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A transcriptomic approach toward understanding PAMP-driven macrophage activation and dietary immunostimulation in fish

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Si supiese qué es lo que estoy haciendo,
no le llamaría investigación, ¿verdad?

Albert Einstein

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Abstract

Fish are by far the most successful and diverse group of vertebrates, representing 40% of all vertebrate species and displaying an amazing level of diversity in several biological aspects. They exhibit a number of genomic particularities unique among vertebrates that present fish as a very interesting model to gain an insight into a wide variety of disciplines, in particular those related to evolution. Therefore some fish species have played important roles in the latest years to increase the knowledge of vertebrate genome speciation. On the other hand, they are of tremendous importance as food for people, becoming the aquaculture industry an essential food-producing sector all around the world. The goal of the present study has been to characterize several molecular and functional aspects of the immune system of two evolutionary distant fish species, *Sparus aurata* (gilthead sea bream) and *Oncorhynchus mykiss* (rainbow trout), with specific emphasis on their transcriptomic responses to different pathogen-related challenges. To that end, *in vivo* and *in vitro* analyses were combined to evaluate global immune mechanisms of these teleosts.

The macrophage cell lineage represents an important group of cells which play a central role in the initiation and coordination of the immune response. A primary culture of *in vitro* differentiated macrophages of gilthead sea bream was developed and characterized; therefore aspects as morphology, phagocytic capacity and response to lipopolysaccharides (LPS) of these cells were investigated. In parallel, CD83, a cell surface membrane used as standard surface marker for dendritic cells in mammals, was cloned and then analyzed from the gilthead sea bream macrophages using Q-PCR (real-time quantitative-PCR). Once this *in vitro* model was characterized and validated, differentiated macrophages of gilthead sea bream were compared with those of rainbow trout to evaluate their differences in the activation of antiviral-related pathways upon LPS induction and the implications of the presence of contaminants in commercial LPS preparations when analyzing regulation of gene expression. Expression of antiviral genes in macrophages stimulated with different LPS preparations were quantified with Q-PCR. To further address rainbow trout macrophages immune responses, their transcriptomic regulation in response to bacterial LPS and viral Poly I:C was studied using a salmonid-specific cDNA microarray platform enriched in immune-related genes and validated with Q-PCR,

together with the analysis of the release of the pro-inflammatory cytokine tumor necrosis factor α (TNFα) by western blot. Finally, the cortisol regulation and transcriptomic response of teleost fish to immuno-modulation were investigated via the administration of immunostimulant diets, which are commonly utilized in aquaculture. Using the salmonid-specific microarray platform, *in situ* hybridizations and quantification of plasma cortisol levels by radioimmunoassay, we studied tissue specific (head kidney, spleen, intestine and gills) responses in rainbow trout fed for four weeks with a commercial immunostimulant diet, in a basal situation and following a challenge with LPS. The results obtained are presented and discussed in this report.

1 General introduction

Evolution of immunity: the immune system in fish

The immune system can be defined as a complex network of organs, cells, and specialized molecules distributed throughout the organism to defend it from foreign invaders that cause infection or disease. The ability to protect oneself is a key element in survival, and all species, starting from prokaryotes, have mechanisms for recognition and elimination of pathogens. Furthermore, the immune system has a crucial role in controlling the integrity of the multicellular organism, thus it contributes to maintain the homeostasis removing senescent or damaged cells and misfolded or changed proteins. The defence response is then critical to all individuals on earth and therefore many changes have taken place during evolution to generate variability and speciation, though immune system has conserved over millions of years of evolution some important features that are common for all species.

Fish, the most successful vertebrate group, are a heterogeneous group composed of 28000 species that include the agnathans (lampreys and mysines), chondryctians (sharks and rays) and teleosteans (bony fish) [4]. They aroused 450 millions years ago, and the range of environmental conditions to which they have adapted induced very variable physiologies, body shapes and lifestyles. Fish are ectothermic vertebrates that live in an aquatic environment, which contain high a concentration of pathogen organisms, and physical conditions, like temperature, oxygen or salinity, are also very variable. Therefore, they have to defend themselves against a wide range of pathogens in different situations [5].

The immune system of fish has cellular and humoral immune responses, and organs whose main function is involved in immune defence. Most of the generative and secondary lymphoid organs present in mammals are also found in fish, except for the lymphatic nodules and the bone marrow. Instead, the anterior part of kidney, usually called head kidney or pronephros, possesses hematopoietic functions and has been described as the organ analog to the bone marrow of mammals [6]. Other functions as phagocytosis, antigen processing activity and formation of IgM and

immune memory have been described [7]. Head kidney is also an important endocrine organ, homologous to mammalian adrenal glands, releasing corticosteroids and other hormones [8].

The innate response is the basis of immune defence of invertebrates and lower vertebrates. In fish, the innate immune response has been considered the essential component in combating pathogen invasions due to the limits placed on the adaptive immune response by their poikilothermic nature, the limited antibody repertoires, affinity maturation and memory and relatively slow lymphocyte proliferation [9]. The component mechanisms are constitutive and responsive, providing protection to the host by preventing attachment, invasion or multiplication of the pathogens or in the tissues through physical and chemical barriers, that is, they are inducible by external molecules but at the same time are constitutive and react within a very short time scale. Relevant innate responses in fish are phagocytosis, opsonisation, lytic and cytotoxic cellular activity, interferon, lysozyme and acute-phase proteins, which have some considerable physicochemical and functional similarities to those observed in mammals, though a number of different properties can be found [10, 11]. The innate defences against viruses include the action of non-specific cytotoxic cells to virus-infected cells and the interferon production, in addition to the activation of pathways like complement. The IFN-mediated antiviral response is able to respond during the early stages of viral infection, inducing the release of antiviral molecules like Mx or Protein Kinase P1 [12], whereas the natural killer (NK) cells limit the spread of virus by lysing infected cells [13]. The innate defences against bacteria comprise production of microbial substances and acute phase proteins, complement activation, release of anti-bacterial peptides and cytokines, inflammation and phagocytosis (reviewed in [5]).

In teleosts, the lymphoid tissue associated to teguments is distributed around the skin, gills and intestine, thus complementing the physical and chemical protection provided by the structure. These external defences act as the first barrier against infection, therefore have a significant role in control of entry of pathogens. Beside the physical protection, they produce mucus, proteases, and antimicrobial molecules. Mucus traps pathogens and prevent bacteria from attaching to the epithelium, and it also contains antibacterial substances. Teleosts lack organized gut-associated lymphoid tissue such as the Peyer's patches of mammals, though there is evidence that skin, gills

and intestine contains populations of leucocytes [7] and innate and adaptive immunity act in case of attack of microorganisms [5, 14].

Cytokines are simple polypeptides or glycoproteins of less than 30 kDa, which are released when innate immune response is activated acting as signalling molecules. These molecules play a significant role in initiating and regulating the inflammatory process, which is an important function performed by the innate immunity. Fish appear to possess a repertoire of cytokines similar to that of mammals and to date, several cytokine homologues have been cloned in fish species (reviewed in [15]. Important cytokines described in fish are for example Tumor necrosis factor-alpha (TNFα), Interleukin-1β (IL-1β), Interleukin-6 (IL-6) or Interferons (IFN). TNFα is an important cytokine shown to induce apoptosis, enhance neutrophil migration and macrophage respiratory burst activity. It has been cloned in various fish species and expression studies have demonstrated that TNFα expression and regulation in fish is similar to that observed in mammals, though its functional role is still not completely understood [16]. The interleukin-1 family of cytokines comprises molecules known to have an important role in inflammation and host defence. IL-1β has been identified in different species of teleost exhibiting a function in immune regulation through stimulation of T cells, which is analogous to mammalian IL-1β [17]. IL-6 is a highly pleotropic cytokine and has a significant role in both innate and adaptive immunity. In fish, it has been identified in several species, displaying structural characteristics shared with other vertebrates therefore supporting the notion of an important function for this cytokine in fish [18]. IFNs are secreted proteins, which induce vertebrate cells into a state of antiviral activity by transcriptional regulation of several hundred IFN-stimulated genes. A number of IFNs have been cloned in fish, which showed structural and functional properties similar to mammalian IFNs [12].

The innate immune system is present in all metazoans while the emergence of adaptive immunity is thought to have originally evolved in the vertebrate lineage, probably at the level of early jawed vertebrates (~500 mya) [14]. Despite the fact that dividing immune system into the innate (non-specific) and the acquired (specific) immunity is a common practice, recent studies in both fish and mammalian immunology demonstrate that these are combined systems rather than independent systems [3]. Thus, the innate immune response is also important in activating the acquired immune response [19]. Natural antibodies are known in teleosts and play a key role in the innate immune response and the link to the adaptive immune response.

Teleosts, in general, have IgM as their main immunoglobulin and are capable of eliciting effective specific humoral antibody responses against various antigens. For IgM, only one gene can generate as many as six structural isoforms and, therefore, diversity would be the result of structural organization rather than genetic variability [20]. However, though until 1997 teleosts were thought to possess only this type of Ig, subsequent research has provide the evidence for the existence of IgD/IgZ/IgT in fish [21-24]. A summary of fish immune system is shown in figure 1.

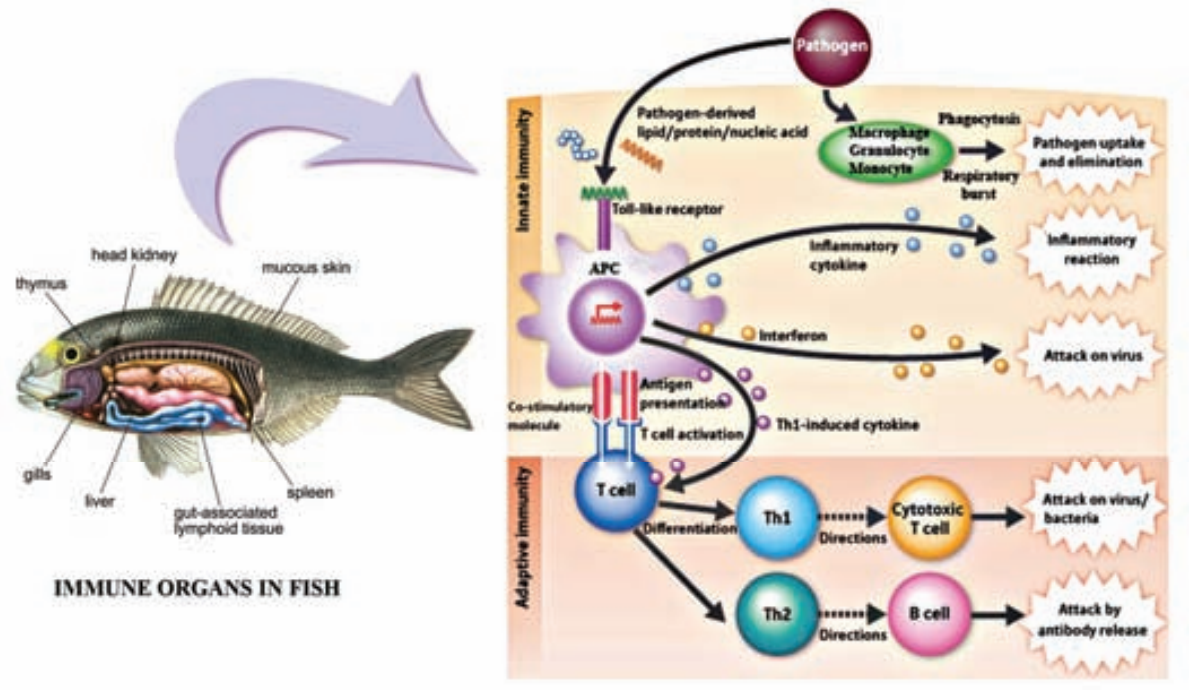

Figure 1: **immune system in fish**. Cross-talk between innate immunity and adaptive immunity

Fish immune cells show the same main features as those of other vertebrates, and lymphoid and myeloid cell families have been described. The lymphoid system is a recent evolutionary development since most animals prior to vertebrates rely on non-lymphoid cells. Key cell types involved in non-specific cellular defence responses of teleost fish include the phagocytic cells monocytes/macrophages, the non-specific cytotoxic cells and granulocytes (neutrophils). Epithelial and antigen presenting cells also participate in the innate defence in fish, and some teleosts have been reported to have both acidophilic and basophilic granulocytes in peripheral blood in addition to

the neutrophils [15]. Furthermore, recently it has been observed that basophilic granular cells (acidophilic/eosinophilic granule cells or mast cells) of fish belonging to the Perciformes order, the largest and most evolutionarily advanced order of teleosts, are endowed with histamine, which is biologically active in these fish and able to regulate the inflammatory response by acting on professional phagocytic granulocytes [25]. On the other hand, T and B lymphocytes are the known cellular pillars of adaptive immunity. T lymphocytes are primarily responsible for cell-mediated immunity, and B lymphocytes are responsible for humoral immunity, but they work together and with other types of cells to mediate effective adaptive immunity [26, 27]. Interestingly, B cells from rainbow trout have high phagocytic capacity, suggesting a transitional period in B lymphocyte evolution during which a cell type important in innate immunity and phagocytosis evolved into a highly specialized component of the adaptive arm of the immune response in higher vertebrates [28].

Immune recognition: PAMPs, DAMPs and PRRs

The activation of the immune response is initiated by the recognition of a range of agents, therefore pathogen distinction is one of the most basic and important properties of the immune system. An effective response requires that the organism recognizes the existence of specific, structurally conserved components that are produced by certain broad groups of potentially pathogenic microorganisms. These components, which are absent in multicellular hosts, are usually called pathogen-associated molecular patterns, PAMPs. Typical PAMPs are lipopolysaccharides (LPS) of Gram-negative bacteria, peptidoglycan and its structural component muramyl dipeptide (MDP) of Gram-positive bacteria, fungal beta-glucans, lipoproteins, flagellin, bacterial CpG and double-stranded RNA (dsRNA). The initial recognition and biological response to PAMPs is mediated by genotypically encoded pathogen recognition receptors, PRRs. Structurally and functionally similar PRRs are shared between vertebrates, invertebrates, and even plants. PRRs are toll-like receptors (TLRs), beta2-integrins, members of the nuclear oligomerization domain (NOD) receptor family, scavenger receptors (SR), etc., that are expressed predominantly in leukocytes. The diversity of the stimuli that are recognized by the immune system is determined not only by the activation of some PRRs but also by the interaction between different receptors. In addition, receptor interactions with neighbouring cells,

as well as with extracellular matrix and constituents of plasma, increase the complexity of this system. The recognition of PAMPs by the PRRs allows the innate immune system to distinguish among classes of pathogens and orchestrate appropriate innate and adaptive immune responses that are mediated by signalling through the induction of inflammatory cytokines, chemokines and co-stimulatory molecules. These pathways promote events such as the recruitment of neutrophils and activation of macrophages that lead to direct killing of the microorganisms [29-31].

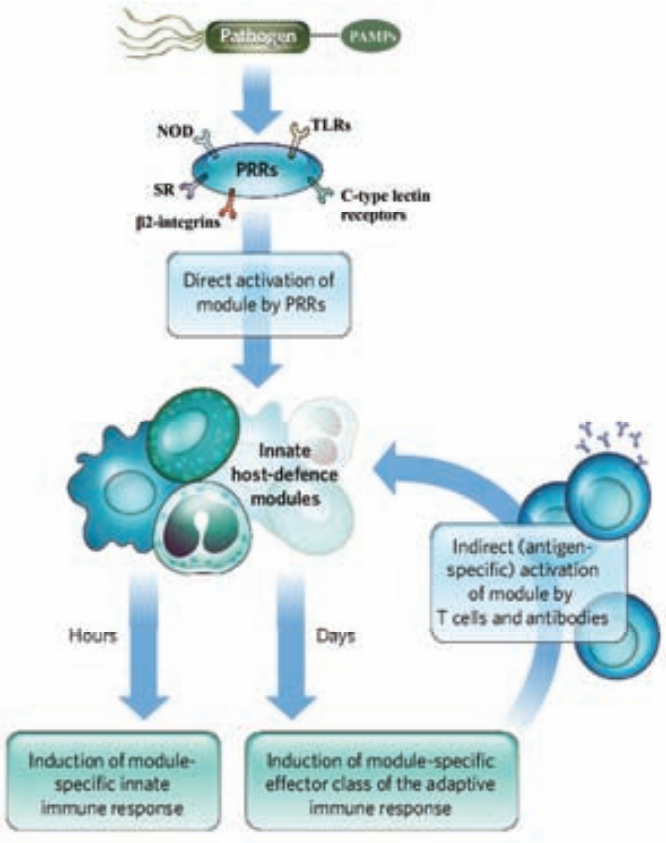

Figure 2: Activation of host-defence mechanism. Defence response is initiated by engagement of PRRs or by T cells and antibodies (Figure adapted from [3])

However, initiation of defence mechanism is not driven only by one pathway, as the innate immune system is not a single entity. It is a collection of distinct subsystems, or modules, that carry out different functions in host defence. Therefore, in addition to the activation by PRRs, immune response can be induced by different modules and also by T cells and antibodies. Each module is characterized by distinct

antimicrobial defence mechanisms and can modulate the adaptive system to initiate a specific response (figure 2) [3].

TLRs are a major class of PRRs. They are type-I transmembrane proteins with extracellular leucine-rich repeat (LRR) motifs and an intracellular Toll/interleukin-1 receptor (TIR) domain. Members of the TLR family contribute both to cell–cell interactions and to signalling, linking extracellular signals to specific gene-expression programmes. Phylogenetic studies point to an ancient origin of TLR genes about 700 millions years ago. The phylogeny of each major vertebrate TLR family recapitulates the phylogeny of vertebrate species and TLR sequence analyses show that, as mentioned above, during evolution critical immune features have been conserved, thus all vertebrate TLRs evolve at about the same slow rate, suggesting strong selection for maintenance of function [32].

Phylum	Subphylum	Class	Common name	Scientific name	Number TLRs (pseudogene)
Chordata	Vertebrata	Mammals	Human	*Homo sapiens*	**10** (1); TLR1–10 (TLR11)
Chordata	Vertebrata	Mammals	Mouse	*Mus musculus*	**12** (1); TLR1–13 (TLR10)
Chordata	Vertebrata	Aves	Chicken	*Gallus gallus*	**13** [4]; TLR1a, b, c, 2a, b, 3, 4, 7a, b, 8, 15, 21
Chordata	Vertebrata	Amphibian	Xenopus	*Xenopus tropicalis*	**19**; TLR1a,b,c, 2–5, 7–9, 11, 13,14a,b,c,d, 16, 21,22
Chordata	Vertebrata	Actinopterygii	**Zebrafish**	***Danio rerio***	**17**; TLR1–3, 4a,b, 5a,b, 7, 8a,b, 9, 18, 20a,b, 22
Chordata	Vertebrata	Actinopterygii	**Japanese puffer fish**	***Takifugu rubripes***	**12** [1]; TLR1–3, 5, 7–9, 14, 21–23 [TLR5S]
Chordata	Vertebrata	Actinopterygii	**Green spotted puffer fish**	***Tetraodon nigroviridis***	**10**; TLR1a,b, 2, 3, 5, 8, 9, 21–23
Chordata	Urochordata	Ascidiacea	Solitary tunica	*Ciona savignyi*	**7–19**
Chordata	Cephalochordata	-	Amphioxus	*Branchiostoma floridae*	**42**
Echinodermata	Eleutherozoa	Echinoidea	Purple sea urchin	*Strongylocentrotus purpuratus*	**222**
Arthropoda	Hexapoda	Insecta	Fruit fly	*Drosophila melanogaster*	**9**; Toll1–9
Nematoda	-	Secernentea	Round worm	*Caenorhabditis elegans*	**1**; Tol-1

Table 1: Toll-like receptor (TLR) genes in representative species with a sequenced genome (table adapted from [2]).

The table 1 shows a summary of the distribution and diversification of TLR genes among the animal kingdom, indicating the ancient evolutionary origin of these receptors. For example, the genome sequence of the sea urchin *S. purpuratus* reveals an enormous expansion of three classes of innate immune recognition proteins, including TLRs, NLRs and scavenger receptors. There have been 222 TLR genes identified [33]. On the other hand, the genome analysis of the cephalochordate amphioxus (*Branchiostoma floridae*) showed also the existence of large number of TLRs and 42 TLR genes have been reported [26]. The distinct number of TLRs within species and the recent accumulation of genomic and functional data in several organisms indicate major differences in TLR function among species, suggesting that different TLRs might have evolved independently to mediate analogous immune functions [2].

In addition to PAMPs, organisms are able to detect molecular patterns exposed through damage of the host own tissues due to infection, necrotic changes and natural cell death. These are host molecules that are not normally expressed on the cell surface, including host DNA, RNA, heat shock proteins and other chaperons, which are released after injury, infection or inflammation [34]. They have recently been called "alarmins", and together with PAMPs constitute the larger family of damage-associated molecular patterns (DAMPs) [35].

The activation of the fish immune system is undoubtedly mediated by the recognition of PAMPs by PRRs. Due to the rapid progress of genome sequencing projects, the amount of sequence data information has exponentially increased. Recently, there have been several studies that have identified some fish orthologs of mammalian TLRs (table 1), and some of these orthologs seem to be functionally analogous. Searching the public genome databases has observed the presence of TLRs in fugu, zebrafish, japanese flounder, goldfish and rainbow trout [32]. However, there appear to be considerable differences in the function of certain TLR members. For instance in TLR4, the central part of the receptor complex that is involved in the activation of the immune system by LPS and critically involved in LPS-induced septic shock [36]. Fish are resistant to the toxic effects of LPS and in many *in vitro* studies on leukocytes from different fish species, extremely high concentrations of LPS have been used to induce immune responses. The different function of TLR4 may explain the differences in the immune responses to LPS of lower vertebrates, and fish in

particular, compared to mammals [37]. Therefore, it is assumed that fish recognize PAMPs by TLRs and other PRRs, though functions and signalling pathways might not be equivalent to those of mammals.

Macrophages and their role in immune response

Haematopoiesis and macrophage differentiation

The monocyte/macrophage cell lineage represents an important group of cells which play a central role in the initiation and coordination of the immune response. They also exhibit an important function in the inflammatory response, in the clearance of senescent cells and tissue remodelling after processes causing damage [38]. The mechanism responsible for the formation of macrophage cells is called haematopoiesis. It is defined as the complex process of cell formation, whereby pluoripotent hematopoietic stem cells are guided to develop into distinct blood cell lineages, therefore it is also the mechanism thereby cells that have expired or are damaged can be replaced through normal cellular turnover [39]. Up-regulation of the hematopoietic machinery during stress and immune-compromised conditions is critical to the establishment of cellular and humoral defences against invading pathogens and the maintenance of the organism integrity. In mammals, haematopoiesis occurs in the bone marrow, whereas in fish the major organ with hematopoietic activity is the head kidney, therefore it has been suggested that head kidney is the organ analog to the mammalian bone marrow [6].

Haematopoiesis is regulated by transcription factors and regulatory signals that specify whether hematopoietic stem cells differentiate down the myeloid, lymphoid or erythromegakaryocytic lineages [40]. Fish possess the major types of myeloid cells involved in the innate immune response including different types of polymorphonuclear granulocytes and mononuclear phagocytes, and many hematopoietic transcription factors have been also described. Macrophage cell culture systems have been developed following mammalian protocols for several fish species and have successfully been exploited to investigate biological responses [41-43]. It appears that hematopoietic process and macrophages cells display some characteristics similar to those of mammals, however marked differences are also found, therefore

fish macrophages have been shown to be about 1000 times less sensitive to LPS than mammalian macrophages [44]

Macrophage and immune recognition

Macrophages play a key role in the host immune system. They are in the first line of defence, participating in detection and identification of potential pathogens. As part of the mechanisms involved in the innate system, they respond to stimuli activating phagocytosis and releasing reactive oxygen and nitrogen species which destroy microbes. They also initiate the inflammatory response through cytokine production, and furthermore, macrophages act as a link between the innate and adaptive immune responses acting as antigen-presenting cells to prime T cells [29].

A broad range of plasma membrane receptors, such as TLRs and other PRRs, are expressed on the surface of these cells. After these receptors recognize the specific altered-self components of the host as well as microbial products, they trigger surface changes, uptake, and a well-defined signal transduction cascade. Resident macrophages in the tissues are very heterogeneous and express very different phenotypes, reflecting specialization within particular microenvironments. These cells are present in large numbers at portals of entry of the organism, where they are constantly exposed to foreign invaders [5]. Transcriptional programmes in fish macrophages are similar to those of mammals in terms of overall transcriptional activity [41]. Nevertheless, as mentioned above, expression of LPS-induced gene programmes at maximum levels in all fish cells is found only after stimulation with LPS concentrations that are orders of magnitude higher compared to mammalian cells [37, 44]. Therefore, many aspects of fish macrophage physiology remains unclear, and more detailed studies are necessary for an adequate understanding of immune recognition and mechanisms involved in innate immunity of fish.

Immune regulation by dietary administration of PAMPs

Immunostimulants (IS) are molecules that can be obtained from a natural source in large amounts and are able to modulate the immune system. Some IS contains units of a certain moiety which are abundant in microbes, that is, some of them are made with types of PAMPs. Many immunostimulants are commercially

introduced in aquaculture as feed additives, as a consequence of the general belief that immunostimulation introduced by dietary administration should induce a PAMP-PRR host response and increase the activity of the immune system in fish. Currently, dietary immunomodulation is a commonly accepted practice in aquaculture, mainly based in diets containing β-glucans [45].

β-glucans are major structural components of yeast and fungal cell walls, and consist of glucose units linked through β1-3 and β1-6 glycosidic linkage. Different receptors have been reported to bind β-glucans and acting as PRRs for this PAMP. Dectin-1 is a nonclassical, C-type, lectin-like receptor which bind predominantly protein ligands, and is the major β-glucan receptor (βGR) on myeloid cells mediating the biological effects of β-glucans. The binding of β-glucans to Dectin-1 promotes the production of reactive oxygen species (ROS) and may trigger the production of cytokines and responses associated with Th1 immunity. Such responses are critical for the defence against many pathogens, as shown in figure 3. Although it seems that Dectin-1 is the main recognition receptor that can mediate their own signalling, it can synergize with other receptors to initiate specific responses to β-glucans [46]. The complement receptor Type 3 (CR3; CD11b/CD18) belongs to the family of β-2 integrins and, in addition to the function as a receptor for the opsonic iC3b fragment of C3, it has been is characterized as β-glucan receptor [47]. TLRs, mainly TLR2, can also bind fungal ligands and inducing signalling through the MyD88 pathway and subsequent release of cytokines [46], and together with Lactosylceramide and Scavenger receptors, they have been classified as receptors which recognize β-glucan carbohydrates providing anti-fungal immunity [1]. Therefore, β-glucans may initiate immune response by activating Dectin-1 and also by the collaboration between Dectin-1, TLRs and other receptors.

Several studies have analyzed the recognition of β-glucans in fish. Receptor activity for β-glucans were found on Atlantic salmon macrophages [48], in channel catfish neutrophils [49] and in fathead minnow (*Pimephales promelas*) [50], and TLR2 gene sequences have been found in zebrafish, pufferfish, Japanese flounder and catfish [51-54]. However, the global knowledge of the receptors and mechanisms involved in β-glucans recognition in fish, as well as the orchestration of the appropriate immune response, remains unclear and needs further analysis. Since β-glucans are the main component of the IS-diets used in aquaculture, a deeper

understanding of the transcriptional regulation activated by this PAMP is of critical importance.

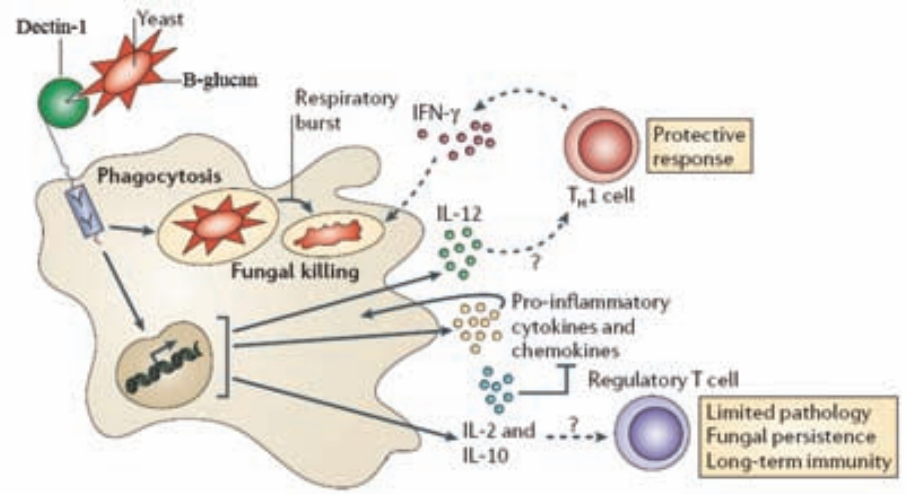

Figure 3: **Immune response activated by β-glucan recognition** (Figure adapted from [1])

It is generally accepted that dietary administration of immunostimulants can modulate and increase the immune defence of the animal, improving the resistance to disease and immunosuppressive processes [45]. However, the efficacy based on dietary administration to reduce infectious processes in fish remains controversial. In crustaceans, the application of immunostimulants to activate the innate immune system has been widely accepted as a good alternative to improve health of cultured animals, and studies of immune-related gene expression in response to β-glucans dietary administration have been conducted [55]. In non-piscine vertebrate culture species, the administration of IS-diets is not a common practice, although some studies about efficiency in growth performance and immunity in pigs and chicken fed with β-glucans have been performed [56, 57]. In fish, such diets were previously thought to act upon the immune system by enhancing non-specific defence mechanisms, such as phagocytic cell activity, pathogen killing, lymphocyte activation or antibody production, and they may also be able to increase the growth rate (reviewed in [45, 58]. However, though several studies reported a consistent trend to increased activation of innate immune system [59, 60], also toxicity processes and

negative effects have been reported [61-63]. So far there is no established explanation of why β-glucans should have positive effects in the immune system of fish.

Genomics revolution in fish: functional genomics

Fish as model for research

In an experimental background, fish possess a number of advantages for the study of the biology in general and molecular genetics in particular. For instance, fugu possess a very small genome size (one of the smallest vertebrate genomes), providing the major advantage to rapidly gain access to a large catalogue of genes. On the other hand, the zebrafish model counts with the attractive qualities of short generation time, easy maintenance, and external development of a transparent embryo, which allow performing an easy genetic screening in order to identify novel genes of interest. Therefore fish are a very interesting model to gain an insight into a wide variety of disciplines, in particular those related to evolution, and several fish species have played important roles in the latest years to increase the knowledge of vertebrate genome speciation [64]. Through genetic diversification that has affected fish over 450 millions years of evolution, they show variable gene organization in the genome, displaying changes such as polyploidy, gene and chromosome duplications, loss of such duplications, or abundance in transposable elements, that in addition to the complexity of genetic information present in all vertebrates, point to fish species as a useful model to investigate a broad range of genomic subjects [64]. Despite these excellent particularities, the current understanding in different disciplines of fish biology remains relatively limited, though the emergence of the genomic revolution and high-throughput technologies aim to change this situation. However, a major problem is that only a small sample of the about 28000 known fish species has been investigated. Most studies have been conducted in fish species with: a) high economic value, such as rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), common carp (*Cyprinus carpio*) or channel catfish (*Ictalurus punctatus*); b) fish species used as models for developmental biology, such as the zebrafish *Danio rerio* and the Japanese medaka *Oryzias latipes*, two well-established complementary models for the study of different aspects of vertebrate organogenesis; c) pure genomics models as the pufferfishes *Takifugu rubripes* (Torafugu) and *Tetraodon nigroviridis* (spotted green

pufferfish). Some of them have been studied at the genetic and genomic levels, and they are or will be subjects of whole-genome sequencing projects [65].

The two subjects of analysis in the present study were *Oncorhynchus mykiss* (rainbow trout) and *Sparus aurata* (gilthead sea bream). They belong to the class actinopteygii (ray-finned fishes), by far the most evolved and diversified group of fish, though *O. mykiss* is classified in the order salmoniformes and *S. aurata* belongs to the order perciformes, existing more than 200 millions years of evolution between both species [66].

Functional genomics in fish

Genomics can be defined as the pathway and functional information analysis to elucidate effects and responses in the organisms into the entire genome's network, whereas functional genomics is the field that attempts to make use of the vast amount of data produced by genomic projects to describe gene functions and interactions. Functional genomics focuses on the dynamic aspects instead of static aspects of genomic information, using mostly high-throughput techniques to characterize the abundance of gene products such as mRNAs. Because of the large quantity of data produced by these techniques and the desire to find biologically meaningful patterns, which is the real aim of genomics research, bioinformatics is crucial to this type of analysis. In this context, one of the typical technologies in functional genomics is the use of microarray platforms. It allows researchers to conduct simultaneous expression analysis on thousands of genes in organisms subjected to a variety of different conditions, providing the possibility to address many cellular functions, metabolic and regulatory pathways in a single assay (for reviews see The Chipping Forecast *Nature Genetics* vol. 21 supplement,[67]).

However, microarray analysis has a number of critical parameters not yet totally clarified. The generation of large amounts of microarray data presents challenges for data collection, annotation, exchange and analysis. Reliability of data produced by these experiments and their reproducibility are not always assured. Errors can be made during hybridization and data generation process, and the choice of the platform or the evaluation method can influence the quality of the results. The complexity in the quantity of data generated in different sources, areas and databases is solved in part by the use of Gene Ontology. In origin, ontology is the branch of

philosophy that is concerned with basic questions about reality and existence of basic categories, with particular emphasis on their relations and how these can be grouped and related within a hierarchy. Gene Ontology (GO) attempts to describe genes and all their relations, comprising a set of well-defined terms with well-defined relationships. Thus, three categories of GO have been described, these are Biological process, Molecular function and Cellular component, all attributes of genes, gene products or gene-product groups and which are able to reflect the biological reality that a particular protein may function in several processes [68].

Research in functional genomics in fish is a reality, with the explosion in the amount of DNA sequence data available and the development of microarrays platforms for several species that aim to elucidate fish immune responses, physiology and evolution [69]. However, as discussed above, functional genomics has focused in a small group of selected species. In this thesis we have used the targeted cDNA arrays SFA 2.0 [70], the new generation of the platform SFM 1.0, which has been repeatedly assessed in diverse experiments across a panel of responsive tissues in salmonid fish [71].

Overview and aim of this thesis

In this thesis we have studied two evolutionary distant fish species, *Oncorhynchus mykiss* (rainbow trout) and *Sparus aurata* (gilthead sea bream), as models to address the following objectives:

- Characterize sea bream macrophage populations in primary culture differentiated *in vitro*, through their morphologic changes, phagocytic capacity, activation and ability to respond to Lipopolysaccharide (LPS). Cloning and expression analysis of the myeloid marker CD83.

- Address the ability of both species to enhance the expression of antiviral-related genes upon LPS stimulation, using primary cell cultures of differentiated macrophage-like cells. Evaluate the contribution of contaminants in commercial LPS preparations in the activation of gene expression.

- Compare the differential gene expression patterns of rainbow trout macrophage cells in response to the bacterial component LPS and the virus analog Poly(I:C), assessed by microarray analysis and TNFα western blot analysis.

- Investigate in rainbow trout transcriptomic changes induced by a commercial immunostimulant diet at two major sites of mucosal immunity, the gills and the intestine, analyzed by microarray analysis and *in situ* hybridization.

- Evaluate in rainbow trout transcriptomic changes induced by a commercial immunostimulant diet at two organs with key immune function in fish, head kidney and spleen, assessed by microarray analysis and *in situ* hybridization.

- Compare in rainbow trout differential gene expression profiles induced by a LPS challenge between fish fed with a basal diet and fish fed with a commercial immunostimulant diet, at two organs acting as portals of entry in fish, gills and intestine. Analyze the effects of the immunostimulant diet in cortisol regulation.

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2 CD83 expression in sea bream macrophages is a marker for the LPS-induced inflammatory response

Abstract

Gilthead sea bream macrophages were obtained in primary culture from head kidney homogenates. Myeloid type cells became adherent to the plastic culture dish surface and differentiated spontaneously. During 7 days in culture, adherent cells modified morphology and size, reaching the typical macrophage phenotype. The terminally differentiated macrophages were responsive to LPS stimulation and possessed a high phagocytic capacity, and to further characterize gilthead sea bream macrophage, we searched for myeloid markers. Mammalian CD83 is a 45 kDa cell surface membrane glycoprotein member of the Ig superfamily. It is predominantly expressed on Langerhans cells, circulating DC and interdigitating DC present in T-cell zones of lymphoid organs, and it is well known that Monocyte-derived DC express high levels of CD83 after stimulation with inflammatory cytokines. Therefore is commonly used as standard surface marker for dendritic cells. In the present work the cloning of CD83 from gilthead sea bream macrophages using degenerate primers against conserved motifs of known CD83 sequences is reported. The obtained cDNA contains an open reading frame of 669 nucleotides that translate into a 222 amino acid putative peptide. The deduced protein sequence shows conservation of features shared by vertebrate CD83 and multiple alignment with fish CD83 sequences reveals high homology. In cultured sea bream macrophages CD83 mRNA expression was significantly enhanced in a dose- and time-dependent fashion after stimulation with *Escherichia coli* LPS. These results indicate that in fish, macrophages express high levels of CD83 mRNA after LPS exposure and CD83 is therefore a good marker for activated mature myeloid cells in fish. In mammals, CD83 it is able to regulate the development of cellular immunity because immobilized CD83 was shown to promote

the activation of CD8+ T cells in peripheral blood mononuclear cells through monocytes, and CD83-deficient mice showed reduced CD4+ T cell generation. To know whether fish CD83 share same functions needs further investigation.

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Introduction

Mammalian CD83 is a 45kDa cell surface membrane glycoprotein member of the Ig superfamily which in mammals is predominantly expressed in Langerhans cells, circulating dendritic cells (DC) and interdigitating DC present in T-cell zones of lymphoid organs [1, 2]. CD83 has also been reported in activated B lymphocytes, T lymphocytes and in monocytes [3]. In monocyte-derived DC, CD83 is highly expressed after stimulation with inflammatory cytokines such as TNF-α and IL-1β [4]. Due to this limited cellular expression profile CD83 is been commonly used as a standard surface marker for dendritic cells.

Up-regulation of CD83 together with the co-stimulatory molecules, CD80 and CD86, during DC maturation suggest an important functional role of CD83 in the induction and regulation of immune responses. Diverse actions of recombinant CD83 proteins have been reported including binding and activation of CD8+ T cells [5] and amplification of antigen-specific CD8+ T cells [6]. In DC, CD83 engagement inhibits both DC maturation and DC-mediated T-cell proliferation [4] and induces changes in the DC cytoskeleton [7]. Inhibition of CD83 cell surface expression on mature DC leads to a significant reduction of their T-cell stimulatory capacity [8] and HSV-1 infected PBMC-derived DC show a dramatically reduced T-cell-stimulatory capacity [9]. In whole animal studies, CD83-deficient mice showed reduced CD4+ T cell generation [10].

Little is known about the existence of DC and their markers in fish. To date CD83 has been cloned in rainbow trout (*Oncorhynchus mykiss*) and nurse shark (*Gynglymostoma cirratum*) [11], atlantic salmon (*Salmo salar*) [12], japanese flounder (*Paralichthys olivaceus*) [13] and zebrafish (*Danio rerio*) (unpublished sequence). As in mammals, the CD83 genes of both rainbow trout and nurse shark contain a split Ig V domain that represents a unique sequence feature for CD83 genes [11]. The current

paper describes a full-length CD83 homolog from the gilthead sea bream (*Sparus aurata*) which is highly inducible in LPS-stimulated macrophages *in vitro*. Expression studies in fish tissues indicate a ubiquitous expression at low levels.

Material and Methods

Animals

Adult gilthead sea bream *Sparus aurata* of approximately 100 g were obtained from the commercial fishery Granja Marina Masnou (Barcelona, Spain). The fish were acclimatized to laboratory conditions for 15 days before being used for experiments. They were held in tanks with recirculated water circuits under a photoperiod of 12h light/12h dark and natural conditions of temperature, and fed with a commercial gilthead sea bream diet.

Cell culture, phagocytosis assay and stimulation

Gilthead sea bream macrophages were isolated using a modification of the protocol previously described [14]. Briefly, head kidneys were homogenized using 100 μm nylon mesh cell-strainers in the presence of DMEM (PAA Laboratories) containing high glucose, 10% heat inactivated FCS (PAA Laboratories) and the antibiotic Primocin (100 μg/ml, Invivogen). The homogenates were plated on 6 wells poly-D-lysine (Sigma) treated cell culture plates, 2 ml per well. The cultures were kept in an incubator at 18 º C and 5% CO_2. Non-adhering cells were removed after 24 hours and new medium was added. The adherent cells were incubated for another 6 days and pictures were taken using Olympus IX70 microscope and Olympus camedia C-3030 camera at 200x.

For the phagocytosis analysis, cells were incubated the 7th day with Alexa fluor 488 *E. coli* fluorescent bioparticles and Alexa fluor 488 Zymosan (*S. cerevisae*) fluorescent bioparticles (Molecular Probes) for 1h at 18 º C and 5% CO2, with a ratio of 25 particles per macrophage. Cells were then repeatedly washed with PBS at low-speed agitation in order to remove the particles which have not been phagocyted. Phase contrast and fluorescent pictures were captured at 100x and 600x using Leica DMRB microscopy and Leica DC200 camera. Wells without cells were used as control.

For stimulation, the medium of each well was removed and fresh medium containing the required concentration of lipopolysaccharide (LPS) from *E. coli* (Sigma) was added and the cultures were incubated for 4h.

RNA extraction and cDNA production

Total RNA was extracted from the cultures using 1 ml of TriReagent (Molecular Research Center) per three wells, following the manufacturer's instructions, and verified for quantity and integrity by denaturing electrophoresis gel for RNA. 2 μg of RNA was used to synthesize cDNA with the SuperScript II RNase Transcriptase (Invitrogen) and oligo-dT primer (Promega).

PCR, cloning and sequencing

cDNA was used as template for PCR reactions using a G-Storm thermocycler. Amplification of CSF-1R (a macrophage marker, [15]), TNFα and IL-1β, genes known to be up-regulated upon LPS activation in macrophages, was performed using primers of Table 1, with a step of 94º 5 min, 30 cycles of 94º 45 sec, 56ºC 45 sec and 72º 1 min, followed by 1 cycle of 72º for 7 min. As control, 18S gene was amplified from the same cDNA samples using 18S Fw and 18S Rv. Products were visualized on a 1% agarose gel containing 1 μg/ml of ethidium bromide under UV light.

For the cloning of CD83, an initial PCR was performed with the Fw1 and Rv1 degenerate primers designed against conserved motifs of known CD83 sequences (Table 1). The cycling reaction was performed for 1 cycle of 94ºC for 5 min, 35 cycles of 94ºC for 45 sec, 51ºC 45 sec and 72ºC for 1 min, followed by 1 cycle of 72ºC for 7 min. PCR products were visualized under UV light in a 1% agarose gel containing 1μg/ml ethidium bromide, purified using MiliElute gel purification system (Quiagen), cloned into PGEM-T Easy Vector (Promega) by T/A cloning and transfected into competent *Escherichia coli* JM 109 cells (Promega). Plasmid DNA was isolated by Nucleospin Quickpure (Marcherey Nagel), digested with EcoRI (Promega) to verify the appropriate insert size and sequenced with T7 primers (Sistemas Genómicos, Spain).

Name		Nucleotide sequence
CSF-1R	CSF-1R Fw	5' CTGCCCTACAATCAGAAGTGG 3'
	CSF-1R Rv	5' TCAGACATCAGAGCTTCCCTC 3'
TNFα	TNFα Fw	5' TCGTTCAGAGTCTCCTGCAG 3'
	TNFα Rv	5' AAGAATTCTTAAAGTGCAAACACACCAAA 3'
IL-1β	IL-1β Fw	5' ATGCCCCAGGGGCTGGGC 3'
	IL-1β Rv	5' CAGTTGCTGAAGGGAACAGAC 3'
Initial PCR	Fw1	5' TACYGGGCGGTGAGGTGGTACARG 3'
	Rv1	5' CCCCAGASGTAAATCAACATTAAATC 3'
RACE reactions	5'race	5' GCTCCACCTCTCGCTCCACGCC 3'
	5'Nested	5' CGACCCTGTACCACCTCACCGCCC 3'
	3' race	5' GGACGCGCCGCTCAAACCTCTTG 3'
	Universal primer mix (UPM)	5'CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT3' 5' CTAATACGACTCACTATAGGGC3'
	Nested universal primer (NUP)	5' AAGCAGTGGTATCAACGCAGAGT 3'
	BD SMART Oligonuclotide	5' AAGCAGTGGTATCAACGCAGAGTACGCGGG 3'
	3' RACE CDs	5' AAGCAGTGGTATCAACGCAGAGTAC$(I)_{30}$
	5' RACE CDs	5' $(I)_{25}$V N3'
Full length reactions	FL Fw	5' GGGGACTCAACTGCCTCTTG 3'
	FL Rv	5' CCTGTAGACCATTTCATCACCG 3'
Expression studies	Exp Fw	5' GGGCGGTGAGGTGGTACAGGGTCG 3'
	Exp Rv	5' CAAGAGGTTTGAGCGGCGCGTCC 3'
Real-Time PCR	Q-PCR Fw	5' GAGACGCACAACATCCTCCT 3'
	Q-PCR Rv	5' CGTCACCAGGGTTTCTCTGT 3'
18 S	18s Fw	5´ CGAGCAATAACAGGTCTGTG 3'
	18s Rv	5' GGGCAGGGACTTAATCAA 3'

Table 1: specific primers used in experiments

Based on the partial gilthead sea bream CD83 sequence obtained, primers 5'race, 5'nested and 3'race were designed (Table 1) in order to obtain the 3' and 5' ends by rapid amplification of cDNA ends (RACE-PCR) with the Smart RACE cDNA Amplification Kit (BD Biosciences). In 3' RACE, cDNA was produced using 3'RACE CDs primer adaptor and PCR was performed with Universal Primer mix and the gene specific primer 3'race. For the 5' RACE, cDNA was synthesized with 5' CDs primer adaptor and SMART II Oligonucleotide. PCR was carried out with a gene specific primer 5'race and the Universal Primer Mix. A Nested PCR was performed using a second gene specific primer 5'nested and the Nested Universal Primer. All products were purified, cloned and sequenced as described previously.

After all the products were sequenced, FL Fw and FL Rv primers were designed (Table 1) to amplify the entire cDNA coding region to ensure accuracy. PCR was carried out using High Fidelity DNA Polymerase (Roche) with an step of 94ºC for 5 min, 30 cycles of 94ºC 45 sec, 55 ºC 45 sec, 72 ºC 1 min and a final cycle of 72ºC for 7 min.

The assembled full-length cDNA sequence was entered in the GenBank with accession number **EF183503**. Basic Local Alignemnt Search Tool (BLASTX) in the National Center for Biotechnology Information was used in order to search similarities with known genes (http://www.ncbi.nlm.nih.gov/). Translation and protein analysis were performed using ExPASy (http://www.expasy.org) tools and SMART (http://smart.embl-heidelberg.de) program. Multiple sequence alignments were generated with Clustal W Multiple Sequence Alignment through EMBL-EBI (European Informatics Institute) (http://www.ebi.ac.uk/clustalw/index.html), and identities and similarities were analyzed with BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A maximum likelihood (ML) analysis was conducted on vertebrate CD83 sequences with PHYLIP v3.65 [16] using the JTT model. *Gallus gallus* was chosen as an outgroup. Confidence in estimated relationships of ML tree topologies was evaluated by a bootstrap analysis with 1000 replicates.

