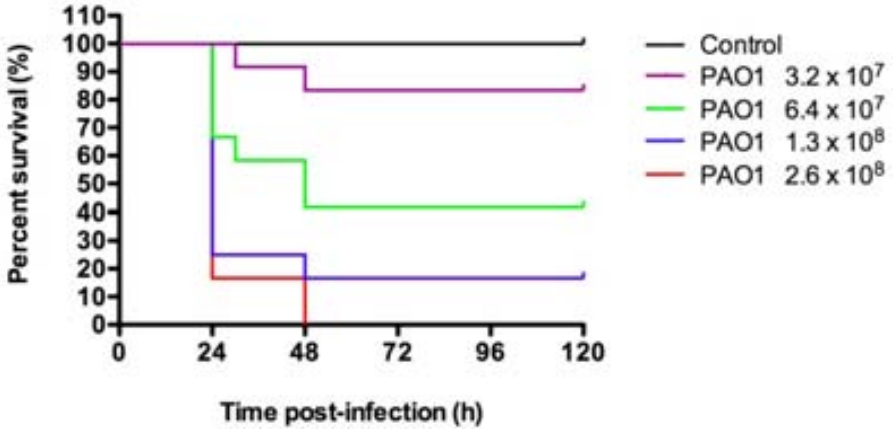

Figure 1. **Survival of adult zebrafish after challenge with *P. aeruginosa* (PAO1) by i.p. injection for LD_{50} determination.**

Fish were challenged with *P. aeruginosa* by i.p. injection of 20 µl of a bacterial suspension at concentrations ranging from 3.2×10^7 to 2.5×10^8 cfu/dose. Survival was recorded daily until 120 h post-injection. LD_{50} was determined to be 5.3×10^7 cfus.

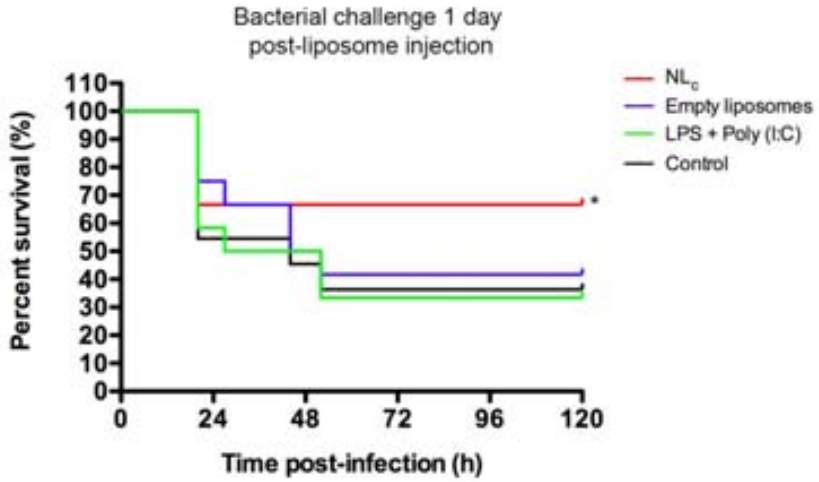

Figure 2. **Survival of adult zebrafish after i.p. injection of NL_c liposomes and challenge with *P. aeruginosa* (PAO1) at 1 day post-injection.**

Fish were immunised with NL_c liposomes, empty liposomes or free Poly (I:C)/LPS by i.p. injection 1 day before being challenged with PAO1 at the LD_{50}. Untreated zebrafish infected with PAO1 at the LD_{50} were used as mortality control. Differences were analysed using log rank test. * $p < 0.05$.

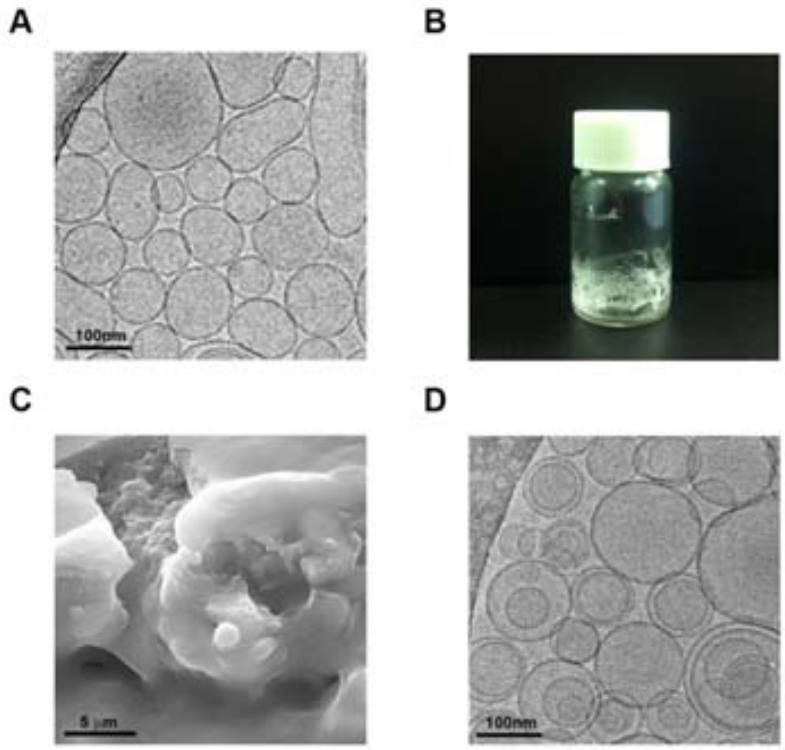

Figure 3. **Characterisation of the lyophilised NL_c liposomes.**

In order to prevent degradation of the lipidic formulation and to study its long-term conservation, the NL_c liposomes were lyophilized in the presence of the cryoprotectant trehalose. The overall morphology of the rehydrated NL_c liposomes was evaluated. **(A)** Representative Cryo-TEM image of the non-lyophilised NL_c liposomes. **(B)** Photograph showing the overall aspect of the lyophilised NL_c liposome cakes. **(C)** SEM image of the lyophilised NL_c liposomes in the presence of trehalose (lipid:carbohydrate ratio = 1:5) **(D)** Cryo-TEM image of the NL_c liposomes after re-hydration with PBS. Images show that both morphology and size were maintained, although some degree of unilamellarity and homogeneity was lost during lyophilisation (compare A to D). Importantly, after 4 months of storage at RT the lyophilised preparation retained good dispersion and solubility properties, and no signs of leakage of the encapsulated products were observed.

	Formulation	Days before challenge	Type of challenge	Mean RPS ± SD (%)
Injection	NL_c liposomes	1 day	Bacterial	33,2 ± 17,9 *
	NL_c liposomes	7 days	Bacterial	47,1 ± 16,9*
	NL_c liposomes	30 days	Bacterial	36,3 ± 4,3 *
	Empty liposomes	1/7 days	Bacterial	-3,6 ± 14,1
	Free Poly (I:C) + LPS	1/7 days	Bacterial	-4,4 ± 4,2
	NL_c liposomes	7 days	Viral	42,3 *
	Empty liposomes	7 days	Viral	0,0
	Free Poly (I:C) + LPS	7 days	Viral	7,7
Immersion	NL_c liposomes	7 days	Viral	33,3 **
	Empty liposomes	7 days	Viral	20,0
	Free Poly (I:C) + LPS	7 days	Viral	21,4

*, p <0.05; **, p <0.01

Table 1. **Mean increase in the survival of zebrafish after immunisation with different formulations.**