Expression studies

Gilthead seabream CD83 mRNA expression was determined *in vitro* and *in vivo*. For dose response experiments, macrophages were incubated during 4 hours with medium containing FCS, Primocin and different LPS concentrations. Control cells were supplied with identical medium free of LPS. For time course analysis, cultures were incubated with 50μg/ml of *E.coli* LPS and samples were collected at different time points. Three independent experiments were performed.

For *in vivo* expression of CD83, 3 gilthead sea bream were killed and gonads, kidney, intestine, heart, liver, head kidney, spleen, brain, thymus and gills were dissected out. After RNA extraction, the final sample used for each tissue was a pool of RNA from the 3 specimens.

cDNA was synthesized from RNA as described above and used as template for PCR reactions. Amplification was performed with Exp Fw and Exp Rv primers,

with an step of 94º 5 min, 30 cycles of 94º 45 sec, 62º 45 sec and 72º 1 min, followed by 1 cycle of 72º for 7 min. As a control, the 18S gene was amplified from the same cDNA samples using 18S Fw and 18S Rv. Products were visualized on a 1% agarose gel containing 1 μg/ml of ethidium bromide under UV light.

In order to quantify CD83 mRNA expression, real time PCR (Q-PCR) was carried out. cDNA was diluted 1:50 for CD83 and 1:100 for 18S and used as a template with the primers Q-PCR Fw and Q-PCR Rv (Table 1). Wells (20 μl final volume) contained 10 μl of iQ™ SYBR Green Supermix (Bio-Rad), 500 nM concentration of forward and reverse primers and 5 μl of cDNA. Controls lacking cDNA and controls containing RNA were included. Reactions were run in a MyiQ thermocycler (BioRad) under the following protocol: 5 min initial denaturation at 95ºC, followed by 40 cycles of 10 sec denaturation at 95ºC and 30 sec at 60ºC, and a final melting curve of 81 cycles (from 55ºC to 95ºC). All samples were run in triplicate and fluorescence was measured at the end of every extension step. C_T (threshold cycle) values for each sample were expressed as "fold differences", calculated relative to untreated controls and normalized for each gene against those obtained for 18S.

Results and discussion

It has been previously shown that head kidney cells isolated from fish and incubated for various days in primary culture spontaneously differentiate into mature macrophages that display cytokine expression with LPS stimulation and increased phagocytic capacity [14, 17]. Gilthead sea bream head kidney cells were plated on 6 wells poly-D-lysine treated cell culture plates in order to investigate their morphologic characteristics and phagocytic activity, since both characteristics have been described as indicators of macrophage function. Myeloid type cells became adherent to the plastic culture dish surface and differentiated spontaneously. During 7 days in culture, macrophage-like cells modified morphology and size, reaching the typical phenotype: elongated, significant branched extensions and rounded morphology (Figure A1). The cell population obtained is homogenous (90% of macrophage-like cells, some residual lymphocytes) due to the removal of non-adherent cells by washing. The terminally differentiated macrophages do not proliferate in culture (data not shown), possess a high phagocytic capacity and are highly responsive to LPS stimulation.

Previous studies have demonstrated that monocyte/macrophages is one of the phagocytic leukocyte types present in gilthead sea bream head kidney [18]. The phagocytic capacity of gilthead sea bream macrophages was evaluated by incubating cells with fluorescent bioparticles from both yeast (Zymosan, *S. cerevisie*) and Gram-negative bacteria (*E. coli*) for 1h. Macrophage cells phagocytosed a significant number of Zymosan and *E. coli* particles (Figure1A 2,3).

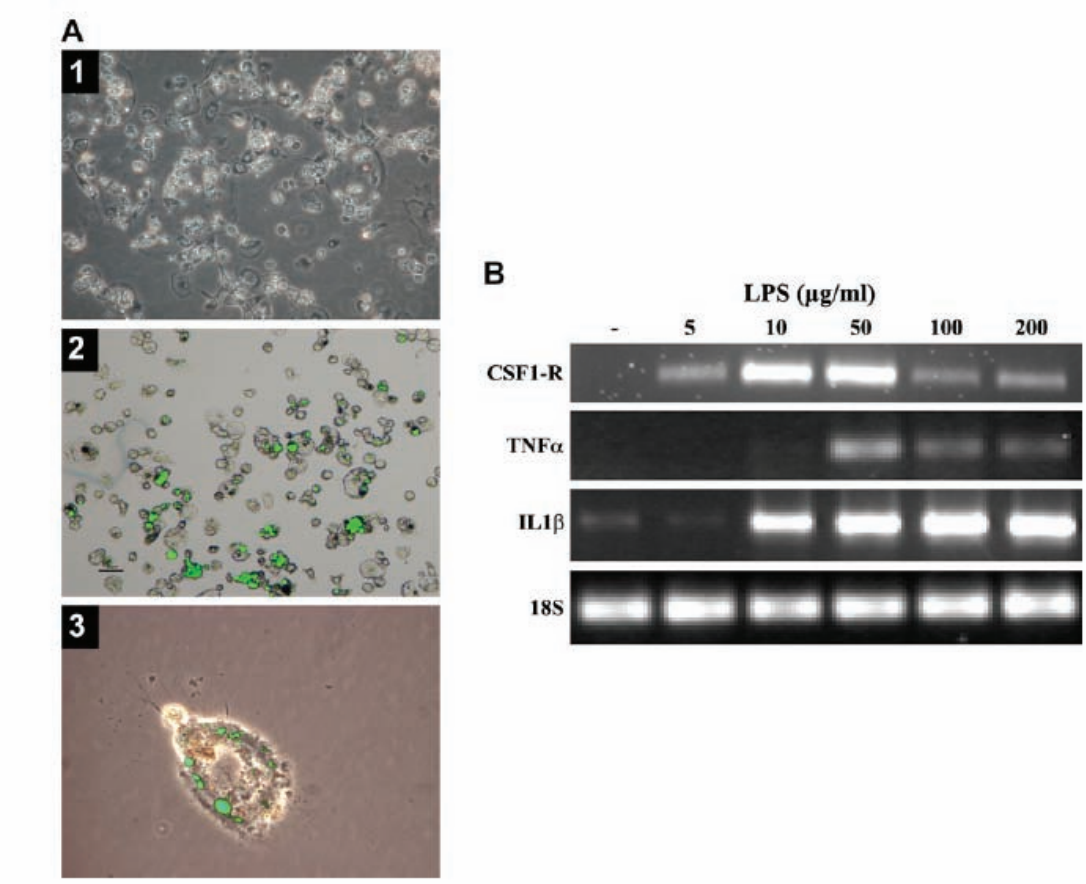

Figure 1: Characterization of gilthead sea bream macrophages differentiated in vitro during 7 days. (A) 1. Microscopic analysis of morphology (x200), 2. Analysis of phagocytic capacity of gilthead sea bream macrophages incubated with Zymosan (*S. cerevisiae*) bioparticles (x100) and 3. *E. coli* bioparticles (x600) assays were for 1h, fluorescent images were superimposed over phase contrast images. B: Dose response induction of CSF-1R, TNFα and IL-1β1 analyzed by RT-PCR stimulated with different LPS concentrations for 4h. 18S was used to show equivalency in the amount of cDNA of the samples. Results are representative of three independent experiments.

RT-PCR was performed to analyze mRNA expression of CSF-1R, TNFα and IL-1β in dose response experiments. As shown in Figure 1B, LPS induced expression of CSF-1R, with similar results to those reported in mammals [19], and TNFα and IL-1β, with similar results to rainbow trout macrophages [17].

Morphology, phagocytosis activity and gene expression analysis results, including the macrophage marker CSF-1R, show that purified head kidney leukocytes adhere to poly-D-lysine-coated culture dishes and differentiate over a period of 7 days to a mature and functional macrophage-like cell.

A full-length CD83 was obtained from the gilthead sea bream (*S.aurata*) by homology cloning from gilthead sea bream macrophages *in vitro*, followed by 3' and 5' RACE-PCR. The size of the complete cDNA is 1248 bp (**EF183503**), containing a 669bp ORF, a 38bp 5'UTR and a 541bp 3' UTR including a polyadenylation signal. The CD83 protein sequence contains 222 amino acids (AA) with a predicted molecular weight of about 23.9kDa (Figure 2). Functional implications for the observed variability in the deduced length, pufferfish, sea bream, trout CD83 (222AA and 218AA respectively) against flounder, shark (192 and 194AA) and mouse and dog (196AA), of vertebrate CD83 proteins is unknown. Analysis of the putative peptide domains reveals a conservation of functional structure relative to other fish species (Figure 3). Two N-linked glycosylation sites are conserved (the first is not present neither in salmon nor in pufferfish) and three cysteines involved in the tertiary structure of CD83. Interestingly, one cysteine residue at the beginning of Ig domain (C_{34} in flounder, C_{27} in salmon and C_{30} in trout) common to most vertebrates is not found in the *S.aurata* CD83 protein (Figure 3). As expected amino acid identity showed higher homology to fish (54.4% identity and 77% similarity; Japanese flounder) than to mammalian peptides (28.3% identity and 43.5% similarity; human). Although the CD83 protein possesses features conserved throughout evolution, in general, CD83 homologies are not high [11, 20]. The rooted phylogenetic tree shows the clustering of all CD83 sequences (Figure 4). Both the separate clustering of fish and higher vertebrates within the CD83 branch and the matching of genic and organismal phylogeny in each cluster [21-23] suggests a scenario of evolutive diversification that supports the homology of the *S. aurata* CD83 with the selected fish sequences.

Figure 2: Full-length *Sparus aurata* CD83 cDNA sequence. Start and Stop codons are in bold print. Conserved N-linked glycosilation sites are underlined. Vertical lines represent the limits between the signal peptide, IG domain, transmembrane region and cytoplasmic domain. The polyadenylation signal is highlighted in grey

In our macrophage cell culture, CD83 mRNA expression was significantly up regulated after stimulation with LPS and increased with concentration, reaching maximal levels with 50μg/ml of LPS (Figure 5A). Time course studies show that LPS

strongly induced CD83 expression from 4h to 12h (13.8 and 7.9 fold respectively) which then declined 24h post-LPS (Figure 5B). These results are consistent with previous mammalian data which showed that CD83 protein was rapidly induced in dendritic cells, 2h after stimulation with 0.5μg/ml of LPS [24]. Interestingly, the sea bream macrophage response over time is highly similar to that observed in mammalian DC whereas the response of trout macrophages is significantly slower (Doñate et al, data not shown). Responses of fish macrophages to LPS are distinct in evolutionary distant fish species [14, 25] with more modern species showing a significant similarity to higher vertebrate responses. Nevertheless, CD83 expression was induced at 50ug/ml LPS, that is several orders of magnitude higher compared to mammalian dendritic cells and macrophages [24]. Rainbow trout macrophages have been shown to be about 1000 times less sensitive to LPS than mammalian macrophages and the presence of TLR4 receptor and associated signal transduction mechanisms questioned [26]. Our results corroborate this observation for sea bream macrophages and therefore question the presence of LPS-TLR4 specific activation mechanisms in fish.

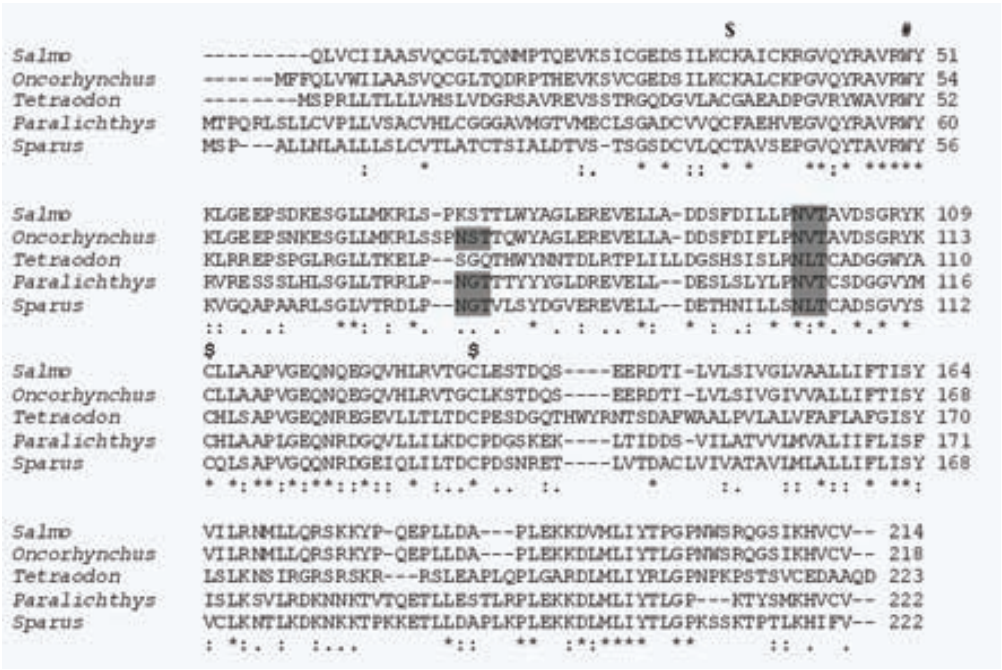

Figure 3: Multiple amino acid alignment of Sparus aurata CD83 with other known CD83 from fish species. Cysteines involved in the tertiary structure are indicated with a $, a conserved tryptophan with a # and N-linked glycosylation sites are highlighted in grey. Identical (*) and similar (: or .) amino acids are shown.

Our studies show that activated gilthead sea bream macrophages express high levels of CD83 mRNA, which is a question that has been controversial to date in mammals. Some studies report that only DC and not macrophages express CD83 [27], although CD83 expression has been observed in other cell types [3, 10, 28, 29] and also in macrophages [24, 30]. The inhibition of DC-mediated T cell stimulation and the maturation of DCs by soluble CD83 suggests an important immunosuppressive function at a systemic level [31]. The observed up-regulation of CD83 in fish macrophages suggests that CD83 may also play an important role in the regulation of immune responses in fish and this cell type may represent an important source of soluble CD83 in fish.

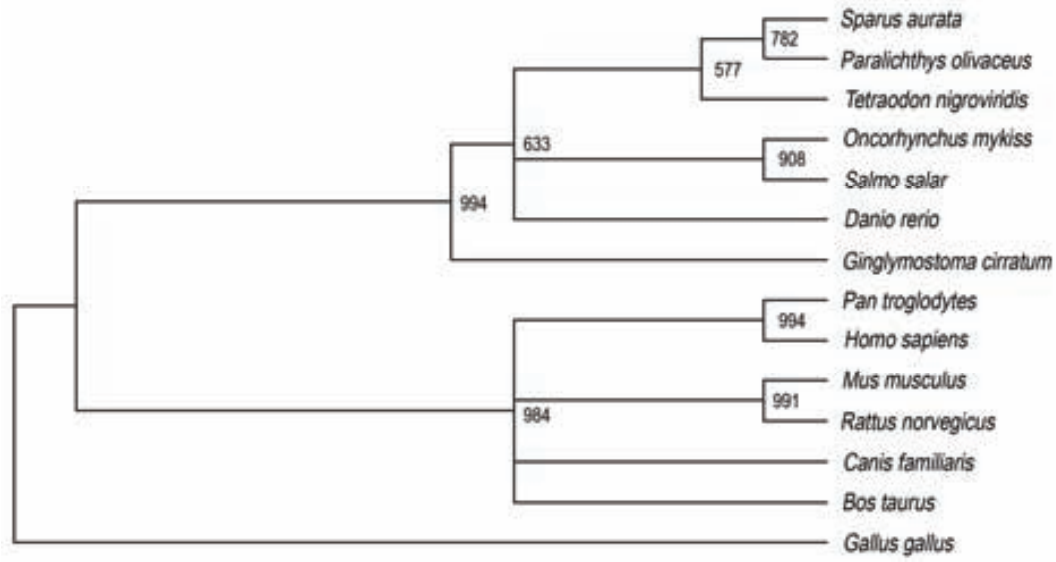

Figure 4: Protein maximum likelihood (ML) rooted phylogeny of CD83. Bootstrap values are shown for major nodes with support over 50%.

In vivo, CD83 was detected at low levels in brain, head kidney, heart, intestine, kidney, liver and thymus. Higher transcript levels were observed in gills, gonads and spleen (Figure 5C). The tissue distribution is similar to that studied by northern blot in the nurse shark [11] and slightly differs to mouse and rainbow trout patterns [11, 32].

In conclusion we have identified a *S.aurata* CD83 homolog whose expression is highly induced in activated macrophages in culture in a time and dose dependent fashion. Therefore CD83 expression is of use as a marker for macrophage activation processes in fish. Furthermore due to the reported functions of CD83 in higher

vertebrates, fish CD83 may represent an important tool to further develop understanding of immune activation processes in lower vertebrates with reference toward the regulation of lymphocyte activation.

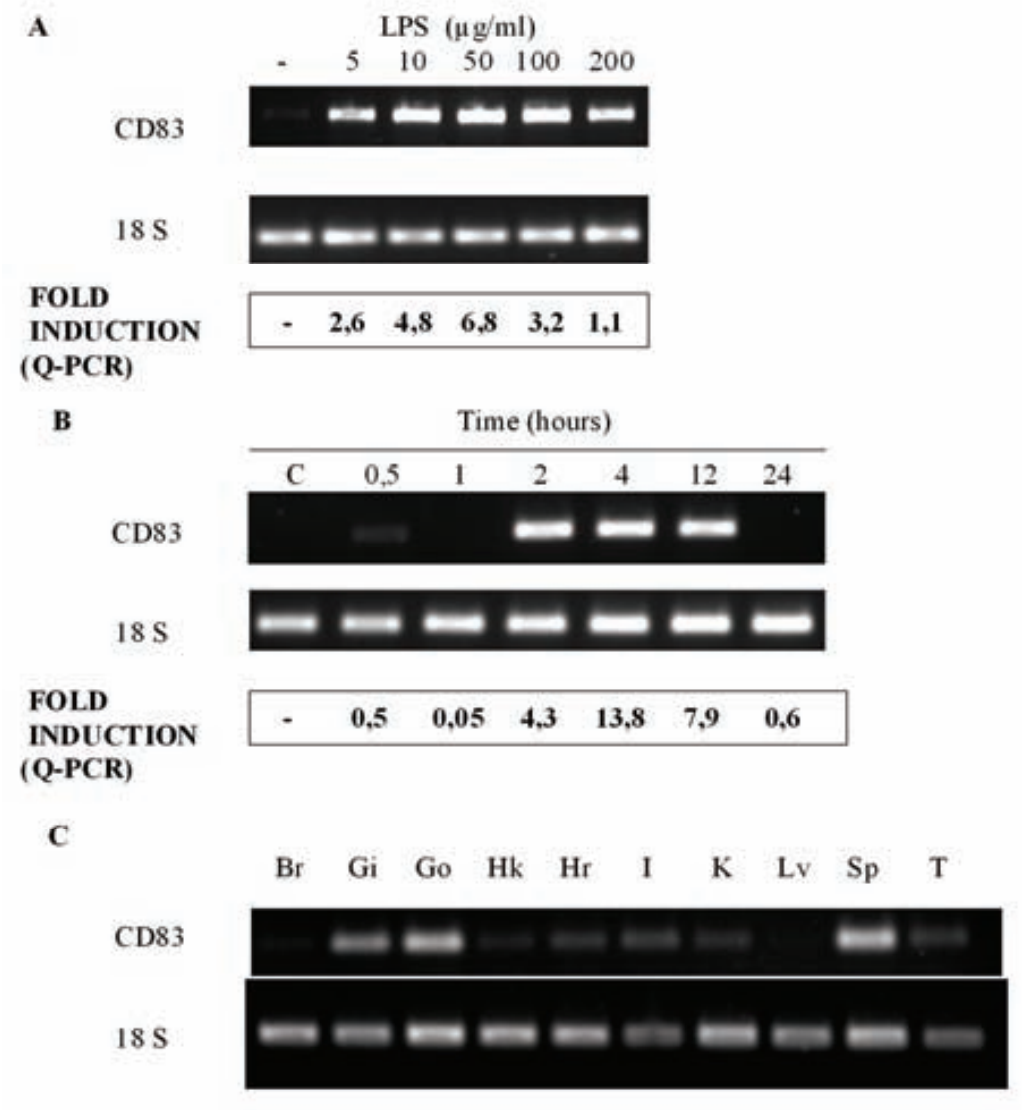

Figure 5: CD83 expression analyzed by RT-PCR and SYBR Green Q-PCR analysis (A) Dose response in head kidney macrophages incubated during 7 days. (B) Time response in head kidney macrophages incubated during 7 days. Data are presented as mean fold change relative to untreated control of values from triplicate measurements (C) Expression in different tissues from healthy gilthead sea bream (Br, brain; Gi, gills; Go, gonads; Hk, head kidney; Hr, heart; I, intestine; K, kidney; Lv, liver; Sp, spleen; T, thymus). The line below shows 18S amplification used as control. A and B results are representative of three independent experiments. C results were obtained from a RNA pool of three specimens.

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3 Differences between two distant fish species in the anti-viral signalling induced by LPS

Abstract

Potential pathogens possess signatures, unique to some classes of microorganism, collectively known as pathogen-associated molecular patterns (PAMPs). These PAMPs are the targets of the recognition activity mediated by the innate system, and are detected by host pattern recognition receptors (PRRs). The toll-like receptors (TLRs) are a family of cell surface proteins that have been shown to detect and bind PAMPs inducing intracellular signalling cascades. Among the common organisms encountered by the host, Gram-negative bacteria constitute an important group as a result of their ubiquitous occurrence in the environment. Lipopolysaccharide (LPS), an integral component of the outer membrane of Gram-negative bacteria, is a typical PAMP. It appears that three-quarters of the bacterial surface consists of LPS, the remaining area being filled by proteins. When bacteria multiply, die or lyse, LPS is set free from the surface. The recognition of LPS by mainly TLR4 and other receptors results in the production of inflammatory mediators and anti-viral molecules and ultimately is a major factor responsible for the toxic manifestations of severe Gram-negative infections and generalized inflammation. Whether fish have the ability to enhance the expression of antiviral-related genes upon LPS stimulation mediated by TLR4 activation is a question that remains controversial.

LPS derived from different groups of Gram-negative bacteria consists of a hydrophilic heteropolysaccharide and a covalently bound lipid component, termed lipid A. The lipid A component constitutes the toxic and immunomodulating agent of LPS, however, when studying the LPS-mediated signalling many preparations of LPS have been shown to contain low concentrations of highly bioactive contaminants.

These contaminants have been suggested to be responsible for some signalling observed upon LPS stimulation.

To investigate the effect of contaminants in LPS preparation when studying immunity of fish, and the anti-viral signalling induced by LPS in 2 evolutionary distant fish species, primary cell cultures of differentiated macrophage-like cells from gilthead sea bream and rainbow trout were stimulated with ultra-pure LPS and normal LPS preparations. Pure LPS increased expression of the antiviral genes Mx and IRF-1 in sea bream macrophages, whereas rainbow trout macrophages did not up-regulate Mx1 and IFNγ expression. In addition, results obtained from cultures stimulated with nLPS suggest that contaminants in nLPS preparations contribute to the activation of gene expression.

Introduction

Innate immune recognition relies on the existence of receptors known as pathogen recognition receptors, PRRs [1] which recognize conserved components, pathogen-associated molecular patterns or PAMPs produced by microbial pathogens [2]. One of the major classes of PRRs are the Toll-like receptors, TLRs. Activated TLRs transmit signals that activate different intracellular signalling cascades that may eventually result in pathogen-specific cellular responses [3]. Lipopolysaccharides (LPS), a typical PAMP, is the major constituent of the external layer of the membrane of Gram-negative bacteria and, in mammals and birds, is recognized by TLR4 [4, 5].

The activation of TLR4 recruits several different adaptor molecules including MyD88 and TIR-domain containing adapter protein (TIRAP) (MyD88-dependent pathway), that causes nuclear translocation of the transcription factor NF-κB resulting in the transcription of a wide array of genes which include proinflammatory cytokines such as TNFα, IL-1β and IL-12. Secondly, TRIF-related adapter molecule (TRAM) and TIR-domain-containing adapter-inducing IFN-β (TRIF) are also recruited (MyD88-independent pathway), inducing the phosphorylation of IFN regulatory factor 3 (IRF3) and a delayed NF-κB response, that ultimately results in the production of IFN-β, IP-10, and RANTES [6, 7].

As in mammals, the activation of the fish immune system is undoubtedly mediated by TLRs. Recently, there have been several studies in fish that have identified many fish orthologs of mammalian TLRs [8-11], including a TLR4 [10].

However considerable differences in the activation of certain TLR members have been reported suggesting potential species-specific functions. In the case of the LPS-specific receptor, TLR4 may provide a molecular background to explain key differences in the observed response and tolerance to LPS in lower vertebrates, in particular in fish, as compared to mammals. Thus, (a) Fish are resistant to the toxic effects of LPS [12]. (b) High concentrations of LPS, ug/ml range, are required to induce immune responses in *in vitro* studies on leukocytes from different fish species [11, 13]. (c) A TLR4-mediated interferon response has not been described in fish. Therefore, it has been hypothesized that TLR4-mediated recognition of endotoxin may be absent in fish [14, 15]. Furthermore and importantly few studies addressing TLR activation have reported the possibility that commercial LPS preparations used in experiments may be contaminated with other components [16] which are known to possess potent bioactivity [17]. As a consequence assigning cellular responses to the LPS component of a particular preparation may be confounded by the presence of these contaminants.

The objective of the current study was to further investigate whether fish possess the ability to enhance the expression of antiviral-related genes upon LPS stimulation. We have used primary cell cultures of differentiated macrophage-like cells from 2 evolutionary distant fish species, the Gilthead sea bream, *S.aurata*, [18] and the rainbow trout, *O.mykiss*, [13] and ultra-pure LPS(pLPS) and normal LPS(nLPS) preparations to address this question. Dose-dependent stimulation with pLPS led to increased expression of the antiviral genes Mx and IRF-1 in the gilthead sea bream macrophage cultures whereas rainbow trout macrophages did not up-regulate Mx1 and IFNγ expression. In addition, results obtained from cultures stimulated with nLPS suggest that contaminants in nLPS preparations contribute to the activation of gene expression.

Material and Methods

Animals

Adult gilthead sea bream (*Sparus aurata*) of approximately 100g were obtained from the commercial fishery Granja Marina Masnou (Barcelona, Spain). Adult rainbow trout (*Oncorhynchus mykiss*) of approximately 120g were purchased from the

commercial fishery, Piscifactoria St. Privat (Girona, Spain). Fish were acclimatized to laboratory conditions for 15 days before being used for experiments. They were held in tanks with recirculated water circuits under natural conditions of light and temperature and fed with commercial diets.

Cell culture and stimulation

Gilthead sea bream and rainbow trout macrophages were isolated using the protocols previously described [13, 18]. Briefly, fish were killed by overanesthetization in 2-phenoxyethanol and head kidneys were dissected, then placed and homogenized in sterile 100 μm nylon mesh cell-strainers in the presence of DMEM (PAA Laboratories) containing high glucose, 10% heat inactivated FCS (PAA Laboratories) and Primocin antibiotic (100 μg/ml, Invivogen). The homogenates were plated on 60 mm poly-D-lysine (Sigma) treated cell culture Petri plates, 3 ml per plate. The cultures were kept in an incubator at 16 ° C and 5% CO_2. Non-adhering cells were removed after 24 hours and new medium was added. The adherent cells were incubated for another 6 days. After 7 total days of culture, medium was removed and cells were stimulated adding fresh medium containing different concentrations (5-200 μg/ml) of normal LPS(nLPS) from *E. coli* (serotype 026:B6, Sigma). In order to verify whether the results could be due to contamination of the LPS by other bacterial components, parallel cultures were induced with 50 μg/ml of Ultra Pure E. coli 0111:B4 LPS (pLPS) (Invivogen). Medium free of LPS was added in control plates. Gilthead sea bream and rainbow trout cultures were incubated for 4h and 12h respectively, time points of maximal level of expression [13, 18]. Three independent experiments were performed for each condition.

RNA extraction, cDNA generation and PCR

Total RNA was extracted from the cultures using 1 ml of TriReagent (Sigma) per plate, following the manufacturer's instructions. Quantity and integrity was analyzed by Experion RNA StdSens Analysis Kit (Bio-Rad). 1 μg of RNA was used to synthesize cDNA with SuperScript III RNase Transcriptase (Invitrogen) and oligo-dT primer (Promega). cDNA was used as template for PCR reactions using a G-Storm thermocycler. Amplification of genes related to antiviral responses (IRF-1 and Mx in *S. aurata*, and IFNγ and Mx in *O. mykiss*), was performed using primers of Table 1,

with a step of 94º 5 min, 30 cycles of 94º 45 sec, Xº 45 sec and 72º 1 min, followed by 1 cycle of 72º for 7 min. X temperature is indicated in Table 1. TNFα, cytokine known to be up-regulated upon LPS activation in macrophages [13, 18], was amplified as control of LPS stimulation and 18S gene was used as loading control. Products were visualized on a 1% agarose gel containing 1 μg/ml of ethidium bromide under UV light.

Specie	Name and Temp.		Nucleotide sequence	Ref
S. aurata	**IRF-1 56º C**	IRF1 Fw	5' CATGAAGAGACATGATGAAGTGC 3'	[19]
		IRF1 Rv	5' CAAACACACAAACAAAACAATCTG 3'	
	Mx 68ºC	Mx Fw	5' AGTCTGGAGATCGCCTCTCCTGATGTTCCG 3'	[20]
		MX-Rv	5'CTCTCTCCATCAGGATCCACTTCCTGTGC-3	
	TNFα 56º C	TNFα Fw	5' TCGTTCAGAGTCTCCTGCAG 3'	[21]
		TNFα Rv	5' AAGAATTCTTAAAGTGCAAACACACCAAA 3'	
	Q-PCR IRF-1	Q-IRF1 Fw	5' CCTGCCACCATCTTTTCCAT 3'	designed
		Q-IRF1 Rv	5' GGGGGATAAGGAACGCTTTCTG 3'	
O. mykiss	**IFNγ 55º C**	t-IFNγ Fw	5' CAGTGAGCAGAGGGTGTTGA 3'	designed
		t-IFNγ Rv	5' TGGACTGTGGTGTCACTGGT 3'	
	Mx1 56º C	t-Mx1 Fw	5' ATGCCACCCTACAGGAGATGAT 3'	[22]
		t-Mx1 Rv	5' TAACTTCTATTACATTTACTATGCAA 3'	
	TNFα 56º C	t-TNFα Fw	5' GGATCCATGGAGGGGTATGCGATG 3'	[14]
		t-TNFα Rv	5' AAGCTTTCATAGTGCAAACACACC 3'	
Both species	**18 S**	18s Fw	5´ CGAGCAATAACAGGTCTGTG 3'	designed
		18s Rv	5' GGGCAGGGACTTAATCAA 3'	

Table 1: specific primers used in experiments

Quantitative real-time PCR (Q-PCR)

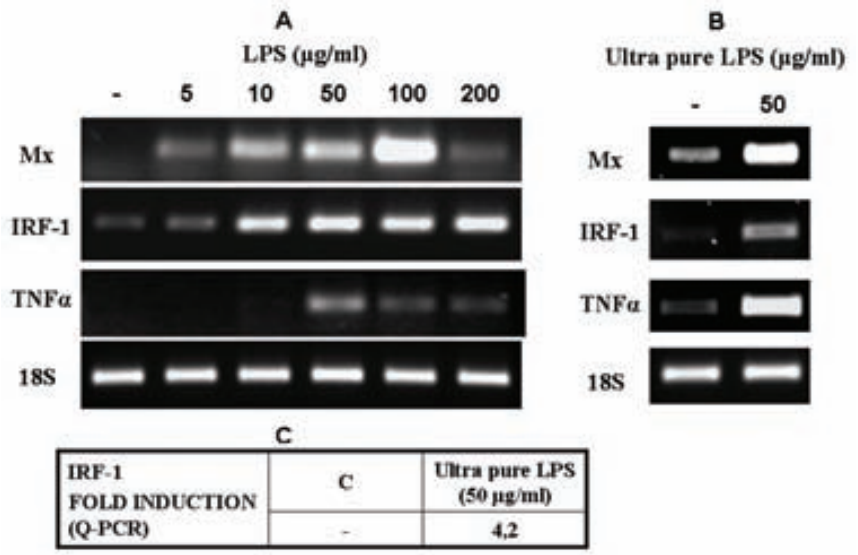
To verify the results obtained in gilthead sea bream from the semiquantitative RT-PCR analysis, real-time PCR (Q-PCR) was performed to quantify IRF-1 mRNA. cDNA was diluted 1:50 and used as template using the primers Q-IRF1 Fw and Q-IRF1 Rv (Table 1). Wells (20 μl final volume) contained 10 μl of iQ™ SYBR Green Supermix (Bio-Rad), 500 nM concentration of forward and reverse primers and 5 μl of cDNA. Controls lacking cDNA and controls containing RNA were included. Reactions were run in a MyiQ thermocycler (BioRad) under the following protocol: 5

min initial denaturation at 95ºC, followed by 40 cycles of 10 sec denaturation at 95ºC and 30 sec at 60ºC, and a final melting curve of 81 cycles (from 55ºC to 95ºC). All samples were run in triplicate and fluorescence was measured at the end of every extension step. C_T (threshold cycle) values for each sample were expressed as "fold differences", calculated relative to untreated controls and normalized for each gene against those obtained for 18S.

Results and discussion

In mammals, the activation of the immune system is mediated by TLRs. LPS is recognized by TLR4, and its activation results in the expression of immune genes including proinflammatory cytokines and genes specifically involved in the antiviral response [7]. The general cellular and molecular features of the fish immune system resemble those of mammals [23]. However, fish macrophages have been shown to be about 1000 times less sensitive to LPS than mammalian macrophages and the presence of TLR4 receptor and associated signal transduction mechanisms has been questioned [14, 15]. Our results corroborate that the LPS-concentration dependence observed in fish leukocytes to initiate inflammatory responses at the gene expression level is orders of magnitude higher compared to mammalian macrophages, but question the absence of the LPS-induced antiviral pathway in all fish species.

Simple semi-quantitative RT-PCR was performed to analyze mRNA abundance of antiviral proteins, Mx and IRF-1 in gilthead sea bream macrophages and Mx and IFNγ in rainbow trout macrophages in dose response experiments. As shown in Fig. 1A, the mRNA expression of the antiviral protein Mx and the interferon regulatory factor IRF-1 was induced after 4h of incubation with 5 and 10 μg/ml of nLPS respectively.



C

IRF-1 FOLD INDUCTION (Q-PCR)	C	Ultra pure LPS (50 µg/ml)
	-	4,2

Figure 1: Response of gilthead sea bream macrophages differentiated in vitro to nLPS and pLPS stimulation. A) Dose response induction of Mx, IRF-1 and TNFα analyzed by RT-PCR in macrophages stimulated with different LPS concentrations for 4h. B) Induction of same genes in macrophages stimulated with ultra-pure LPS. C) Q-PCR results or IRF-1 expression in macrophages induced with pLPS. 18S was used to show equivalency in the amount of cDNA of the samples. Results are representative of three independent experiments.

The proinflammatory cytokine TNFα, used as control of macrophage activation, was also increased, and 18S expression show equivalency in the amount of cDNA in all samples. A similar response was also observed after stimulation with 50 µg/ml of pLPS (Fig.1B) and was quantified by RTQ-PCR for IRF-1 (4.2 fold induction, Fig. 1C) to avoid a qualitative interpretation of data. These results confirmed that re-purified LPS, free of other bacterial components, enhanced the expression of antiviral proteins in sea bream macrophages. Interestingly, the response of trout macrophages was significantly different. The relative mRNA abundance of Mx and IFNγ transcripts increased after 12h of stimulation with nLPS (Fig. 2A) however pLPS treatment, although able to activate a proinflammatory expression profile exemplified by increased abundance of TNFα transcripts, clearly failed to induce the Mx and IFNγ antiviral response (Fig.2B).

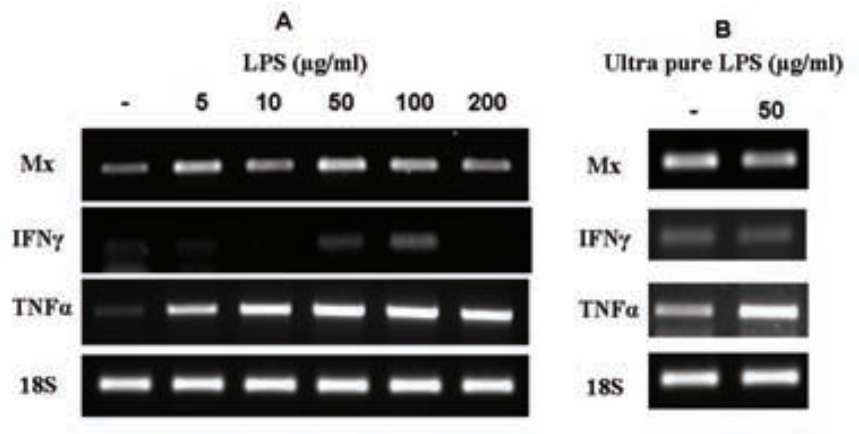


Figure 2: Response of rainbow trout macrophages differentiated in vitro to nLPS and pLPS stimulation. A) Dose response induction of Mx, IFNγ and TNFα analyzed by RT-PCR in macrophages stimulated with different LPS concentrations for 12h. B) Expression of same genes in macrophages stimulated with ultra-pure LPS. 18S was used to show equivalency in the amount of cDNA of the samples. Results are representative of three independent experiments.

So far, the ability of LPS to induce antiviral responses has not been demonstrated in fish, and few publications in fish have focused upon the effect of LPS on IFN-induced proteins with antiviral activity such as Mx or transcription factors of antiviral pathways such as IRF-1. *In vitro* Mx and IRF-1 expression has been investigated in rainbow trout gonad cells, which did not up-regulate neither Mx nor IRF-1 after LPS stimulation [24]. In *in vivo* studies, the absence of an Mx response in Atlantic salmon individuals challenged with *E. coli* LPS has been reported [25], however a significant increase in Mx expression was observed in salmon after intraperitoneal injection of *Listonella anguillarum* LPS [26] and also in gilthead sea bream after intraperoniteal challenge with *E. coli* LPS [27]. Microarray experiments have demonstrated stimulation of some anti-viral genes in LPS-stimulated macrophages in trout (see next chapter) and IRF-1 expression in cultured kidney cells after exposure to LPS [28].

These results suggest that fish are able to increase the expression of antiviral molecules after LPS induction. Our analysis in differentiated trout macrophages in primary cell culture suggests that in previous studies activation of genes involved in the antiviral response by non-repurified LPS may have been driven by contaminants, as it is have been reported in mammals [16].

Sea bream macrophage stimulated with ultra pure LPS responded with similar results to those reported in mammals, although using LPS concentrations that are orders of magnitude higher [7]. These data confirm that fish macrophages are less sensitive to LPS than mammalian macrophages, but also suggest a parallel mechanism to up-regulate antiviral molecules after LPS treatment. The differences between sea bream and rainbow trout macrophages suggest a specie-specific different response to LPS, supporting the evidence that evolutionary distant fish species are distinct, with more modern species showing a significant similarity to higher vertebrate responses. However, it has recently been reported that LPS consistently failed to activate the MyD88-independent induction of IFNβ in chicken cells, suggesting that chicken lack a functional LPS-specific TRAM-TRIF signalling pathway, which may explain their different response to LPS compared with mammalian species [5]. Therefore, it appears that both fish [15] and birds [5] lack the MyD88-independent signalling route that underlies the LPS-induced production of IFNβ in the mammalian species. Nevertheless, the results presented in the current study suggest conservation of the LPS-induced activation pathways including a parallel function in anti-viral signalling between sea bream and mammals that confers specificity of the innate immune response to stimulation with LPS. Thus, the exact nature of sea bream response to LPS through antiviral pathways remains unclear and needs further investigation.

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4 Comparison of transcriptomic profiling of rainbow trout macrophage cells following stimulation with bacterial LPS or viral ds(RNA) analog

Abstract

Macrophages initiate the innate immune response by recognizing pathogens and their PAMPs and secreting inflammatory, antiviral and cell differentiation mediators. Bacterial PAMPs are often components of the cell wall, such as lipopolysaccharide (LPS) or peptidoglycan. In the case of virus, the targets of innate immune recognition are viral nucleic acids, which have specific structural features that are unique to viral RNA and DNA and therefore the host can differentiate them from own nucleic acids.

The aim of the current study was to investigate the response of *in vitro* differentiated trout macrophages, a primary cell culture system widely used in fish immunology research, to lipopolysaccharide (LPS) and to the viral ds(RNA) analog Poly I:C. To that end we have used the salmonic-specific cDNA microarray SFA 2.0. This microarray contains 1800 unique clones which were selected by their functional roles, therefore the platform is enriched in a number of functional classes such as immune response, cell communication, signal transduction, receptor activity, apoptosis, cell cycle, protein catabolism, protein folding and response to oxidative stress. Microarray results were validated by quantification of the expression of selected genes analyzed with SYBR Green Q-PCR. In order to evaluate differences between LPS- and Poly (I:C)- stimulated macrophages at the protein level, the presence of TNFα in the supernatants was analyzed by western blot, using an anti-TNFα polyclonal antibody.

The results clearly suggest that the molecular mechanisms involved in the response of macrophages to LPS and Poly (I:C) are specific in some signalling pathways related to cell communication, signal transduction and kinase cascades. Nevertheless, macrophages stimulated with bacterial or viral PAMPs also activate common transcription factors through differentiation of immune cells and inflammatory response mediated by some cytokines, chemokines and receptors. Microarray results also indicated that LPS enhances the expression of genes and functional Gene Ontology categories involved in inflammation and innate immune mechanisms, confirmed with the earlier detection of TNFα in the supernatants of cells induced with LPS, whereas Poly (I:C) regulates gene expression towards the adaptive response and MHC I antigen presentation.

Introduction

The monocyte/macrophage cell lineage represents an important group of cells which play a central role in the initiation and coordination of the immune response, as well as in the inflammatory reaction. It is well known that fish possess the major types of myeloid cells involved in the innate immune system, including macrophage cells. Fish macrophages differentiated *in vitro* can be induced by pathogens to produce inflammatory cytokines, chemokines, reactive oxygen (respiratory burst) and nitrogen species and to activate the adaptive immune system through antigen presentation [1]. The utility of this *in vitro* system used to characterize the regulation of the immune response has been widely demonstrated in several fish species [2-5].

Pathogen recognition is one of the most basic and important properties of the immune system. This process relies on the detection of PAMPs (pathogen-associated molecular patterns), specific and conserved components produced by certain groups of pathogenic microorganisms which are absent in multicellular hosts [2, 6]. PAMPs are lipopolysaccharides (LPS) of Gram-negative bacteria, peptidoglycan and the structural component muramyl dipeptide (MDP) of Gram-positive bacteria, fungal beta-glucans, and double-stranded RNA (dsRNA). The initial recognition and biological response to PAMPs is mediated by pathogen recognition receptors, PRRs [7]. The activation of PRRs in response to pathogens triggers distinct transcriptomic programmes, including transcription factors such as NFkB, AP-1 and IRF-1/3/5 and

7, which result in the cellular/tissue response [8]. The recognition of certain PAMPs by PRRs elicits pathogen-specific reactions. Thus, activation of TLR2 and TLR4 by lipoproteins induces MAPK and NFκB signalling, resulting in the expression of antibacterial peptides, proinflammatory cytokines and other molecules involved in inflammation [9], whereas activation of TLR3 by double stranded RNA (or its analog Poly(I:C)), results in IRF3/7-driven induction of type I interferon (IFN) which further activates STAT-1-dependent antiviral responses [10].

As in all metazoans, the activation of the fish immune system is undoubtedly mediated by PRRs. This activity has been commonly investigated using PAMP preparations, such as LPS and Polyinosine-polycytidylic acid (Poly(I:C)). LPS is the major constituent of the external layer of the outer layer of Gram-negative bacteria and is responsible for the endotoxic shock observed in mammals. Poly(I:C) is a synthetic double-stranded RNA that is a potent inducer of type I IFN genes in vertebrates [11]. This chemical has been used as a viral analog for the study of the immune response to virus. As in higher vertebrates, teleost and teleost macrophage cells recognize LPS and Poly(I:C) and induce a coordinated response activating specific cytokine and chemokine release. However there appear to be considerable differences. For instance, fish (and all non-mammalian species) seem to possess a remarkable tolerance to LPS challenge, in comparison to mammals [12].

In recent years, gene chip technology has been developed allowing the analysis of large sets of genes, thereby providing a global vision of the physiological-immune response of an organism to different pathogens and PAMPs. A number of projects have used SFM 1.0 and SFA 2.0 cDNA microarray platforms to evaluate immune responses in salmonid fish [13-22].

Tumor necrosis factor α (TNFα) is an inflammatory cytokine produced by monocytes/macrophages during acute inflammation and is responsible of many signalling events within cells. Indeed, its role as an important mediator in the response against parasitic, bacterial and viral infections has been widely described [23]. Several fish TNFα gene sequences have been reported in the last years, however, currently no further information is available concerning its regulation at a protein level.

The objective of the current study was to compare in primary cultures of rainbow trout macrophage cells differential gene expression patterns induced by the bacteria component *E.coli* LPS and the virus analog poly(I:C), assessed by microarray analysis and TNFα western blot analysis. The results lead to further understand two

different types of responses in fish to two distinct immune agents, viral ds(RNA) and bacterial cell wall component.

Material and Methods

Animals

Adult rainbow trout (*Oncorhynchus mykiss*) of approximately 120g were purchased from the commercial fishery, Piscifactoria St. Privat (Girona, Spain). Fish were acclimatized to laboratory conditions for 15 days before being used for experiments. They were held in tanks with recirculated water circuits under natural conditions of light and temperature and fed with commercial diets.

Cell culture, stimulation and RNA extraction

Trout macrophages were isolated using the protocol previously described [24]. Briefly, fish were killed in water with a lethal concentration of MS-222, 100 ppm, stage III of anaesthesia [25]. Head kidneys were dissected, placed in sterile 100 μm nylon mesh cell-strainers and homogenized in the presence of DMEM (PAA Laboratories) containing high glucose, 10% heat inactivated FCS (PAA Laboratories) and Primocin antibiotic (100 μg/ml, Invivogen). The homogenates were plated on 60 mm poly-D-lysine (Sigma) treated cell culture Petri plates, 3 ml per plate. The cultures were kept in an incubator at 16 º C and 5% CO_2. Non-adhering cells were removed after 24 hours and new medium was added, then adherent cells were incubated for another 4 days. After 5 total days of culture, medium was removed and test cells were stimulated adding fresh medium containing 10 μg/ml of LPS from *E. coli* (serotype 026:B6, Sigma) or 10 μg/ml of Poly (I:C) (Invivogen), whereas medium free of stimulant was added in control plates. All culture plates were incubated for 12h. Six independent experiments (six different fish) were performed for each condition.

After 12h of stimulation, the supernatant was extracted and kept at -80º for western blot analysis, whereas for microarray analysis total RNA was extracted from the cultures using 1 ml of TriReagent (Sigma) per plate, following manufacturer's instructions. Quantity and integrity was analyzed by Experion RNA StdSens Analysis Kit (Bio-Rad).

Microarrary analysis

The salmonid fish cDNA microarray SFA2.0 immunochip contains 1800 unique clones printed each in six spot replicates. The genes were selected by their functional roles and the platform is enriched in a number of functional classes such as immune response (236 genes), cell communication (291 genes), signal transduction (245 genes) and receptor activity (126 genes), apoptosis (120 genes), cell cycle (76 genes), protein catabolism (90 genes) and folding (70 genes) and response to oxidative stress (39 genes). The platform was submitted to NCBI GEO repository, accession number GPL6154.

RNA was pooled within treatments (n=6), and 15 μg of control and test were labelled with Cy3- and Cy5-dCTP (Amersham Pharmacia) using SuperScript III reverse transcriptase (Invitrogen) and oligo(dT) primer (Promega), and cDNA was purified with Microcon YM30 (Millipore). Dye swap method was used, therefore each sample was hybridized to two microarrays. For the first slide, test and control cDNA were labelled with Cy5 and Cy3 respectively, and for the second array dye assignments were reversed. The slides were pre-treated with 1% BSA, fraction V, 5× SSC, 0.1% SDS (30 min at 50 ◦C), washed with 2× SSC (3 min) and 0.2× SSC (3 min) and hybridized overnight in cocktail containing 1.3× Denhardt's, 3× SSC, 0.3% SDS, 0.67 μg/μl polyadenylate and 1.4 μg/μl yeast tRNA. All chemicals were from Sigma–Aldrich. Scanning was performed with Axon scanner 4000B and images were processed with GenePix Pro 6.0. The measurements in spots were filtered by criteria $I/B \geq 3$ and $(I-B)/(SI+SB) \geq 0.6$, where I and B are the mean signal and background intensities and SI, SB are the standard deviations. Results were submitted to NCBI GEO repository, accession number: **GSE13197.** After subtraction of mean background, and LOWESS normalization [26] was performed. To assess differential expression of genes, the normalized log intensity ratios were analyzed with Student's t-test ($p<0.01$) and genes were ranked by log(p-level). The Bayesian modification to the false discovery rate (FDR) was used to correct for multiple comparison tests, estimating the *q*-value for the set of differentially expressed genes [27]. Differentially expressed genes ($p<0.01$) were grouped by Gene Ontology categories [28] and mean log(expression ratio) were analyzed by Student's t-test ($p<0.05$).

Q-PCR

Real-time PCR (Q-PCR) was performed in order to verify the results obtained in microarrays. 4 μg of the same pooled RNA used for microarray was used to synthesize cDNA, which was diluted 1:50 and used as template using the primers of additional file 1. Wells (20 μl final volume) contained 10 μl of iQ™ SYBR Green Supermix (Bio-Rad), 500 nM concentration of forward and reverse primers and 5 μl of cDNA. Controls lacking cDNA and controls containing RNA were included. Reactions were run in a MyiQ thermocycler (BioRad) under the following protocol: 5 min initial denaturation at 95ºC, followed by 40 cycles of 10 sec denaturation at 95ºC and 30 sec at 60ºC, and a final melting curve of 81 cycles (from 55ºC to 95ºC). All samples were run in triplicate and fluorescence was measured at the end of every extension step. C_T (threshold cycle) values for each sample were expressed as "fold differences", calculated relative to untreated controls and normalized for each gene against those obtained for 18S. In parallel, Q-PCR was performed with the individual samples in order to test biological variability. Thus, microarray results and Q-PCR from pools results were confirmed by means of real time PCR from RNA tested on individual macrophage cultures. 2 μg of RNA from each treatment was used to synthesize cDNA and Q-PCR was performed as described above. Expression of each gene was normalized to that obtained for 18S and fold changes set to the control of each fish. Transcripts were sequenced to ensure amplification was specific: products were visualized under UV light in a 1% agarose gel containing 1 mg/ml ethidium bromide, purified using MiliElutegel purification system (Quiagen), cloned into PGEM-T Easy Vector (Promega) by T/A cloning and transfected into competent Escherichia coli JM 109 cells (Promega). Plasmid DNA was isolated by Nucleospin Quickpure (Marcherey Nagel), digested with EcoRI (Promega) and sequenced with T7 primer.

Western assay

For western blotting analysis, samples were run on 15% polyacrylamide gel, and were electrophoretically transferred to a PVDF membrane. The membrane was blocked overnight at 4ºC with 5% dry milk in TBS buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4), followed by overnight incubation with antibody against TNFα at a

dilution of 1:500 in TBS buffer containing 5% dry milk at 4 °C. The membrane was washed three times for 10 min each in TTBS buffer (150 mM NaCl, 25 mM Tris pH 8,3 mM KCl, 0.1% Tween20) and then incubated 1h at room temperature with 1:5000 diluted Goat anti-rabbit HRP (Jackson Immunoresearch). Membrane was washed three times for 10 min each in TTBS buffer and detection was performed using SuperSignal chemiluminescent substrate (Thermo scientific) and finally exposed to Chemidoc (Biorad).

Results and discussion

Macrophage cell culture systems in fish has been widely used, to identify a large number of gene sequences using EST and SSH approaches [29], to evaluate the immunostimulatory properties of different PAMPs [2, 4] and to analyze transcriptomic modulation in response to LPS and cortisol stimulation [3]. The objective of the present work was to further characterize the biological response of trout macrophages to different immune agents. Thus we analyzed and compared the immunomodulatory effects of the bacterial cell wall component LPS and the viral ds(RNA) analog Poly I:C in rainbow trout macrophages, using a salmonid-specific microarray platform enriched with immune-related genes (SFA 2.0) [30] and TNFα western blot. The comparison of the transcriptomic profiles obtained together with the different pattern in the release of TNFα revealed significant differences in the macrophage response to bacterial or viral components.

Global effects of LPS and Poly (I:C) in differential gene expression profiles of macrophages

The transcriptomic profiles of macrophages stimulated with LPS or with poly(I:C) were compared with control cells (figure 1). The total number of differentially expressed genes identified ($p<0.01$) was similar for both treatments, 575 genes for LPS and 537 for Poly (I:C) (additional file 2). Applying a selection criteria based upon a classical cut-off value of >2 fold change (FC) over the differentially expressed genes ($p<0.01$), LPS induced the regulation of 401 transcripts (70% of the total ranked genes), 196 genes were up-regulated and 205 were down-regulated. In Poly (I:C) stimulated macrophages, 219 genes (41% of the total ranked genes) displayed a fold change (FC)>2, of those 119 were increased and 100 were decreased

(figure 1). The magnitude of the transcriptomic response, measured as the total number of differentially expressed genes, was similar between treatments, however the intensity of the response, in terms of genes with a FC>2, was greater in LPS treated cells. On the other hand, the values of the FC were much higher in macrophages stimulated with Poly (I:C) (table 1). All these data together emphasizes the different mechanism used by fish macrophages in response to bacterial or viral challenge. The global response of macrophages to LPS and Poly (I:C) evaluated by transcriptomic profiles correspond to those programmes observed in mammalian systems [31, 32].

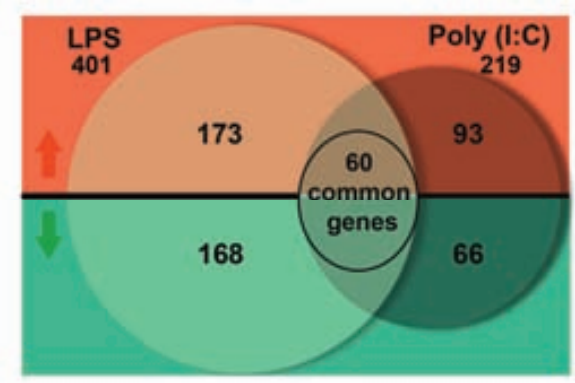

Figure 1: Total number of differentially expressed genes. Differentially expressed genes with a FC>2 (t-student, p<0.01) in rainbow trout macrophages stimulated for 12h with LPS or Poly (I:C).

Interestingly, 226 transcripts were found differentially expressed in both treatments of which 75 showed reverse pattern of expression for LPS and Poly (I:C) (additional file 2). 41 genes were down-regulated in LPS and up-regulated in Poly (I:C), 34 were increased in LPS and decreased in Poly (I:C). Applying the cut-off value of FC>2, 60 genes were common for both groups, where 11 were down-regulated in LPS and up-regulated in Poly (I:C), 8 were increased in LPS and decreased in Poly (I:C), and 15 and 26 were up-regulated and down-regulated respectively in both stimulations (table 1).

The differentially expressed genes in both LPS and Poly (I:C) stimulated macrophages were analyzed by interrogating the functional classes of Gene Ontology (GO) [28], and compared by the mean log(expression ratio) of ranked genes (Student's t-test, p<0.05) (Figure 2, Additional file 4). As in the ranked genes, a

number of GO categories were common for both treatments, including some groups which showed opposite pattern of expression. The categories Cell communication, Signal transduction, Defence and Immune response, Response to biotic stimulus and the Protein kinase and I-kappaB kinase NF-kappaB cascades were reduced in LPS challenge although induced in Poly (I:C), whereas the Sodium ion transporter activity was enhanced in LPS and declined in Poly (I:C). Heme biosynthesis and categories related to Caspase activation were up-regulated in both stimulations, and functional groups involved in Ribosome and Protein biosynthesis were down-regulated also for both LPS and Poly (I:C). Besides to the common GO groups, some categories were specifically modulated. Cells stimulated with LPS induced functions involved in Heme metabolism, Endomembrane system, Receptor activities related to Steroid hormone and Protein kinase, Inhibition of protease activity, Transcription and RNA metabolism. In contrast to Poly (I:C) responses, LPS reduced signalling cascades, such as JNK cascade, MAP kinase activity and Stress-activated protein kinase signalling, in addition to Peptidase activity. On the other hand, Poly (I:C) increased significantly important immune categories such as Antigen processing, MHC I receptor activity, Extracellular matrix and Signalling cascades including JAK-STAT and Intracellular signalling cascade. Categories related to Protein activity, Peroxisome, Zinc-Magnesium or Monosaccharide binding, Regulation of immune response and Cell cycle were also enhanced. Down-regulation was observed in Haemoglobin complex, groups related to DNA and Chromosome, Antigen binding, Microsome and Superoxide metabolism.

A

LPS FC	Gene Name	PIC FC
-2.53	Interferon-induced guanylate-binding protein 2	13.09
-2.47	TNF receptor superfamily member 5 precursor	9.35
-4.59	T-Cell activation protein	8.66
-2.01	Peroxisomal acyl-coenzyme A thioester hydrolase 1	7.97
-2.38	Tax1 binding protein 1	4.68
-2.98	Ras activator RasGRP	4.15
-17.60	Interleukin-1 receptor-associated kinase 1-2	3.58
-5.33	Transport-associated protein	3.08
-2.13	GrpE protein homolog 1, mitochondrial precursor	2.79
-2.07	Pyrin-2	2.30
-9.56	IL-8 receptor	2.00

B

LPS FC	Gene Name	PIC FC
2.92	Eukaryotic translation elongation factor 1 alpha 1	-2.02
2.81	60S ribosomal protein L23	-2.04
2.87	Complement factor D	-2.16
2.61	Complement component 1, Q subcomponent binding	-2.66
2.39	Ubiquitin-like protein SMT3A-1	-2.86
2.30	C type lectin receptor C	-2.99
3.89	Unknown-79	-3.01
2.59	Core promoter element binding protein	-4.45

C

LPS FC	Gene Name	PIC FC	LPS FC	Gene Name	PIC FC
4.65	Unknown-227	410.25	-5.28	Unknown-163	-2.00
2.76	CCL4	283.55	-10.46	Fibronectin receptor beta	-2.05
8.69	TNF decoy receptor	208.08	-2.72	Cathepsin B-1	-2.21
15.33	Matrix metalloproteinase-13	16.02	-2.64	Thymosin beta-4-2	-2.21
2.07	Eukaryotic translation initiation factor 2 subunit 1	14.91	-5.56	DeltaB	-2.27
4.92	Interleukin-1 receptor-like protein 2	14.41	-2.33	Ubiquinol-cytochrome C reductase complex 11 kDa prot, mit precursor	-2.28
2.10	Signal transducer and activator of transcription 1-alpha/beta	14.38	-2.11	Cathepsin Z precursor	-2.33
3.90	CGI-112 protein	9.63	-2.12	CD63	-2.41
2.82	FYVE finger-containing phosphoinositide kinase	7.18	-2.53	MHC class II alpha chain	-2.66
2.98	Matrix metalloproteinase-9	5.92	-2.58	Receptor-interacting serine/threonine-protein kinase 2	-2.82
2.14	Matrix metalloproteinase 9-2	3.64	-3.10	Cathepsin D-1	-3.06
2.86	Unknown-122	3.30	-2.62	Thymosin beta-4-1	-3.16
2.16	Aminolevulinate, δ synthetase 1-2	2.83	-3.92	Galectin-1	-3.30
2.00	NF-kappaB inhibitor alpha-3	2.39	-3.57	Arachidonate 5-lipoxygenase-2	-3.74
2.12	Galectin-9 (VHSV-induced prot)-2	2.25	-2.61	Calmodulin-3	-3.80
			-2.40	Unknown-100	-3.94
			-4.53	Cathepsin D-2	-3.99
			-2.09	Toll-like receptor 20a	-4.07
			-2.71	Unknown-6	-4.77
			-2.19	Hemoglobin beta chain	-5.46
			-2.31	Annexin A1-1	-5.68
			-2.38	Tolloid-like protein (nephrosin)-1	-5.96
			-2.38	Chemokine receptor-2	-7.93
			-9.11	Tolloid-like 2 protein (nephrosin)	-11.90
			-6.90	Tolloid-like protein (nephrosin)-2	-12.54
			-7.17	Unknown-11	-43.24

Table 1: Common differentially expressed genes in LPS- and Poly (I:C)-stimulated macrophages. Differentially expressed genes for both LPS and Poly (I:C) (PIC) treatments, with a FC>2 (t-student, $p<0.01$) in rainbow trout macrophages stimulated for 12h

Microarray results were validated by quantifying the expression of a number of genes with quantitative real-time Q-PCR, using same RNA pools used in the microarray hybridization (Additional file 3) and subsequently the individual samples to further evaluate the biological variability between fish (data not shown). Results obtained by Q-PCR were similar to the previous results and confirmed data obtained from microarray analysis.

Differential regulation induced by LPS and Poly (I:C)

As described above, when gene expression profiling of macrophages stimulated with LPS or Poly (I:C) were analyzed with control cells many immune related genes were modulated by both preparations (Figure 1, additional file 5), although there were also notable differences (Figure 2, Additional files 6 and 7). A number of immune related genes and immune GO categories were up-regulated by Poly (I:C) and repressed by LPS, including the functional groups Cell communication and Signal transduction, together with the Protein kinase and I-kappaB kinase NF-kappaB cascades, and Defence and Immune response. At a single gene level, several transcripts were observed to be regulated in agreement with the GO analysis (table 1). Thus, related to signalling, Interferon-induced guanylate-binding protein 2 (GBP2), Interleukin-1 receptor-associated kinase 1-2 (IRAK 1-2) and IL-8 receptor were highly increased in Poly (I:C) and decreased in LPS. GBP2 acts as an antiviral GTPase in response to interferon-gamma (IFN-gamma) [33]. GBPs have been previously described in fish and seen to be increased in rainbow trout cells after Poly (I:C) stimulation [34]. IRAK 1-2 activation is a signalling module for IL-1R and TLR signal transduction and is a gene partially responsible for IL1-induced up-regulation of the transcription factor NF-kappa B. In mammals, IRAK 1 has prominent role in the inflammatory response to LPS mediated by TLR4 and also in type-I IFN induction upon engagement of TLR7 or TLR9 [35]. IL-8 receptors characterization have been reported in fish, and in agreement with our results, mRNA expression was decreased after LPS stimulation and increased with Poly (I:C) [36]. Our results confirm that GBP, IRAK 1 and IL-8 receptor are induced in fish cells by viral-analog challenge, though after LPS exposure macrophage attenuate mRNA expressions of these genes involved in viral response, probably due to the aim of these cells to carry out an activation of specific internal cellular functions against bacterial challenge.

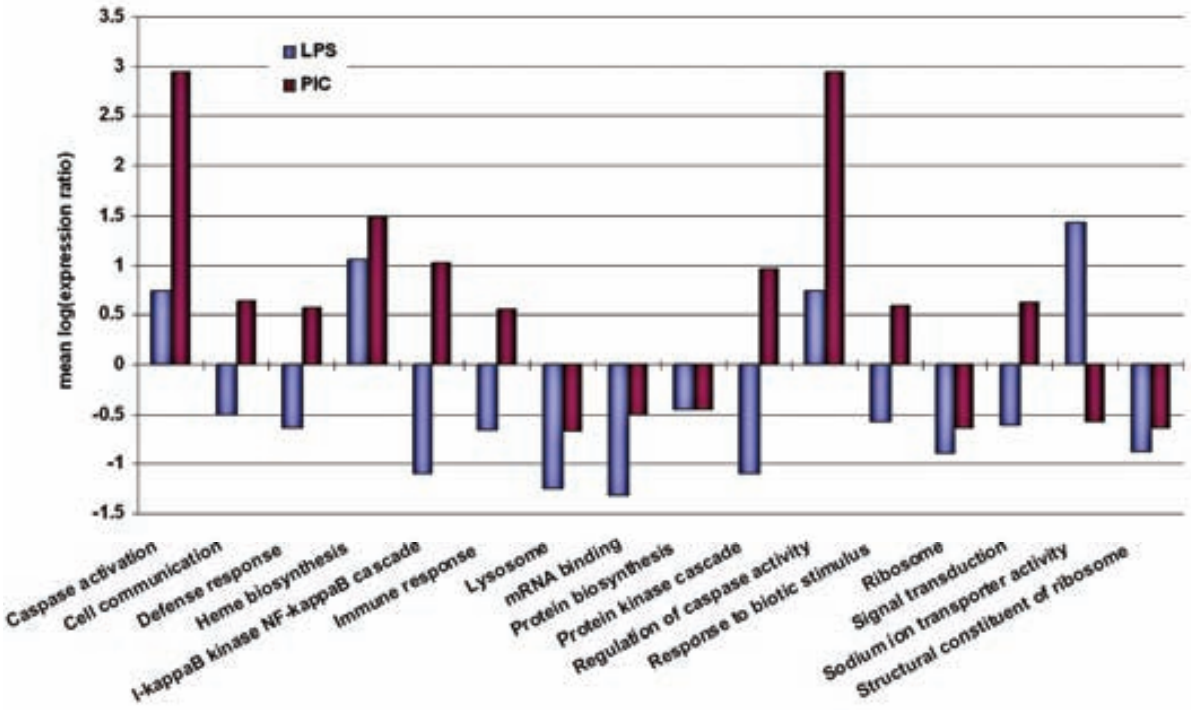

Figure 2: Common functional categories of Gene Ontology for LPS- and Poly (I:C)-stimulated macrophages. Analysis of GO categories between control macrophage and macrophages induced with LPS or Poly (I:C) for 12h. Differentially expressed genes ($p<0.01$) were grouped by Gene Ontology categories and mean log(expression ratio) were analyzed by Student's t-test ($p<0.05$)

Some genes known to be related to cell activation and differentiation were also induced by Poly (I:C) and reduced by LPS. Tumor necrosis factor receptor superfamily member 5 (CD40) is a receptor involved in cellular signalling and lymphocyte maturation and activation. It was observed to be up-regulated in Japanese flounder kidney by the VHSV G-protein DNA vaccine at 1 and 3 days post-immunization [37], and contrary to our microarray results, increased in Japanese flounder normal PBLs after LPS treatment [38]. Ras activator RasGRP, a regulator of small GTPase mediated signal transduction related to cell differentiation and T-cells and B-cells development [39], followed same pattern of expression.

Genes involved in inflammatory response at the level of cytokinesis and cytoskeleton, such as MAPRE2 microtubule-associated protein (T-Cell activation

protein), Tax1 binding protein 1 (human T-cell leukemia virus type I) and Pyrin-2, were also up-regulated in Poly (I:C) induced cells and down-regulated in LPS induced cells. MAPRE2 microtubule-associated protein has a role in microtubule dynamics, maintaining cell shape and signal transduction [40], whereas Tax1 binding protein 1 play a role in cytokinesis, cell shape, and specialized functions such as secretion and capping [41]. Expression of Tax 1 binding protein is increased by IL-1β in the fish cell line RTS-11 [42], and regulation of cytoskeleton, including MAPRE2 microtubule-associated protein and Tax 1 binding protein, are observed in salmon after viral infections [30]. These data together suggest that the differential regulation induced by Poly(I:C) and LPS might possibly be involved with the distinct changes provoked in the cell morphology following activation of trout macrophages with these PAMPs.

On the other hand, 8 genes with a FC>2 were increased in LPS and decreased in Poly (I:C) (table 1). Among these, two complement factors, Complement factor D and Complement component 1 Q subcomponent binding were also differentially expressed in LPS and Poly (I:C). Furthermore, although with a FC<2, more complement factors following the same pattern for both treatments can be found in the list of all common ranked genes (additional file 5). Significant extra-hepatic synthesis of complement components have been demonstrated in rainbow trout [43], and analysis of its regulation have reported an increase in complement expression in trout macrophages challenged with LPS [3] in agreement with the up-regulation observed in channel catfish after infection with a Gram-negative bacterium [44]. C type lectin receptor C was ranked as well in the category of genes enhanced by LPS and decreased by Poly (I:C). Lectins and their receptors are also produced in large quantities during infection to identify bacterial surfaces [45]. In fish, like in mammals, this response is stimulated by factors such as LPS [46], data confirmed with our results. The transcriptomic profiles clearly indicate that acute phase response is enhanced by LPS, though macrophage stimulated with Poly (I:C) down-regulated complement to switch expression towards the adaptive response.

In addition to those genes, many transcripts were specific for each treatment, including many unknown genes (Additional files 6 and 7). Thus, LPS up-regulated the expression of genes involved in innate immunity as lysozyme g-2, Serum amyloid P-component-2, Scavenger receptor with C-type lectin type I, Heat shock protein HSP 90-alpha or Interleukin 13 receptor alpha-2, and down-regulated genes involved in

adaptive response, as well as several Immunoglobulin chains, genes belonging to signalling kinase cascades and to processes related to cell-cell and cell-matrix interactions. In contrast, Poly (I:C) challenge triggers regulation towards viral-specific and the adaptive response, and increased the expression of many interferon regulator factors, Immunoglobulin chains, proteins of MHC class I complex or related to it, lymphocyte-cell activators and kinases involved in immune signalling, coordinated with a down-regulation of cathepsins, genes of the haemoglobin complex and genes involved in basic cellular functions (Additional files 6 and 7).

Common regulation between LPS and Poly (I:C) challenges

In addition to the common GO categories Heme biosynthesis and Caspase activation, at a single-gene level 15 up-regulated genes followed same pattern for both LPS and Poly (I:C) (table 1 and figure 2). Transcripts falling within this category were mainly genes involved in immune signalling and in extracellular matrix proteolysis. Thus, the chemokine CCL4, TNF decoy receptor, which is a TNF receptor that lacks a death domain resulting in the inhibition of the ligand-induced death response, Interleukin-1 receptor-like protein 2, signal transducer and activator of transcription 1-α-β (STAT 1), that mediates signalling by interferons, FYVE finger-containing phosphoinositide kinase, involved in intracellular signalling, the NF-kappaB inhibitor alpha-3 and Galectin-9 (VHSV-induced protein)-2, a galactoside-binding protein which acts like a positive regulation of I-kappaB kinase/NF-kappaB cascade. Collagenases, such as matrix metalloproteinase 9 (MMP-9), MMP-9 2 and MMP-13, which are genes known to have important roles in remodelling of the extracellular matrix and also involved in the trafficking of macrophages, were induced by both LPS and Poly (I:C) in trout macrophages, although the intensity of this regulation, measured in terms of FC, was much higher in macrophages treated with Poly (I:C) (table 1).

Functional categories involved in Ribosome and Protein biosynthesis in the GO analysis, together with 26 genes with a FC>2 were down-regulated for both PAMPs. Most of these differentially expressed genes were involved in cytoskeleton, cell adhesion and cell differentiation. Interestingly, although MMPs were increased with both agents, the lysosomal cysteine proteases Cathepsin B-1, Z precursor, D-1 and D-2 were decreased, as well as Thymosin beta-4-2 and Thymosin beta-4-1, which

play an important role in the organization of the cytoskeleton. Fibronectin receptor beta, Delta B, CD63, Galectin-1, Annexin A1-1, and the metallopeptidases Tolloid-like protein (nephrosin)-1, Tolloid-like 2 protein (nephrosin), Tolloid-like protein (nephrosin)-2, all of them involved in cell adhesion and/or cell differentiation, were repressed. These results suggest a coordinated expression of regulatory genes that may orchestrate the reorganization of the cytoskeleton, which in mammals is an essential step in lymphocyte activation [47]. High regulation of proteases and molecules with a key role in the organization of the cytoskeleton seems to be one of the most characteristic effects of bacterial and viral pathogens in fish [48]. Other immune-related genes of interest were immune receptors, such as Receptor-interacting serine/threonine-protein kinase 2, Toll-like receptor 20a and Chemokine receptor-2.

Therefore it appears that in vitro differentiated trout macrophages respond differentially to LPS and Poly (I:C) in some categories and genes involved in cell communication, signal transduction and some kinase cascades, as described above. Nevertheless, some important cytokines, chemokines, receptors and signalling proteins shared the same pattern of expression and are elevated in both treatments (see also additional file 5). This is not surprising, since as in mammals, after specific pathogen recognition mediated by different PRRs, in many cases Toll-like receptors, fish requires a robust response to cope with any viral or bacterial infection, activating mechanisms that are coordinated by common cytokine and chemokine release [44, 48-50]. Ultimately, the expression of signal transduction results in the expression of transcription factors through different signalling pathways to drive specific biological responses against microorganisms [8, 51].

Expression of the MHC antigen processing/presenting machinery

The viral-analog treatment induced a typical MHC Class I expression, increasing genes such as the MHC class I antigen, MHC class I heavy chain-2, MHC class 1b antigen and MHC class I heavy chain-1 (Additional files 5-7), together with the GO category MHC I receptor activity (figure 2), and the Tapasin-1 and Tapasin-2 genes, transmembrane glycoproteins which mediate interaction between newly assembled MHC class I molecules and the transporter associated with antigen processing (TAP), required for the transport of antigenic peptides across the endoplasmic reticulum membrane [52]. On the other hand, MHC class II alpha chain

was repressed by Poly (I:C) (table 1), and LPS down-regulated expression of both MHC class I antigen and MHC class II alpha chain (Additional files 5-7).

Fish possess MHC class I and II complexes, therefore antigen presenting machinery appears to be similar to that of mammals. As in the mammalian counterparts, the interaction of MHC molecules with T cell receptors (TCR) seems to activate subsets of cytotoxic T lymphocytes (CTL) and T helper cells (Th) [53]. Similar to the results presented here for the viral analog Poly (I:C), IHNV challenge in trout enhanced CD8 response coupled with activation of MHC Class I antigen presentation following IHNV infection, together with a down-regulation in MHC Class IIB mRNA in head kidney and spleen of infected trout [54], and same pattern was observed in a cohabitant model of fish viral infection [30]. In agreement with the Tapasin regulation observed in our results, transcription of Tapasin genes was increased in trout during acute infection with infectious hematopoietic necrosis virus (IHNV) [55] and during early infectious salmon anemia virus (ISAV) infection and poly I:C stimulation *in vivo* and *in vitro* in atlantic salmon [56]. Furthermore, repression of MHC II mRNA expression has been reported in differentiated trout macrophages in response to *E.coli* LPS [3] and in head kidney, liver and spleen of turbot after *Vibrio anguilarum* infection [57]. Taken together, the data indicate that viral infections decrease MHC class II genes favouring MHC class I pathway whereas bacterial pathogens repress expression of genes related to both classes of MHC.

TNFα release in macrophages stimulated with LPS and Poly (I:C)

As shown in figure 3, a positive band of 17kDa corresponding to TNFα was detected by western blot analysis in stimulated macrophage supernatants, although with different pattern of expression for LPS and Poly (I:C). LPS induced cells released TNFα protein 12h after challenge and the production was maintained over 24h, whereas in Poly (I:C) induced cells TNFα protein expression was observed 24h after stimulation. The clear difference in the time response of TNFα protein production confirms that some specific responses observed between LPS and Poly (I:C) at the transcriptomic level persist at the protein level.

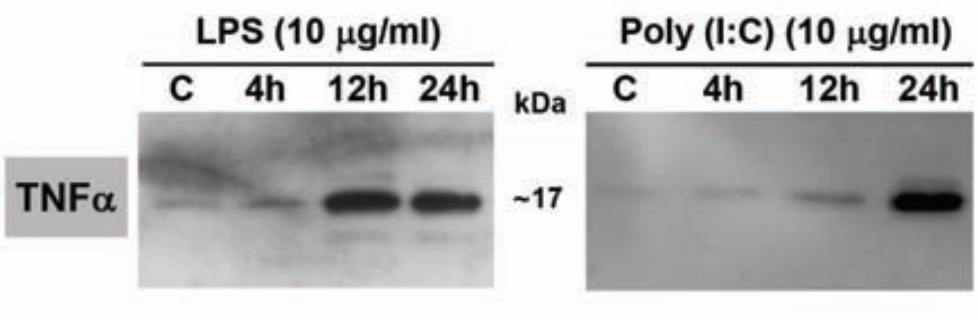

Figure 3: Western blot analysis of TNFα release in the supernatants of LPS- and Poly (I:C)-stimulated macrophages. Macrophages were incubated for different times with LPS or Poly (I:C). TNFα was detected using a specific polyclonal antibody.

In mammals, the recognition of PAMPs by PRRs triggers an intracellular signalling pathway which culminates in the induction of proinflammatory cytokines, chemokines and type I interferon. The receptors for bacterial lipopolysaccharide activate transcription factors that results in the secretion of proinflammatory cytokines such as TNFα and IFN regulatory factor 3 (IRF3) and subsequent induction of IFN-b and IFN-inducible genes. However, the recognition of double-stranded (ds)RNA is mediated by TLR3, which lack the MyD88-dependent pathway that activates NF-kB and subsequently induction of genes encoding inflammatory cytokines. Recognition of Poly (I:C) by TLR3 activates the IRF3 followed by the induction of the expression of IRF3-dependent genes [8]. Figure 3 reveals that fish macrophages, as mammalian cells, show clear differences after the recognition of bacterial or viral PAMPs. However, it appears that whereas macrophages incubated with LPS possess similar signalling pathways that culminate in TNFα secretion, the late response of trout macrophages to Poly (I:C) challenge may indicate that TNFα production is not directly induced after TLR3-IRF3 activation but after triggering all the signalling pathways activated by Poly (I:C).

Conclusions

Overall, the response of differentiated trout macrophages to LPS and Poly (I:C) involves an increase in the ability of the macrophages to respond to external bacterial or viral stimuli and processes involved in cellular differentiation and adhesion and matrix remodelling, at the same time that there is a coordinated decrease in basic cellular functions and in the immune responses that are not specific for each treatment. LPS enhances the expression of genes involved in the mechanisms activated in the innate response, whereas Poly (I:C) decreases innate response and increases gene expression towards the adaptive response and MHC I antigen presentation. In addition, at a protein level, LPS triggers a faster release of the proinflammatory cytokine TNFα than the viral component.

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Annex: additional files

A.1 Table 1: primers used in Q-PCR

GENE	Nucleotid sequence (5'- 3')	Amplicon size
CASP8 and FADD-like apoptosis regulator precursor	Fw: GAGGGACATCAGCAGGGTTGAC Rv: GGATGGAGTCCTGATCCACTG	134 bp
High affinity immunoglobulin ε receptor α	Fw: AAGCACACAACACACCCTCCAC Rv: GGACACGACACATCAGGGTCAT	125 bp
Pyrin-2	Fw: GGAGGGCCAGGTCTCCTTCTA Rv: GAGTGATGACCAGTGGGGTTG	147 bp
Alpha-blobin I-2	Fw GGCCCCCGGTTCCGCTCCAG Rv: GGCGGCAATGACCACAATTAG	190 bp
MHC class II alpha chain	Fw: CAGGTGGACCAGGAACAATC Rv: GGGGTGAAGGTCAGACTGGAG	109 bp
IRAK 1-2	Fw: GAGAGGAGAGAGAGACGGAC Rv: GGAGCAGCAAGTGGAGTGGAA	173 bp
Alpha-globin I-1	Fw: CTGGAGCGGAACCGGGGGCC Rv: CAGCTAAGGACAAAGCCAACGTG	160 bp
MMP13	Fw: GCTGAGGTGGACAACTCCAT Rv: AGCTGAAGCTGAAGGACTCG	238 bp
IRF 1-2	Fw: ACCAGACCACCCCACAGACT Rv: CAGCCGCAGCTCTATTTCCT	205 bp
Cathepsin B-2	Fw: TCTGCTGCGTGGGACTTCTG Rv: GTGCCGTTGACATGGTGCTC	116 bp
Hemoglobin β chain	Fw: CCGCCGTTTTCAATGCTGATAC Rv: GCACAGGTTGCAGCCTAACAG	162 bp

A.2 Table 2: Total number of differentially expressed genes. Ranked genes with a FC>1 (t-student, p<0.01) in rainbow trout macrophages stimulated for 12h with LPS or Poly (I:C).

	COMMON	SPECIFIC	TOTAL	UP-REGULATED			DOWN-REGULATED		
				FC>1	FC>1.5	FC>2	FC>1	FC>1.5	FC>2
LPS	226 (60	349	575	290	269	196	285	268	205
Poly(I:C)	FC>2)	311	537	217	163	119	320	181	100

41 down-regulated in LPS and up-regulated in Poly (I:C): **11 with FC>2**
34 up-regulated in LPS and down-regulated in Poly (I:C): **8 with FC>2**
151 same pattern of expression: **41 with FC >2, 15 up-regulated and 26 down-regulated**

A.3 Table 3: Q-PCR validation for microarray results. Selected genes expressions were analyzed by SYBR Green Q-PCR analysis and normalized to the abundance of 18s. Data are presented as fold change of stimulated macrophages relative to control macrophages. All values are from triplicate measures.

Agent	GENE NAME	Q-PCR FOLD CHANGE	MICROARRAY FOLD CHANGE
Poly (I:C)	**CASP8 and FADD-like apoptosis regulator precursor**	18.79	31.05
Poly (I:C)	**High affinity immunoglobulin ε receptor α**	7.26	7.42
Poly (I:C)	**Pyrin-2**	16.79	2.30
Poly (I:C)	**Alpha-blobin I-2**	-2.81	-5.48
Poly (I:C)	**MHC class II alpha chain**	-3.54	-2.66
Poly (I:C)	**IRAK 1-2**	3.36	3.58
Poly (I:C)	**Alpha-globin I-1**	-8.42	-5.61
Poly (I:C)	**MMP13**	31.52	16.02
LPS	**Alpha-globin I-1**	-5.68	-1.70
LPS	**IRF 1-2**	-2.39	-2.81
LPS	**MHC class II alpha chain**	-6.56	-2.53
LPS	**Cathepsin B-2**	-2.90	-2.50
LPS	**Hemoglobin β chain**	-5.15	-2.19
LPS	**MMP13**	11.97	15.33

A. 4 Table 4: Gene Ontology analysis in macrophages induced with LPS or Poly (I:C) for 12h. Control cells and stimulated cells showed significant differences in functional groups. GO categories were compared pairwise by the mean log(expression ratio) of differentially expressed genes ($p<0.01$). Significance was determined with Student's t test ($p<0.05$) (Diff. exp.: differential expression).

LPS			GO category	Poly (I:C)		
Diff. exp.	**N genes**	**P Student**		**Diff . exp.**	**N genes**	**P Student**
0.736	3	0.049	Caspase activation	2.950	3	0.023
-0.503	79	0.010	Cell communication	0.637	92	0.003
-0.635	81	0.002	Defence response	0.577	96	0.005
1.059	3	0.021	Heme biosynthesis	1.481	2	0.007
-1.096	12	0.036	I-kappaB kinase NF-kappaB cascade	1.021	24	0.033
-0.661	73	0.002	Immune response	0.562	91	0.008
-1.244	8	0.000	Lysosome	-0.673	16	0.015
-1.316	3	0.005	mRNA binding	-0.513	4	0.019
-0.462	44	0.038	Protein biosynthesis	-0.451	53	0.005
-1.103	21	0.005	Protein kinase cascade	0.956	33	0.010
0.736	3	0.049	Regulation of caspase activity	2.950	3	0.023

-0.579	102	0.001	Response to biotic stimulus	0.584	121	0.001
-0.898	28	0.001	Ribosome	-0.643	40	0.000
-0.601	69	0.004	Signal transduction	0.626	84	0.004
1.422	3	0.024	Sodium ion transporter activity	-0.569	2	0.009
-0.878	35	0.001	Structural constituent of ribosome	-0.641	49	0.000
1.204	2	0.048	5-aminolevulinate synthase activity			
-1.000	7	0.018	Cysteine-type peptidase activity			
0.680	3	0.001	Endomembrane system			
1.059	3	0.021	Heme metabolism			
-1.675	6	0.046	Hemopoietic or lymphoid organ development			
-1.930	4	0.023	JNK cascade			
1.055	3	0.030	Ligand-dependent nuclear receptor activity			
-1.898	2	0.013	MAP kinase kinase kinase activity			
0.674	2	0.034	Nuclear membrane			
1.550	4	0.016	Pre-mRNA splicing factor activity			
1.264	7	0.031	Protease inhibitor activity			
0.791	16	0.015	RNA metabolism			
-1.417	2	0.038	Single-stranded RNA binding			
1.055	3	0.030	Steroid hormone receptor activity			
-1.930	4	0.023	Stress-activated prot kinase signalling pathway			
0.894	10	0.040	Transcription from RNA polymerase II promoter			
0.970	3	0.040	Transmembrane receptor prot. kinase activity			
			Alpha-type channel activity	-0.524	2	0.010
			Antigen binding	-1.523	5	0.001
			Antigen processing	2.033	5	0.045
			ATP biosynthesis	-0.526	3	0.010
			Chromatin	-1.437	7	0.015
			Chromatin assembly or disassembly	-1.572	5	0.046
			Chromosome	-1.447	8	0.006
			Copper ion binding	-0.441	2	0.017
			DNA packaging	-1.360	6	0.041
			Electrochemical potential-driven transporter activity	-0.870	3	0.008
			Extracellular matrix	1.491	8	0.020
			Hemoglobin complex	-2.370	5	0.001
			Intracellular protein transport	1.545	10	0.008
			Intracellular signalling cascade	0.747	56	0.004
			Ion channel activity	-0.524	2	0.010
			JAK-STAT cascade	3.154	3	0.016
			M phase	-0.936	3	0.007
			Magnesium ion binding	0.562	6	0.038

MHC class I receptor activity	0.959	4	0.015
Microsome	-2.491	4	0.009
Mitochondrial membrane	-0.543	6	0.043
Monosaccharide binding	2.441	6	0.003
Nuclear transport	1.935	7	0.014
Nucleotide metabolism	-0.634	6	0.001
Peroxisome	2.368	3	0.029
Porter activity	-0.870	3	0.008
Positive regulation of immune response	0.477	2	0.001
Protein folding	0.525	27	0.046
Protein import	2.192	7	0.003
Protein localization	1.026	11	0.031
Protein targeting	1.945	8	0.004
Protein transport	1.048	10	0.045
Protein-nucleus import	2.310	6	0.006
Receptor activity	0.774	38	0.042
Receptor signalling protein activity	1.423	17	0.013
Regulation of cell cycle	1.245	14	0.025
Regulation of immune response	0.477	2	0.001
Spliceosome complex	1.162	2	0.017
Structural molecule activity	-0.576	63	0.000
Superoxide metabolism	-0.827	4	0.023
Zinc ion binding	0.892	23	0.025

A.5 Table 5: Common differentially expressed genes for LPS- and Poly (I:C)-stimulated macrophages. Differential expression was analyzed with Student's t-test ($p<0.01$). Values are expressed as fold change (FC).

Gene name	LPS FC	Poly(I:C) FC	LPS P_Student	Poly (I:C) P_Student
Sulfotransferase 2	-1.72	7.19	7.4E-04	5.7E-05
DnaJ homolog subfamily A member 1	-1.74	5.68	6.6E-03	3.7E-03
Myristoylated ala-rich protein kinase C substrate	-1.38	5.38	3.3E-03	1.9E-08
Synapse associated protein	1.88	3.82	1.2E-03	4.3E-03
C-Jun protein	1.46	3.53	2.4E-03	8.3E-05
MHC class I antigen	-1.68	2.23	1.2E-03	2.3E-09
Transposase-57	1.77	1.95	3.8E-03	8.7E-06
14-3-3C2	1.95	1.94	3.9E-05	4.7E-06
Plasma glutathione peroxidase precursor	2.51	1.92	1.9E-05	8.8E-04
Suppressor of cytokine signalling 3	-10.74	1.91	2.8E-04	1.9E-03
Secretory granule proteoglycan core protein	1.81	1.89	2.6E-03	1.5E-11
Unknown-75	3.49	1.87	7.6E-06	1.5E-03
Adenosine kinase 2	-9.55	1.86	5.0E-04	2.2E-11

Chemokine-like factor family member 7	1.62	1.79	7.8E-04	1.2E-03
Na/K ATPase alpha subunit-1	3.34	1.75	1.5E-08	9.0E-08
Unknown-183	-2.18	1.73	4.2E-03	2.7E-04
Toll-like receptor 3 -1	-2.04	1.72	1.4E-03	3.5E-03
Unknown-176	-2.56	1.71	2.2E-05	3.3E-03
Cathepsin C-1	-4.88	1.68	1.5E-04	1.6E-10
Proteasome subunit alpha type 3	-2.49	1.68	2.9E-03	1.4E-04
Unknown-5	1.51	1.67	1.7E-05	9.0E-10
Unknown-112	2.12	1.62	7.4E-05	1.2E-05
Beta-2-microglobulin-2	-1.50	1.58	4.1E-04	1.2E-06
Unknown-70	3.39	1.56	5.0E-05	4.8E-04
Complement factor MASP-3	-13.61	1.49	5.5E-03	7.0E-04
T-cell lymphoma associated antigen se33-1	1.56	1.47	7.3E-03	8.2E-03
Transposase-23	-1.84	1.44	1.7E-05	2.0E-03
Cathepsin C-3	1.27	1.44	2.5E-04	3.9E-04
5'-3' exoribonuclease 2	2.63	1.43	2.1E-04	7.9E-05
Ubiquitin	-2.07	1.42	3.4E-05	9.6E-09
Transcription regulator protein BACH1	1.35	1.40	6.8E-03	2.6E-05
Unknown-207	1.51	1.40	5.6E-03	2.3E-03
Hpa repeat-2	6.68	1.39	2.8E-09	3.7E-03
NF-kappaB inhibitor alpha-2	-5.81	1.39	7.1E-04	5.4E-03
Toll-like receptor 3-2	-4.10	1.39	6.1E-04	1.2E-03
Heat shock protein HSP 90-beta-2	5.88	1.36	2.0E-11	1.2E-05
All-trans-13,14-dihydroretinol saturase	1.77	1.35	5.1E-04	7.2E-05
Microtubule-associated protein RP/EB	-2.06	1.31	4.0E-05	5.4E-06
Hyperosmotic protein 21	2.21	1.29	5.1E-06	5.7E-04
MHC class I heavy chain-like	-3.11	1.29	3.7E-05	5.0E-04
Procathepsin L-1	-1.50	1.29	7.5E-05	2.3E-07
Cytochrome c oxidase subunit I-1	-17.48	1.26	1.3E-04	2.4E-06
Na/K ATPase alpha subunit-2	3.28	1.25	1.5E-09	7.7E-06
Unknown-92	-4.00	1.22	9.5E-03	2.1E-04
LPS binding protein	-2.52	1.19	5.5E-04	1.8E-03
Tyrosine-protein kinase HCK	4.31	1.18	2.2E-08	1.2E-04
Estrogen-responsive B box protein	-2.89	1.18	4.4E-04	5.4E-03
Transposase-45	-1.46	1.17	4.1E-03	4.8E-04
Unknown-231	-2.02	1.15	7.3E-03	6.4E-03
Thioredoxin	-2.31	1.15	9.3E-04	1.0E-04
Growth arrest DNA-damage-inducible prot α-2	2.06	1.14	1.7E-04	1.5E-03
Unknown-180	-5.50	1.13	3.5E-04	9.9E-03
Unknown-170	-1.75	1.11	3.0E-04	7.3E-03
Unknown-83	-2.41	1.06	1.1E-06	8.8E-03
Cyclophilin-3	-3.62	-1.09	1.1E-05	2.0E-04
Ubiquitin and ribosomal protein S27a-2	-1.31	-1.11	5.7E-03	5.1E-03
Transposase-55	-1.46	-1.12	2.9E-04	8.2E-04
Transposase-41	1.51	-1.13	6.9E-04	2.4E-04

Collagen alpha 1(I) chain-1	1.20	-1.14	9.9E-03	1.3E-03
Unknown-109	1.58	-1.16	3.9E-05	6.2E-04
Myosin heavy chain, skeletal, fetal	-2.61	-1.16	9.4E-05	2.9E-03
T-complex protein 1, gamma subunit	1.63	-1.16	3.1E-03	3.4E-04
DnaJ homolog subfamily C member 9	-3.18	-1.17	4.5E-05	2.7E-03
60S ribosomal protein L37a	-6.98	-1.17	2.4E-03	1.6E-03
60S ribosomal protein L7a-1	-1.69	-1.17	8.1E-03	7.7E-07
Similar to GTP-binding protein ragB	-1.62	-1.18	9.6E-03	6.8E-03
Unknown-276	1.26	-1.18	3.8E-03	2.7E-06
Unknown-133	1.66	-1.19	1.3E-04	1.3E-06
TNF ligand superfamily member 13B	-4.79	-1.19	2.9E-03	8.7E-03
Polyadenylate-binding protein 4	-2.83	-1.20	7.8E-04	8.4E-03
Nuclease sensitive element binding protein 1-2	-1.19	-1.20	3.6E-03	8.8E-08
Full-length cDNA clone CS0DC006YH13 of Neuroblastoma of Homo sapiens	-2.05	-1.20	8.9E-06	8.8E-03
Unknown-236	-5.47	-1.20	3.2E-04	4.2E-03
PNAS-106	-3.30	-1.21	2.7E-03	2.1E-04
Egl nine homolog 2	-4.47	-1.21	4.4E-06	1.6E-05
Heat shock cognate 71 kDa protein	-9.59	-1.21	2.4E-04	3.1E-05
Unknown-238	-2.58	-1.21	6.4E-04	2.2E-06
Interferon regulatory factor 1-2	-2.81	-1.22	1.0E-06	1.5E-03
Transposase-17	-1.66	-1.22	8.3E-04	8.3E-06
Unknown-272	-1.72	-1.24	1.3E-03	9.8E-06
Granule cell differentiation protein (myotrophin)	-3.45	-1.24	4.7E-05	1.9E-04
MAPK/ERK kinase kinase 6	-2.07	-1.24	2.7E-03	2.7E-05
TATA-binding protein associated factor 2N	-2.52	-1.24	1.0E-03	1.1E-06
BAG-family molecular chaperone regulator-4	-3.38	-1.25	1.1E-03	8.4E-03
MAPK/ERK kinase kinase 5-1	-3.63	-1.27	6.0E-04	8.5E-06
C-terminal binding protein 1	-5.29	-1.27	7.7E-05	4.2E-03
Fructose-bisphosphate aldolase A	-1.73	-1.27	2.3E-03	7.1E-04
T-complex protein 1, subunit 2	-2.01	-1.27	8.6E-03	2.0E-03
Transposase-52	-1.39	-1.28	5.3E-03	4.7E-07
Actin, cytoplasmic 2	1.37	-1.28	2.2E-06	4.5E-07
Transposase-60	-4.20	-1.29	1.2E-03	4.4E-07
Adenosine deaminase 3	-1.59	-1.29	1.0E-03	4.2E-07
Histone H33-2	3.76	-1.31	3.9E-06	1.0E-04
Ribosomal protein L35-1	-2.40	-1.32	5.3E-06	6.8E-05
P21-activated kinase 2	-1.65	-1.32	1.8E-03	2.9E-04
Unknown-76	-1.56	-1.33	4.8E-03	6.5E-08
Superoxide dismutase [Mn], mitocho. precursor	-4.04	-1.33	9.0E-05	7.5E-04
60S ribosomal protein L18a	-2.37	-1.33	6.0E-05	2.3E-03
Rab GDP dissociation inhibitor beta	1.26	-1.34	7.9E-04	1.1E-05
Serine/threonine-protein kinase 25	-3.06	-1.34	4.8E-04	2.5E-03
Wiskott-Aldrich syndrome protein	-1.68	-1.35	5.4E-03	8.4E-07
Ubiquitin ligase SIAH1	-2.86	-1.35	3.2E-04	9.1E-06

60S ribosomal protein L26	-1.40	-1.37	9.9E-03	4.0E-07
Drebrin-like protein	-7.10	-1.37	5.9E-04	1.7E-04
40S ribosomal protein S7	-7.04	-1.37	1.5E-03	3.1E-07
Cathepsin B-2	-2.50	-1.39	2.0E-05	1.2E-11
60S ribosomal protein L14	2.37	-1.39	6.1E-05	6.1E-05
Glyceraldehyde-3-phosphate dehydrogenase-6	1.83	-1.40	7.4E-04	1.7E-08
60S ribosomal protein L17	-2.68	-1.41	2.1E-04	3.1E-09
Complement component C3-3-2	1.47	-1.42	8.8E-03	6.9E-03
60S ribosomal protein L35a-2	-2.32	-1.42	5.0E-04	4.3E-06
Histone 3A-ATP synthase F0 6	-2.71	-1.44	4.6E-03	6.5E-07
Annexin IV	1.54	-1.44	5.9E-06	2.5E-07
14-3-3 B1-like	5.83	-1.44	1.4E-09	1.8E-03
40S ribosomal protein S15-1	-1.70	-1.45	5.4E-03	6.6E-03
Cold inducible RNA binding protein-2	5.20	-1.45	3.1E-09	1.8E-06
Gastrointestinal glutathione peroxidase 2	-22.27	-1.45	2.7E-04	2.1E-04
Lysozyme g-3	-2.24	-1.45	2.2E-04	4.9E-04
T-complex protein 1, zeta subunit	-10.37	-1.47	3.2E-05	5.8E-03
Metallothionein-IL	-3.83	-1.50	1.9E-04	8.5E-07
Cdc45	2.62	-1.53	2.4E-04	8.1E-05
Acidic leu.-rich nuclear phosphoprotein 32 A-2	1.48	-1.54	5.3E-03	1.0E-05
ATP synthase beta chain-2	-27.38	-1.54	7.8E-05	1.1E-04
Heat shock 70 kDa protein 1	1.43	-1.55	4.7E-03	8.9E-10
Cornichon homolog	-4.56	-1.55	5.4E-04	3.6E-04
Reverse transcriptase-like-1	-1.68	-1.55	4.6E-06	8.7E-07
Ribosomal protein L36a-like-2	-2.16	-1.56	7.4E-05	6.9E-05
40S ribosomal protein S9-3	-2.72	-1.57	2.0E-04	2.2E-07
Eukaryotic translation initiation fact. 3 subunit 5	3.17	-1.59	1.8E-06	2.8E-05
Complement factor H-related protein 1	-2.62	-1.60	2.2E-04	2.9E-03
40S ribosomal protein S30	-2.42	-1.60	8.8E-04	6.2E-04
Goodpasture antigen-binding protein	-2.36	-1.60	2.7E-05	6.9E-03
Eukaryotic translation initiation factor 5	2.77	-1.62	1.2E-03	1.6E-04
Similar to rRNA (Vangl2)	-1.87	-1.63	8.6E-03	1.6E-10
Ribosomal protein L35-2	-2.27	-1.64	5.5E-04	1.4E-04
Tyrosine-protein kinase SYK	-1.28	-1.69	6.1E-03	5.4E-04
40S ribosomal protein S2	-3.46	-1.71	1.4E-03	2.7E-07
60S ribosomal protein L36	-2.53	-1.71	6.3E-03	7.1E-03
Unknown-116	3.79	-1.72	9.7E-08	1.3E-05
60S ribosomal protein L10-1	-1.82	-1.72	2.2E-03	4.9E-04
40S ribosomal protein S15-2	-18.79	-1.74	2.2E-05	2.1E-03
60S ribosomal protein L5-1	2.61	-1.74	1.3E-05	3.7E-04
Unknown-118	1.54	-1.75	1.6E-03	6.1E-08
Complement factor B/C2-B	1.53	-1.76	9.1E-03	2.4E-03
Cathepsin S	-1.54	-1.78	2.2E-03	8.8E-10
MHC class II invariant chain-like protein 1	-1.83	-1.78	2.8E-03	6.8E-13
Ependymin related protein-1	-1.53	-1.80	1.6E-04	8.5E-08

Eukaryotic translation elongation factor 1 δ 3	-1.46	-1.80	3.5E-03	6.2E-10
Mitogen-activated protein kinase 6	-2.05	-1.82	4.7E-03	5.6E-07
Transaldolase	1.20	-1.88	5.1E-03	1.8E-09
40S ribosomal protein S21	-1.62	-1.89	1.8E-03	4.6E-04
Cyclin G1	-3.23	-1.92	3.1E-03	3.3E-05
Leukemia virus receptor 1-1	-3.34	-1.95	8.9E-04	4.6E-05
Glutathione S-transferase P-2	-3.29	-1.99	5.9E-03	1.1E-05
Elongation factor 1-beta	-1.78	-2.07	1.0E-03	2.0E-04
Profilin-1	-1.73	-2.13	7.2E-04	2.2E-08
ATP-binding cassette transporter 1	-1.64	-2.14	1.5E-03	9.2E-06
Galectin-3	1.48	-2.26	6.8E-03	7.0E-06
Ig heavy chain V-III region HIL	1.95	-2.72	5.6E-03	1.6E-03
Elongation factor 1-alpha 2	-1.68	-2.86	6.9E-03	1.2E-09
Annexin A1-2	-1.38	-3.76	3.7E-03	6.2E-12
Alpha-globin I-2	-1.70	-5.48	1.7E-05	2.1E-08
Coronin-1B	-1.90	-5.73	2.0E-06	4.5E-11
Selenium-binding protein 1	-1.94	-8.29	3.4E-03	1.4E-05

A.6 Table 6: Specific differentially expressed genes in LPS-stimulated macrophages. Genes with FC>2 expressed upon LPS induction but not regulated with Poly (I:C) treatment. Differential expression was analyzed with Student's t-test ($p<0.01$). Values are expressed as fold change (FC). A) Genes up-regulated. B) Genes down-regulated

A		B	
FC	**Gene name**	**FC**	**Gene name**
6.92	Hypothetical-fish 31	-2.08	Xaa-Pro aminopeptidase 2
6.38	Hypothetical-fish 44	-2.10	T-complex protein 1, subunit 5
5.91	Transcription factor jun-B-1	-2.11	Aldehyde dehydrogenase 1A2
5.51	Unknown-66	-2.13	40S ribosomal protein S5
5.19	Lysozyme g-2	-2.18	Myelin basic protein-1
5.17	Unknown-38	-2.19	D-3-phosphoglycerate dehydrogenase
4.76	Serum albumin precursor	-2.21	Over-expressed breast tumor prot.-like
4.41	N-myc downstream regulated protein-2	-2.21	Ig kappa chain V-III region VG
4.39	Unknown-127	-2.22	Granulins
4.32	D-type cyclin-interacting protein 1	-2.23	Phospholemman precursor
4.18	Mpv17	-2.34	Unknown-107
4.17	Unknown-21	-2.45	Peroxiredoxin 6
4.09	Hypothetical-fish 17	-2.46	Prostaglandine D synthase
3.96	Unknown-209	-2.55	Angiotensin I converting enzyme
3.90	Peptidyl-prolyl cis-trans isomerase 2-1	-2.59	Hypothetical-fish 26
3.85	Sox-like transcriptional factor	-2.65	Unknown-247
3.84	Sp1 transcriptional activation factor	-2.67	Cytochrome P450 2F1
3.81	Organic anion transporter	-2.71	CREB-binding protein

3.80	Unknown-81	-2.71	Unknown-280
3.70	Hypothetical-fish 30	-2.75	Guanine nucleotide-binding protein subunit 2-like 1
3.52	NADH-ubiquinone oxidoreductase B15	-2.76	Frizzled 8 precursor
3.50	Unknown-113	-2.77	Creatine kinase, B chain
3.42	Mitochondrial inner membrane translocase Tim17 A	-2.78	Ovarian fibroin-like substance-2
3.37	Unknown-111	-2.78	Unknown-233
3.36	Nuclear matrix protein NMP200	-2.83	Heat shock protein 4
3.30	Unknown-262	-2.84	Unknown-268
3.18	Glycogen phosphorylase-2	-2.88	Malate dehydrogenase, cytoplasmic
3.17	Hypoxanthine-guanine phosphoribosyltransferase	-2.88	Unknown-161
3.10	Proteasome subunit alpha type 7-1	-2.89	Heterogeneous nucl ribonucleoprot. A0
3.04	Hypothetical UPF0193 protein EVG1	-2.89	Heat shock 70kDa protein 8
2.91	CD6, T cell surface glycoprotein	-2.94	Unknown-235
2.89	ATP-binding cassette, sub-family F, member 2	-2.97	Unknown-174
2.88	Hypothetical-fish 5	-2.99	60S ribosomal protein L39
2.85	Beta enolase-4	-3.08	GTP-binding protein GEM
2.83	Haptoglobin	-3.11	Unknown-28
2.76	Scavenger recep. with C-type lectin type I	-3.12	Unknown-229
2.75	Serum amyloid P-component-2	-3.13	Notch1
2.73	COP9 signalosome complex subunit 6	-3.13	Unknown-3
2.70	Eukaryotic initiation factor 4A-I	-3.13	Lysozyme C precursor
2.68	NADH dehydrogenase subunit 2	-3.14	Unknown-195
2.66	ATP synthase coupling factor 6, mitochondrial prec.	-3.15	ADAM 9
2.66	Septin 7 (CDC10 protein homolog)	-3.23	Transposase-2
2.58	Latent transforming growth factor beta binding	-3.24	Cytochrome c oxidase subunit II
2.58	Unknown-52	-3.29	Troponin C-1, skeletal muscle
2.55	CD97	-3.31	Unknown-152
2.54	Cofactor required for Sp1 activation, subunit 7-2	-3.34	Ah receptor interacting protein
2.53	Nonhistone chromosomal protein HMG-14-2	-3.47	Bone morphogenetic protein 4
2.52	Synaptic glycoprotein SC2-2	-3.49	Ik-B kinase complex-associated prot-2
2.52	Protein kinase C, alpha type	-3.54	Unknown-155
2.46	5-aminolevulinate synthase	-3.60	40S ribosomal protein S9-2
2.43	Hypothetical-fish 16	-3.70	MAP/ERK kinase-like
2.42	ReO_6-2	-3.75	Transposase-30
2.41	Membrane associated progesterone recept. comp. 2	-3.84	CC chemokine CCL1
2.40	Multidrug resistance protein 3-2	-3.87	Troponin T-3, fast skeletal muscle
2.38	Transposase-59	-3.88	40S ribosomal protein S9-1
2.38	CD166	-4.01	Transposase-50

2.37	Unknown-88
2.35	Hypothetical-fish 19
2.34	Unknown-138
2.33	ADP-ribosylation factor-like protein 7
2.33	CD9
2.32	ATP-binding cassette, sub-family F, member 1
2.32	Splicing factor arginine/serine-rich 11
2.31	Unknown-261
2.31	Phospholipid hydroperox. glutathione peroxidase A
2.31	Cell death activator CIDE-B
2.29	Matrix metalloproteinase 2
2.28	Mitochondrial 39S ribosomal prot. L51
2.27	Coatomer zeta-1 subunit
2.27	Unknown-244
2.26	Anti-silencing function 1B
2.25	CD18
2.23	Protein phosphatase methylesterase 1
2.23	Unknown-114
2.20	Aminopeptidase N
2.20	Transposase-56
2.18	Unknown-119
2.18	Hypothetical-fish 3
2.15	Regulator of nonsense transcripts 1
2.12	Unknown-74
2.12	28S ribosomal protein S16, mitochondrial precursor
2.12	B-cell-specific coactivator OBF-1
2.11	Membrane-bound transcription factor site 2 protease
2.09	Baculoviral IAP repeat-containing protein 6
2.09	Cell division control protein 42 homolog
2.08	ReO_6-1
2.02	Unknown-140
2.01	Heat shock protein HSP 90-alpha
2.00	Interleukin 13 receptor alpha-2
2.00	TEL/JAK2 fusion protein
-4.14	Unknown-36
-4.15	SCHIP-1
-4.37	Unknown-104
-4.38	Unknown-167
-4.50	Bcl2-associated X protein
-4.70	ADAMTS-8
-4.96	Tumor necrosis factor receptor superfamily member 19
-5.07	Cytochrome P450 27
-5.46	Interferon inducible protein 1
-5.91	Hypothetical-fish 12
-6.22	Transposase-49
-6.36	Unknown-230
-6.54	Arachidonate 5-lipoxygenase-1
-6.57	60S ribosomal protein L18
-6.64	Cytokine inducible SH2-containing prot. 5
-6.89	p53-like transcription factor
-6.98	Acyl-CoenzymeA dehydrogen. long chain
-7.59	Unknown-196
-7.68	TGF beta-inducible nuclear protein 1
-7.72	Unknown-197
-7.93	Transposase-51
-8.12	Gamma crystallin B-1
-8.74	Immunoglobulin-binding protein 1
-8.84	Histone deacetylase 4
-8.90	Unknown-162
-9.07	Properdin
-10.18	FLJ21439 protein
-10.24	Ig kappa chain V-IV region Len
-10.60	IgG VH protein
-11.40	Unknown-164
-11.88	Creatine kinase, sarcomeric mitoch. prec.
-12.24	Transposase-24
-14.86	Mannan-binding lectin serine protease 2-1
-16.54	Histone H33-1
-16.61	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2
-21.27	Engulfment and cell motility protein 2
-34.55	Unknown-166

A.7 Table 7: Specific differentially expressed genes in Poly (I:C)-stimulated macrophages. Genes with FC>2 expressed upon Poly-IC induction but not regulated with LPS treatment. Differential expression was analyzed with Student's t-test ($p<0.01$). Values are expressed as fold change (FC). A) Genes up-regulated. B) Genes down-regulated

A		B	
FC	**Gene name**	**FC**	**Gene name**
144.17	Interferon-induced protein 44-2	-2.00	IL-1 receptor-associated kinase 4
112.42	High affinity immunoglobulin γ Fc receptor I prec.	-2.00	GRB2-related adaptor protein 2
93.99	C166_BRARE CD166 ag homolog	-2.03	Ferritin H-3
44.76	Seryl-tRNA synthetase	-2.05	High affinity immunoglobulin ε receptor γ
41.60	Tyrosine-protein kinase FRK	-2.09	Hyperosmotic glycine rich protein
31.05	CASP8 and FADD-like apoptosis regulator prec.	-2.10	Ribosomal protein S2
27.14	Phospholipase A-2-activating protein	-2.10	Double-strand break repair protein MRE11A
25.12	Interferon-induced protein 44-3	-2.10	Glutathione peroxidase-gastrointestinal
23.51	DnaJ homolog subfamily B member 11 precursor	-2.11	Ig kappa chain V-IV region JI
18.61	Tapasin-2	-2.19	40S ribosomal protein S29
18.13	TNF receptor superfamily member 9	-2.19	Cathepsin K-2
17.11	Ds RNA-specific adenosine deaminase	-2.20	Cytochrome b-245 light chain
14.44	Pyrin-1	-2.22	Semaphorin 7A
12.79	Pre-B cell enhancing factor	-2.25	Ribosomal protein L6-1
12.66	Galectin-9 (VHSV-induced protein)-3	-2.25	Phosphatidylinositol 3,4,5-trisphosph.-dependent Rac exchanger 1 prot
11.95	HCF-binding transcription factor Zhangfei	-2.29	Tumour necrosis factor receptor
11.58	Trans-2-enoyl-CoA reductase	-2.31	CCAAT/enhancer binding protein beta
10.34	Galectin-3 binding protein	-2.35	Engulfment and cell motility protein 1
9.46	Proteasome subunit beta type 9 precursor	-2.36	Ribosomal protein L6-2
9.12	Interferon regulatory factor-like	-2.38	Unknown-12
9.09	Signal transducer and activator of transcription 3	-2.40	Complement receptor 1-1
8.99	Vitellogenin-2	-2.44	Ig kappa chain V-IV region B17-1
8.65	Tapasin-1	-2.49	Ras-related C3 botulinum toxin substrate 2
8.45	Ran-binding protein 2	-2.54	Polyposis locus protein 1
7.71	Guanylate-binding protein	-2.55	Cathepsin Y
7.66	Interferon regulatory factor 1-1	-2.59	Cathepsin K-1
7.42	High affinity immunoglobulin epsilon receptor α	-2.71	Caspase recruitment domain protein 4
7.00	Mitogen-activated protein kinase 9-2	-2.75	Beta-globin

6.89	GWSC6486
6.80	NF-kappaB inhibitor alpha-1
6.51	Galectin-9 (VHSV-induced protein)-1
6.12	VHSV-induced protein
5.74	Estradiol 17 beta-dehydrogenase 4
5.58	Unknown-278
5.51	Adenosine deaminase 2
5.48	Galectin like 1
5.39	Signal transducer/activator of transcription Stat1
5.11	Tumor necrosis factor receptor associated factor 2
4.78	Sulfotransferase 3
4.69	Unknown-51
4.56	Unknown-39
4.27	Unknown-120
4.02	Unknown-226
3.98	Unknown-283
3.89	CC chemokine SCYA106
3.81	Cyclophilin-like 6
3.81	StAR-related lipid transfer protein 3
3.50	AHA1, activator of heat shock 90kDa prot. ATPase
3.45	G1/S-specific cyclin D2
3.23	Unknown-17
3.23	C3a anaphylatoxin chemotactic receptor
3.23	Regulator of G-protein signalling 1-2
3.20	Sialoadhesin
3.14	Fibromodulin
3.02	MHC class I heavy chain-2
3.01	Catalase-1
3.00	Transforming growth factor-beta type II receptor
2.95	78 kDa glucose-regulated protein precursor
2.85	Lymphocyte antigen 75
2.76	Protoporphyrinogen oxidase
2.74	MHC class 1b antigen
2.64	MAPK/ERK kinase kinase 2
2.62	Unknown-37
2.48	Tissue inhibitor of metalloproteinase 2
2.46	Growth arrest specific 6
-2.80	Complement factor Bf-1
-2.89	Lysosomal acid lipase/cholesteryl ester hydrol
-3.06	Lymphocyte pore forming protein
-3.39	Plasminogen precursor-2
-3.42	γ-IFN inducible lysosomal thiol reductase
-3.53	High mobility group protein 2
-3.56	Gastrulation specific protein G12
-3.56	Ig mu heavy chain disease protein
-3.63	Histone H14
-3.71	X-box binding protein 1
-3.73	Nonhistone chromosomal protein HMG-14-1
-3.76	UDP-glucuronosyltransferase 2B17 precursor
-3.93	Keratin, type II cytoskeletal 8
-3.93	Ig kappa chain V-IV region B17-2
-3.98	Unknown-172
-4.00	Cytokeratin 8
-4.08	Heme oxygenase-2
-4.10	Deoxyribonuclease II-1
-4.35	Transcription factor jun-B-2
-5.11	Cytochrome P450 2J2
-5.61	Alpha-globin I-1
-6.00	Xaa-Pro dipeptidase
-6.61	Unknown-106
-8.00	Hemoglobin alpha chain
-8.35	Unknown-14
-10.32	Histone H10
-10.76	Unknown-214
-12.71	Heme oxygenase-1

2.39	Nucleolar protein Nop56-2
2.38	Peroxisome proliferator activated receptor gamma
2.35	Unknown-94
2.33	Cullin homolog 1
2.30	Calpain 2, large [catalytic] subunit precursor
2.29	Splicing factor, arginine/serine-rich 12-1
2.26	Cathepsin L2
2.21	Tyrosine-protein kinase Jak1-1
2.21	Caspase 2
2.19	Splicing factor 3B subunit 1
2.18	Galectin like 2
2.16	Regulator of G-protein signalling 1-1
2.10	Proteasome activator complex subunit 2
2.09	Cytokine receptor common gamma chain
2.09	MHC class I heavy chain-1
2.07	Annexin 11a
2.05	Glucose-6-phosphatase
2.05	Ubiquitin-conjugating enzyme E2-18 kDa
2.04	Unknown-149
2.02	Heat shock protein HSP 90-beta-1
2.00	Matrix metalloproteinase 9-1

5 The effects of immunostimulation through dietary manipulation in the Rainbow trout; evaluation of mucosal immunity

Abstract

Immunostimulant (IS)-containing diets are commonly used in husbandry practices in aquaculture to enhance the resistance of cultured fish to disease and stress during critical life cycle, environmental and production periods. Although widespread in use there have been conflicting results published where several reports confirm improved protection against bacterial and viral diseases whereas others have not found beneficial effects or even have reported toxicity. Most of the commercial IS-diets contain β-glucans, which may have a potential to activate fish Th17 cells that, in turn, should increase mucosal disease resistance. Surprisingly, very little is known about the regulation by dietary immunostimulation of immune related genes in tissues key to mucosal immunity in fish.

In teleosts, external barriers act as the first barrier against infection, having a key role in the control of entry of potential pathogens. The lymphoid tissue associated to teguments is localized in skin, gills and intestine, complementing the physical and chemical protection provided by the structure. Using a salmonid-specific microarray platform enriched with immune-related genes and *in situ* hybridization (ISH), we investigated dietary acclimation in 2 organs relevant to mucosal immunity in the rainbow trout (*Oncorhynchus mykiss*). Immunostimulant diet feeding significantly changed gene expression profiles and gene distribution in a tissue-specific manner: genes and functional Gene Ontology categories involved in immunity were differently expressed at portals of entry, the intestine and gills, where significant changes in genes and functional groups related to remodelling processes and antigen presentation were

observed. Furthermore, Matrix metalloproteinase 9-1 (MMP 9-1), Pyrin-2, MHC II alpha chain and Macrophage C-Type lectin (Mincle) in gills, and Myeloid leukemia factor 1 (MLF-1) and CXC chemokine receptor transcript variant B (CXCR B) in Intestine, which are genes involved in tissue remodelling, cell differentiation, antigen presenting capacity and chemotaxis, were localized by ISH in both gills and intestine.

Herein we present the identification and characterization of the response of rainbow trout to Immunostimulant diets by transcriptomic analysis and ISH analysis. In conclusion, IS-diets induce immune-modulation at the portals of entry of the animal. One of the main effects of in trout is the increase of the expression of some genes involved in antigen recognition in epithelial cells of gills.

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Introduction

The manipulation of the immune response in aquatic organisms by the diet is a commonly accepted practice in many intensive aquaculture systems. Immunostimulant-containing diets (IS diets) are commonly utilized in aquaculture in order to enhance the resistance of cultured fish and invertebrate species to disease and stress. Such diets were previously thought to act mainly upon the innate immune system by enhancing the activity of phagocytic cells [1] however a renewed more generic definition has been proposed to embrace the full potential of the immune response and its corresponding cellular players [2]. An immunostimulant is a naturally occurring composite which has a modulatory effect upon the immune system. To this end IS diet additives form a diverse range of bioactive compounds ranging from synthetic chemicals such as Levamisole, an anthelminthic used to treat nematode infections in mammals [3], bacterial lipopolysaccharides [4], nucleotides (for review see; [5, 6]), a range of polysaccharides, animal and plant extracts, specific dietary nutrients such as Vitamin C and certain hormones and cytokines (for review see [1]). However commercial immunostimulant diets are mainly limited to the addition of β1-3 and β1-6 glucans.

A number of studies which address the efficacy of potential IS compounds exist in which a large number of species (>12 fish species) have been studied at

different life stages [1, 2]. In salmonid fish, a wide range of experimental approaches to test potential and commercial IS composites have been applied mainly based upon *in vivo* (enteral formulations or intra-peritoneal administration) experimentation subsequently followed up on one hand with analysis of classical immune activation parameters such as lysozyme and complement activity, respiratory burst, phagocytosis and inflammatory gene expression in different tissue samples [4, 7-9] or by direct disease challenge [7, 10]. A clear picture of the host response has not been achieved because even toxicity processes have been reported [11], although a consistent trend to increased activation of innate immune system is apparent. However the efficacy of enteral formulations which are based upon β-glucan supplementation to reduce infectious complications in salmonid fish remains questionable.

The major portals of entry for pathogen penetration into fish are considered to be gills, intestine and skin [12] and recently fin bases have been shown to be the major portal of entry for IHNV [13]. The mucosal immune system in fish encompasses the above mentioned tissues and presents the primary barrier to repel pathogen invasion. These tissues are in direct contact with the external milieu and therefore with the antigenic matter containing potential pathogens. These tissues mediate the innate and adaptive responses to potential invasors and also function to limit the intensity of activation to avoid tissue damage. In mammals the mucosal immune system is considered to function independently from systemic immunity [14]. In teleost fish, the gill and intestinal epithelium like the mammalian mucosal epithelium is functionally diverse. Whilst the gill epithelium together with its mucus layer acts as an important physical barrier [15], there is no evidence of lymphoid tissue architecture similar to the mammalian gut-associated lymphoid tissues (GALT) such as the Peyer's patches, although teleost gut contains important populations of leucocytes [15] and there is evidence of cutaneous innate and adaptive immunity in fish [16, 17].

The IS component of diets contain molecules (PAMPs; pathogen associated molecular patterns) which will interact with a diverse set of cellular receptors collectively termed Toll-like Receptors (TLR) in the mucosa of the intestine which then elicit specific cellular responses and orchestrate the initiation of an immune response to pathogenic microbes [18]. Thus, immunostimulation via enteric formulation aims induce a PAMP-TLR host response and increase the activity of the immune system over a determinate time period.

In this study we address changes in the transcriptome induced by a commercial immunostimulant diet at two major sites of mucosal immunity; the gills and the intestine. The SFM 1.0 and SFA 2.0 targeted cDNA arrays have been repeatedly assessed in diverse experiments across a panel of responsive tissues in salmonid fish undergoing cortisol-related stress responses, exposure to aquatic contaminants and bacterial and viral immune insults [19-28] Here we report that differential gene expression is induced with an IS diet and furthermore we localize differentially expressed genes identified by microarray analysis in both tissues by *in situ* hybridization which contribute to important immune functions such as inflammation and antigen presentation in the tissues under study.

Material and Methods

Diets

The standard commercial diet Gama Delta 26, 3-mm pellets, from Proaqua S.A., was used as control diet. Gama Qualistar 26, 3-mm pellets, which include specific immunostimulants for salmonids, was used as the IS diet.

Fish and feeding trial

Adult rainbow trout (*Oncorhynchus mykiss*) of approximately 120g. were purchased from a commercial fishery, Piscifactoria Andrés, in Sant Privat (Girona, Spain). The fish were randomly distributed in four fibreglass tanks (25 fish per tank) with recirculated water circuits under a photoperiod of 12h light/12h dark and natural conditions of temperature. They were acclimatized to laboratory conditions for 15 days before being used for experiments. During the feeding trial, trout were hand-fed once a day with either the control diet or the immunodiet, in duplicate tanks, following manufacturer's indication of 1 g food/fish for 4 weeks. After the 4 weeks, 4 fish of each tank (8 for each diet) were sampled for microarray analysis, and 3 fish of each tank (6 for each diet) were sampled for ISH. Fish were killed in water with a lethal concentration of MS-222 (Sigma), 100 ppm, stage III of anaesthesia [29] intestine and gills and were collected. Tissues removed for RNA extraction were frozen in liquid nitrogen and stored at -80 ºC. Tissues sampled for ISH were fixed in

neutral buffered formalin (4%, pH 7.2), embedded in paraffin and cut in 10 μm sections.

Microarray analysis

The platform uses was the salmonid fish cDNA microarray SFA2.0 immunochip (see chapter 4). 100 mg from gills and 100 mg from the medium part of the intestine were used for total RNA extraction, using 1 ml of Tri Reagent (Sigma) per 100 mg of tissue, following the manufacturer's instructions and quantity and integrity was analyzed by Experion RNA StdSens Analysis Kit (Bio-Rad). RNA was pooled within treatments (n=8), and 15 μg of control and test were hybridized to the immunochip as described in chapter 4. Results were submitted to NCBI GEO repository, accession number: **GSE10981**. After subtraction of mean background, and LOWESS normalization [30] was performed. To assess differential expression of genes, the normalized log intensity ratios were analyzed with Student's t-test ($p<0.01$) and genes were ranked by log(p-level). The Bayesian modification to the false discovery rate (FDR) was used to correct for multiple comparison tests, estimating the *q*-value for the set of differentially expressed genes [31]. The functional categories of Gene Ontology [32] were compared with regulated genes ($p<0.01$) by the sums of ranks (Student's t-test, $p<0.05$). The statistical significance of over represented functional categories, showing the differential expression in the experiment grouped by functional classes compared with all genes and GO categories from the chip, was assessed using the Yates correction to Chi square test (corrected $p<0.05$).

Real-time PCR

In order to verify microarray results, real time PCR (Q-PCR) was carried out. 4 μg of the same pooled RNA used for microarray was used to synthesize cDNA with SuperScript III RNase Transcriptase (Invitrogen) and oligo-dT primer (Promega). cDNA was diluted 1:100 for the amplification of selected genes and 1:1000 for 18S, and used as a template with primers designed for Q-PCR (Additional file 2). Amplification was carried out as described in chapter 4. Values for each sample were expressed as "fold differences", calculated relative to control diet and normalized for each gene against those obtained for 18S. In parallel, Q-PCR was performed with the individual samples in order to test biological variability. Thus, microarray results and

Q-PCR from pools results were confirmed by means of real time PCR from RNA tested on individual fish in each group. 4 μg of RNA from each fish was used to synthesize cDNA and Q-PCR was performed as described above. Expression of each gene was normalized to that obtained for 18S and fold changes were arbitrarily set to one of the fish. Differences between IS-fed fish and control fish were analyzed with Student's t test ($p<0.05$, $n=8$ each group).

Transcripts were sequenced to ensure amplification was specific: products were visualized under UV light in a 1% agarose gel containing 1 mg/ml ethidium bromide, purified using MiliElutegel purification system (Quiagen), cloned into PGEM-T Easy Vector (Promega) by T/A cloning and transfected into competent Escherichia coli JM 109 cells (Promega). Plasmid DNA was isolated by Nucleospin Quickpure (Marcherey Nagel), digested with EcoRI (Promega) and sequenced with T7 primer.

In situ hybridization

In situ hybridization was performed to characterize the mRNA distribution of some interesting differentially expressed genes. Matrix metalloproteinase 9-1 (MMP 9-1), Pyrin-2, MHC II alpha chain and Macrophage C-Type lectin (Mincle) were monitored in gills, and Myeloid leukemia factor 1 (MLF-1) and CXC chemokine receptor transcript variant B (CXCR B) were monitored in Intestine. Hybridizations of each gene were performed in several fish. Probes were amplified with specific primers (Additional file 2) from cDNA, (60ºC, 30 cycles) and cloned into a bacterial plasmid vector (pGEM-T Easy Vector, Promega), except for MHC II and Macrophage C-Type lectin which were amplified from clones of a cDNA library (INRA-SCRIBE, Rennes, France) accession numbers BX081858 and BX870419 respectively. The DNA insert was sequenced in order to check identity and insert orientation. Probes were amplified using a PCR amplification of clones (Jump Start system, Sigma) followed by a PCR product purification using a Microcon PCR filter kit (UFC7PCR50, Millipore). Sense (S) and Antisense (AS) riboprobes were generated using Riboprobe in vitro Transcription Systems (Promega) according to manufacturer's instruction, with 0.35 mM digoxigenin-UTP (Roche). The RNA probes were kept –80ºC until use.

Sections were deparaffined in the robot Robostainer Microm, 15 min at 56º to eliminate paraffin followed by wash of toluene for 10 min and hydration in decreasing concentrations of ethanol (100 and 95 % for 3 min each and 70 % and destilled water for 5 min each), and washed in TBS. Hybridization was performed in the robot *In Situ* Pro VS (Intavis), using consecutive sections for S and AS probes. Slides were washed five times 2h in TBS, then twice 5 min in TBS-Tween (TBS-T, 150 mM NaCl, 50 mM Tris pH 7.4, 0. 05 % Tween 20, Sigma), and fixed two times for 10 min in 4% paraformaldehyde in 0.01 M PBS. Sections were washed again ten times for 3 min in TBS-T, and incubated four times for 5 min at 37°C in TBS-2 mM $CaCl_2$ and 3 µg/ml proteinase K (Sigma). The reaction was stopped in 50 mM Glycine (Prolabo) 50 mM tris pH 7.4 twice for 5 min, and sections were washed 5 min four times in TBS-T. Sections were fixed again twice for 5 min in 4% paraformaldehyde in 0.01 M PBS, and washed 5 min four times in TBS-T. Slides were prehybridized for 2h at 60°C in a hybridization buffer (50% formamide, 10% dextran sulfate, 2× SSC, 1× Denhardt's reagent, 250 µg/ml yeast transfer RNA, all products from Sigma). Sections were incubated at 60ºC with riboprobes (3 ng/µL in hybridization buffer). Slides were washed 10 min six times in 2× SSC, incubated twice for 15 min at 37ºC in 10 µg/mL RNAse A (Sigma) in 2× SSC. They were then washed eight times for 10 min in 2× SSC and eight times for 10 min in 0.1× SSC at 60°C, and twice for 30 min in TBS-T at room temperature. Non-specific sites were blocked three times for 40min in blocking solution (TBS containing 1% sheep serum, Sigma), and sections were incubated 2 times for 2h in 1:1000 diluted alkaline phosphatase conjugated anti-digoxigenin antibody (Roche) in blocking solution. Sections were washed 10 min 10 times in TBS-T and then incubated four times for 10 min in detection buffer, following by the revelation with NBT (nitroblue tetrazolium chloride, 4.5 µl/ml, Roche) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate, 4 toluidine salt, 3.5 µl/ml, Roche) seven times for 3h. Sections were mounted with Mowiol and pictures were taken using Leica DMRB microscope and Olympus DP71 camera at 20x or 40x.

Results

The effects of a commercial IS diet upon global gene expression profiles in two tissues of the rainbow trout (*O.mykiss*), the gills and intestine, which represent major portals of pathogen entry in fish were evaluated using a salmonid-specific

targeted cDNA microarray platform enriched with immune-related genes (SFA 2.0) [28] . Gene expression profiles obtained show feeding with an IS diet during a 4 week period significantly alters gene expression at portals of entry in a tissue-specific manner. Applying a selection criteria based upon a classical cut-off value of >2 fold change (FC) over the ranked selected genes ($p<0.01$), 8% of the ranked selected genes (a total of 305 genes) displayed a fold change (FC)>2 in the intestine (Additional file 1). The % of regulated ranked selected genes was 42% and 58% for up-regulated or down-regulated respectively. In gills, 166 genes, 40% upregulated and 60% downregulated, were differentially expressed ($p<0.01$) in which 57 displayed a FC>2 (34% of ranked selected genes, p-value <0.01) (Additional file 1). Applying the cut-off value of >2 fold change over the ranked selected genes ($p<0.01$) emphasizes the stronger global induction of gene expression in gills after dietary adaptation. On the other hand the magnitude of the transcriptomic response, measured as the number of differentially expressed genes (ranked selected genes $p<0.01$) highlights the intestine as the tissue with increased transcriptomic remodelling. Interestingly, the global tendency in both tissues analysed with SFA 2.0 shows an overall decrease in gene expression with specific emphasis upon genes related to immunity in response to the dietary stimulation with immunostimulant diets.

The log (*p*-level) ranked up or down-regulated genes in the gills were analyzed by interrogating the functional classes of Gene Ontology (GO) [32] and compared by the sums of ranked genes (Student's t-test, $p<0.05$) (Figure 1, Additional file 3). GO categories significantly downregulated included carbohydrate metabolism, signal transduction, cell communication and two categories which include genes involved in synaptic transmission. Up-regulated categories involve structural molecule activity encompassing protein and ribosome biosynthesis, cytoskeleton and primary active transporter (Figure 1). In addition and due to the large number of rank selected genes (166), the statistical significance of over represented functional categories (Figure 2) was also assessed using the Yates correction to Chi square test (corrected $p<0.05$). This test analyses the significantly regulated GO categories analyzed by their total representation on the platform. Analysis of over-represented functional GO categories ($p<0.05$) included structural molecule activity, structural constituent of ribosome and protein biosynthesis, and inhibition in functional categories related to cell communication and signal transduction (Figures 2A) similar to that obtained with

functional GO analysis. Analysis at the single-gene level, rank selected genes ($p<0.01$, FC>2), in the gills reveals genes involved in antigen detection, immune activation and regulation of some unknown genes (Additional file 4).

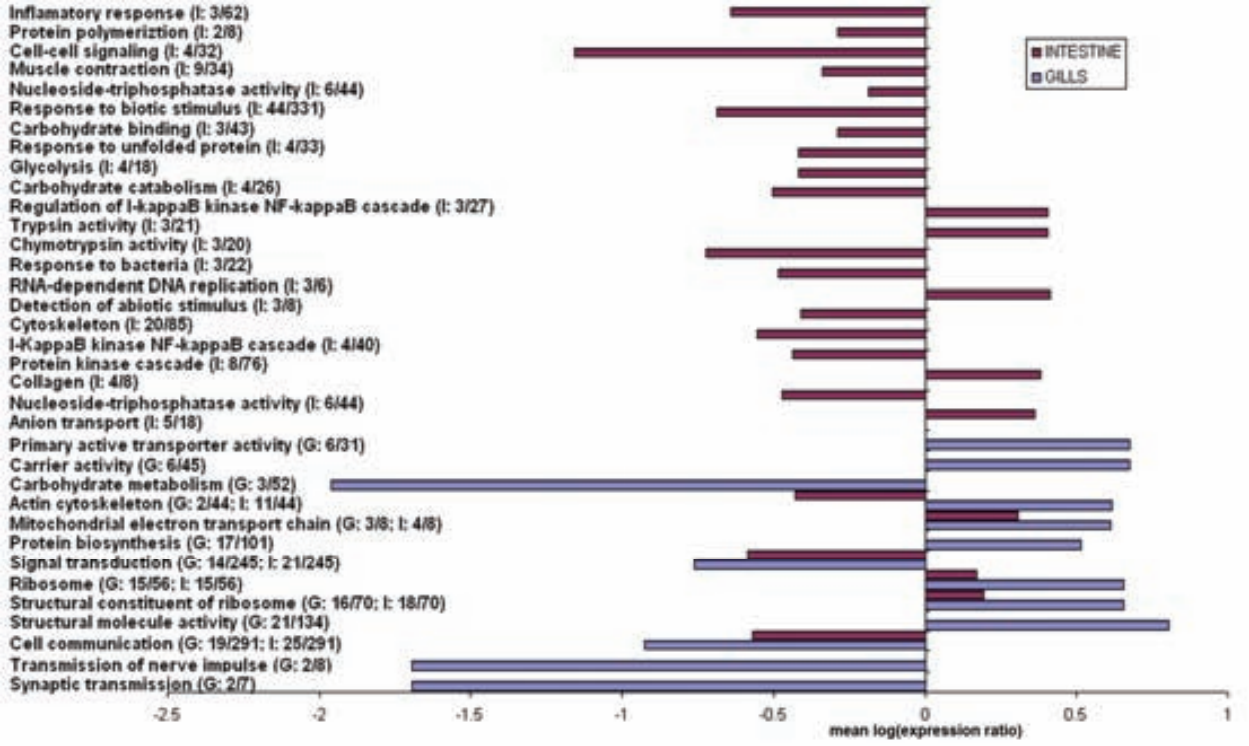

Figure 1: **Functional categories of Gene Ontology in gills and intestine that discriminate between control fish and fish fed with immunostimulant diet for 4 weeks**. Differentially expressed genes ($p<0.01$) were grouped by Gene Ontology categories and mean log(expression ratio) were analyzed by Student's t-test ($p< 0.05$). The number of differentially expressed genes and genes for each category on the microarray is shown in parenthesis.

In agreement with identified GO categories observed for the gills, a number of regulated genes belonging to Protein kinase cascades were identified. MAPK/ERK kinase 3 and MAPK/ERK kinase 4-2, Protein tyrosine kinase 2 beta and Mitogen-activated protein kinase 1 were all significantly reduced. Expression of surface proteins related to lymphocyte populations such as the B-cell receptor CD22-2, which mediates B-cell B-cell interactions or T-cell surface glycoprotein CD3 zeta chain, were

also decreased, as well as genes encoding proteins involved in structural remodelling, such as the metalloproteinase, MMP 9-1. In mammals, MMP 9 has been implicated in the signal processing and maturation of dendritic cells, IL-8 mediated activation of neutrophils , leukocyte migration and undifferentiated hematopoietic stem cells [33, 34]. Pyrin 2 or marenostrin was also highly down-regulated, this gene product is key to FEMV and has been shown to have an important role in the innate immune response regulating IL-1β production and caspase-1 activity [35]. On the other hand, several immune system related genes were up-regulated in fish fed with the IS diet in comparison to controls. Key antigen presentation molecules such as MHC II was highly up-regulated as was the Macrophage C-type lectin, Mincle, which is involved in endocytosis and the C/EBP-driven inflammatory response [36]. Interestingly and in accordance with GO analysis, the inductor of apoptosis, Apolipoprotein E-2 was significantly enhanced (Additional file 4). In short, an apparent global down-regulation of both innate and adaptive immune responses was observed in parallel to an increase in antigen presentation capacity.

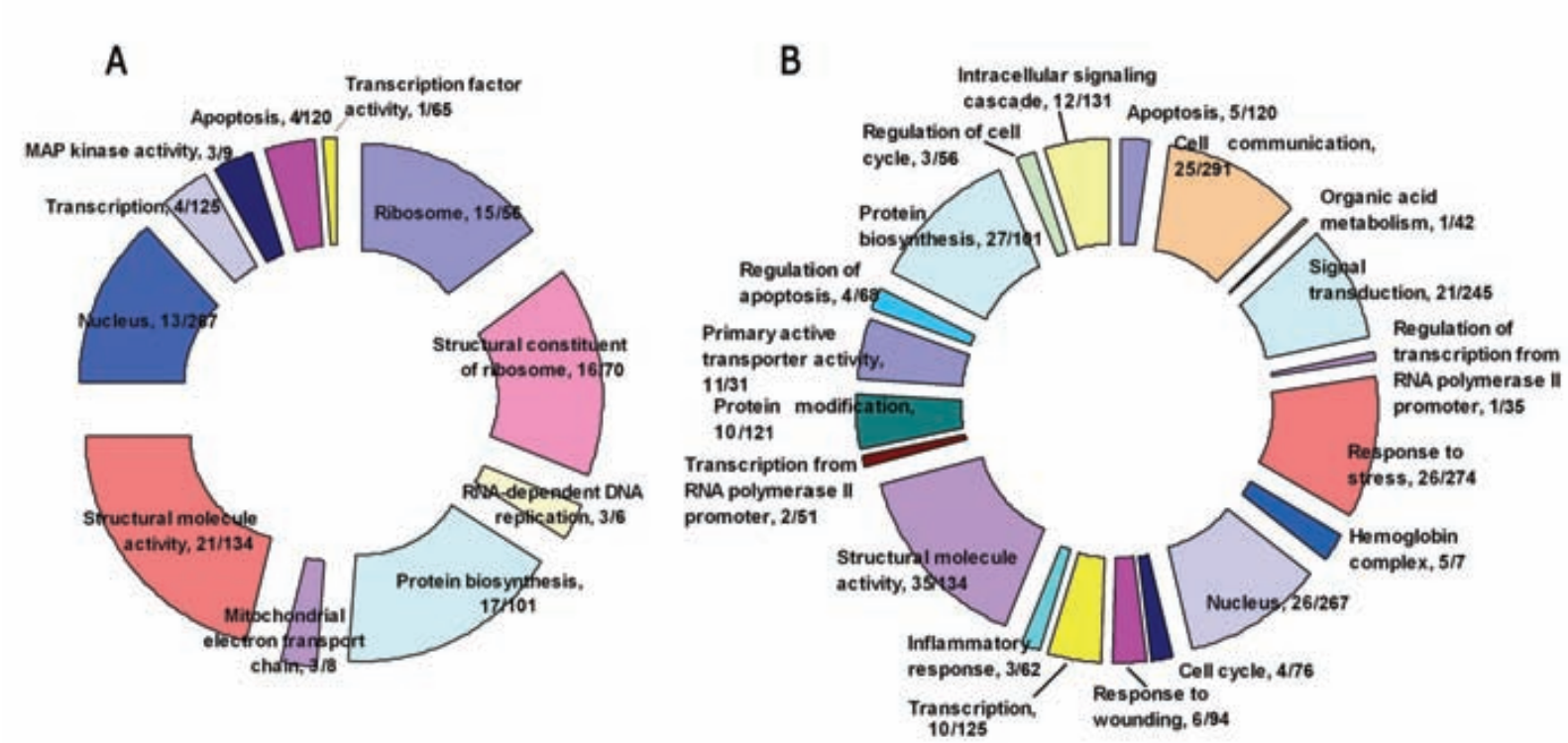

Figure 2: **Over representation of Gene Ontology functional categories** in gills (A) and intestine (B) of fish fed with immunostimulant diet. Categories were selected with Yates corrected Chi squared ($p<0.05$). The number of differentially expressed genes and genes for each category on the microarray is shown in parenthesis.

In the intestine, IS diets induced a significant change in terms of magnitude in the transcriptome, 305 ranked selected genes ($p<0.01$), although the intensity of the changes was relatively low, 8% of ranked genes had a FC of >2. GO categories found to be downregulated ($p<0.05$) (Figure 1) include response to biotic stimulus, cell communication, regulation of I-kappaB kinase NF-kappaB cascade, signal transduction and cytoskeleton (Figures 1, Additional file 3). Analysis of the over representation of functional GO categories shows that more biological processes are affected by the IS diet in comparison to the gills and highlights the presence of inflammatory, response to stress, apoptotic and structural remodelling processes (Figure 2B). Parallel single-gene analysis (Additional file 5) shows down-regulation of Myeloid leukemia factor 1(MLF1), an oncogene which promotes myleoid cell differentiation from erythroid-myeliod precursors by interfering with erythropoietin-driven differentiation processes [37]. N-myc downstream regulated protein-1 (NDRG-1) also plays a role in cellular differentiation and has been suggested to be involved in axonal survival [38]. Interestingly the Apoptosis regulator, Bcl-X, which has anti-apoptotic activity is significantly down-regulated whereas Baculoviral IAP repeat-containing protein 2 also an inhibitor of apoptosis which functions by interfering with the tumor necrosis factor receptor-associated factors TRAF1 and TRAF2 [39] is up-regulated. The Myosin regulatory light chain MRCL2-2 was found to be the most highly regulated gene in response to IS diets. This gene is involved in the structural reorganisation of the cytoskeleton in non-muscle cells [40]. The CXC chemokine receptor transcript variant B (CXCR3 or CD183) is primarily a receptor for three interferon-inducible chemokines and participates in leukocyte trafficking with preference for activated lymphocytes [41].

Quantitative PCR was carried out to validate microarray data both in the pools used for microarray analysis and then subsequently in the individual samples ($n=8$) to further investigate the biological variability between individuals used to constitute pools (Table 1). Results obtained from the respective pools, essentially technical replicates, of total RNA validate and reflect data obtained from the microarray analyses. In the individual fish samples the means of fold change for MHC II alpha chain, macrophage C-type lectin (Mincle) and Pyrin-2 in gills, and CXCRB in intestine were significantly different (Table 1, $p<0.05$), in agreement with the previous results. Thus the data obtained from pooled analyses were not due to the presence of a small number of highly activated individuals within the population and the effects of

the IS diets were ubiquitous across experimental fish as shown by the low variance between individuals.

Organ	Gene name	Microarray FC	Q-PCR FC pools	Mean FC control fish ± SEM	Mean FC IS-fish ± SEM	p (t-test)
Gills	**MHC class II alpha chain**	7,81	4,40	1.191 ± 0.52	6.757 ± 2.22	0.039
Gills	**MO C-type lectin (Mincle)**	3,71	5,43	0.456 ± 0.13	7.656 ± 2.31	0.012
Gills	**Pyrin-2**	-6,02	-1,84	8.147 ± 2.17	1.513 ± 0.36	0.010
Intestine	**CXC recept. variant B**	2.07	2,35	4.257 ± 1.91	13.717 ± 3.34	0.027
Intestine	**MMP 9-1**	-2.12	-3.33	2.435 ± 0.90	1.824 ± 0.76	No sign.
Intestine	**Alpha-globin I-1**	-1,78	-1,58			
Intestine	**Alpha-globin I-2**	-1,66	-1,18			

Table 1: Q-PCR validation for microarray results. Selected genes expressions were analyzed by SYBR Green Q-PCR analysis. For pooled samples, data are presented as mean fold change (FC) of fish fed with immunostimulant diet relative to control fish. For individual samples, fold changes were arbitrarily set to one of the fish and data are presented as mean fold change of fish fed with immunostimulant diet or with control diet ± standard errors of the mean (mean ± SEM). Differences between IS-fed fish and control fish were analyzaed with the individual samples using Student's test ($p<0.05$, $n=8$ each group). Values are from triplicate measurements.

From results obtained from the microarray analysis and subsequent Q-PCR validation we selected a number of target mRNAs, four genes in gills and two genes in intestine, using a criteria of high differential expression and gene function to further investigate gene function by localising these mRNAs in the tissues under study. In the gills, Matrix metalloproteinase 9-1 (MMP9) and Pyrin-2 were selected for down-regulated mRNAs whereas MHC class II alpha chain and Macrophage C-type lectin (Mincle) were selected for up-regulation (Additional file 4). For *in situ* studies in the intestine Myeloid leukemia factor 1 (down-regulated) and CXC chemokine receptor transcript variant B (up-regulated) were selected (Additional file 5).

In the gills a strong increase, in accordance with both microarray and Q-PCR data, in signal for MHC II alpha chain mRNA was detected on both primary and secondary lamellae in fish fed the immunostimulant diet (Figure 3A). The MHC II

alpha+ve reactive cells were more abundant in the filaments of the secondary lamellae than in the primary lamellae and were observed to be consistent with epithelial and antigen presenting/macrophage cellular morphologies. In the secondary lamellae the signal was mainly concentrated upon the external edge of the cells in direct contact with the external milieu. *In situ* hybridisation (ISH) with the Macrophage C-type lectin (Mincle) probe shows a similar localisation (Figure 3B) to that of the MHC II alpha+ve reactive cells in the secondary lamellae. Indeed cells that contact with the external milieu appear to be both MHC and Mincle +ve's. Furthermore Mincle+ve cells can also be observed in the arterioles of the primary lamellae and in the capillaries which feed into the secondary lamellae suggesting the recruitment of leukocytes to the sites of contact with the external milieu (Figure 3B). MMP9-1 positive cells were identified and found to be distributed across all primary and secondary lamellae, being more abundant in inter-lamellar cells within the primary filaments. Although the signal obtained was weaker than that of both MHC and Mincle+ve cells we were able to observe that epithelial cells, mucous cells or extracellular cartilaginous matrix did not express MMP 9-1 (Additional file 6). No difference was observed in the distribution or expression of MMP 9-1 mRNA in gills of fish from both diet groups. ISH studies on the localization of Pyrin-2 also did not identify any significant changes in localisation of the mRNAs signal although a stronger signal was detected in the epithelial cells at the extreme of the secondary lamellae (Additional file 6).

In the intestine the ISH localization profiles of CXC chemokine receptor transcript variant B (CXCR3) and Myeloid leukemia factor 1 (MLF-1) did not significantly differ between fish fed with either diet. CXCR3 mRNA expression could be localized to the epithelium and the serous membrane, whilst the lamina propia and mucous cells lack reactivity. A stronger signal can be appreciated in areas close to the serous membrane and the lamina propia (Figure 3C). MLF-1 positive signals were detected in the intestinal epithelium, however cells expressing MLF-1 mRNA in lamina propia were not detected (Additional file 6). Stronger hybridization signals could be observed in the epithelial cells in the areas nearest to the lamina propia.

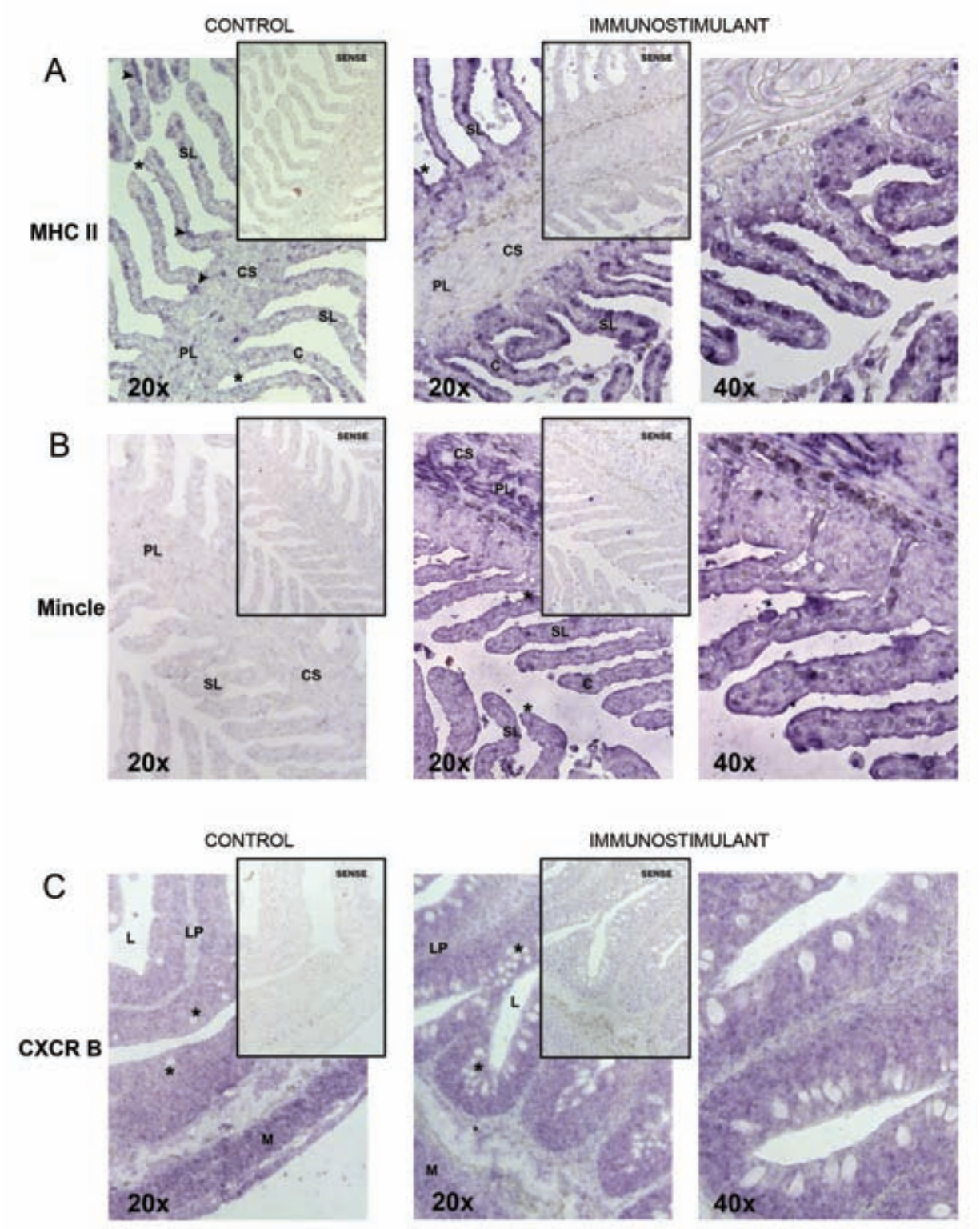
CONTROL
IMMUNOSTIMULANT
A
MHC II
SENSE
SL
CS
PL
C
20x
40x
B
Mincle
C
CXCR B
L
LP
M

Figure 3 (previous page): ISH analysis. Representative photos of several fish at 20x and 40x magnification illustrating the localization by *in situ* hybridization of mRNA expression in gills and intestine in fish fed with control diet and fish fed with immunostimulant diet. Positive reaction shows blue. Representative pictures of A: MHC II alpha chain expression in gills. In control fish hybridization signals were observed in few epithelial cells (arrowheads) in the secondary lamellae. In immunostimulant fish strong signal is evident in epithelial cells distributed across all secondary lamellae in the areas in contact with exterior and in some probable APC in secondary lamellae. B: Mincle expression in gills. In control fish no signal was detected. In fish fed with immunodiet positive hybridizations are localized across secondary lamellae in epithelial cells (PL: Primary lamellae, SL: Secondary lamellae, asterisk: mucous cells, CS: cartilaginous support, C: capillary). C: CXCR B expression in intestine. mRNA appeared in cells in the intestinal mucosal epithelium and in the membrane. Differences between diets are not significant (L: lumen, M: membrane, LP: lamina propia, asterisk: mucous cells).

Discussion

Using a salmonid-specific microarray platform enriched with immune-related genes (SFA 2.0) and *in situ* hybridizations, we investigated the effect of a commercial immunostimulant diet upon gene expression profiles in the gills and intestine which both represent major portals of pathogen entry in fish and in our animal model, the rainbow trout (*Oncorhynchus mykiss*). To the best of our knowledge, this is the first time that a transcriptomic analysis using a targeted cDNA microarray enriched for genes related to the immune system and its activation has been used to address the response at major sites of mucosal immunity to dietary immunostimulant administration.

The differential gene expression counts obtained for both tissues when combining the rank selected genes ($p<0.01$) and a fold change of >2 were 57 and 26 genes for gills and intestine respectively. If rank selected genes are used alone then the numbers of differentially expressed genes are 166 and 305 respectively. Thus the intensity of the response is higher in the gills whereas the magnitude greater in the intestine. If compared to recent studies, using the SFA 2.0 platform where fish were challenged with infectious salmon anemia virus [28] and infectious hematopoietic necrosis virus [23] the magnitude of the response, in terms of highest differential gene expression counts, to immunostimulant diets is significantly lower although is significantly higher than responses observed in the ovaries of trout challenged

intraperitoneally with bacterial lipopolysaccharide [25]. In addition, no significant tissue damage could be appreciated in any of the histological sections from the IS diet adapted fish which suggests that no long term damage is induced by the IS in the diet. This is not surprising as fish as a general rule are highly tolerant to challenge with bacterial lipopolysaccharide preparations [42]. Responses to β-glucan, the major PAMP present in commercial immunostimulant diets [2], in trout macrophages in primary culture have been shown to play a 'priming' role and in themselves are unable to induce an inflammatory response. However when applied with a bacterial LPS, β-glucans can act synergistically to increase the intensity and longevity of the inflammatory response to LPS [43].

The analysis of the gene profiles reveals that the effect of the IS diet surprisingly seems to be an overall reduction in the gene expression profiles measured (Additional files 4 and 5). Decreased transcription of individual genes in response to inflammatory stimuli have been observed for several reasons, including economy of transcriptional activity [44]. Extensive bidirectional remodelling of transcriptional processes has been reported in a number of studies addressing immunity in fish [23, 28, 45-47] and is representative of the extensive shifts in tissue and cellular function during immune system activity [48].

The majority of the DEGs (FD>2, $p<0.01$) identified in our study were tissue-specific and only 2 genes were directionally altered in both tissues (Matrix metalloproteinase 9-1 and G1/S-specific cyclin D2). Subsequent Q-PCR analyses of individual gill and intestine and total RNA samples validated targets identified by microarray analyses (Table 1). This highlighted the fact that all experimental fish were responding to the experimental treatment thus confirming the effect of IS-diet over the group and validating the design of the experiment. This specific tissue-effect of the diet in the fish likely represents direct contact of the IS compound with the intestine and indirect, cytokine-mediated, effects in the gill. However comparison of responses by functional classes revealed an overlapping of function that can not be seen at a single-gene level analysis: cell communication and signal transduction were decreased in both organs, GO categories related to structural constituent of ribosome and ribosome were increased (figures 1-2 and Additional file 3).

Many of the regulated genes on the array encode structural proteins of the extracellular matrix and the cytoskeleton (Additional files 4 and 5). In the intestine, Matrix metalloproteinase 9-1 was decreased, but GO analysis shows that the

functional category "collagen" increased (figures 1-2 and Additional file 5). In previous studies, member of the MMP gene family were induced by LPS in trout macrophages [24] and MMP9 was increased in trout leucocytes after induction with both LPS and TNFα [49]. We localized by ISH analysis MMP 9-1 and Pyrin-2 mRNAs in the primary and secondary lamellae of gills, although spatial expression patterns obtained were unable to discern a change in localization of these mRNAs (Additional file 6). The MMPs are implicated in the migration of myeloid cells and remodelling of the extracellular matrix [50]. Interactions between cells and the extracellular matrix affect cell adhesion, migration, proliferation and differentiation, and it may also serve as a binding site for the colonization of microorganisms [51]. Our data suggests that IS diets induce an extracellular matrix remodelling process and leukocyte movement in both gills and intestine which is further supported by GO analysis (figures 1-2, Additional files 3-5). Extracellular matrix remodelling and regulation of structural proteins of cytoskeleton, which are likely to be involved in the activation programs of different cell types of the immune system, have been seen in trout in stress responses [21], chemical contamination [19] and IHNV challenge [23].

Both MHC II and Macrophage C-type lectin (Mincle) were enhanced in the gills of IS diet fish (figure 3, Additional file 4). MHC class II protein is an important heterodimeric cell surface receptor involved in antigen peptide presentation during the adaptive immune response. Trout macrophages like those of mammals appear to constitutively express this molecule [52], and morphological localization has been studied in the Atlantic salmon [53]. In our experiments the MHC II alpha chain was significantly up-regulated suggesting a role for adaptive immunity after dietary immunostimulation in fish. C-type lectins possess multiple functions by recognizing carbohydrate moieties and thereby having a role in macrophages adhesion and receptor-mediated internalization of pathogens [36]. The Macrophage inducible C-type lectin (Mincle), is strongly induced in response to several inflammatory stimuli [36]. Localization of both mRNAs in the gill tissue show a significant increase in MHC class II and Macrophage C-type lectin (Mincle) bearing cells, either resident APCs such as tissue macrophages and epithelial cells, associated with the immunostimulant diet (figure 3). Therefore, the external epithelial cell layer up-regulates the expression of these receptors, or possibly macrophage and other antigen presenting cells are actively recruited to outermost cellular layer in contact with the external milieu increasing the antigen presentation capacity of the gills.

Functional groups including inflammatory response, response to bacteria, response to unfolded protein and response to biotic stimulus were reduced in intestine, whereas detection of abiotic stimulus was induced (figures 1-2, Additional file 3). ISH analysis localized both the CXC chemokine receptor transcript variant B (CXCRB) mRNA and Myeloid leukemia factor 1 (MLF1) mRNA to the epithelium and the serous membrane, whilst the lamina propia and mucous cells lack reactivity (figure 3, Additional file 6). In humans, CXCRB is a receptor for CXCL4 and also mediates the inhibitory activities of CXCL9, CXCL10 and CXCL11 on the growth of human microvascular endothelial cells (HMVEC), showing expression restricted to activated T lymphocytes [54]. CXCRB has been identified in fish and found expressed in monocytes and/or phagocyte and granulocytes, although the exact function of the receptor in fish needs to be elucidated [55]. The second gene, MLF1, could be involved in normal hemopoietic differentiation as well as in erythroid/myeloid lineage switching in humans [56] a role supported by over represented GO analysis which highlights haemoglobin complex regulation. Increased CXCR B transcription and decreased MLF1 transcription in intestine reflect regulation of chemotaxis and cellular differentiation.

Although a number of studies have been carried out to characterize responses in salmonids to IS compounds [4, 8, 9, 57] only a few of these studies have used oral administration of IS compounds which undoubtedly is the method of choice for producers. In salmon, *Salmo salar*, infected with AGD (Amoebic Gill Disease) the dietary administration of β-glucans through feed was shown to have no effect upon resistance to disease outcome [7]. Bacterial lipopolysaccharide coated feed also did not significantly reduce mortality in Atlantic salmon fry when challenged with *Vibrio salmonicida* [4]. On the other hand a number of reports demonstrate that dietary β-glucan administration increases resistance to infection over a short time periods (weeks) in the chinook salmon (*Onchorhnchus tshawytscha*), the african catfish (*Clarius gariepinus*), the Indian carp (*Labeo rohita*) and in the gilthead sea bream (*Sparus aurata*) [58-61]. Furthermore, a recent study highlighted the importance of the timing of β-glucan administration, in this case intra-peritoneal injection, upon the induced resistance to the microsporidian, *Loma salmonae*, in the rainbow trout [10] .

In our study the objective was to assess changes in molecular determinants at the portals of entry in the rainbow trout after dietary administration, subsequent challenges will be the subject of further papers. Treatment of organisms with specific

PAMPs will induce different physiological states which likely determine subsequent periods of protection. In order to provide comprehensive protection against pathogens which are problematic in aquaculture it may be recommendable to administer PAMPs which will protect against a specific pathogen group. This is the gold standard well understood in vaccination. Most published reports in which positive results have been obtained in cultured fish apply bacterial challenges after IS administration. However many reports use challenge and IS administration routes which are non-physiological and effectively 'jump' the mucosal immune barriers such as intra-peritoneal injection [62]. Such studies are useful to measure changes in the global immune response to a PAMP however may not allow for a efficacious evaluation of mucosal immunity which is key to the treatment and the survival of the organism.

In conclusion our data provides an insight into molecular determinants and biological processes which are regulated in the trout after dietary administration with an immunostimulant. In two major sites of mucosal immunity, the gills and the intestine, differential tissue-specific responses described by suites of genes were observed at the level of the transcriptome. Only two genes were found to directionally regulate in both tissues. In the gills a significant increase in antigen presenting capacity coupled to a tissue remodelling and cell recruitment response was observed.

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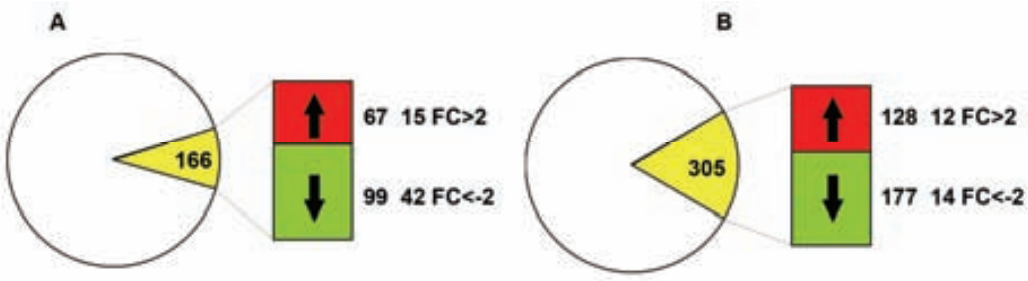
Annex: additional files

A.1 Figure 1: Total number of differentially expressed genes in fish fed with immunostimulant diet (t-student, p<0.01). A: gills. B: Intestine. (FC: fold change)



A.2 Table 1: primers used in Q-PCR

GENE	Nucleotid sequence (5'- 3')	Amplicon size
MHC class II alpha chain	Fw: CAGGTGGACCAGGAACAATC	109 bp
	Rv: GGGGTGAAGGTCAGACTGGAG	
Macrophage C-type lectin Mincle	Fw: GGAGAGAGGAGCAGACCTGGTG	190 bp
	Rv: CCCATTAGGGCCTGCATTATC	
Pyrin-2	Fw: GGAGGGCCAGGTCTCCTTCTA	147 bp
	Rv: GAGTGATGACCAGTGGGGTTG	
CXC chemokine receptor transcript variant B	Fw: GCATCAGTCTGGACCGCTACC	168 bp
	Rv: GTCTCGTCTGGTGTCCCTCAC	
Alpha-globin I-1	Fw: CTGGAGCGGAACCGGGGGCC	160 bp
	Rv: CAGCTAAGGACAAAGCCAACGTG	
Alpha-blobin I-2	Fw GGCCCCCGGTTCCGCTCCAG	190 bp
	Rv: GGCGGCAATGACCACAATTAG	
Matrix metalloproteinase 9-1	Fw: CTGTGCCACTTCCCTTTTTCC	168 bp
	Rv: TTGCTGTTTCCGTCGAATGTG	
Myeloid leukemia factor 1	Fw: CCCACTCATTCAGCTCCTCCTC	128 bp
	Rv: CAGGGACTGCCGAGTCTCCTTA	

A.3 Table 2 (next page): Functional Gene Ontology categories. Gene Ontology analysis in gills (A) and intestine (B) that discriminate between control fish and fish fed with immunostimulant diet for 4 weeks. Functional categories compared pairwise by the sums of ranks of differentially expressed genes (p<0.01) Significance was determined with Student's t test (p<0.05).

A

GO category	Differential expression	PStudent
Synaptic transmission	-1.69516	0.000152
Transmission of nerve impulse	-1.69516	0.000152
Cell communication	-0.92701	0.000237
Structural molecule activity	0.802854	0.000599
Structural constituent of ribosome	0.655653	0.00098
Ribosome	0.654144	0.001911
Signal transduction	-0.76292	0.00989
Protein biosynthesis	0.516239	0.010422
Mitochondrial electron transport chain	0.612715	0.011318
Actin cytoskeleton	0.616127	0.019227
Carbohydrate metabolism	-1.96057	0.037048
Carrier activity	0.675427	0.040974
Primary active transporter activity	0.675427	0.040974

B

GO category	Differential expression	PStudent
Anion transport	0.363803	0.000521
Cell communication	-0.57042	0.000855
Nucleoside-triphosphatase activity	-0.47231	0.002002
Collagen	0.381303	0.002475
Pprotein kinase cascade	-0.43712	0.003369
Signal transduction	-0.58741	0.003665
I-kappaB kinase NF-kappaB cascade	-0.55417	0.005274
Cytoskeleton	-0.41082	0.005333
Detection of abiotic stimulus	0.41402	0.006258
RNA-dependent DNA replication	-0.48302	0.007138
Structural constituent of ribosome	0.191666	0.010532
Response to bacteria	-0.72198	0.012508
Chymotrypsin activity	0.40635	0.01341
Trypsin activity	0.40635	0.01341
Mitochondrial electron transport chain	0.305792	0.015209
Regulation of I-κB kinase NF-κB cascade	-0.50469	0.024866
Carbohydrate catabolism	-0.41545	0.026199
Glycolysis	-0.41545	0.026199
Response to unfolded protein	-0.28883	0.027038
Carbohydrate binding	-0.68816	0.029371
Response to biotic stimulus	-0.18525	0.032321
Actin cytoskeleton	-0.43042	0.032966
Muscle contraction	-0.33704	0.041056
Cell-cell signalling	-1.15673	0.044975
Ribosome	0.171008	0.046657
Protein polymerization	-0.28946	0.046939
Inflammatory response	-0.63881	0.048465

A.4 Table 3: Differentially expressed genes in gills. Differentially expressed genes with fold chage >2 in gills of fish fed with immunostimulant diet. Differential expression was analyzed with Student's t-test ($p<0.01$). Values are expressed as fold change, FC. A: genes up-regulated. B: genes down-regulated.

A

Clone ID	P_Student	FC	Gene name
HKT0001_B03	0.000194	10.713	Alpha 2 type I collagen-1
CA379977	8.93E-07	7.811	MHC class II alpha chain
HK0002_B08	1.64E-05	4.326	Unknown-166
HK0002_G02	0.006904	4.119	Creatine kinase, sarcomeric mitochon. precursor
HK0003_C02	3.62E-06	3.800	Collagen alpha 1(I) chain-1
CA348066	0.006197	3.711	Macrophage C-type lectin Mincle
HK0001_G02	0.000433	3.657	ATP synthase beta chain-2
HK0001_H06	0.001413	3.614	40S ribosomal protein S7
CA384134	0.00446	2.599	G1/S-specific cyclin D2
HK0001_H08	0.007523	2.411	Gastrointestinal glutathione peroxidase 2
HK0002_F06	0.003104	2.169	Histone 3A-ATP synthase F0 6
HKT0001_H06	0.000275	2.156	60S ribosomal protein L18
HK0002_C04	9.78E-07	2.149	Apolipoprotein E-2
CA366489	0.008404	2.077	Multidrug resistance protein 3-1
HK0002_A01	0.005092	2.043	Unknown-163

B

Clone ID	P_Student	FC	Gene name
EST1-3A_G09	0.009433	-2.118	Unknown-75
est03d09	0.0036	-2.147	Unknown-40
EXOB1_E09	0.000235	-2.165	Transposase-57
EST1-3A_F04	0.000464	-2.175	ReO_6-2
EXOB3_E03	0.0001	-2.255	Unknown-114
EXOB3_F04	0.00035	-2.303	Unknown-119
CA365034	3.06E-05	-2.322	Transposase-42
CA353086	1.77E-06	-2.328	Unknown-279
CA343327	0.00305	-2.344	Transposase -61
EST1-3A_E05	0.005629	-2.462	Hpa repeat-2
HK0003_C01	0.001736	-2.521	Unknown-188
EXOB2_H11	0.001043	-2.526	Glucose-6-phosphatase
est01c09	0.003462	-2.560	Unknown-12
EXOB1_A08	0.005199	-2.658	Mitochondrial ribosomal protein L4, isoform a
EXOB2_A02	3.18E-05	-2.788	Nucleoside diphosphate kinase, mitochon. prec.
CA383564	0.000577	-2.869	Coatomer epsilon subunit 1
CA344798	0.007519	-2.913	T-cell surface glycoprotein CD3 zeta chain

KVkm2_A12	0.001085	-2.929	Unknown-220
EST1-3A_D05	0.000995	-2.930	Unknown-70
CA342227	0.004281	-2.945	Protein tyrosine kinase 2 beta
CA384513	0.009316	-2.996	MAPK/ERK kinase kinase kinase 4-2
CA384559	0.005942	-3.032	B-cell receptor CD22-2
CA349456	0.000457	-3.237	Mitogen-activated protein kinase 1
CA369440	0.000156	-3.239	NAD(P)H menadione oxidoreductase 1, dioxin-inducible
EXOB1_F08	0.003404	-3.249	Unknown-88
HK0001_D09	0.00314	-3.365	Cytochrome P450 2F1
EXOB3_D08	8.48E-05	-3.397	Histone H33-2
CA366296	0.002442	-3.604	Src kinase-associated phosphoprotein 55-related protein
EXOB3_H01	0.000299	-3.658	Matrix metalloproteinase-13
CA366512	0.000959	-3.660	Alcohol dehydrogenase [NADP+]
CA377287	0.000797	-3.714	MAPK/ERK kinase kinase kinase 3
EXOB1_D10	3.95E-05	-3.739	Unknown-85
est04a05	0.000963	-3.805	Ectonucleotide pyrophosphatase/phosphodiesterase 1
Hete0002_E09	0.000122	-4.673	Transposase-56
EXOB2_F03	0.000784	-5.198	Nuclear cap binding protein subunit 2
CA372761	0.001548	-6.021	Pyrin-2
CA342769	0.000797	-6.129	Matrix metalloproteinase 9-1
EST1-3A_D08	0.003688	-6.377	Glyceraldehyde-3-phosphate dehydrogenase-6
CA388340	0.002045	-6.660	Beta-2-glycoprotein I
EXOB1_C06	0.001177	-8.692	Nucleolar protein Nop56-1
utu04f03	0.008994	-11.969	NB thymosin beta
EXOB4_G05	0.001667	-53.083	10 kDa heat shock protein, mitochondrial

A.5 Table 4: Differentially expressed genes in intestine. Differentially expressed genes with fold change >2 in intestine of fish fed with immunostimulant diet. Differential expression was analyzed with Student's t-test ($p<0.01$). Values are expressed as fold change, FC. A: genes up-regulated. B: genes down-regulated.

A

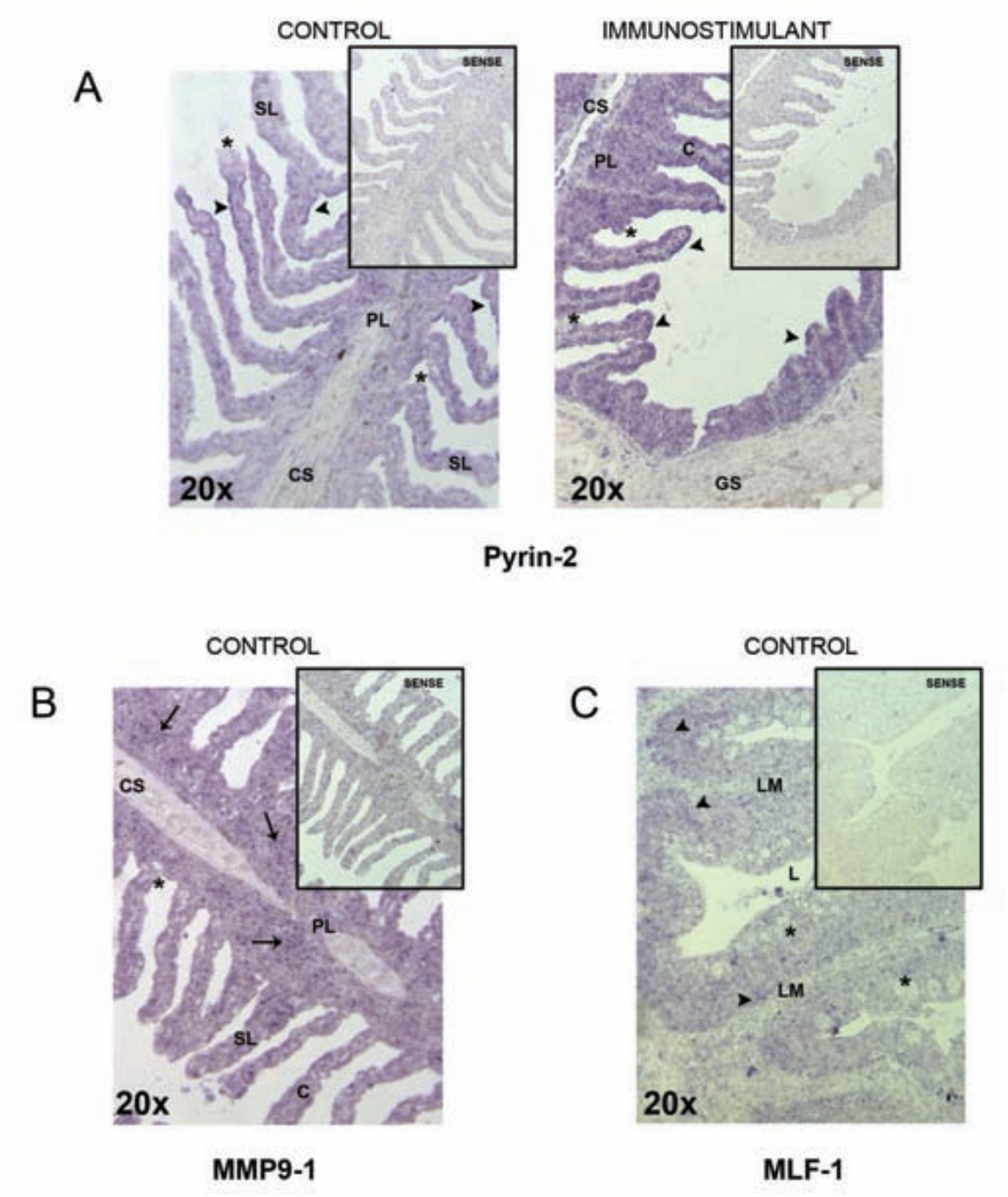
Clone ID	P_Student	FC	Gene name
KVkm2_F12	0.002926	23.101	Basic leucine-zipper protein BZAP45-2
EXOB3_C10	0.00397	3.932	G1/S-specific cyclin E1
CA374698	0.001035	3.852	High-glucose-regulated protein 8
CA368411	0.003627	3.102	Phosphatidylethanolamine N-methyltransferase, isoform 1
Hete0002_A07	2.16E-07	2.428	Metallothionein-IL
EST1-3A_E12	0.004607	2.411	Hypothetical-fish 3
EST1-3A_H08	0.003831	2.361	Sp1 transcriptional activation factor

EXOB1_G05	0.007891	2.328	Unknown-89
CA343700	0.005745	2.079	CXC chemokine receptor transcript variant B
EXOB1_B08	0.009488	2.076	Unknown-82
CA384134	2.02E-09	2.058	G1/S-specific cyclin D2
CA344820	0.009753	2.019	Baculoviral IAP repeat-containing protein 2

B

Clone ID	P_Student	FC	Gene name
CA347787	2.13E-09	-2.024	Transposase-15
CA368764	1.13E-05	-2.069	Xaa-Pro aminopeptidase 2
CA342769	0.004248	-2.123	Matrix metalloproteinase 9-1
CA362691	0.001193	-2.177	N-myc downstream regulated protein-1
CA349657	0.000118	-2.327	Ah receptor interacting protein
CA366462	0.002308	-2.677	Apoptosis regulator Bcl-X
CA376673	0.000788	-2.708	Pre-B cell enhancing factor
utu04f08	0.000146	-2.934	Actin, alpha skeletal 5
HST0001_F03	0.004985	-3.570	Unknown-215
CA374085	0.009425	-3.665	Myeloid leukemia factor 1
EXOB1_G12	0.000524	-3.868	Thioredoxin
CA377768	0.004477	-6.417	Phospholipase D1
CA363521	0.007041	-6.826	Coatomer beta subunit
utu02d09	0.005203	-34.916	Myosin regulatory light chain MRCL2-2

A.6 Figure 2 (next page): ISH Analysis. Representative photos of several fish at 20x magnification illustrating the localization by *in situ* hybridization of mRNA expression in gills and intestine in fish fed with control diet and fish fed with immunostimulant diet. Positive reaction shows blue. A: Pyrin-2 expression in gills in control fish and in fish fed with immunodiet. Positive hybridizations are localized across primary and secondary lamellae. Stronger hybridization signals were observed in epithelial cells at the extreme of secondary lamellae (arrowheads). B: MMP9-1 expression in gills fed with control diet. Positive cells are distributed across primary and secondary lamellae, more abundant in interlamellar cells (arrow) (PL: primary lamellae, SL: secondary lamellae, GS: gill support, asterisk: mucous cells, CS: cartilaginous support, C: capillary). C: MLF-1 expression in intestine of trout fed with control diet. Strong signal is present in the epithelium near the lamina propia (arrowhead) (L: lumen, LP: lamina propia, asterisk: mucous cells).


A
CONTROL
IMMUNOSTIMULANT
SENSE
SL
PL
CS
C
GS
20x
Pyrin-2
B
CONTROL
MMP9-1
C
CONTROL
LM
L
MLF-1

6 The effects of immunostimulation through dietary manipulation; evaluation of haematopiesis and immune response

Abstract

Following the study of the effect of immunostimulation through dietary administration of PAMPs at the portals of entry of fish, here we report the evaluation of transcriptomic regulation in two organs which act in the regulation of the immune mechanisms of fish, head kidney and spleen.

Immunostimulants (IS) have been used as feed additives for many years in aquaculture, and yeast β-glucan may be the most utilized. IS-diets were previously thought to activate the innate immune system by increasing non-specific defence mechanisms, such as inflammation, phagocytosis or pathogen killing, therefore information is available in several reports describing the effects of β-glucan on direct disease challenge or classical immune activation parameters such as lysozyme and complement activity, respiratory burst or phagocytic activity. Surprisingly, no approach has been performed to elucidate the effects of dietary administration of β-glucans in the transcriptomic regulation of the processes involved in immune activation modulated by head kidney and spleen, and no attention has been paid to the potential effects of immunostimulants in the hematopoietic activity in the head kidney.

Using the salmonid-specific microarray platform SFA 2.0, which is enriched with immune-related genes, and localization of selected genes by *in situ* hybridization (ISH) analysis, dietary acclimation of rainbow trout (*Oncorhynchus mykiss*) was evaluated in 2 organs relevant to immunity, head kidney and spleen. A larger reduction of genes and GO categories rather than induction was obtained for both tissues, though head

kidney was weakly affected and major changes in gene expression were observed in spleen. ISH analysis was performed to further analyze the role of IS-diets, thus mRNA distribution of CXCR4 and PU.1, involved in haematopoiesis activity, were monitored in head kidney, whereas MHC II, CASP8 and FADD-like apoptosis regulator precursor and Cathepsin D-1 were studied in spleen. Transcriptomic profiles and ISH analysis revealed that administration of a commercial immunostimulant diet for four weeks induced differential expression profiles in both organs.

Introduction

The use of Immunostimulant-containing diets (IS diets) has been widely accepted in aquaculture in order to enhance the resistance of cultured fish and invertebrate species to disease and stress. An immunostimulant is a naturally occurring composite which has a modulatory effect upon the immune system, thus the components used as immunostimulants range from chemicals such as Levamisole[1], bacterial lipopolysaccharides [2], nucleotides (for review see; [3, 4]), a range of polysaccharides, animal and plant extracts, specific dietary nutrients such as Vitamin C and certain hormones and cytokines (for review see [5]). Some of these components are conserved components produced by microbial pathogens, commonly called PAMPs, pathogen associated molecular patterns. PAMPs interact with a diverse set of cellular receptors known as PRR, pathogen recognition receptors, activating distinct transcriptomic programmes which result in the cellular/tissue response [6, 7]. Thus, IS diets containing PAMPs should induce a PAMP-PRR host response and increase the activity of the immune system in fish.

One of the earliest applications of immunostimulants in aquaculture was the use of glucans in salmon diets, as it was believed that IS-diets were effective treatment for stressful events and also had some benefit in reducing sea lice settlement [8]. Currently, dietary-immunomodulation is a commonly accepted practice in both salmonid and non-salmonid species. It is thought that IS-diets act mainly upon the innate immune system by enhancing non-specific defence mechanisms, such as phagocytic cell activity, pathogen killing, lymphocyte activation or antibody production [5]. Commercial immunostimulant diets are mainly limited to the addition of β1-3 and β1-6 glucans, although the efficacy of the enteral formulations based on

dietary administration to reduce infectious processes in salmonid fish remains questionable.

A number of studies have addressed the potential benefits of commercial IS-diets in salmonid fish, mainly based upon *in vivo* (enteral formulations or intra-peritoneal administration) experimentation subsequently followed by classical analysis of immune activation parameters such as lysozyme and complement activity, respiratory burst, phagocytosis and inflammatory gene expression in different tissue samples [2, 9-11] or by direct disease challenge [9, 12]. Whereas several works reported a consistent trend to increased activation of innate immune system [13, 14], also toxicity processes and negative effects have been reported [15, 16].

Head kidney and spleen are two of the main organs with a key role in the immune system of fish. The head kidney possesses hematopoietic functions [17, 18], contains phagocytic cells [19] and is responsible in part of antigen processing and formation of IgM and immune memory [20, 21]. The lymphoid parenchyma of head kidney comprises a rich population of leukocytes, including lymphocytes and macrophages. Accumulations of melanomacrophages are prominent in the hematopoietic tissue and these cells have been shown to retain antigen for long periods [22]. Although it is also an important endocrine organ, homologous to mammalian adrenal glands, releasing corticosteroids and other hormones [23], it has been well characterized for immune activity [21].

The spleen is one of the main peripheral lymphoid organs [24]. It can be divided into a red pulp, a reticular cell network including populations of erythrocytes and macrophages, and white pulp, containing melanomacrophage accumulations and ellipsoids [25]. Melanomacrophage centers of the spleen are major sites of erythrocyte destruction and have been considered to be metabolic dumps [26]. Ellipsoids appear to have a specialized function for plasma filtration and the trapping of blood-borne substances, particularly immune complexes [27].

The utility of microarray analysis to investigate immune mechanisms of fish has been widely demonstrated. SFM 1.0 and SFA 2.0 targeted cDNA arrays have been repeatedly used to analyze several immune responses in salmonid fish [28-37]. The aim of the present work was to address changes in the transcriptome induced by a commercial immunostimulant diet in two organs relevant in the immune system of fish: the head kidney and spleen. Differential gene expression is induced with an IS diet and furthermore we localize differentially expressed genes identified by

microarray analysis in both tissues by *in situ* hybridization which contribute to important immune functions such as inflammation, defence response and hematopoietic activity.

Material and Methods

Diets, fish and feeding trial

Same diets, fish and feeding trial of chapter 5 were used for head kidney and spleen analysis. Fish were killed in water with a lethal concentration of MS-222 (Sigma), 100 ppm, stage III of anaesthesia [38], and head kidney and spleen were collected. Tissues removed for RNA extraction were frozen in liquid nitrogen and stored at -80 ºC. Tissues sampled for ISH were fixed in neutral buffered formalin (4%, pH 7.2), embedded in paraffin and cut in 10 μm sections.

Microarray analysis

Platform, RNA extraction, hybridization and microarray analysis are described in chapters 4 and 5. Results were submitted to NCBI GEO repository, accession number: **GSE11949**. After subtraction of mean background, LOWESS normalization [39] was performed. To assess differential expression of genes, the normalized log intensity ratios were analyzed with Student's t-test ($p<0.01$) and genes were ranked by log(p-level). The Bayesian modification to the false discovery rate (FDR) was used to correct for multiple comparison tests, estimating the *q*-value for the set of differentially expressed genes [40].The functional categories of Gene Ontology [41] were compared with regulated genes ($p<0.01$) by the sums of ranks (Student's t-test, $p<0.05$) and the statistical significance of over represented functional categories was assessed using the Yates correction to Chi square test (corrected $p<0.05$).

Real-time PCR

In order to verify microarray results, real time PCR (Q-PCR) was carried out. Real-time protocol was performed as discussed in chapter 5. Primers used are shown in additional file 1. Values for each sample were expressed as "fold differences", calculated relative to control diet and normalized for each gene against those obtained

for 18S. In parallel, Q-PCR was performed with the individual samples in order to test biological variability. Thus, microarray results and Q-PCR from pools results were confirmed by means of real time PCR from RNA tested on individual fish in each group. 4 μg of RNA from each fish was used to synthesize cDNA and Q-PCR was performed as described above. Expression of each gene was normalized to that obtained for 18S and fold changes were arbitrarily set to one of the fish. Values were transformed to logarithmic data to obtain homogeneity of variances (except for MHC II, where variances were homogeneous). Differences between IS-fed fish and control fish were analyzed with Student's t test ($p<0.05$, $n=8$ each group). Transcripts were sequenced to ensure amplification was specific (chapter 4 and 5).

In situ hybridization

In situ hybridization was performed to characterize the mRNA distribution of some interesting differentially expressed genes. MHC II alpha chain, Cathepsin D-1, and CASP8 and FADD-like apoptosis regulator precursor were monitored in spleen, and PU.1 and CXCR4 were monitored in head kidney. Hybridizations of each gene were performed in several fish.

Probes were amplified from clones of a cDNA library (INRA-SCRIBE, Rennes, France) accession numbers BX081858 (MHC II), CU068018 (Cathepsin D-1), BX872761 (PU.1) and CU068212 (CXCR4). CASP8 and FADD-like apoptosis regulator precursor probes were amplified with specific primers (Additional file 1) from cDNA, (60ºC, 30 cycles) and cloned into a bacterial plasmid vector (pGEM-T Easy Vector, Promega). The DNA insert was sequenced in order to ensure identity and insert orientation. Probes were amplified using a PCR amplification of clones (Jump Start system, Sigma) followed by a PCR product purification using a Microcon PCR filter kit (UFC7PCR50, Millipore). Sense (S) and Antisense (AS) riboprobes were generated using Riboprobe in vitro Transcription Systems (Promega) according to manufacturer's instruction, with 0.35 mM digoxigenin-UTP (Roche). The RNA probes were kept –80ºC until use. Hybridization protocol is described in chapter 5.

Results

In order to evaluate the gene expression profiles of rainbow trout fed with immunostimulant diets, a salmonid-specific microarray platform enriched with immune-related genes was used, and the organs with relevant function in the immune system, head kidney and spleen were studied. Head kidney was not highly affected, showing a total of 32 differential expressed genes ($p<0.01$), of which 13 genes were up-regulated and 19 were down-regulated. Of the genes induced, 2 possessed a fold induction >2; of the genes repressed, 2 had a fold repression >2 (Additional file 2). However, diets caused a stronger effect in the gene expression profiles in spleen: a total of 168 genes ($p<0.01$) showed a differential expression level, of which 73 genes were increased and 95 repressed. Of the genes induced, 10 possessed a fold induction >2; of the genes repressed, 27 had a fold repression >2 (Additional file 2).

The differentially expressed genes found ($p<0.01$) were analyzed by comparison in the GO functional categories ($p<0.05$), results are shown in figure 1 and additional file 3. In head kidney, the GO categories I-kappaB kinase NF-kappaB cascade, Intracellular signalling cascade and Protein kinase cascade were increased, whereas Structural molecule activity, Structural constituent of ribosome, Protein biosynthesis and Ribosome were decreased. The over representation analysis of functional categories in the platform, which highlight the significantly regulated GO categories compared with their total representation in the platform, shows that such processes are significantly representative ($p<0.05$). In addition, other groups as chemokine binding, lysosome or Protein homodimerization activity, containing few genes, are also representative processes (figure 2). Applying a selection criteria of fold change (FC) >2, 4 genes were found to be modulated (Additional file 4). Nucleolysin TIAR (2.2 FC), a protein involved in apoptosis and defence response is a member of a family of RNA-binding proteins and possesses nucleolytic activity against cytotoxic lymphocyte target cells, and Transposase-3 (2.1 FC), which recombine DNA segments, as up-regulated. On the other hand, Chemokine receptor CXCR4 (-19.8 FC), a chemokine receptor involved in haematopoiesis, and 78 kDa glucose-regulated protein precursor (-7.2 FC), with a function of folding and assembly of proteins in the endoplasmic reticulum, were down-regulated.

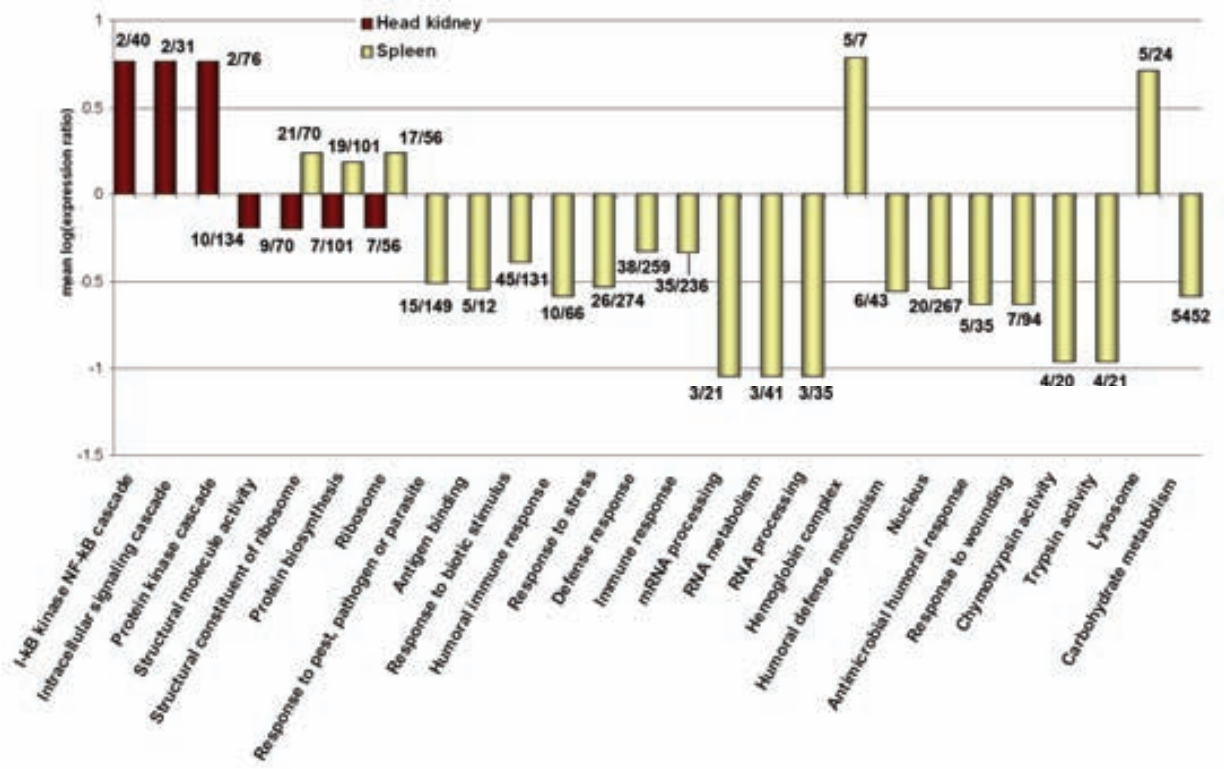


Figure 1: **Functional categories of Gene Ontology in head kidney and spleen**. Different responses in GO groups were observed in control fish and fish fed with immunostimulant diet for 4 weeks. Differentially expressed genes ($p<0.01$) were grouped by Gene Ontology categories and mean log(expression ratio) were analyzed by Student's t-test ($p<0.05$). The number of differentially expressed genes and genes for each category on the microarray are shown in the bars.

Many more functional categories and genes were differentially modulated in spleen. Thus, groups related to Ribosome and Protein biosynthesis, the Haemoglobin complex or Lysosome were enhanced. However, large amount of categories were reduced, including GO groups related to the immune system (Response to pesticide, pathogen or parasite, Antigen binding, Response to biotic stimulus, Humoral immune response, Response to stress, Defence response, Immune response, Humoral defence mechanism, Antimicrobial humoral response, Response to wounding), groups involved in RNA processing and groups with function in metabolism and trypsin activity (figure 1 and additional file 3). In addition, other groups related to metal homeostasis, peptidase activity or cell wall catabolism in spleen, with a smaller number

of genes, are also representative as indicated by the over-representation analysis (figure 2).

The analysis at a single-gene level ($p<0.01$) shows the up-regulation of genes which translate to proteins involved in apoptosis, such as CASP8 and FADD-like apoptosis regulator precursor (CFLAR, 25.5 FC) and Receptor-interacting serine/threonine-protein kinase 2 (2.2 FC), the proteins with proteolytic and peptidolytic function Cathepsin D-1 (7.1 FC) and Cathepsin D-2 (3.1 FC) and proteins involved in oxygen transport, such as Heme oxygenase-1 (2.9 FC), Alpha-globin 1-3 (Hemoglobin alpha chain, 2.7 FC) or Beta-globin (Hemoglobin gamma-1 chain, 2.0 FC). Within the down-regulated group, Heat shock proteins as HSP 90-beta-2 (-6.2 FC) and 60 kDa heat shock protein-1 (-3.2 FC); Nucleolysin TIA-1 (member as nucleolysin TIAR-1 of a RNA-binding protein family with apoptotic activity and nucleolytic activity against cytotoxic lymphocyte target cells), was reduced (-6.0 FC); proteins involved in tissue remodelling and cytoskeleton as Matrix metalloproteinase-13 (-4.4 FC), Plasminogen precursor-1 (-3.1 FC), Actin alpha skeletal 4 (-2.3 FC) or Dynein light chain 2 (-2.3 FC); Immunoglobulin receptors, as High affinity immunoglobulin epsilon receptor alpha (-3.0 FC) and High affinity immunoglobulin gamma Fc receptor I precursor (-2.1 FC), and several genes involved in RNA processing (Additional file 5).

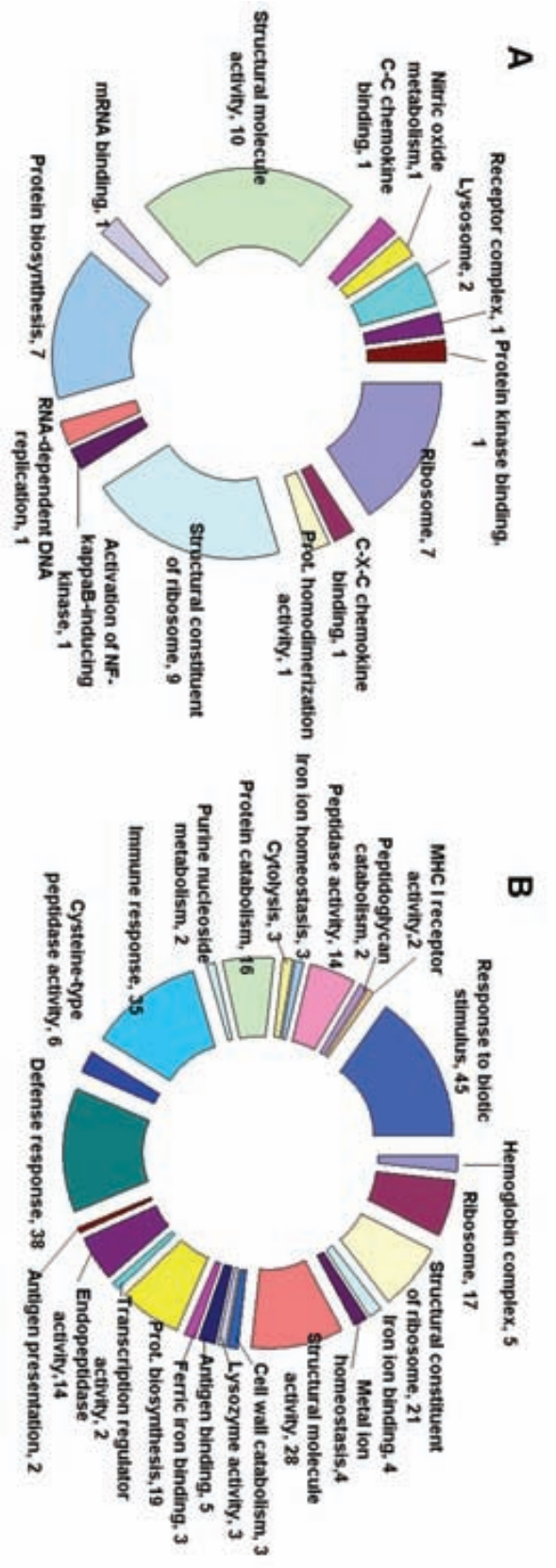


Figure 2: Over representation of Gene Ontology functional categories in head kidney (A) and spleen (B) of fish fed with immunostimulant diet. Differentially expressed categories were analyzed with Yates corrected Chi squared ($p<0.05$). The number of differentially expressed genes for each group is shown in parenthesis.

Microarray expression data were confirmed using real-time quantitative Q-PCR (table 1). The expression of selected genes was quantified in the same pools of RNA analyzed on the microarray and then subsequently in the individual samples (n=8) to further investigate the biological variability between individuals used to constitute pools. The results obtained from the pools, showing a similar profile in both microarray quantification and Q-PCR, validate and reflect data obtained from the microarray analyses. In the individual fish samples the means of fold change for MHC II alpha chain, and cathepsin B-2 in spleen, and Nucleolysin TIAR and IRAK-1 in head kidney were significantly different (Table 1, $p<0.05$), in agreement with the previous results. Thus the data obtained from pooled analyses were due to the effects of the IS diets within the population as shown by the low variance between individuals.

Organ	Gene name	Microarray FC	QPCR FC pools	Mean FC control fish ± SEM	Mean FC IS-fish ± SEM	P (t-test)
Spleen	**MHC II**	1.62	4.90	5.433	11.945	0.040
Spleen	**Alpha-globin I-1**	1.28	1.24			
Spleen	**Cathepsin B-2**	1.39	3.27	-0.409	0.450	0.047
Spleen	**Cathepsin D-1**	7.15	2.45			
Spleen	**CASP8/FADD-like apoptosis reg. prec.**	25.49	1.84	2.478	3.807	No sign.
Head kidney	**Nucleolysin TIAR**	2.25	2.35	-0.131	0.529	0.028
Head kidney	**IRAK-1**	1.69	4.95	0.135	0.756	0.036
Head kidney	**Cathepsin B-2**	1.41	1.35			

Table 1: Q-PCR validation for microarray results. Selected genes expressions were analyzed by SYBR Green Q-PCR analysis. For pooled samples, data are presented as fold change of fish fed with immunostimulant diet relative to control fish. For individual samples, fold changes were arbitrarily set to one of the fish and data are presented as mean fold change of fish fed with immunostimulant diet or with control diet ± standard errors of the mean (mean ± SEM). Values were transformed to logarithmic data to obtain homogeneity of variances (except for MHC II, where variances were homogeneous). Differences between IS-fed fish and control fish were analyzaed with the individual samples using Student's test ($p<0.05$, n=8 each group). Values are from triplicate measurements.

From the results obtained from the microarray analysis, some genes were selected to analyze their localization in both tissues by ISH to further understand the role of Immunostimulant diets. Two genes were chosen to investigate their distribution in head kidney and three genes were chosen in intestine. Therefore, mRNA distribution of CXCR4, down-regulated in fish fed with IS-diet (Additional file 4), and PU.1, not detected in microarray analysis but chosen for its role in haematopoiesis [42], were monitored in head kidney. MHC II, CASP8 and FADD-like apoptosis regulator precursor and Cathepsin D-1, whose expression was increased in animals fed with immunostimulant diet (Additional file 5), were studied in spleen.

In situ hybridisation revealed the presence of scattered CXCR4 and PU.1 positive cells in the haematopoietic and endothelial cells of head kidney (figure 3). Furthermore, PU.1 distribution included clusters of haematopoiesis showing strong signal. mRNA expression was not detected in endocrine cells, the adrenal equivalent of mammals in teleosts. Melanomacrophage centers (MMCs) are distributed across the tissue, but detection of CXCR4 or PU.1 in these cells is difficult to validate due to the high amount of cytoplasmic vacuoles filled with dark-brown dense pigment granules in the cells. A significant difference in abundance, distribution and intensity of CXCR4 and PU.1 signal was seen between fish fed with control diet and fish fed with immunostimulant diet (Figure 3), with a clear reduction in the signal in head kidney of fish fed with IS-diet.

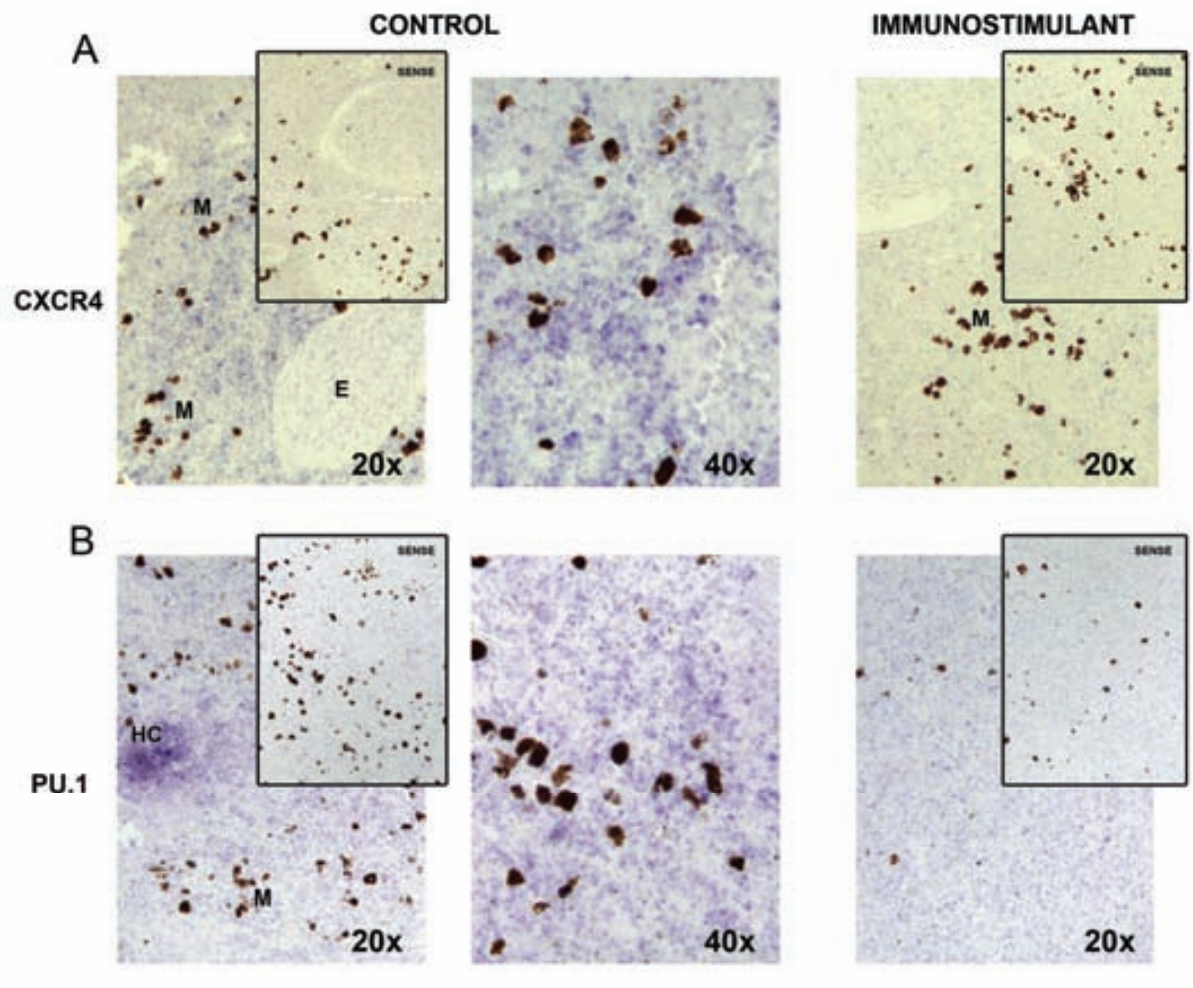
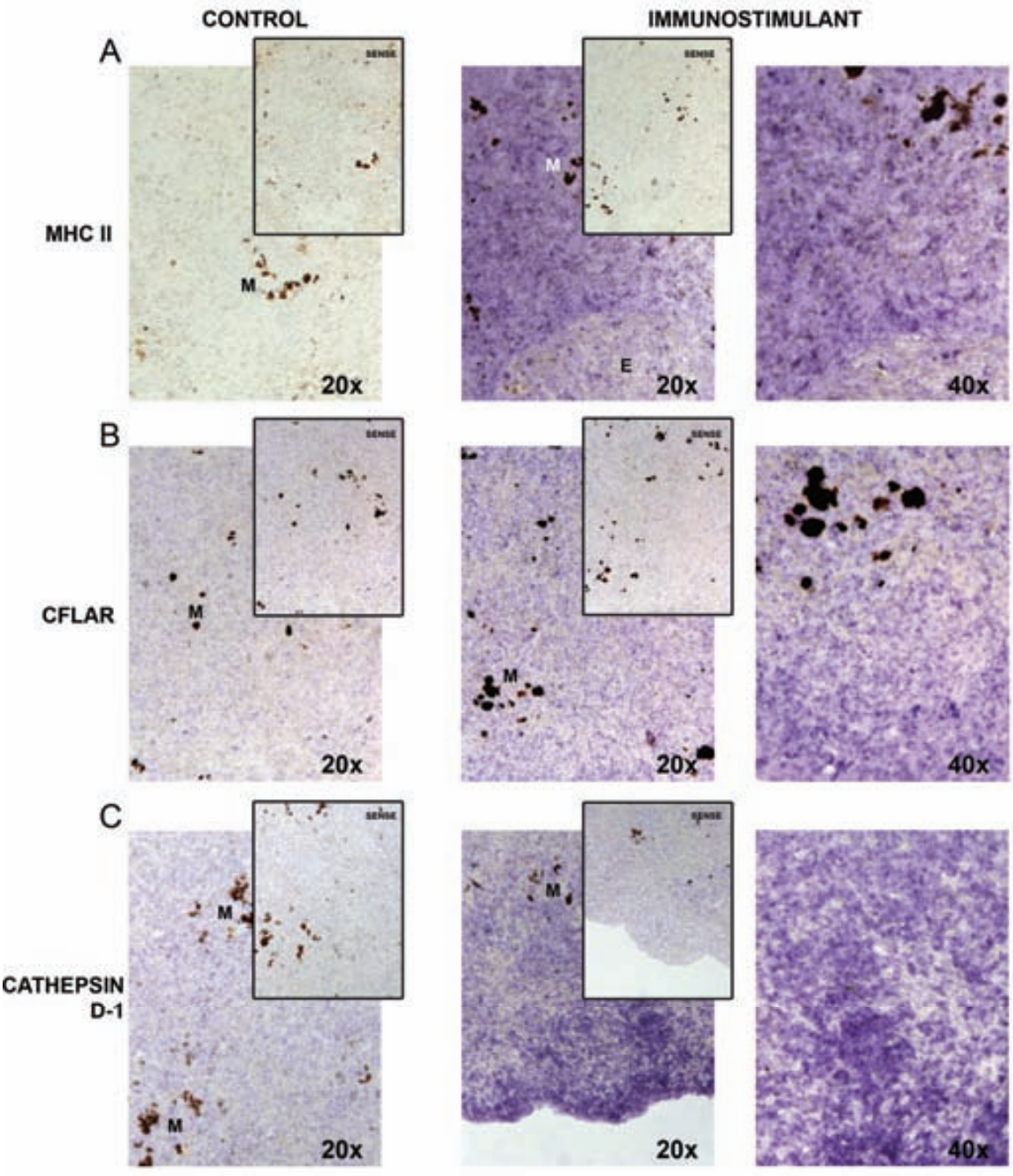


Figure 3: ISH analysis. Representative pictures of several fish at 20x and 40x magnification illustrating the localization by *in situ* hybridization of mRNA expression in head kidney of fish fed with control diet and fish fed with immunostimulant diet. Positive reaction shows blue. Pictures are representative of independent hybridizations with different fish. A: CXCR4. Positive cells were distributed in the head kidney of control fish throughout the parenchyma of the organ. A cluster of erythrocytes can be seen in the right part of the picture, showing no staining. In IS-diet fish cells showing hybridization signal were not present. B: PU.1. In control fish expression was detected in cells scattered across the tissue. A hematopoietic cluster showing strong signal can be observed. In fish fed with immunodiet positive hybridizations were not detected (M: melanomacrophage center, E: erythrocytes, HC: hematopoietic cluster).

In situ hybridisation in spleen (figure 4) shows that, although the boundary between white pulp (lymphocyte-rich) and red pulp (erythrocyte-rich) portions in fish is not clear [43], MHC II alpha chain mRNA signals seem to be in cells across the

white pulp. CASP8 and FADD-like apoptosis regulator precursor (CFLAR) and Cathepsin D-1 positive signals were identified in cells scattered in the splenic pulp. Erythrocytes were unstained and MMCs were present throughout the tissue but, as in head kidney, signal in these cells can not be distinguished. An increase in MHC II alpha chain, CFLAR and Cathepsin D-1 bearing cells was detected associated with the diet containing immunostimulants (figure 4), in agreement with the previous microarray and Q-PCR data.

Figure 4 (next page): ISH analysis. Representative pictures of several fish at 20x and 40x magnification illustrating the localization by *in situ* hybridization of mRNA expression in spleen of fish fed with control diet and fish fed with immunostimulant diet. Positive reaction shows blue. Pictures are representative of independent hybridizations in different fish. A: MHC II alpha chain. mRNA expression was not detected in control fish, whereas positive cells were distributed throughout the tissue in fish fed with IS-diet. Note that erythrocytes were unstained. B: CASP8 and FADD-like apoptosis regulator precursor (CFLAR). mRNA signals were detected in cells scattered in the splenic pulp in fish fed with immuno-diet. Staining was weak in spleen of control fish. C: Cathepsin D-1: mRNA signals are present in cells across the pulp in fish fed with immunostimulants, expression was weak in control fish (M: melanomacrophage center, E: erythrocytes).


CONTROL
IMMUNOSTIMULANT
A
MHC II
B
CFLAR
C
CATHEPSIN D-1
SENSE
M
E
20x
40x

Discussion

The aim of the present study was to analyze the global effects of immunostimulant diets in the gene expression patterns in the organs known to possess important roles in the immune system of rainbow trout. Head kidney is the major hematopoietic organ in fish [44] and has been well characterized for immune activity [21], whereas spleen is one of the main peripheral lymphoid organs [24] but surprisingly relatively few studies have investigated it. Transcriptomic profiles and ISH in head kidney and spleen of fish fed for four weeks with a commercial immunostimulant diet were compared with fish fed with a control diet in order to analyze the general physiological/immunological response of fish to immuno-diets.

The diet containing immunostimulants induced differential expression profiles in both head kidney and spleen. The global effect of the IS-diet in these two immune organs was similar to those results obtained for the portals of entry of fish (Doñate et al., accepted in BMC genomics; see chapter 5): a larger reduction of genes and GO categories rather than induction was obtained. However, genes and functional classes showed different or even opposite responses in the two tissues examined. Head kidney was weakly affected: applying the selection criteria of FC value >2 ($p<0.01$) to address the most marked changes, only 4 genes were regulated (two with FC>2 and 2 with FC<2) (Additional file 2 and 4). Major changes in gene expression were observed in spleen, where 10 genes with a fold change >2 and 27 genes with a fold change <-2 were found (additional file 2 and 5). Many more functional GO categories were also modulated in spleen (figure 1), showing that IS-diet caused a strong effect in the immune activity of spleen but did not elicit a drastic change in transcripomic profiles of head kidney. The over represented functional categories (Figure 2), analyzed using the Yates correction to Chi square test (corrected $p<0.05$), supported the GO results revealing that more over-represented categories were identified in spleen. The global down-regulation obtained did not affect the both organs in the same manner: GO categories related to ribosome and synthesis of proteins, decreased in head kidney, were some of the few categories increased in spleen. These results highlight the tissue-specific effect of the IS-diet.

Microarray data showed that there was no common differentially expressed genes for both organs, though Nucleolysin TIAR was up-regulated in head kidney (2.25 FC) and Nucleolysin TIA-1, TIA-1-related protein, was down-regulated in

spleen (-6.01 FC). The Over representation of GO functional categories in head kidney and spleen, which show the significant regulated GO categories ($p<0.05$) analyzed by their total representation in the platform revealed that the most significantly representative processes on the platform did not overlap in both tissues (figure 2). Therefore, all the data of the single gene analysis, the GO analysis and the over-representation GO analysis revealed that the effect of Immunostimulant diets was a down-regulation and was tissue-specific, inducing different transcriptional programs in the two organs analyzed. This response is not totally surprising because in fish, different organs (including immune related organs) display different gene profiles, under normal situation [45] and also following application of bacterial vaccine [46] or bacterial infection [47]. Furthermore, the results of chapter 5 showed different profiles to those obtained for head kidney and spleen in the organs involved in mucosal immunity, gills and intestine (Doñate et al., accepted BMC genomics). The different expression pattern found between spleen and head kidney supports the hypothesis that these organs play different roles in the immune system of fish, and therefore they possess different functions in response to immunostimulants introduced in the organism by the diet.

As described above, the GO groups involved in synthesis of proteins were decreased in head kidney. Within the list of genes, 78 kDa glucose-regulated protein precursor (also called GRP78 or Heat shock 70 kDa protein 5, -7.2 FC), which possesses a function of folding and assembly of proteins in the endoplasmic reticulum, was down-regulated. Two heat shock proteins (HSPs) were also found in spleen, HSP 90-beta-2 (-6.2 FC) and 60 kDa heat shock protein-1 (-3.2 FC) (Additional file 5). Heat shock proteins are highly conserved proteins that are associated with tissue damage and stress [48], and they have been implicated in immune responses in fish [49, 50]. Differential gene expression of some HPSs was reported in trout macrophage after LPS stimulation [51], in transcriptome profiling of adult zebrafish at the late stage of chronic tuberculosis [52] or in rainbow trout head kidney after LPS and viral challenge [53]. Although the reliability of HSP as an indicator of pathologic/immune disturbances has been criticized [54], microarray analysis indicates that diets containing immunostimulants induce a repression in HSP mRNA expression in both head kidney and spleen.

The second gene showing a severe down-regulation in head kidney in response to immunostimulant feeding was CXCR4 (-19.8 FC). In agreement with our

results, CXCR4 gene was significantly down-regulated in response to LPS in rainbow trout [53] and also in channel catfish [55]. The cluster containing CXCR4, heat shock proteins 70 and 90, and growth differentiation factor 5 was proposed as a CD14-independent model for LPS recognition [56]. The high repression in GRP78, member of the heat-shock protein-70 family and CXCR4 suggest that IS-diets caused a down-regulation in this cluster involved in recognition. In addition, the chemokine receptor CXCR4 has been described to possess a critical role in response to colony stimulating factor (G-CSF), leading to the mobilization of hematopoietic cells into the peripheral blood [57]. The hematopoietic activity is critical in the establishment of cellular and humoral defences against invading pathogens and the maintenance of the organism integrity. Therefore, cytokines and chemokines act upon hematopoietic progenitor cells regulating cellular proliferation [58] and moreover, hematopoietic stem cells and precursors produce a large number of immunomodulatory molecules and contribute directly in the outcome of inflammatory episodes [59]. In order to further understand the effect of immunostimulant diets in the hematopoietic activity of head kidney, the transcription factor PU.1, which plays a key role in hematopoietic lineage development [42, 60], and CXCR4, were analyzed by ISH. *In situ* hybridization studies of PU.1 have been performed in zebrafish embryos [61, 62], and in rainbow trout head kidney of control animals compared to LPS-treated fish, where no significant differences were found [42]. Concerning CXCR4, ISH analysis of two genes have been described in zebrafish development [63]. In this report, distribution of PU.1 and CXCR4 in head kidney was investigated in fish fed with a diet containing immunostimulants and was compared to control fish (figures 3 and 4). Both hematopoietic genes showed a significant decrease in abundance and intensity of signal in IS-fed fish, indicating that the supplement of immunostimulants in the diet to rainbow trout for 4 weeks reduced hematopoietic activity in head kidney.

Although down-regulation was the general observed response, intracellular signalling related to the I-kappaB kinase NF-kappaB cascade was enhanced in head kidney. NF-kappaB is a transcription factor involved in the control of a large number of processes, such as immune and inflammatory responses, developmental processes, cellular growth and apoptosis [64]. At a single gene level, Nucleolysin TIAR (nucleolysin TIA-1 related protein), which possesses apoptotic functions, was also enhanced (2.25 FC). Interestingly, TIA-1 was down-regulated in spleen (-6.01 FC), although other apoptotic promoters, such as CASP8 and FADD-like apoptosis

regulator precursor (25.5 FC) and Receptor-interacting serine/threonine-protein kinase 2 (which promotes activation of NF-kappaB, 2.2 FC) were increased. These results suggest that immunostimulant diet induced a high modulation in processes related to apoptosis in the head kidney and spleen of rainbow trout. Apoptosis is a biological process that removes unnecessary, superfluous, damaged or harmful cells [65]. As in mammals, apoptosis in fish is an important phenomenon in defence and immune response [51, 53]. In order to understand the apoptotic process in rainbow trout, CASP8 and FADD-like apoptosis regulator precursor was studied by ISH in spleen. This gene, which regulates the activation of caspases [66], has been identified in the genomic database of zebrafish, Tetraodon and fugu [67], although this is the first time that its distribution in spleen has been reported. The spleen parenchyma in fish can be divided into red (erythrocyte-rich portions) and white pulp (lymphocyte-rich) [43]. Red pulp, which may occupy the majority of the organ, is dominated by blood sinuses with populations of erythrocytes and macrophages, while the white pulp consists of stretches of lymphoid tissue divided into two compartments: the melanomacrophage centers (MMCs) and the ellipsoids [24]. CASP8 and FADD-like apoptosis regulator expression was distributed throughout the spleen, without a clear association with the white or the red pulp neither with MMCs. The significant difference observed by ISH between control-fed fish and immuno-fed fish confirmed by microarray and Q-PCR data, suggest that diets containing immunostimulants provoked an effect of high regulation in apoptosis activity in the spleen.

GO analysis in spleen reveals that the category haemoglobin complex was enhanced. Within the differentially expressed genes identified, Heme-oxygenase-1 (2.9 FC), Alpha-globin 1-3 (Haemoglobin alpha chain, 2.7 FC) or Beta-globin (Haemoglobin gamma-1 chain, 2.0 FC) were increased. Regulation of haemoglobin genes has been described in rainbow trout after LPS and virus infection [53], and a moderate reduction was observed after starvation [68]. Spleen is an organ with important immune functions by taking, filtering and trapping blood-borne substances from the circulation. MMCs are major sites of erythrocyte destruction, therefore they have been proposed to act as metabolic dumps [24]. The reason of the up-regulation of haemoglobin complex elicited by the immunostimulants of the diet is not clear. In fact, we have observed that haemoglobin complex is also highly regulated by the effect of the IS-diets in gills and intestine, organs acting in mucosal immunity (see chapters 5

and 7). Further investigation is necessary to elucidate the role of the haemoglobin complex in immune mechanisms.

Recently we have seen that one of the main effects of IS-diets in portals of entry of trout is the increase of genes involved in antigen recognition (Doñate et al. BMC genomics and chapter 5). In order to examine the possibility that spleen display a similar response, MHC II alpha chain, which showed an up-regulation of 1.62 FC in microarray evaluation, was analyzed by Q-PCR and ISH. Q-PCR confirmed elevation of MHC II mRNA although with even higher value (4.90 FC, table 1). Furthermore, ISH analysis showed that mRNA expression is localized in the red as well as in the white pulp, corresponding to immunoreactive cells detected previously by Immunohistochemistry [69]. However, our results revealed strong differences between control fish and fish fed with diet containing immunostimulants, since the latter showed many more positive cells all across the tissue (figure 4). In addition, two Cathepsin D forms, Cathepsin D-1 and (7.1 FC) and Cathepsin D-2 (3.1 FC), which belong to a family of cysteine and aspartic proteases implicated in endosomal protein degradation, tissue remodelling and generation of peptides for Ag processing [70, 71] were found as differentially expressed genes (Additional file 5). Tissue localization of Cathepsin D-1 was analyzed by ISH, finding Cathepsin+ cells distributed throughout the spleen in fish fed with IS-diet (figure 4). Another member of this family, Cathepsin B-2, was observed to be differentially up-regulated in microarray and Q-PCR data (table 1 and Additional file 5). It has been reported that Cathepsins D and B enhance their expression in zebrafish with a *Mycobacterium marinum* infection [52]. In this study, the increase of several cathepsins and MHC II mRNAs confirmed by microarray analysis, Q-PCR and ISH may indicate that IS-diet caused an increasing in antigen-presenting capacity in spleen.

Interestingly, other proteins involved in tissue remodelling and cytoskeleton, such as Matrix metalloproteinase-13 (-4.4 FC), Plasminogen precursor-1 (-3.1 FC), Actin alpha skeletal 4 (-2.3 FC) or Dynein light chain 2 (-2.3 FC) were reduced. Extracellular matrix remodelling and regulation of structural proteins of cytoskeleton have been seen in trout in stress responses [31] and chemical contamination [29], in *Mycobacterium marinum* infection in zebrafish [52] and in LPS or IHNV challenge in trout [53]. The results presented here indicate that trout decreased genes related to cell-tissue remodelling activity in spleen in response to IS-diet.

Immunoglobulin receptors (High affinity immunoglobulin epsilon receptor alpha, -3.0 FC, and High affinity immunoglobulin gamma Fc receptor I precursor, -2.1 FC), also appear as transcriptionally down-regulated. This reduction is in agreement with the global suppression found in the GO analysis, where classes related to immune system, such as Response to pesticide, pathogen or parasite, Antigen binding, Response to biotic stimulus, Humoral immune response, Response to stress, Defence response, Immune response, Humoral defence mechanism, Antimicrobial humoral response or Response to wounding (figure 1 and additional file 3).

In conclusions, our results increase the knowledge of the molecular and biological processes which are modulated in organs with a relevant immune role in the trout, head kidney and spleen, after acclimation to an IS-diet. Microarray analysis, Q-PCR and ISH showed that transriptome regulation is tissue-specific. The differences observed in the gene expression patterns of spleen and head kidney support the hypothesis that these organs display different functions in the fish immune system and suggest a more important role for the spleen.

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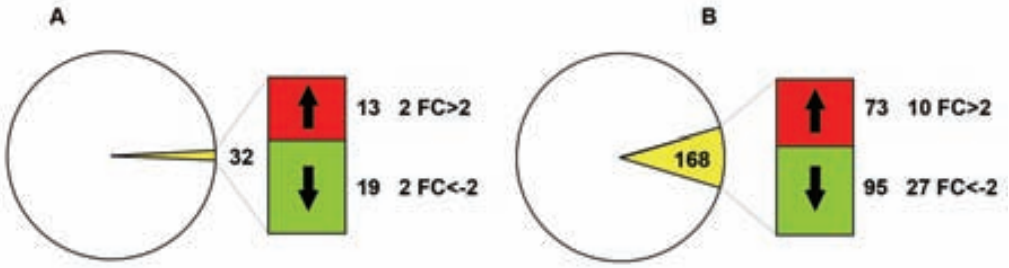
Annex: additional files

A.1 Table 1: primers used in Q-PCR

GENE	Nucleotid sequence (5'- 3')	Amplicon size
MHC II	Fw: CAGGTGGACCAGGAACAATC	109 bp
	Rv: GGGGTGAAGGTCAGACTGGAG	
Alpha-globin I-1	Fw: CTGGAGCGGAACCGGGGGCC	160 bp
	Rv: CAGCTAAGGACAAAGCCAACGTG	
Cathepsin B-2	Fw: TCTGCTGCGTGGGACTTCTG	116 bp
	Rv: GTGCCGTTGACATGGTGCTC	
Cathepsin D-1	Fw: GCAAAATCCAAGTGTGGGGAAA	191 bp
	Rv: CCAACACCACCCTCCATGTTTT	
CASP8 /FADD-like apoptosis reg. precursor	Fw: GAGGGACATCAGCAGGGTTGAC	134 bp
	Rv: GGATGGAGTCCTGATCCACTG	
Nucleolysin TIAR	Fw GGGAAAATATCGGATGCTCG	162 bp
	Rv: GTTGCCCAGTTGGTCCTGATC	
IRAK-1	Fw: GAGAGGAGAGAGAGACGGAC	173 bp
	Rv: GGAGCAGCAAGTGGAGTGGAA	

A.2 Figure 1: General view of differentially expressed genes in fish fed with immunostimulant diet. Total number of differentially expressed (t-student, $p<0.01$). A: head kidney. B: spleen (FC: fold change)



A.3 Table 2 (next page): Functional Gene Ontology categories. Gene Ontology analysis in head kidney (A) and spleen (B). Control fish and fish fed with immunostimulant diet for 4 weeks showed significant differences in functional groups. GO categories were compared pairwise by the sums of ranks of differentially expressed genes ($p<0.01$) and significance was determined with Student's t test ($p<0.05$).

A

GO category	Differential expression	PStudent
I-kappaB kinase NF-kappaB cascade	0.76172	4.26E-05
Intracellular signalling cascade	0.76172	4.26E-05
Protein kinase cascade	0.76172	4.26E-05
Structural molecule activity	-0.189506	0.00408029
Structural constituent of ribosome	-0.197612	0.00685285
Protein biosynthesis	-0.189349	0.03530822
Ribosome	-0.189349	0.03530822

B

GO category	Differential expression	PStudent
Structural constituent of ribosome	0.235047	3.34E-07
Ribosome	0.238578	1.09E-05
Response to pest, pathogen or parasite	-0.505544	0.00021125
Antigen binding	-0.55034	0.00052049
Response to biotic stimulus	-0.39064	0.00065775
Humoral immune response	-0.583779	0.00088248
Response to stress	-0.529336	0.00129303
Protein biosynthesis	0.185233	0.00186021
Defence response	-0.324162	0.00221436
Immune response	-0.330277	0.00321478
mRNA processing	-1.04211	0.01047213
RNA metabolism	-1.04211	0.01047213
RNA processing	-1.04211	0.01047213
Hemoglobin complex	0.783017	0.01640912
Humoral defence mechanism	-0.556039	0.02272918
Nucleus	-0.542429	0.02843809
Antimicrobial humoral response	-0.62765	0.03003051
Response to wounding	-0.634429	0.03936624
Chymotrypsin activity	-0.962651	0.04081347
Trypsin activity	-0.962651	0.04081347
Lysosome	0.708942	0.04507131
Carbohydrate metabolism	-0.587678	0.04632761

A.4 Table 3 (next page): Differentially expressed genes in head kidney. Differentially expressed genes with fold change >2 in head kidney of fish fed with immunostimulant diet compared to control fish. Differential expression was analyzed with Student's t-test ($p<0.01$). Values are expressed as fold change, FC. A: genes up-regulated. B: genes down-regulated.

A

Clone ID	P_Student	FC	Gene name
CA361443	0.00407628	2.252	Nucleolysin TIAR
est03a04	0.00770516	2.112	Transposase-3

B

Clone ID	P_Student	FC	Gene name
CA368961	0.00908587	-7.231	78 kDa glucose-regulated protein precursor
CA374193	0.00414123	-19.854	Chemokine receptor CXCR4

A.5 Table 4: Differentially expressed genes in spleen. Differentially expressed genes with fold change >2 in spleen of fish fed with immunostimulant diet compared to control fish. Differential expression was analyzed with Student's t-test ($p<0.01$). Values are expressed as fold change, FC. A: genes up-regulated. B: genes down-regulated.

A

Clone ID	P_Student	FC	Gene name
CA366608	0.00449928	25.493	CASP8 FADD-like apoptosis regulator precursor
CA347041	0.00022079	7.150	Cathepsin D-1
CA365458	0.00039752	3.167	Cathepsin D-2
CA342675	4.94E-05	2.941	Heme oxygenase-1
EXOB4_H06	4.68E-11	2.714	Alpha-globin 1-3
EXOB4_A03	0.00029094	2.399	Carbonic anhydrase
CA348053	1.07E-09	2.300	Ferritin H-3
CA356686	0.00091423	2.264	Receptor-interacting serine/threonine-prot kinase 2
HST0001_C08	4.83E-05	2.188	Unknown-214
HST0001_D08	3.19E-11	2.012	Beta-globin

B

Clone ID	P_Student	FC	Gene name
EXOB1_E09	0.00285649	-2.042	Transposase-57
CA368203	0.00351816	-2.047	Transposase-55
CA381440	0.00506087	-2.068	Ds RNA-specific adenosine deaminase
EST1-3A_B09	0.00704617	-2.077	Lysozyme g-2
EXOB4_C11	8.46E-06	-2.117	High affinity Ig gamma Fc receptor I precursor
CA378393	0.00587902	-2.146	P-selectin glycoprotein ligand 1
CA373525	1.24E-05	-2.186	Cytochrome B-245 heavy chain-2
est02f08	5.16E-10	-2.202	Serine protease-like protein-1
EXOB3_G04	0.00229227	-2.218	Tyrosine-protein kinase HCK
EXOB1_D11	0.00083258	-2.283	Dynein light chain 2, cytoplasmic

utu04d04	0.00755489	-2.333	Actin, alpha skeletal 4
HK0001_B08	0.00115014	-2.338	Putative pre-mRNA splicing factor RNA helicase
est04e05	1.67E-06	-2.407	Glutathione peroxidase-gastrointestinal
CA354833	0.00591364	-2.524	Anti-silencing function 1B
est04b01	2.24E-13	-2.624	Similar to rRNA (Vangl2)
CA348284	0.00594812	-2.763	CCAAT/enhancer binding protein beta
CA349350	0.00279225	-2.796	Serum/glucocorticoid-regulated kinase
EXOB2_H06	1.47E-08	-3.046	High affinity Ig epsilon receptor alpha
HK0003_E01	0.00017649	-3.170	Plasminogen precursor-1
CA384637	0.00235186	-3.232	60 kDa heat shock protein-1
CA370339	0.00202548	-3.518	Nucleophosmin 1
CA376673	0.00503344	-3.643	Pre-B cell enhancing factor
EXOB3_H01	0.00010313	-4.376	Matrix metalloproteinase-13
CA354056	8.34E-05	-6.011	Nucleolysin TIA-1
EST1-3A_F08	0.0015097	-6.235	Heat shock protein HSP 90-beta-2
est01f12	8.94E-05	-11.689	DNA-directed RNA polymerase II 23 kDa polypeptide
KVkm2_D01	1.82E-05	-16.143	Vacuolar ATP synthase 16 kDa proteolipid subunit

7 Evaluation of mucosal immunity following a LPS challenge in fish previously acclimated to an immunostimulant diet

Abstract

Fish diseases and states of stress are the major problems in aquaculture causing significant economic losses, therefore they have been considered the most critical factors affecting sustainable development of this sector. A number of solutions have been proposed, such as the use of immunostimulants or probiotic to enhance the immune system of fish. Currently, dietary-immunomodulation is a commonly accepted practice in fish farms, where commercial immunostimulant diets are mainly based in β1-3 and β1-6 glucans. These diets are believed to activate the innate immunity providing broad-spectrum resistance to infections. However, the real efficacy of dietary administration of immunostimulants to reduce infectious and stress processes in fish remains controversial, and little is known concerning their effects at the molecular level.

The major portals of entry for pathogen penetration into fish are gills and intestine, therefore an effective immunostimulant should increase the mucosal immunity of these barriers. On the other hand, stress is known to adversely affect growth and immune function, thus the shutdown of cortisol release associated with stress might be other mechanism of beneficial influence of the IS-diets. LPS, the major constituent of the external layer of the outer layer of Gram-negative bacteria, has been used to threat the animal in order to evaluate the differential gene expression at the portals of entry and cortisol regulation in fish fed with a control diet and fish fed for 4 weeks with an IS-diet. Transcriptomic profiles analyzed with a salmonid-specific microarray platform enriched with immune-related genes (SFA 2.0) and plasma cortisol concentrations measured by radioimmunoassay (R.I.A) revealed that

immunostimulant diet caused important changes in the major sites of mucosal immunity of fish and in cortisol regulation when fish were challenged with LPS. Gills of fish fed with IS-diet increased in response to LPS, compared with control fish, the expression of genes related to antigen processing-presentation, antioxidant activity and protein biosynthesis, and decreased the haemoglobin complex and the transposon activity. Intestine of IS-fish showed 24h after LPS challenge, compared to control fish, a more intense response characterized by a mixture of adaptive (MHC I, Ig) and innate (complement, lysozyme) immunity, coordinated with a down-regulation in the response to stress (protein folding, response to oxidative stress), metabolism, apoptosis, immune signalling and cell activation, in a background of muscle-matrix remodelling.

Introduction

Pathogen recognition is one of the most basic and important properties of the immune system. This process relies on the existence of specific, structurally conserved components that are produced by certain broad groups of potentially pathogenic microorganisms. These components, which are absent in multicellular hosts, are usually known as pathogen-associated molecular patterns, PAMPs [1]. The initial recognition and biological response to PAMPs is mediated by diverse set of cellular receptors called PRR, pathogen recognition receptors, activating distinct transcriptomic programmes which will result in the cellular/tissue response [2, 3]. Immunostimulants (IS) are often naturally occurring molecules that can be obtained from a natural source in large amounts and are able to modulate the immune system. The IS component of these diets may contain PAMPs, thus immunostimulation introduced by dietary administration should induce a PAMP-PRR host response and modulate the activity of the immune system in fish. It is generally accepted that the administration of an immunostimulant diet to fish can improve the innate defence of the animal providing resistance to potential pathogens [4]. Currently, dietary-immunomodulation is a commonly accepted practice in both salmonid and non-salmonid species. Such diets were previously thought to act upon the immune system by enhancing non-specific defence mechanisms, such as phagocytic cell activity, pathogen killing, lymphocyte activation or antibody production [4]. Commercial immunostimulant diets are mainly based in β1-3 and β1-6 glucans, although their

efficacy based on dietary administration to reduce infectious processes in salmonid fish remains controversial. It has been published that β-glucans induce a priming effect in fish macrophages [3], however, whereas several studies reported a consistent trend to increased activation of innate immune system [5, 6], also toxicity processes and negative effects have been observed [7, 8].

In addition to fish diseases, accumulative states of stress are one of the major problems in fish culture. Although an organism may appear to recover from a stressful experience, its ability to survive may be reduced [9]. In fish, stress caused by handling, crowding or transport are known to adversely affect growth [10] and immune function [11], and elevations in plasma cortisol has been widely used as an indication of stress [12]. A shutdown of cortisol release associated with stress has been proposed as one of the mechanisms by which immunostimulant diets caused a beneficial influence in the fish immune system [13].

The major portals of entry for pathogen penetration into fish are gills, intestine and skin [14]. These tissues are in direct contact with the external milieu and therefore possess a significant role in control of entry of pathogens. In teleosts, the mucosal immune system is distributed around the above mentioned tissues, providing a primary barrier to avoid potential pathogen invasions [15]. These tissues mediate the innate and adaptive response and also act to limit the intensity of activation to avoid tissue damage. Gill and intestinal epithelium, like the mammalian mucosal epithelium, is functionally diverse, though there is little evidence of lymphoid tissue architecture similar to the mammalian gut-associated lymphoid tissues (GALT) such as the Peyer's patches. However, teleost gut contains important populations of leucocytes [15] and there is evidence of cutaneous innate and adaptive immunity in fish [16, 17].

LPS is the major constituent of the external layer of the outer layer of Gram-negative bacteria, is a PAMP-preparation which induces potent immune responses in fish [3] and is also known to affects cortisol regulation [18] therefore widely used to study immune and endocrine-immune responses in fish. *In vivo* intra-peritoneal injections of bacterial lipopolysaccharide (*E. coli* LPS) have been extensively used to investigate immune responses in fish, reporting that challenges with high concentrations of LPS in fish does not result in endotoxin-mediated mortality [19].

The utility of microarray analysis to investigate immune mechanisms of fish has been widely demonstrated. SFM 1.0 and SFA 2.0 targeted cDNA arrays have been repeatedly assessed to analyze several immune responses in salmonid fish [20-29]. The

objective of the current study was to investigate changes in the transcriptome induced by Lipopolysaccharide (LPS) challege, analyzing differences between fish fed with a basal diet and fish fed with a commercial immunostimulant diet, at the two of the organs acting as portals of entry in fish, gills and intestine. Comparison of differential gene expression at the portals of entry and cortisol regulation reveals that feeding with either a basal diet or an immunostimulant diet results in a significant different immune-defence response to LPS.

Material and Methods

Diets, fish and feeding trial

Diets and fish were described in chapter 5. During the feeding trial, trout were hand-fed once a day with either the control diet or the immunostimulant diet, following manufacturer's indication of 1g food/fish for 4 weeks. After the 4 weeks, 5 fish of each diet were intraperitoneally injected either with 6 mg/Kg of LPS (Sigma, L8274, 026:B6) or with same volumes of phosphate saline buffer (PBS). At 24h, fish were killed in water containing MS-222 (Sigma) with a lethal concentration of 100 ppm, stage III of anaesthesia [30]. Blood was collected for cortisol levels analysis from the caudal vein and was centrifuged at 800 g for 5 min. Plasma was collected and stored at −80 °C. Gills and intestine were collected for microrarray analysis, frozen in liquid nitrogen and stored at -80 ºC.

Microarray analysis

Platform, RNA extraction, hybridization and microarray analysis are described in chapters 4 and 5. Results were submitted to NCBI GEO repository, accession number: **GSE12961**. After subtraction of mean background, LOWESS normalization [31] was performed. To assess differential expression of genes, the normalized log intensity ratios were analyzed with Student's t-test and genes were ranked by log(p-level). The Bayesian modification to the false discovery rate (FDR) was used to correct for multiple comparison tests, estimating the *q*-value for the set of differentially expressed genes [32].The functional categories of Gene Ontology [33] were compared with regulated genes ($p<0.01$) by the sums of ranks (Student's t-test, $p<0.05$).

Real-time PCR

In order to verify microarray results, real time PCR (Q-PCR) was carried out. Real-time protocol was performed as described in chapter 5

Radioimmunoassay

As an index of the stress response, cortisol secretion was measured by radioimmunoassay (R.I.A) [34]. The antibody used for the assay was purchased from Biolink, S.L. (CostaMesa, CA, USA) and used at a final dilution of 1:6000. This antibody cross-reacts 100% with cortisol, 11.40% with 21-desoxycorticosterone, 8.90% with 11-deoxycortisol and 1.60% with 17αhydroxyprogesterone. The radioactivity was quantified using a liquid scintillation counter. Differences among time (0h, 24h, 72h), treatment (PBS, LPS) and Diet (Control, IS) groups were assessed by means of analysis of variance (ANOVA), type V decomposition, followed by Duncan post-hoc test to analyze particular differences between groups. Values (n=5 for each groups) are shown as means±standard errors of the mean (mean±S.E.M).

Results and discussion

The aim of the present study was to analyze the global effects of the LPS administration after 4 weeks of Immunostimulant dietary supplementation in rainbow trout. Trout acclimated during 4 weeks to either a control diet or to a IS diet were stimulated with 6 mg/kg of LPS and gene expression profiles from gills and intestine, which represent major portals of entry in fish, were evaluated using a salmonid-specific microarray platform enriched with immune-related genes (SFA 2.0) [20]. In parallel, plasma cortisol concentrations were measured by radioimmunoassay (R.I.A). The analysis of the transcriptomic profiles obtained together with the significant differences in cortisol values revealed that immunostimulant diet caused important changes in the major sites of mucosal immunity of fish and in cortisol regulation when fish were challenged intraperotineally with LPS.

Global effects of LPS in differential gene expression profiles

Transcriptomic profiles from gills and intestine of fish injected with LPS were compared with those of sham fish injected with PBS, for both control diet and-IS diet (Additional file 1). The total number of differentially expressed genes ($p<0.01$) was similar for both diets: in gills, 623 and 714 for control diet and IS-diet respectively, in intestine 763 and 780. However, applying selection criteria based upon a classical cut-off value of >2 fold change (FC) over the ranked selected genes ($p<0.01$), the results highlight the differential transcriptomic response to LPS for both diets: in control-diet gills, 63 genes (10% of the total regulated genes) displayed a FC>2, 51 were up-regulated and 12 were down-regulated. For IS-diet gills, the profile was reversed: 77 genes possessed a FC>2 (11% of total ranked genes), though 18 were increased and 59 were decreased. In intestine, a higher difference between diets was observed. In control fish, 66 genes had a FC>2 (9% over total regulated genes), 32 and 34 for up-regulated or down-regulated respectively, whereas in fish fed with IS-diet 312 genes displayed a FC>2 (40 % over total ranked genes), 169 enhanced and 143 reduced. Therefore transcriptomic profiles of IS-diet from intestine emphasize a stronger induction of gene expression in this organ. Fold changes of some of the differentially expressed genes were validated by Q-PCR quantification (Additional file 2).

When gene expression profiling of fish injected with LPS were analyzed with those of fish injected with PBS, the magnitude of the response, in terms of number of differentially expressed genes was, not surprisingly, high. Compared to recent studies using either SFA 2.0 or SFM 1.0 platforms, the magnitude of the response upon LPS challenge was similar to infection with salmon anemia virus [25] or with hematopoietic necrosis virus [25], and significantly higher than head kidney response after LPS stimulation [25], macrophage activity upon LPS or/and cortisol treatment [26] or ovary response to LPS [27]. It should be noted that the new platform possesses many more transcripts, including immune related genes and thus more regulated genes could be detected, though clearly LPS stimulation caused large changes at the level of the transcriptome in two major sites of mucosal immunity, the gills and the intestine. The intensity of the response was also high, measured as number of genes with a FC>2: 63-77 genes for control diet and IS-diet respectively in gills and 66-312 in intestine, therefore highlighting, mainly in intestine, a greater transcriptional change in response to LPS in fish fed with IS-diet. This effect can be associated to the previously

described priming effect of the β-glucans [3], thereby after 4 weeks of administration of a diet containing β-glucans, a second challenged carried out by LPS induced a more robust and intense transcriptomic response compared to control animals.

Innate vs adaptive response

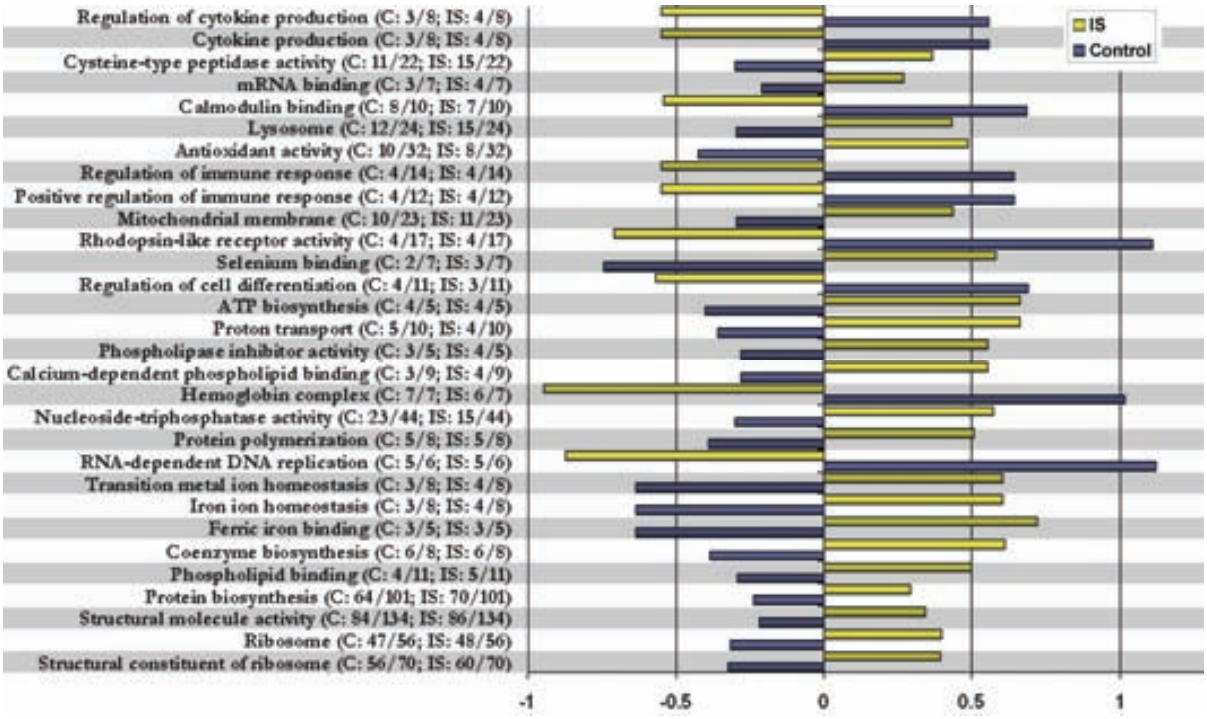
The differentially expressed genes found ($p<0.01$) were ranked applying a selection criteria of FC>2 and analyzed by comparison in the Gene Ontology GO [33] functional categories ($p<0.05$) (figures 1-2, additional files 3-6). After LPS administration, differential tissue-specific responses were observed in terms of suite of genes for both control fish or immunostimulant fed fish.

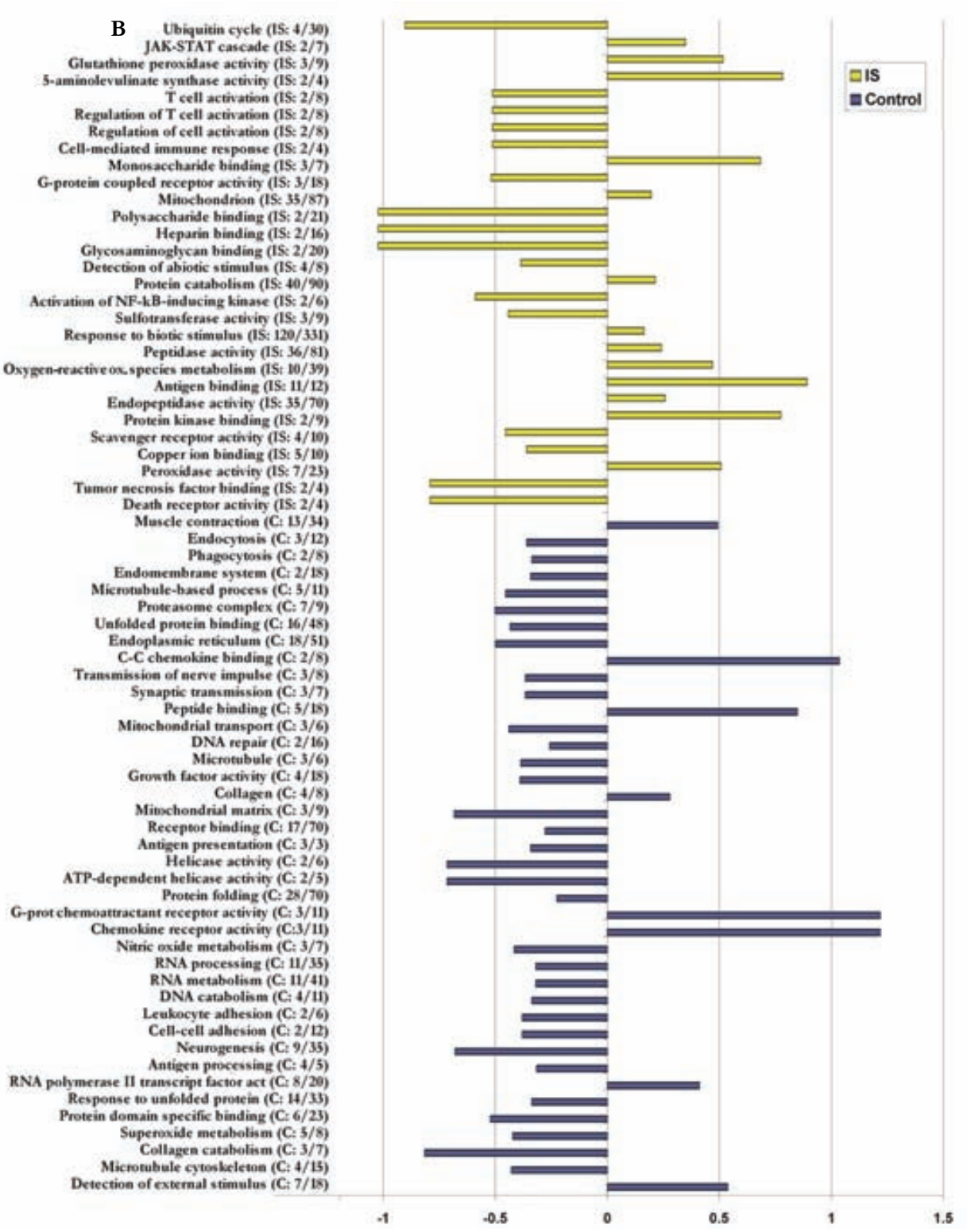
Gills, portal of entry for pathogens and therefore a primary site of many infections, showed opposite responses after LPS treatment in animals fed with control diet compared with animals fed with IS-diet. Thus, many genes and functional GO categories expressed a reversed pattern of expression for both diets. The gills response in control fish, but not IS-fish, was characterized by an increase in the levels of transcripts involved in recruitment and differentiation of immune cells, indicated by elevation in receptors as Chemokine 5a receptor-like, Chemokine receptor CXCR4 and Tumor necrosis factor receptor superfamily member 14 and in immune signalling genes as Regulator of G-protein signalling 1-2, Interferon regulatory factor 1-2, Mitogen-activated protein kinase 13 and Diacylglycerol kinase delta2. The transcript Serine protease-like protein-1, involved in complement activation, was also up-regulated (Additional file 5). The list of functional GO categories ($p<0.05$) increased include Regulation of cell differentiation, Chemokine binding, Regulation of immune response and Cytokine production (figure 1). The high modulation of these genes and functional groups represents an inflammatory response which is not present in IS-fish, as the same or similar GO categories and genes were down-regulated (figure 1, additional file 5), as well as the complement component C7. On the other hand, a large group of genes with roles in antigen processing and presentation, as MHC I heavy chain I and several Ig chains, and also GO functional category Antigen binding, were up-regulated in gills of fish fed with the IS-diet but not in fish fed with control diet, where MHC I and categories such as Antigen processing and presentation were down-regulated. In mammals, there are five Ig isotypes that possess distinct effector functions for secretory immunity, whereas mammalian MHC class I molecules are

involved in presenting antigenic peptides derived from endogenous proteins at the cell surface for recognition by the TCR/CD8 complex of cytotoxic T lymphocytes [35]. Fish possess MHC class I and II complexes and three Ig isotypes has been described, therefore antigen presenting machinery seems to be similar to that of mammals [36, 37]. Our data reveal that control fish activate inflammatory activity whereas the overall effect of immunostimulant diet in gills seems to be the faster increase of adaptive immunity. Similar patterns related to antigen processing-presentation were obtained for intestine (figure 2 and additional file 6). Thus, the genes Lymphocyte antigen 75 and Gamma-interferon inducible lysosomal thiol reductase, which are related to the MHC II complex, the MHC I heavy chain-1 and the GO category MHC class I receptor activity were down-regulated in control fish, whereas MHC I heavy chain-1 and MHC I heavy chain-2 were up-regulated in fish fed with IS-diet, as well as several Ig (figure 2 and additional file 6). These results agree and extend the information found in previous studies, where Atlantic salmon fed with an IS-supplemented diet for 8 weeks had significantly enhanced specific antibody production compared to fish fed the basal diet [38]. This modulation may be very important as some pathogens, such as *Neoparamoeba perurans*, agent of the Amoebic gill disease (AGD) in atlantic salmon, or *Micobacterium marinum*, which cause tuberculosis infection in zebrafish, inhibit the acquired immunity in gills by suppressing MHC I and antigen presenting machinery to create infection [39, 40]. Furthermore, it has been seen that MHC I pathway was activated earlier in blue catfish (*Ictalurus furcatus*), which are resistant to infection with *Edwardsiella ictaluri*, than in the closely related specie channel catfish (*Ictalurus punctatus*), which is susceptible to infection, suggesting that this molecular regulation may be a determinant for resistance [41]. In this context, the early activation of the adaptive response due to the previous administration of an IS-diet confers an important advantage against the attack of pathogens.

Figure 1 (this and next page): Differentially expressed GO categories in gills of both control fish and IS-fed fish 24h after LPS intraperitoneal injection. Differentially expressed genes ($p<0.01$) were grouped by Gene Ontology categories and mean log(expression ratio) were analyzed by Student's t-test ($p<0.05$). A: common categories. B: specific categories for control diet and IS diet.

A




B
Ubiquitin cycle (IS: 4/30)
JAK-STAT cascade (IS: 2/7)
Glutathione peroxidase activity (IS: 3/9)
5-aminolevulinate synthase activity (IS: 2/4)
T cell activation (IS: 2/8)
Regulation of T cell activation (IS: 2/8)
Regulation of cell activation (IS: 2/8)
Cell-mediated immune response (IS: 2/4)
Monosaccharide binding (IS: 3/7)
G-protein coupled receptor activity (IS: 3/18)
Mitochondrion (IS: 35/87)
Polysaccharide binding (IS: 2/21)
Heparin binding (IS: 2/16)
Glycosaminoglycan binding (IS: 2/20)
Detection of abiotic stimulus (IS: 4/8)
Protein catabolism (IS: 40/90)
Activation of NF-kB-inducing kinase (IS: 2/6)
Sulfotransferase activity (IS: 3/9)
Response to biotic stimulus (IS: 120/331)
Peptidase activity (IS: 36/81)
Oxygen-reactive ox. species metabolism (IS: 10/39)
Antigen binding (IS: 11/12)
Endopeptidase activity (IS: 35/70)
Protein kinase binding (IS: 2/9)
Scavenger receptor activity (IS: 4/10)
Copper ion binding (IS: 5/10)
Peroxidase activity (IS: 7/23)
Tumor necrosis factor binding (IS: 2/4)
Death receptor activity (IS: 2/4)
Muscle contraction (C: 13/34)
Endocytosis (C: 3/12)
Phagocytosis (C: 2/8)
Endomembrane system (C: 2/18)
Microtubule-based process (C: 5/11)
Proteasome complex (C: 7/9)
Unfolded protein binding (C: 16/48)
Endoplasmic reticulum (C: 18/51)
C-C chemokine binding (C: 2/8)
Transmission of nerve impulse (C: 3/8)
Synaptic transmission (C: 3/7)
Peptide binding (C: 5/18)
Mitochondrial transport (C: 3/6)
DNA repair (C: 2/16)
Microtubule (C: 3/6)
Growth factor activity (C: 4/18)
Collagen (C: 4/8)
Mitochondrial matrix (C: 3/9)
Receptor binding (C: 17/70)
Antigen presentation (C: 3/3)
Helicase activity (C: 2/6)
ATP-dependent helicase activity (C: 2/5)
Protein folding (C: 28/70)
G-prot chemoattractant receptor activity (C: 3/11)
Chemokine receptor activity (C:3/11)
Nitric oxide metabolism (C: 3/7)
RNA processing (C: 11/35)
RNA metabolism (C: 11/41)
DNA catabolism (C: 4/11)
Leukocyte adhesion (C: 2/6)
Cell-cell adhesion (C: 2/12)
Neurogenesis (C: 9/35)
Antigen processing (C: 4/5)
RNA polymerase II transcript factor act (C: 8/20)
Response to unfolded protein (C: 14/33)
Protein domain specific binding (C: 6/23)
Superoxide metabolism (C: 5/8)
Collagen catabolism (C: 3/7)
Microtubule cytoskeleton (C: 4/15)
Detection of external stimulus (C: 7/18)
IS
Control
-1
-0.5
0
0.5
1
1.5

Tissue remodelling in response to LPS

Upon LPS stimulation, gills of fish of both diets were undergoing a regulation in tissue remodelling processes, expressing differentially up as well as down regulated genes (figure 1, additional files 3 and 5). In control fish, the GO categories Microtubule cytoskeleton, Collagen catabolism, Cell-cell adhesion and Microtubule, together with the genes Perostin precursor, Semaphorin 4D and CD97, involved in matrix and cell adhesion, were reduced, and also the muscle constituent Nebulin. The groups Collagen and Muscle contraction, and the transcripts Connective tissue growth factor, Fibronectin precursor, Connexin 43 and Myosin heavy chain were induced (figure 1, additional files 3 and 5). In intestine, data indicated an elevation in control fish in a set of genes encoding cytoskeletal proteins, collagen and sarcomeric proteins involved in contractile function, such as Myosin heavy chain, Myosin regulatory light chain, Troponin I-2 genes, Cyclins involved in cell cycle, and the GO categories Cytoskeleton and Actin cytoskeleton, Muscle development and Muscle contraction, and Microtubule-based process (figure 2, additional files 4 and 6). At the same time several transcripts encoding regulators of the extracellular matrix were increased, such as Lymphatic endothelium-specific hyaluronan receptor LYVE-1, Cytohesin binding protein HE, Tissue factor pathway inhibitor 2 precursor and Ependymin I. These data suggest that after LPS injection, myofiber, tissue and muscle remodelling were regulated in gills without a clear tendency and were activated in intestine. In contrast, a decrease in extracellular matrix remodelling and leukocyte movement occurs in head kidney 24h after intra-peritoneal LPS injection [25]. Similar pattern of expression than in control gills was obtained in profiles of gills of fish fed with IS-diet. Tissue factor pathway inhibitor 2 precursor, Ependymin I, Sialoadhesin and Cytohesin binding protein HE, which are genes related to matrix and cell adhesion, were reduced, although Microtubule-associated protein RP/EB and Matrix metalloproteinase-13 were enhanced. Interestingly, in intestine some patterns were opposed in fish fed with IS-diet. The GO functional groups Cytoskeleton, Collagen and Cell adhesion were decreased (figure 2 and additional file 4), as several types of collagen genes, several myosin and actin genes, microtubule-related genes (additional file 6). Nevertheless, at a single-gene level some transcripts involved in muscle, cytokinesis or matrix sculpting, as Matrix metalloproteinase 9 (MMP9), were found increased. Previous works have reported induction of the MMP gene family by LPS in trout macrophages

[26] and in trout leucocytes after induction with both LPS and TNFα [42]. In vivo, extracellular matrix remodelling and regulation of structural proteins of cytoskeleton, which are likely to be involved in the activation programs of different cell types of the immune system, have been seen in trout in stress responses [23], chemical contamination [21] and IHNV challenge [25]. We have reported that IS-diet administration during 4 weeks was able to regulate extracellular matrix and cytoskeleton proteins in gills and intestine (Doñate et al. BMC Genomics, chapter 5). Thus it appears that LPS induced modulation in gills and a high induction in intestine in tissue and muscle remodelling activity. This sculpting process is maintained in the gills of fish fed with immunostimulants, though is not so strong or is even repressed in intestine of fish which previously activated remodelling activity in response to the administration of the IS-diet.

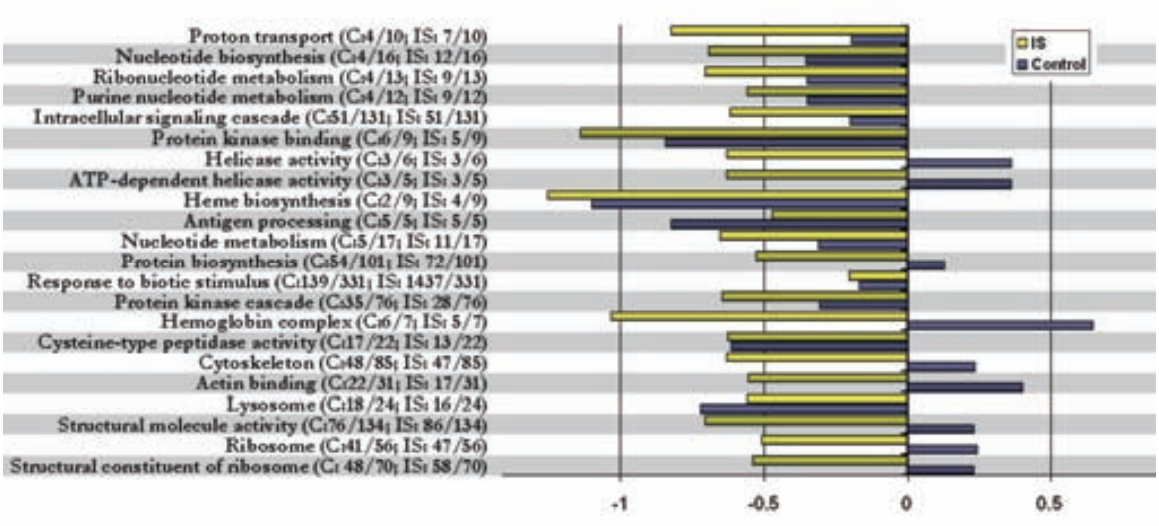
Regulation of transposon activity

Transposases were significantly up-regulated in gills of control fish and repressed in IS-fish (additional file 5). Transposases are proteins that catalyze transposition, a recombination reaction in which a DNA segment translocates from a DNA molecule to another. Transposable elements are drivers of genome evolution as they have been involved in chromosome rearrangements during the evolution of a wide variety of organisms [43]. In contrast to mammals, few transpositions and complete transposase sequences have been detected in the trasposon elements of fish genomes, though active transposons have been found in fish and used for the development of transgenic fish, also have been proposed to be used for identification of genes or improvement of DNA vaccines [44]. In fact, the diversity of transposable elements is much higher in teleost fish than in mammalian genomes [45].

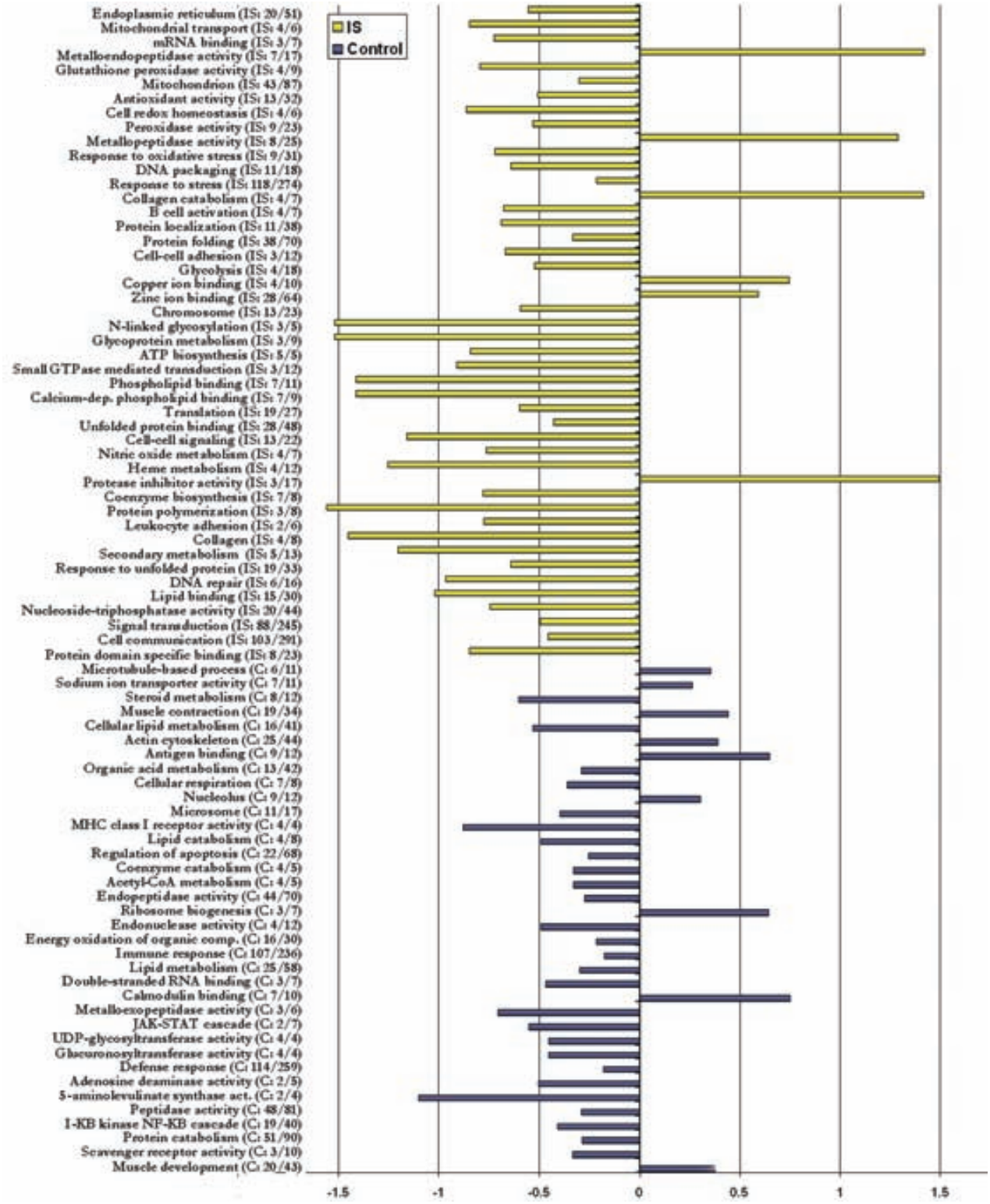
It has been described that transcription of rainbow trout transposons is activated by external stimuli, such as toxicity, stress and bacterial antigens [46], however, the dynamics of expression of transposases upon different conditions are far from being fully understood. Our microarray results suggest that LPS activates transposon activity in gills, though when LPS challenge occurs after immunostimulant dietary administration this activity is reduced.

Figure 2 (this and next page): Differentially expressed GO categories in intestine of both control fish and IS-fed fish 24h after LPS intraperitoneal injection. Differentially expressed genes ($p<0.01$) were grouped by Gene Ontology categories and mean log(expression ratio) were analyzed by Student's t-test ($p<0.05$). A: common categories. B: specific categories for control diet and IS diet.

A



B


IS
Control
Endoplasmic reticulum (IS: 20/51)
Mitochondrial transport (IS: 4/6)
mRNA binding (IS: 3/7)
Metalloendopeptidase activity (IS: 7/17)
Glutathione peroxidase activity (IS: 4/9)
Mitochondrion (IS: 43/87)
Antioxidant activity (IS: 13/32)
Cell redox homeostasis (IS: 4/6)
Peroxidase activity (IS: 9/23)
Metallopeptidase activity (IS: 8/25)
Response to oxidative stress (IS: 9/31)
DNA packaging (IS: 11/18)
Response to stress (IS: 118/274)
Collagen catabolism (IS: 4/7)
B cell activation (IS: 4/7)
Protein localization (IS: 11/38)
Protein folding (IS: 38/70)
Cell-cell adhesion (IS: 3/12)
Glycolysis (IS: 4/18)
Copper ion binding (IS: 4/10)
Zinc ion binding (IS: 28/64)
Chromosome (IS: 13/23)
N-linked glycosylation (IS: 3/5)
Glycoprotein metabolism (IS: 3/9)
ATP biosynthesis (IS: 5/5)
Small GTPase mediated transduction (IS: 3/12)
Phospholipid binding (IS: 7/11)
Calcium-dep. phospholipid binding (IS: 7/9)
Translation (IS: 19/27)
Unfolded protein binding (IS: 28/48)
Cell-cell signaling (IS: 13/22)
Nitric oxide metabolism (IS: 4/7)
Heme metabolism (IS: 4/12)
Protease inhibitor activity (IS: 3/17)
Coenzyme biosynthesis (IS: 7/8)
Protein polymerization (IS: 3/8)
Leukocyte adhesion (IS: 2/6)
Collagen (IS: 4/8)
Secondary metabolism (IS: 5/13)
Response to unfolded protein (IS: 19/33)
DNA repair (IS: 6/16)
Lipid binding (IS: 15/30)
Nucleoside-triphosphatase activity (IS: 20/44)
Signal transduction (IS: 88/245)
Cell communication (IS: 103/291)
Protein domain specific binding (IS: 8/23)
Microtubule-based process (C: 6/11)
Sodium ion transporter activity (C: 7/11)
Steroid metabolism (C: 8/12)
Muscle contraction (C: 19/34)
Cellular lipid metabolism (C: 16/41)
Actin cytoskeleton (C: 25/44)
Antigen binding (C: 9/12)
Organic acid metabolism (C: 13/42)
Cellular respiration (C: 7/8)
Nucleolus (C: 9/12)
Microsome (C: 11/17)
MHC class I receptor activity (C: 4/4)
Lipid catabolism (C: 4/8)
Regulation of apoptosis (C: 22/68)
Coenzyme catabolism (C: 4/5)
Acetyl-CoA metabolism (C: 4/5)
Endopeptidase activity (C: 44/70)
Ribosome biogenesis (C: 3/7)
Endonuclease activity (C: 4/12)
Energy oxidation of organic comp. (C: 16/30)
Immune response (C: 107/236)
Lipid metabolism (C: 25/58)
Double-stranded RNA binding (C: 3/7)
Calmodulin binding (C: 7/10)
Metallo-exopeptidase activity (C: 3/6)
JAK-STAT cascade (C: 2/7)
UDP-glycosyltransferase activity (C: 4/4)
Glucuronosyltransferase activity (C: 4/4)
Defense response (C: 114/259)
Adenosine deaminase activity (C: 2/5)
5-aminolevulinate synthase act. (C: 2/4)
Peptidase activity (C: 48/81)
I-KB kinase NF-KB cascade (C: 19/40)
Protein catabolism (C: 51/90)
Scavenger receptor activity (C: 3/10)
Muscle development (C: 20/43)
-1.5
-1
-0.5
0
0.5
1
1.5

Haemoglobin modulation

Other opposite transcriptional regulations between the two diets comprised the haemoglobin complex. In control gills, Haemoglobin alpha chain (2.94 FC), Alpha-globin I-2 (2.65FC), Haemoglobin beta chain (2.58 FC) and Alpha-globin I-1 (2.29 FC) were increased, in IS gills the same genes were down-regulated with -2.13, -2.11, -2.12, and -2.10 FC respectively (additional files 5-6). Consequently, the GO category Haemoglobin was increased in control fish after LPS challenge and decreased in IS-fish (figures 1-2 and additional files 3-4). Unlike their mammalian counterparts, mature fish erythrocytes are nucleated and can synthesise haemoglobin while circulating in the blood [47, 48], furthermore the regulation of haemoglobin genes has been previously described in rainbow trout in infections processes and starvation [25, 49]. Fish fed with IS-diets were already regulating haemoglobin dynamics (Doñate et al. BMC genomics, chapter 5), and the subsequent response to injection was a decreased in haemoglobin mRNA expression. The reason of the strong regulation of haemoglobin complex in all these processes remains unclear.

Balance in transcription and synthesis of proteins

Whereas gills of control fish repress synthesis of proteins, indicated by the down-regulation in categories and genes related to ribosome-protein biosynthesis and favoured transcription processes, gills of IS-fish showed a coordinated increase in synthesis of proteins together with a reduction in transcription (figure 1). Results indicate that LPS decreased protein synthesis in control fish, though the animals were undergoing transcriptional activation, in accordance with results obtained in the head kidney of rainbow trout injected with LPS [25]. However, profiles of gills of fish previously fed with the IS-diet showed opposite pattern of expression, suggesting that the "priming" effect induced by β-glucans [3] is able to trigger a faster synthesis of proteins. Nevertheless, in IS-fish this activation was not detected in intestine, organ which was in direct contact with the IS-diet.

Regulation of antioxidant system

Several members of the antioxidant systems that keep intracellular redox homeostasis showed reversed pattern of expression in both treatments in gills, but not in intestine. The category antioxidant activity together with the genes Thioredoxin-like

protein p19 precursor and Glutathione peroxidase-gastrointestinal were down-regulated in control fish (figure 1 and additional file 5), whilst the GO groups Antioxidant activity, Peroxidase activity and Oxygen and reactive oxygen species metabolism, including Thioredoxin gene, were increased in IS-fish. On the other hand, the antioxidant system was repressed in intestine for both diets. The genes Aldeyde oxidase and Glutathione reductase in intestine of control fish, and Thioredoxin-like protein p19 precursor, Glutathione peroxidase-gastrointestinal and thioredoxin transcripts, together with the GO categories Response to oxidative stress, Peroxidase activity, Cell redox homeostasis and Antioxidant activity in intestine of IS-fish, were decreased (figure 2, additional file 6). Interestingly, Thioredoxin, in addition to its role in protecting against oxidative stress and maintaining cellular redox status, is also a potential B cell growth factor in fish [50], and high regulation of this gene has been observed in fish upon infectious processes [41, 51-53]. The up-regulation in gills of IS-fish suggests, as results of the antigen presenting capacity, an activation of adaptive cellular immunity. Globally, microarray results indicated that LPS decreased antioxidant system in gills and intestine of rainbow trout, though a prior IS dietary administration is able to increase the antioxidant activity in gills in response to the challenge.

Metabolism, apoptosis, communication and immune-related activities

LPS down-regulated many processes in intestine for both diets, as shown in figure 2 and additional files 4 and 6. Proteolysis, including several types of cathepsins, and apoptosis, including the GO category Regulation of apoptosis and the genes CASP8 and FADD-like apoptosis regulator precursor and Lymphocyte pore forming protein in control, or Programmed cell death proteins and Growth arrest and DNA-damage-inducible peptides in IS-fish, were repressed. This regulation is not surprising, as apoptosis is a conserved immune regulatory mechanism in fish [54], and those set of activities has been found to be regulated in fish upon LPS stimulation [25, 26]. However, only in fish fed with the immunostimulant diet there was also a coordinated down-regulation of other groups, which comprised genes involved in ATP biosynthesis, DNA repair, response to unfolded protein (heat shock proteins, DnaJ homologs, chaperons), cell proliferation (including up-regulation of inhibitors of cell proliferation) or response to virus (additional file 6). Thus, profiles suggest that LPS

induced a shut-down of those categories favouring the activation of other processes such as MHC Class I antigen presentation, complement activation or tissue remodelling.

On the other hand, LPS induced a high regulation in communication and signalling modules involved in the immune system in intestine of both control fish and IS-fish. Thus, the functional categories Intracellular signalling cascade and Protein kinase cascade for both groups, I-KB kinase NF-KB cascade in control fish and Cell-cell signalling in IS-fish were down-regulated (figure 2, additional file 4), as common down-regulated genes as Granulins, Interleukin-1 receptor-associated kinase 1-2 or Phosphotyrosine independent ligand for the Lck SH2 domain p62. Several cytokines, as the Small inducible cytokine SCYA104 in both diets, and CC chemokine SCYA110-2 and Small inducible cytokine B14 precursor in IS fed fish were repressed. However, important genes involved in communication were also increased. For example, Allograft inflammatory factor-1 was detected in both diets. Toll-interacting protein, Cold autoinflammatory syndrome 1 protein and several MAP kinases were increased in IS-fish. Immune cell activation was increased, showed by the regulation of immune-related receptors, as C-type lectin receptor B in both groups, CXCR4 in control fish, and Interleukin-1 receptor-like protein 2, TNF receptor associated factor 1 and T-cell receptor alpha chain V region HPB-MLT precursor in IS-fish.

Signalling cascades, cytokines, chemokines and receptors, involved in inflammatory response, are well known to be regulated in different organs and cells in response to LPS and infectious processes in fish [25, 26, 39, 41, 51]. Here, significantly differential regulation obtained in the transcriptomic profiles indicated that immune signalling and cell activation were modulated in response to LPS, as it could be expected, however the effect of a previous dietary immunostimulation caused a more intense response.

Complement response

The complement system of teleost fish plays conserved roles in sensing and clearing pathogens [55]. Complement related peptides are synthesized primarily in the liver, nevertheless, minor but biologically significant extra-hepatic synthesis of complement components has recently been demonstrated in fish [56]. High enhanced in complement transcription has been detected in liver when channel catfish or blue

catfish infected with *Edwardsiella ictaluri* [41, 51], but also high increase has been described in extra-hepatic organs in rainbow trout upon virus infection [25]. Our results here show a significant expression of complement related proteins in intestine but not in gills 24h after LPS injection (additional files 5 and 6), however this response is only observed in animals fed with IS-diet, suggesting that this enhance may be due to the priming effect of the diet and is restricted to the intestine

Plasma cortisol regulation

Cortisol release induced by PBS or LPS injection was also regulated by the previous administration of an Immunostimulant diet during 4 weeks (figure 3). The ANOVA analysis of the cortisol concentrations measured by R.I.A revealed that the effect of the Diet (Control or IS, p=0.0010), the treatment (PBS or LPS, p= 0.0027) and the combination of both factors (p=0.0073) were significant. Thus, basal cortisol levels (C diet=11.03±4.04 ng/ml, IS diet=11.29±3.89 ng/ml) were similar for both diets in the control groups. However, LPS injection increased significantly cortisol concentrations only in fish fed with control diet over 24h (124.82±28.28 ng/ml) reaching maximum levels at 72h (333.36±158.94 ng/ml). PBS injection also had a stressful effect, although lower than LPS, and enhanced significantly cortisol concentrations in fish fed with control diet at 24h (117.10±56.61 ng/ml) and at 72h (109.41±55.00 ng/ml). Interestingly, the elevation in cortisol values for the fish fed with IS diet was very weak (7.81±2.33 with LPS at 24h, 22.51±9.9 with LPS at 72h), and no significant difference in any of the treatment PBS or LPS over the time was observed (figure 3).

Elevated cortisol levels are indicative of stress [12], therefore the high cortisol levels evident in fish fed with control diet confirmed that the fish were undergoing a stress response caused by intraperitoneal PBS or LPS injection. Lower vertebrates are relatively insensitive to the toxic effects of LPS [57]. Thus, fish macrophages has been shown to be 1000 times less sensitive to LPS than mammalian macrophages [3, 58], and only very high doses of LPS caused an increase in cortisol levels [18, 59]. Here, we report that high concentration of LPS increased plasma cortisol levels, however, fish fed with the immunostimulant diet had significantly lower cortisol values compared to control fish. This can be due to the fact that fish fed with IS-diet rapidly increased adaptive response but decreased innate response, including important cytokines and

ckemokines, therefore there is no activation of cortisol secretion. Offsetting the inhibitory effects of cortisol release associated with stress has been proposed as one of the mechanisms by which dietary nucleotides beneficially influence the fish immune system [13].

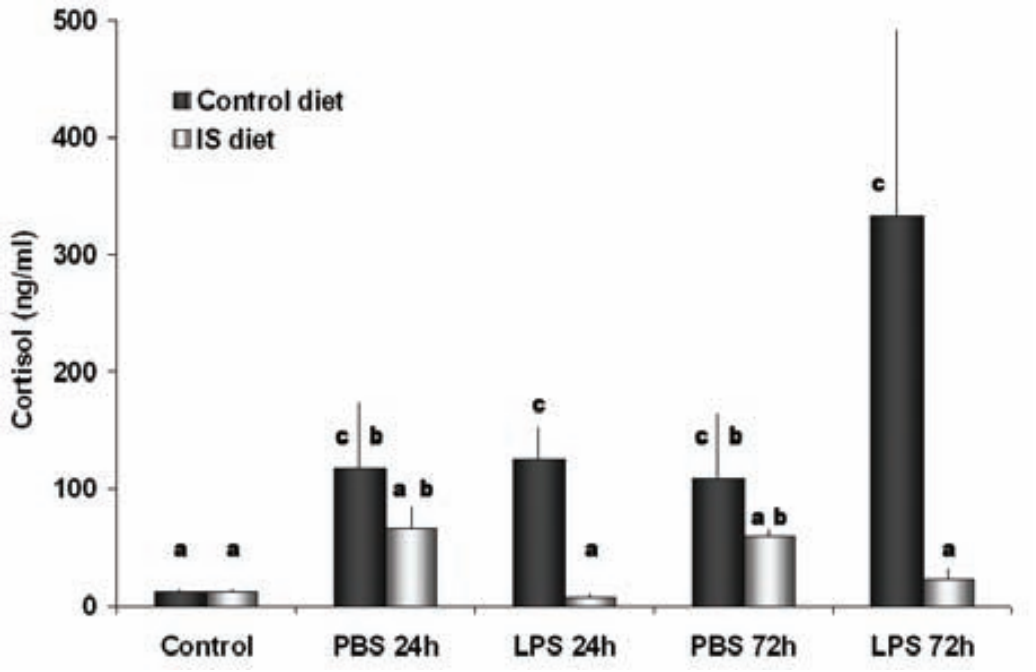


Figure 3: Effect of IS-diet after PBS or LPS intraperitoneal injection in plasma cortisol levels (expressed in ng/mL) over 24 and 72 h. The results are shown as a mean±SEM of five fish for each group. Statistically significant ($p<0.05$) differences among groups are indicated by different letters.

Conclusions

Several studies have been carried out to characterize responses in salmonids to IS compounds, however they have not evaluated the response of the portals of entry of the animal, which have a key role in the survival of the organism. Furthermore, transcriptomic response has not been investigated, and results continue to be controversial [5, 7, 8, 60].

It has been described that β-glucan is able to profoundly modulate LPS-induced cytokine expression in fish leukocytes [3], in agreement with the immunostimulatory effects of β-glucan observed in vivo in fish [61, 62]. Microarray and Q-PCR results indicated that a previous β-glucan dietary administration provokes a priming effect and therefore modulates LPS-induced transcriptomic profiles and cortisol concentrations. Our objective was to assess changes in molecular mechanism in the mucosal immune barriers of rainbow trout after dietary administration and a subsequent challenge with bacterial lipopolysacharide. In conclusion, Immunostimulant diets markedly modulated trancriptomic profiles and plasma cortisol levels of fish upon LPS administration. Whereas in gills the main response to LPS was that fish fed with IS-diet increased the expression of genes related to antigen processing-presentation and antioxidant activity, therefore providing protection conferred by activation of adaptive immunity, intestine of IS-fish responded with greater intensity with a mixture of adaptive (MHC I, Ig) and innate (complement, lysozyme) immunity, together with the regulation in the response to stress (protein folding, response to oxidative stress), metabolism, apoptosis, immune signalling and cell activation, in a background of tissue remodelling.

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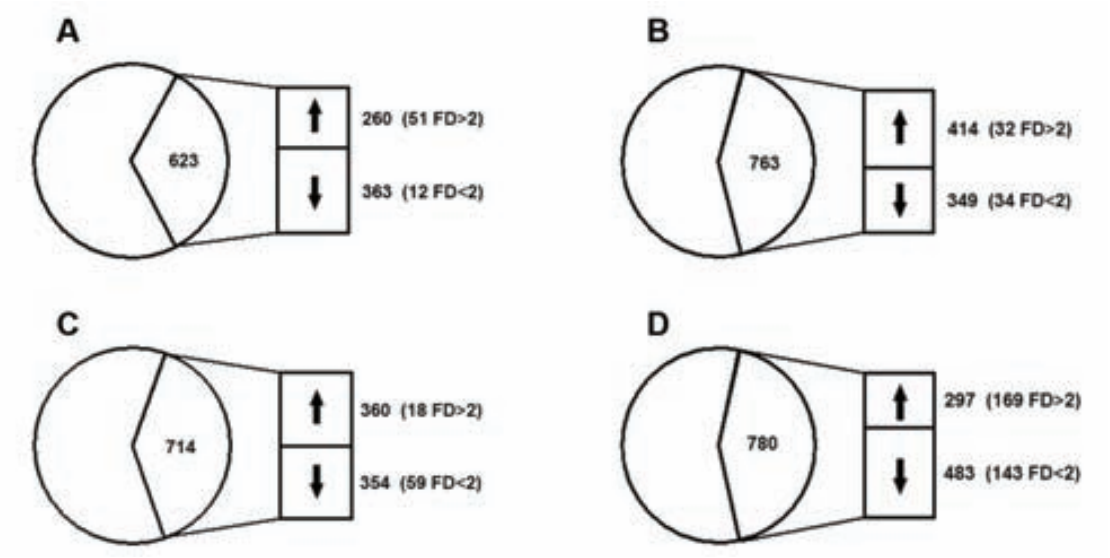
Annex: additional files

A.1 Figure 1: Total number of differentially expressed genes. Ranked genes with a FC>1 (t-student, p<0.01) 24h after LPS injection in gills of fish fed with control diet (A), gills of fish fed with IS diet (B), intestine of fish fed with control diet (C) and intestine o fish fed with IS diet (D). FD: fold difference.



A.2 Table 1: Q-PCR validation for microarray results. Expression of selected genes was analyzed by SYBR Green Q-PCR analysis and normalized to the abundance of 18s. Data are presented as fold change of fish injected with LPS relative to fish injected with PBS. All values are from triplicate measures.

ORGAN	GENE NAME	Q-PCR FC	MICROARRAY FC
Gills C	**Alpha-blobin I-2**	2.57	2.65
Gills C	**Alpha-globin I-1**	2.24	2.29
Gills C	**CXCR4**	1.35	2.16
Gills C	**Cathepsin B-2**	-1.88	-1.65
Gills C	**MHC I heavy chain-1**	-1.57	-2.40
Intestine C	**CXCR4**	1.93	3.67
Intestine C	**High affinity Ig ε receptor α**	1.44	1.93
Intestine C	**Translin**	1.41	1.84
Intestine C	**IRAK 1-2**	-4.19	-3.34
Intestine C	**CASP8 and FADD-like apoptosis regulator precursor**	-3.02	-2.00

Intestine C	**Cathepsin B-2**	-1.93	-1.76
Intestine C	**MHC I heavy chain-1**	-8.02	-2.91
Gills IS	**Translin**	-4.35	-2.01
Gills IS	**Alpha-globin I-1**	-3.66	-2.10
Gills IS	**Alpha-blobin I-2**	-3.77	-2.11
Gills IS	**Hemoglobin β chain**	-2.33	-2.12
Intestine IS	**MMP9-1**	2.90	4.20
Intestine IS	**MHC I heavy chain-1**	7.65	6.48
Intestine IS	**IRAK 1-2**	-1.51	-4.93
Intestine IS	**Hemoglobin β chain**	-1.38	-1.97

A.3 Table 2: Gene Ontology analysis in gill of fish injected with LPS compared with control fish injected with PBS, in animals fed with both control and IS-diet. GO categories were compared pairwise by the mean log(expression ratio) of differentially expressed genes ($p<0.01$). Significance was determined with Student's t test ($p<0.05$). DE: differential expression

CONTROL			GO category	IS		
DE	**N genes**	**P Student**		**DE**	**N genes**	**P Student**
-0.32	56	2.6E-32	**Structural constituent of ribosome**	0.39	60	1.9E-14
-0.32	47	4.0E-26	**Ribosome**	0.40	48	3.1E-11
-0.22	84	9.1E-07	**Structural molecule activity**	0.34	86	2.0E-09
-0.24	64	1.5E-06	**Protein biosynthesis**	0.29	70	6.8E-07
-0.29	4	3.3E-04	**Phospholipid binding**	0.50	5	2.2E-03
-0.39	6	4.9E-04	**Coenzyme biosynthesis**	0.61	6	9.7E-05
-0.63	3	5.7E-04	**Ferric iron binding**	0.72	3	5.4E-03
-0.63	3	5.7E-04	**Iron ion homeostasis**	0.60	4	1.6E-02
-0.63	3	5.7E-04	**Transition metal ion homeostasis**	0.60	4	1.6E-02
1.12	5	7.3E-04	**RNA-dependent DNA replication**	-0.87	5	1.9E-03
-0.39	5	1.2E-03	**Protein polymerization**	0.50	5	1.7E-03
-0.30	23	1.3E-03	**Nucleoside-triphosphatase activity**	0.57	15	1.4E-04
1.02	7	1.7E-03	**Hemoglobin complex**	-0.95	6	1.9E-04
-0.28	3	2.9E-03	**Calcium-dependent phospholipid binding**	0.55	4	2.2E-03
-0.28	3	2.9E-03	**Phospholipase inhibitor activity**	0.55	4	2.2E-03
-0.36	5	3.2E-03	**Proton transport**	0.66	4	1.7E-03
-0.40	4	3.8E-03	**ATP biosynthesis**	0.66	4	1.7E-03
0.69	4	3.9E-03	**Regulation of cell differentiation**	-0.57	3	6.7E-04
-0.74	2	6.9E-03	**Selenium binding**	0.58	3	1.6E-02
1.11	4	7.4E-03	**Rhodopsin-like receptor activity**	-0.71	4	4.0E-02
-0.30	10	1.3E-02	**Mitochondrial membrane**	0.43	11	6.6E-03
0.64	4	1.3E-02	**Positive regulation immune response**	-0.55	4	2.5E-04
0.64	4	1.3E-02	**Regulation of immune response**	-0.55	4	2.5E-04
-0.43	10	1.5E-02	**Antioxidant activity**	0.48	8	3.5E-04

-0.30	12	1.6E-02	Lysosome	0.43	15	0.003
0.68	8	1.6E-02	Calmodulin binding	-0.54	7	0.002
-0.21	3	2.2E-02	mRNA binding	0.27	4	0.012
-0.30	11	2.5E-02	Cysteine-type peptidase activity	0.36	15	0.007
0.56	3	4.7E-02	Cytokine production	-0.55	4	0.000
0.56	3	4.7E-02	Regulation of cytokine production	-0.55	4	0.000
0.54	7	7.5E-03	Detection of external stimulus			
-0.43	4	7.8E-03	Microtubule cytoskeleton			
-0.81	3	8.0E-03	Collagen catabolism			
-0.42	5	8.3E-03	Superoxide metabolism			
-0.52	6	9.3E-03	Protein domain specific binding			
-0.34	14	1.1E-02	Response to unfolded protein			
0.41	8	1.2E-02	RNA polymerase II transcription factor activity			
-0.32	4	7.2E-03	Antigen processing			
-0.68	9	1.6E-02	Neurogenesis			
-0.38	2	1.7E-02	Cell-cell adhesion			
-0.38	2	1.7E-02	Leukocyte adhesion			
-0.33	4	2.0E-02	DNA catabolism			
-0.32	11	2.0E-02	RNA metabolism			
-0.32	11	2.0E-02	RNA processing			
-0.42	3	2.0E-02	Nitric oxide metabolism			
1.22	3	2.3E-02	Chemokine receptor activity			
1.22	3	2.3E-02	G-prot chemoattractant receptor act.			
-0.23	28	2.3E-02	Protein folding			
-0.71	2	2.5E-02	ATP-dependent helicase activity			
-0.71	2	2.5E-02	Helicase activity			
-0.34	3	2.7E-02	Antigen presentation			
-0.28	17	2.9E-02	Receptor binding			
-0.68	3	3.1E-02	Mitochondrial matrix			
0.28	4	3.1E-02	Collagen			
-0.39	4	3.4E-02	Growth factor activity			
-0.38	3	3.5E-02	Microtubule			
-0.26	2	3.8E-02	DNA repair			
-0.44	3	4.2E-02	Mitochondrial transport			
0.85	5	4.3E-02	Peptide binding			
-0.37	3	4.7E-02	Synaptic transmission			
-0.37	3	4.7E-02	Transmission of nerve impulse			
1.04	2	4.9E-02	C-C chemokine binding			
-0.50	18	8.2E-06	Endoplasmic reticulum			
-0.43	16	1.6E-04	Unfolded protein binding			
-0.50	7	5.0E-04	Proteasome complex			
-0.45	5	1.5E-03	Microtubule-based process			
-0.34	2	1.8E-03	Endomembrane system			
-0.33	2	1.9E-03	Phagocytosis			

-0.36	3	5.3E-03	**Endocytosis**		
0.49	13	6.2E-03	**Muscle contraction**		
			Death receptor activity	-0.79	2	0.000
			Tumor necrosis factor binding	-0.79	2	0.000
			Peroxidase activity	0.51	7	0.001
			Copper ion binding	-0.36	5	0.006
			Scavenger receptor activity	-0.45	4	0.007
			Protein kinase binding	0.78	2	0.007
			Endopeptidase activity	0.26	35	0.008
			Antigen binding	0.89	11	0.009
			Oxygen and reactive species metabolism	0.47	10	0.010
			Peptidase activity	0.24	36	0.012
			Response to biotic stimulus	0.17	120	0.015
			Sulfotransferase activity	-0.44	3	0.015
			Activation of NF-kB-inducing kinase	-0.59	2	0.016
			Protein catabolism	0.22	40	0.018
			Detection of abiotic stimulus	-0.38	4	0.025
			Glycosaminoglycan binding	-1.02	2	0.028
			Heparin binding	-1.02	2	0.028
			Polysaccharide binding	-1.02	2	0.028
			Mitochondrion	0.20	35	0.032
			G-protein coupled receptor activity	-0.52	3	0.036
			Monosaccharide binding	0.68	3	0.041
			Cell-mediated immune response	-0.51	2	0.041
			Regulation of cell activation	-0.51	2	0.041
			Regulation of T cell activation	-0.51	2	0.041
			T cell activation	-0.51	2	0.041
			5-aminolevulinate synthase activity	0.78	2	0.046
			Glutathione peroxidase activity	0.52	3	0.046
			JAK-STAT cascade	0.35	2	0.046
			Ubiquitin cycle	-0.90	4	0.047

A.4 Table 3 (next page): Gene Ontology analysis in intestine of fish injected with LPS compared with fish injected with PBS, in animals fed with both control and IS-diet. GO categories were compared pairwise by the mean log(expression ratio) of differentially expressed genes ($p<0.01$). Significance was determined with Student's t test ($p<0.05$). DE: differential expression; N genes: number of genes in each group.

CONTROL			GO category	IS		
DE	N genes	P Student		DE	N genes	P Student
0.24	48	3.3E-10	Structural constituent of ribosome	-0.54	58	6.1E-12
0.25	41	6.6E-09	Ribosome	-0.51	47	4.2E-08
0.23	76	9.2E-07	Structural molecule activity	-0.70	86	1.0E-14
-0.72	18	3.9E-05	Lysosome	-0.56	16	4.9E-03
0.41	22	5.2E-04	Actin binding	-0.55	17	1.6E-02
0.24	48	5.5E-04	Cytoskeleton	-0.63	47	2.8E-04
-0.61	17	1.7E-03	Cysteine-type peptidase activity	-0.63	13	1.5E-03
0.65	6	2.2E-03	Hemoglobin complex	-1.03	5	4.0E-03
-0.31	35	3.7E-03	Protein kinase cascade	-0.65	28	8.7E-03
-0.17	139	5.5E-03	Response to biotic stimulus	-0.20	143	2.4E-02
0.13	54	6.7E-03	Protein biosynthesis	-0.53	72	2.8E-08
-0.31	5	7.7E-03	Nucleotide metabolism	-0.66	11	1.4E-03
-0.82	5	8.9E-03	Antigen processing	-0.47	5	3.4E-03
-1.10	2	1.2E-02	Heme biosynthesis	-1.25	4	4.4E-03
0.36	3	1.4E-02	ATP-dependent helicase activity	-0.63	3	6.6E-03
0.36	3	1.4E-02	Helicase activity	-0.63	3	6.6E-03
-0.84	6	1.5E-02	Protein kinase binding	-1.14	5	2.0E-02
-0.20	51	2.6E-02	Intracellular signalling cascade	-0.62	51	1.9E-04
-0.35	4	3.0E-02	Purine nucleotide metabolism	-0.56	9	2.9E-02
-0.35	4	3.0E-02	Ribonucleotide metabolism	-0.70	9	3.1E-02
-0.35	4	3.1E-02	Nucleotide biosynthesis	-0.69	12	5.6E-03
-0.19	4	4.6E-02	Proton transport	-0.82	7	1.2E-03
0.38	20	2.9E-03	Muscle development			
-0.33	3	4.0E-03	Scavenger receptor activity			
-0.28	51	8.1E-03	Protein catabolism			
-0.41	19	1.0E-02	I-KB kinase NF-KB cascade			
-0.29	48	1.1E-02	Peptidase activity			
-1.10	2	1.2E-02	5-aminolevulinate synthase act.			
-0.50	2	1.3E-02	Adenosine deaminase activity			
-0.18	114	1.3E-02	Defence response			
-0.45	4	1.4E-02	Glucuronosyltransferase activity			
-0.45	4	1.4E-02	UDP-glycosyltransferase activity			
-0.55	2	1.5E-02	JAK-STAT cascade			
-0.71	3	1.6E-02	Metalloexopeptidase activity			
0.75	7	1.6E-02	Calmodulin binding			
-0.47	3	1.8E-02	Double-stranded RNA binding			
-0.30	25	1.8E-02	Lipid metabolism			
-0.17	107	1.8E-02	Immune response			
-0.21	16	2.0E-02	Energy oxidation of organic comp.			
-0.49	4	2.1E-02	Endonuclease activity			
0.64	3	2.1E-02	Ribosome biogenesis			
-0.27	44	2.5E-02	Endopeptidase activity			

-0.33	4	2.8E-02	Acetyl-CoA metabolism
-0.33	4	2.8E-02	Coenzyme catabolism
-0.26	22	3.0E-02	Regulation of apoptosis
-0.49	4	3.3E-02	Lipid catabolism
-0.88	4	3.6E-02	MHC class I receptor activity
-0.40	11	3.8E-02	Microsome
0.30	9	4.2E-02	Nucleolus
-0.36	7	4.3E-02	Cellular respiration
-0.29	13	5.0E-02	Organic acid metabolism
0.65	9	9.8E-05	Antigen binding
0.39	25	1.7E-04	Actin cytoskeleton
-0.53	16	3.0E-04	Cellular lipid metabolism
0.44	19	4.7E-04	Muscle contraction
-0.60	8	1.1E-03	Steroid metabolism
0.26	7	1.1E-03	Sodium ion transporter activity
0.35	6	1.9E-03	Microtubule-based process
			Protein domain specific binding
			Cell communication
			Signal transduction
			Nucleoside-triphosphatase activity
			Lipid binding
			DNA repair
			Response to unfolded protein
			Secondary metabolism
			Collagen
			Leukocyte adhesion
			Protein polymerization
			Coenzyme biosynthesis
			Protease inhibitor activity
			Heme metabolism
			Nitric oxide metabolism
			Cell-cell signalling
			Unfolded protein binding
			Translation
			Calcium-dep. phospholipid binding
			Phospholipid binding
			Small GTPase mediated transduction
			ATP biosynthesis
			Glycoprotein metabolism
			N-linked glycosylation
			Chromosome
			Zinc ion binding
			Copper ion binding
			Glycolysis
			Cell-cell adhesion

-0.85	8	3.3E-05
-0.45	103	3.5E-05
-0.50	88	4.1E-05
-0.74	20	8.3E-05
-1.02	15	1.9E-04
-0.97	6	4.0E-04
-0.64	19	6.9E-04
-1.20	5	8.6E-04
-1.45	4	1.2E-03
-0.78	2	2.0E-03
-1.56	3	2.5E-03
-0.78	7	2.6E-03
1.49	3	3.9E-03
-1.25	4	4.4E-03
-0.76	4	6.6E-03
-1.16	13	8.3E-03
-0.43	28	8.8E-03
-0.60	19	9.0E-03
-1.41	7	9.5E-03
-1.41	7	9.5E-03
-0.91	3	1.1E-02
-0.84	5	1.4E-02
-1.52	3	1.6E-02
-1.52	3	1.6E-02
-0.59	13	1.6E-02
0.59	28	1.8E-02
0.75	4	1.8E-02
-0.52	4	1.8E-02
-0.67	3	2.5E-02

Protein folding	-0.33	38	2.6E-02
Protein localization	-0.69	11	3.0E-02
B cell activation	-0.68	4	3.1E-02
Collagen catabolism	1.41	4	3.3E-02
Response to stress	-0.21	118	3.6E-02
DNA packaging	-0.64	11	3.6E-02
Response to oxidative stress	-0.72	9	3.7E-02
Metallopeptidase activity	1.29	8	3.8E-02
Peroxidase activity	-0.53	9	3.8E-02
Cell redox homeostasis	-0.86	4	4.1E-02
Antioxidant activity	-0.51	13	4.3E-02
Mitochondrion	-0.30	43	4.3E-02
Glutathione peroxidase activity	-0.80	4	4.3E-02
Metalloendopeptidase activity	1.42	7	4.5E-02
mRNA binding	-0.72	3	4.5E-02
Mitochondrial transport	-0.85	4	4.6E-02
Endoplasmic reticulum	-0.55	20	4.6E-02

A.5 Table 4: Differentially expressed genes with a Fold Change >2 or <-2 in gills of fish injected with LPS in comparison with control fish injected with PBS, in animals fed with both control diet immunostimulant diet. Differential expression was analyzed with Student's t-test ($p<0.01$). Values are expressed as fold change (FC). Common genes are highlight in grey

CONTROL			IMMNOSTIMULANT		
P student	**FC**	**Gene name**	**FC**	**Gene name**	**P Student**
		TRANSPOSASES		TRANSPOSASES	
1.10E-12	4.587	Transposase-15	-2.904	Transposase-15	1.02E-08
6.70E-23	2.989	Transposase -63	-2.333	Transposase -63	3.03E-22
2.63E-09	2.705	Transposase-36	-2.032	Transposase-36	6.82E-11
1.02E-09	2.672	Transposase-22			
1.14E-10	2.509	Transposase -64	-2.525	Transposase -64	1.56E-10
1.81E-10	2.505	Transposase-52			
1.45E-07	2.346	Transposase-9			
2.02E-10	2.346	Transposase-6			
1.19E-09	2.267	Transposase-27			
3.54E-13	2.251	Transposase-55	-2.901	Transposase-55	4.69E-10
5.13E-08	2.224	Transposase-1			
1.99E-08	2.177	Transposase-35			
4.64E-07	2.158	Transposase-37			
2.76E-08	2.126	Transposase-46			

2.35E-08	2.107	Transposase-30	-2.616	Transposase-56	1.06E-10
5.69E-09	2.043	Transposase-54	-2.142	Transposase -61	4.35E-09
1.12E-07	2.035	Transposase-42	-2.149	Transposase-18	5.66E-10
7.19E-07	2.019	Transposase-28	-2.092	Transposase-59	1.39E-06
3.30E-09	2.007	Transposase-23			
		HEMOGLOBIN		HEMOGLOBIN	
1.66E-10	2.939	Hemoglobin α-chain	-2.134	Hemoglobin α-chain	2.53E-09
4.12E-10	2.654	Alpha-globin I-2	-2.111	Alpha-globin I-2	9.59E-09
2.00E-14	2.582	Hemoglobin beta chain	-2.124	Hemoglobin beta chain	3.89E-10
2.35E-12	2.291	Alpha-globin I-1	-2.101	Alpha-globin I-1	3.36E-09
		CXC RECEPTOR		CXC RECEPTOR	
0.007986	2.990	Chemokine 5a receptor-like	-2.435	Chemokine 5a receptor-like	0.009035
2.75E-06	2.167	Chemokine receptor CXCR4			
4.95E-05	2.008	Tnf receptor superfamily 14			
		IMMUNE SIGNALLING		IMMUNE SIGNALLING	
2.84E-17	3.756	Reg. of G prot. signalling 1-2			
2.71E-10	2.683	Interferon reg. factor 1-2			
1.07E-12	2.630	Mit. activated prot. kinas 13			
2.81E-07	2.589	Diacylglycerol kinase delta2			
1.12E-07	-2.034	Nucleophosmin 1			
2.32E-11	-2.080	14-3-3C2			
		CELL ADHESION, MATRIX		CELL ADHESION, MATRIX	
0.000227	3.693	Connective tissue growth factor	-4.548	Cytohesin binding protein HE	0.00071
4.04E-07	2.350	Fibronectin precursor	-2.439	Ependymin I	0.001767
1.61E-05	2.014	Connexin 43	-3.162	Sialoadhesin	0.000361
7.47E-08	-2.084	CD97	-2.699	Tissue fact. pathway inh. 2 prec.	0.002863
0.002756	-2.095	Periostin precursor	2.079	Matrix metalloproteinase-13	3.25E-06
0.00535	-5.299	Semaphorin 4D			
		MUSCLE		MUSCLE	
0.000925	2.083	Myosin heavy chain, skeletal adult 1-2	-2.232	Myosin heavy chain, cardiac muscle β isoform	3.99E-05
0.00882	-3.326	Nebulin-2			
		TRANSCRIPTION		TRANSCRIPTION	
7.69E-12	2.675	Reverse transcriptase-like-2	-4.983	T-cell leukemia virus enh. factor	0.001585
3.50E-10	2.584	Reverse transcriptase-like-1	-2.188	Reverse transcriptase-like-1	1.16E-09
			-2.088	Transcription reg. prot. BACH1	2.40E-09
			-3.175	Sp1 transcriptional act. factor	0.000196
			-6.772	Splicing factor arg/ser-rich8	0.000956
			-2.488	PRPF39 protein	8.71E-05
			2.104	Calpactin I light chain	7.02E-08
		Ig		Ig	

6.70E-14	2.386	Ig k chain V-III region VG	3.559	Ig k chain V-IV region Len	1.86E-08
			3.214	Ig mu heavy chain disease prot.	2.06E-10
			3.202	Ig k chain V-IV region B17-2	4.29E-12
			3.060	Ig heavy chain V-III region HIL	5.92E-07
			2.829	Ig mu chain C reg. membr bound	6.02E-08
		COMPLEMENT		COMPLEMENT	
1.26E-06	2.067	Serine protease-like protein-1	-3.709	Complement component C7	0.002431
		METABOLISM		METABOLISM	
0.000162	2.654	Creatine kinase, sarc. mit. prec.	-10.08	UDP-glucuronosyltransferase 1-1	0.003153
8.60E-11	-2.168	Glyc-3-phosph dehydrogenase-6			
		DNA SYNTHESIS		DNA SYNTHESIS	
0.000182	2.173	Nucleoside diphosphate kinase, mit. prec.			
		REDOX HOMEOSTASIS		REDOX HOMEOSTASIS	
2.08E-08	-2.158	Thioredoxin-like prot. p19 prec.	2.142	Thioredoxin	7.79E-05
2.40E-10	-2.402	Glutathione peroxidase-gastroint.			
		CYTOKINE		CYTOKINE	
2.46E-08	-2.150	Small inducible cytokine SCYA104			
		MHC I		MHC I	
1.09E-11	-2.405	MHC class I heavy chain-1	8.918	MHC class I heavy chain-1	1.26E-12
				COAGULATION	
			-2.094	Beta-2-glycoprotein I	0.005799
				PROTEIN FOLDING	
			-2.437	Peptidyl-prolyl cis-trans isom. H	0.004458
			-2.066	Heat shock 27 kDa protein-1	6.06E-13
				CELL CYCLE/CYTOKINESIS	
			-10.567	Gastrulation specific prot G12	0.000787
			-6.989	N-myc downstream reg. prot.-1	9.09E-05
			-2.192	Huntingtin	3.93E-07
			-2.243	Septin 7 (CDC10 prot. homolog)	0.003519
			-2.459	G1/S-specific cyclin E1	0.000526
			-3.286	Ubiquitin ligase protein CHFR	0.000645
			-2.013	Translin	9.21E-06
			2.462	G1/S-specific cyclin D2	6.15E-11
			2.154	Adenosine kinase 2	2.50E-11
			2.001	Tyrosine-prot kin FRK	1.39E-05

			2.025	Microtubule-assoc. prot. RP/EB	9.33E-14
				TRANSPORT	
			-3.667	Organic anion transporter	0.000108
				TRANSLATION	
			3.684	40S ribosomal prot. S3-2	0.006653
			2.994	Nuclear RNA helicase	0.004288
				LYSOZYME	
			2.040	Lysozyme C precursor	1.41E-11
			-3.779	Lysozyme g-2	3.76E-05
				PROEOLYSIS	
			2.111	Proteasome subunit αtype 3	0.002016
				IMMUNE	
			-2.597	Beta-arrestin 2	0.001398
		UNKNOWN		UNKNOWN	
8.55E-07	2.051	Unknown-173	2.139	Unknown-31	0.002103
3.02E-11	2.486	Unknown-273	-2.040	Unknown-273	8.73E-10
0.000306	2.633	Unknown-215	-3.217	Unknown-88	2.58E-05
5.01E-11	2.899	Unknown-235	-4.423	Unknown-39	0.001974
0.003163	2.878	Unknown-274	-5.837	Unknown-274	0.001029
0.003238	3.120	Unknown-3	-5.828	Unknown-243	0.004381
8.73E-05	-2.265	Unknown-282	-2.603	Unknown-42	1.01E-11
			-2.247	Unknown-75	1.21E-07
			-2.137	Unknown-74	1.10E-08
			-2.139	Unknown-70	1.00E-05
			-2.163	Unknown-81	2.94E-06
			-2.061	Unknown-82	0.008864
			-4.671	Unknown-89	0.008153
5.18E-11	2.128	ReO_6-5			
3.39E-06	2.050	ReO_6-2	-2.678	ReO_6-2	3.41E-09
3.36E-12	2.116	Similar to rRNA (Vangl2)			
0.007791	2.014	Orphan nuclear receptor NR4A2			
9.96E-10	2.396	Hpa repeat-1	-2.054	Hpa repeat-1	9.64E-11
			-2.338	Hpa repeat-2	6.32E-10
			-14.586	Hypothet. UPF0193 prot-EVG1	0.00108
			-8.610	Hypothetical-fish 33	0.003904
			-2.582	Hypothetical-fish 31	8.91E-05
			-2.018	Hypothetical-fish 36	1.02E-07

A.6 Table 5: Differentially expressed genes with a Fold Difference (FD) >2 or <-2 in intestine of fish injected with LPS in comparison with control fish injected with PBS, in animals fed with both control diet immunostimulant diet. Differential expression was analyzed with Student's t-test ($p<0.01$). Values are expressed as fold change (FC). Common genes are highlight in grey

CONTROL			IMMNOSTIMULANT		
P student	**FC**	**Gene name**	**FC**	**Gene name**	**P Student**
		CELL ADHESION /MATRIX		CELL ADHESION /MATRIX	
0.00173	10.108	Lymphatic endothelium-specific hyaluronan receptor LYVE-1	-3.292	Alpha 2 type I collagen-1	2.75E-13
0.000533	3.563	Cytohesin binding protein HE	-3.099	Embryonic-type globin2+collagen α2	1.34E-11
5.38E-08	3.219	Tissue factor pathway inhib. 2 prec.	-2.840	Collagen a3(I)-1	7.29E-14
5.43E-05	2.035	Ependymin I	-2.750	C166BRARE CD166 antig. homolog	0.000296
2.89E-05	-6.672	Membrane-type matrix metalloproteinase 1 α	-2.704	Collagen a3(I)-2	3.67E-11
4.31E-07	-6.046	CD97	-2.439	Syntenin 1	9.93E-05
			-2.210	Collagen α1(V) chain prec.	0.000654
			-2.083	ADAM 9	0.003618
			4.851	Matrix metalloproteinase-9	2.57E-07
			3.207	Melanoma derived growth reg. prot.	6.31E-14
			3.008	Reversion-inducing cys-rich prot. with Kazal motifs	3.26E-11
			2.904	Matrix metalloproteinase 9-1	2.57E-05
			2.728	Hyaluronan and proteoglycan link prot. 2 prec.	2.73E-07
			2.645	Matrix metalloproteinase 9-2	1.33E-07
			2.619	Ependymin related protein-1	2.54E-11
			2.473	Tissue inhib. of metalloproteinase 2	4.69E-05
			2.285	B-cell receptor CD22-1	9.83E-09
		IMMUNE RECEPTORS		IMMUNE RECEPTORS	
0.001091	4.272	C type lectin receptor B	3.816	C type lectin receptor B	1.61E-05
2.86E-07	3.673	Chemokine receptor CXCR4	2.975	Interleukin-1 receptor-like protein 2	0.000134
			2.233	TNF receptor associated factor 1	2.85E-07
			2.168	T-cell recept α chain V reg. HPB-MLT precursor (Fragment)	8.94E-13

		TRANSCRIPTION		TRANSCRIPTION	0.000245
6.85E-07	2.655	Splicing fact., arg/ser-rich 8	-5.217	Bromodom. adj to zinc finger dom. 2B	1.29E-06
1.04E-05	2.568	Sp1 transcriptional activation factor	-5.150	HCF-binding transcript fact. Zhangfei	0.001099
9.81E-11	2.267	Transcription factor jun-B-1	-3.015	Cofactor for Sp1 activation subunit 7-1	0.002798
			-2.474	Calpactin I light chain	1.01E-11
			-2.194	Calponin 1	0.003772
			-2.155	COUP transcription factor 2	1.91E-11
			-2.002	A+U-rich element RNA binding fact.	0.004635
			3.057	Poly(RC) binding protein 2	2.25E-10
			2.924	Adenosine deaminase 1	7.05E-13
			2.900	Zinc finger protein 228	8.29E-10
			2.801	Estrogen-responsive B box protein	3.32E-12
			2.788	HIRA-interacting protein 5	3.15E-11
			2.683	Nuclear cap binding protein subunit 2	6.36E-08
			2.603	Leukotriene B4 receptor 1	3.17E-11
			2.441	5'-3' exoribonuclease 2	2.53E-08
			2.400	Heterogen. nucl. ribonucleoprot. A0	3.03E-12
			2.229	Serine/arginine repetitive matrix 1	7.18E-10
			2.103	TATA-binding prot. assoc. factor 2N	0.000245
		Ig		Ig	
1.62E-06	2.306	High affinity Ig gamma Fc receptor I precursor	4.514	Ig heavy chain V-III region HIL	1.08E-05
1.39E-13	2.176	Ig kappa chain V-IV region B17-2	2.282	Ig kappa chain V-IV region B17-1	4.27E-09
			2.116	Ig mu heavy chain disease protein	8.27E-11
			-2.945	Ig kappa chain V-IV region JI	4.58E-07
		LYSOZYME ACTIVITY		LYSOZYME ACTIVITY	
8.69E-10	3.715	Lysozyme g-2			
1.62E-14	3.110	Lysozyme C precursor	3.482	Lysozyme C precursor	1.16E-13
		SIGNALLING		SIGNALLING	
3.60E-12	3.756	Regulator of G-protein signalling 1-2	5.194	Calmodulin-3	
2.24E-10	2.438	Allograft inflammatory factor-1	2.773	Allograft inflammatory factor-1	
			3.906	Toll-interacting protein	
			2.965	Cold autoinflammat. syndrome 1 prot.	
			2.901	Mitogen-activated protein kinase 1	

			2.874	MAPK/ERK kinase kinase 1-1	
			2.613	Glomulin	
			2.568	MAPK7 interacing protein	
			2.442	MAP kinase-like	
			-22.445	Gap junction alpha-3 protein	
0.003399	-3.341	IL-1 receptor-associated kinase 1-2	-4.932	IL-1 receptor-associated kinase 1-2	
1.44E-07	-2.064	C-jun-amino-terminal kinase interacting protein 3	-3.531	Annexin IV	
			-2.831	Suppressor of cytokine signalling 3	
1.71E-09	-2.040	Granulins	-2.702	Granulins	
			-2.661	Unc-13 homolog C	
1.21E-12	-2.174	Phosphotyrosine independent ligand for the Lck SH2 domain p62	-2.024	Phosphotyrosine independent ligand for the Lck SH2 domain p62	
		APOPTOSIS AND DNA REPAIR		APOPTOSIS AND DNA REPAIR	
2.37E-14	2.364	Nuclear protein 1	2.684	Deoxyribonuclease gamma precursor	1.48E-10
7.05E-15	-2.727	Lymphocyte pore forming protein	2.141	BCL2-associated athanogene 1	1.03E-10
0.000813	-2.009	CASP8/FADD apoptosis reg. Prec.	2.116	Astrocytic phosphoprotein PEA-15	1.08E-05
			-6.915	Growth arrest and DNA-damage-inducible GADD45 beta	7.46E-07
			-6.694	Deoxyribonuclease I-like-1	0.002286
			-5.808	Programmed cell death protein 2	0.009981
			-4.088	Anti-silencing function 1B	0.000264
			-3.505	Metastasis-suppressor gene CC3	6.49E-06
			-2.482	Proliferating cell nuclear antigen	9.87E-06
			-2.440	Apoptosis regulator Bcl-X	4.42E-05
			-2.319	Growth arrest and DNA-damage-inducible GADD45 alpha-1	7.94E-05
			-2.120	Growth arrest and DNA-damage-inducible protein alpha-2	4.33E-05
			-2.103	Apoptosis inhibitor 5	0.004363
			-2.036	Programmed cell death protein 5	0.000786
		MUSCLE		MUSCLE	
0.000972	3.102	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	-10.663	Myosin regulatory light chain MRCL2-4	0.00054
6.00E-05	2.583	Troponin I-2, fast skeletal muscle	-3.444	Myosin light chain 2-2	0.00091
0.001354	2.010	Myosin heavy chain, skelet	-3.212	Myosin regulatory light chain	3.95E-07

		adult 1-2		MRCL2-1	
			-2.883	Myosin regulatory light chain MRCL2-3	0.004079
			-2.492	Transgelin	2.96E-10
			-2.298	Cofilin, muscle isoform	2.12E-11
			-2.191	Actin, alpha skeletal 5	2.42E-07
			-2.185	Actin, alpha skeletal 4	1.32E-12
			-2.150	Actin, alpha skeletal 2	1.54E-08
			2.994	Tolloid-like protein (nephrosin)-1	4.77E-12
			2.918	Myosin heavy chain, skeletal, fetal	9.40E-12
			2.830	Troponin T-3, fast skeletal muscle	2.76E-11
			2.817	Parvalbumin alpha-2	5.72E-12
			2.718	Myosin light chain 2-1	3.96E-11
			2.683	Troponin C-1, skeletal muscle	2.90E-06
			2.500	Phospholemman precursor	1.93E-12
			2.385	MIR-interact. saposin-like prot. prec.	2.96E-07
		MITOCHONDRIA /OXYGEN		MITOCHONDRIA /OXYGEN	
0.001230	2.177	Glioma pathogenesis-related prot. 1	-5.774	Thioredoxin-like protein p19 precursor	2.84E-10
1.71E-05	-2.331	Aldeyde oxidase	-4.663	Selenoprotein W	0.008341
1.56E-10	-2.264	Glutathione reductase, mit-2	-4.233	PDZ and LIM domain protein 1	3.67E-10
2.78E-06	-2.017	Ubiquinol-cytochrome C reductase complex 11 kDa prot., mit. prec.	-3.965	Thioredoxin	6.49E-08
			-3.249	Cytochrome c-1	1.76E-07
			-2.835	Glutathione peroxid-gastrointestinal	7.37E-12
			-2.832	Selenoprotein S	1.82E-06
			-2.410	Protoheme IX farnesyltransferase	0.008237
			-2.075	Peroxiredoxin 1-1	2.61E-09
			-2.054	Electron transfer flavoprot.-ubiquinone oxidoreductase	0.001065
			-2.021	Selenoprotein T-3	2.69E-05
			4.327	Cytochrome P450 2K4-1	0.001096
			4.021	Cytochrome P450 4F3	1.31E-09
			2.610	Cytochrome B-245 heavy chain-1	7.19E-07
			2.490	Quinone oxidoreductase	2.46E-11
			2.454	Superox dismutase Cu-Zn extracell.	9.84E-10
			2.253	NADH-ubiquin. oxidoreduct. 15 kDa	2.95E-14

			2.188	Cytochrome B-245 heavy chain-2	5.47E-08
		HEMOGLOBIN		HEMOGLOBIN	
3.00E-08	2.276	Hemoglobin alpha chain	-2.111	Hemoglobin alpha chain	0.000125
		CELL CYCLE		CELL CYCLE, /CYTOSKELETON	
0.000189	2.378	G1/S-specific cyclin E1	-25.771	Thioredoxin-like protein 4A	3.55E-06
0.004818	2.142	D-type cyclin-interacting protein 1	-4.301	Microtubule-associated prot. RP/EB	4.29E-14
			-3.510	Transmembrane 4 superfamily member 4	3.86E-07
			-2.882	Beta actin-1	1.52E-08
			-2.643	Tubulin alpha-ubiquitous chain	7.07E-06
			-2.497	CD63	7.03E-13
			-2.035	Protein phosphatase 2C gamma isoform	4.79E-09
			152.51	Cyclin C	0.001746
			13.363	ADAMTS-8	0.005633
			3.255	Retinoblastoma-like protein 1	5.80E-07
			2.965	T-cell leukemia associated homeodomain protein Tlx3b	0.000208
			2.781	Chromosome-associated kinesin KIF4A	1.61E-12
			2.677	Cyclophilin-1	1.64E-12
			2.256	Myristoylated alanine-rich protein kinase C substrate	5.71E-13
			2.245	G1/S-specific cyclin D2	5.60E-13
			2.157	N-myc downstream regulated protein-1	1.77E-10
			2.125	Fumarate hydratase, mitochondrial precursor	7.59E-08
			2.081	Cyclin A2	1.37E-08
			2.053	Huntingtin	3.01E-06
		TRANSLATION		TRANSLATION	
0.000353	2.006	Zinc finger protein 183	2.519	Eukaryotic translat initiat. fact2 unit2	4.60E-13
			2.501	Mitochondrial ribosomal protein L1	1.35E-08
			2.380	28S ribosomal prot. S16, mit. precursor	3.01E-10
			2.315	39S ribosomal prot.L45, mit. precursor	4.19E-06
			2.032	Tyrosyl-tRNA synthetase	2.67E-07
			2.032	Euk. translat. initiat. fact 3 unit 6-2	5.75E-14
			-6.765	Basic leu-zipper protein BZAP45-2	0.004148
			-3.398	Elongation factor 1-beta (EF-1-beta)	0.000847

			-2.261	40S ribosomal protein S9-2	1.40E-08
			-2.107	Suppressor of initiator codon mutations, related sequence 1	4.03E-11
			-2.089	Basic leucine-zipper prot. BZAP45-1	9.71E-06
		PROTEOLYSIS		PROTEOLYSIS	
3.50E-16	-2.899	Cathepsin Z precursor	-4.151	Serine protease EOS	0.004889
1.90E-12	-2.534	Cathepsin C-1	-2.182	Cathepsin C-1	8.75E-10
6.52E-12	-2.482	Cathepsin L2	-2.426	Proteasome subunit beta type 9 prec.	7.63E-10
2.24E-10	-2.343	Cathepsin Y	-2.394	Cathepsin C-2	1.62E-11
1.26E-11	-2.263	Procathepsin L-1	-2.261	Hypothetical protein FLJ11342	0.000938
			-2.193	Cathepsin S	1.95E-09
			2.035	Proteasome subunit alpha type 6	2.13E-07
			2.007	Dipeptidyl peptidase IV	1.11E-10
		ANTIGEN PROCESSING, PRESENTATION		ANTIGEN PROCESSING, PRESENTATION	
2.12E-12	-2.915	MHC class I heavy chain-1	6.485	MHC class I heavy chain-1	3.02E-14
3.58E-13	-2.743	Lymphocyte antigen 75	2.239	MHC class I heavy chain-2	0.000604
1.76E-17	-2.696	γ-inf induc. lysosomal thiol reduct.	-2.046	MHC II invariant chain-like protein 1	5.06E-05
		COMPLEMENT		COMPLEMENT	
0.000452	-2.068	Complement factor MASP3	-3.934	Complement factor MASP-3	0.001396
			-2.431	Complement factor H-related prot 1	7.69E-07
			4.788	Serine protease-like protein-1	8.05E-15
			4.012	Complement factor H-2	1.79E-07
			3.489	Complement factor B/C2-B	1.55E-10
			3.005	Complement component C3-3-2	4.17E-08
			2.818	Serine protease-like protein-3	9.60E-12
			2.763	Serine protease-like protein-2	2.16E-13
			2.727	Complement factor H-1	2.01E-12
			2.446	Complement factor D	4.17E-05
		CYTOKINE/CXC		CYTOKINE/CXC	
			-3.368	Small inducible cytokine B14 prec.	0.004127
1.03E-07	-5.663	Small inducible cytokine SCYA104	-2.913	Small inducible cytokine SCYA104	0.001547
			-2.581	CC chemokine SCYA110-2	1.75E-08
			4.000	CCL4	9.22E-12
			3.916	Leukocyte cell-derived chemotaxin 2	2.14E-12
		RESPONSE TO VIRUS		RESPONSE TO VIRUS	
			-7.110	Leukemia virus receptor 1-2	0.000152
2.86E-10	-2.703	Interferon-induced protein 44-2	-2.639	Interferon-induced protein 44-2	1.15E-09

			-2.390	Leukemia virus receptor 1-1	0.000359
			-2.364	Barrier-to-autointegration factor	3.74E-06
			-2.251	Interferon-induced protein 44-3	8.20E-06
			-2.249	HIV TAT specific factor 1	0.00081
		TRANSPORT		TRANSPORT	
1.19E-05	2.110	Organic anion transporter	-2.726	Coatomer epsilon subunit 2	9.03E-06
1.83E-12	-2.911	Solute carrier family 10 , member 2	-2.219	14-3-3B1	0.000916
2.89E-14	-2.542	Metallothionein-IL	3.535	Sodium/bile acid cotransporter	3.37E-09
5.09E-14	-2.155	Ferritin heavy chain-1	2.026	Metallothionein A	1.78E-08
3.29E-16	-2.109	Ferritin heavy chain-2			
		HEME BYOSINTHESIS		HEME BYOSINTHESIS	
1.26E-07	-2.175	Aminolevulinate, δ-, synthetase 1-2	-2.495	Aminolevulinate, δ-, synthetase 1-2	6.76E-05
6.50E-09	-2.113	Aminolevulinate, δ-synthetase 1-2	-3.035	Aminolevulinate, δ-synthetase 1-2	5.53E-06
		METABOLISM		METABOLISM	
8.06E-14	-2.601	β-hexosaminidase α-chain prec.	-11.292	Phospholipase A-2-activating protein	0.001228
7.93E-14	-2.474	High density lipoprot.binding prot.	-6.212	Cytosolic phospholipase A2 beta	0.00339
6.72E-08	-2.133	Acid ceramidase precursor	-5.845	Glyoxylate reductase/hydroxypyruvate reductase	0.004642
			-4.343	Ornithine decarboxylase antizyme-1	4.31E-06
			-3.088	Carbonic anhydrase	0.008029
			-3.001	Mannosidase alpha	0.002983
			-2.560	Nonspecific cytotoxic cell recep prot.1	6.18E-09
			-2.539	Apolipoprotein A-IV	2.14E-10
			-2.339	Creatine kinase, M-2	1.75E-08
			-2.288	Creatine kinase, M-3	2.53E-06
			-2.221	Lysosomal acid lipase/cholesteryl ester hydrolase	1.39E-05
			-2.064	Transaldolase	4.82E-07
			3.207	Hypothetical protein LOC122618	0.000169
			2.957	Fructose-1,6-bisphosphatase isozym 2	9.50E-13
			2.943	D-3-phosphoglycerate dehydrogenase	4.49E-15
			2.922	Dermatan-4-sulfotransferase-1-2	4.30E-15
			2.810	Galectin-3	1.31E-12
			2.742	Beta-galactosidase-related protein	1.98E-13
			2.733	Trans-2-enoyl-CoA reductase	8.22E-09
			2.706	Alcohol dehydrogenase	1.54E-11

		[NADP+]
	2.663	Dermatan-4-sulfotransferase-1-1
	2.534	Alanine-glyoxylate aminotransferase 1
	2.471	Prostaglandin D synthase homolog
	2.044	Secretory phospholipase A2 precursor
		RESPONSE TO TOXIN
1.54E-10	-2.574	Epoxide hydrolase 1
		PROTEIN FOLDING
	-4.738	78 kDa glucose-regulated prot. prec.
	-2.760	Cyclophilin-40
	-2.703	Ah receptor interacting protein
	-2.600	60 kDa heat shock protein-2
	-2.552	Heat shock 70kD protein 9B-1
	-2.490	15 kDa selenoprotein
	-2.318	Stress 70 protein chaperone
	-2.182	Cyclophilin-2
	-2.281	DnaJ homolog, subfamily C, memb 3
	-2.096	94 kDa glucose-regulated protein
	-2.051	60 kDa heat shock protein-1
	-2.050	DnaJ homolog subfamily B 11 prec.
	3.333	Serum amyloid P-component 2
	2.611	DnaJ homolog subfamily C member 9
	2.152	Heat shock factor 2-2
	2.133	Heat shock protein 75 kDa-2
		ATP
	-2.558	ATP synthase beta chain-1
	-2.537	ADP,ATP carrier protein T2
	-2.455	ATP synthase beta chain-2
	-2.371	ADP,ATP carrier protein 3
	-2.044	ADP-ribosylation factor 4
		MELANIN
	-2.006	D-dopachrome tautomerase
		DNA SYNTHESIS
	-4.167	Adenosine kinase 2
	-3.518	Ribonucleoside-diphosphate reductase large subunit
	-2.452	Thymidine kinase, cytosolic
	2.161	Histone H14
		SPERM

1.54E-11	
8.05E-14	
1.37E-06	
0.009448	
8.36E-07	
0.000182	
3.73E-06	
2.79E-05	
1.96E-07	
1.71E-07	
1.30E-07	
2.58E-13	
0.000691	
6.28E-12	
2.36E-07	
0.000264	
2.50E-08	
4.92E-12	
8.59E-07	
1.87E-11	
9.33E-11	
6.17E-11	
4.74E-05	
1.30E-10	
8.99E-08	
0.000391	
2.29E-12	
7.74E-08	
0.001548	
6.46E-07	

		UNKNOWN
0.009052	3.115	Unknown-71
0.002056	2.511	Unknown-66
0.000251	2.108	Unknown-141
7.61E-07	2.033	Unknown-116
1.72E-06	-2.708	Unknown-203
7.12E-14	-2.196	Unknown-5

-2.841	Epididymal secretory protein E1	1.07E-09
	TRANSPOSASES	
-2.903	Transposase-15	1.25E-10
2.754	Transposase-44	0.004394
2.679	Transposase-21	2.13E-11
2.446	Transposase-31	2.83E-10
2.271	Transposase-57	4.76E-07
2.074	Transposase-11	1.05E-10
	COAGULATION	
-3.913	Annexin 5	1.24E-13
-3.308	Annexin A3	3.97E-08
-2.048	Red cell acid phosphatase 1	0.000264
3.291	Plasminogen precursor-1	1.02E-12
2.691	Coagulation factor X precursor	1.72E-13
2.246	Beta-2-glycoprotein I	2.41E-05
3.525	Similar to rRNA (Vangl2)	
2.322	Over-expressed breast tumor prot-like	
2.253	FLJ14655	
	UNKNOWN	
8.741	Unknown-271	0.005429
4.182	Unknown-42	4.68E-13
3.647	Unknown-193	4.00E-10
3.429	Unknown-158	1.18E-12
3.370	Unknown-149	2.90E-10
3.291	Unknown-11	8.04E-13
3.123	Unknown-199	2.32E-12
3.117	Unknown-150	2.78E-13
3.113	Unknown-85	2.64E-10
3.111	Unknown-194	5.03E-13
3.091	Unknown-155	2.91E-07
3.041	Unknown-123	9.27E-10
3.036	Unknown-168	2.10E-13
2.955	Unknown-54	1.76E-12
2.928	Unknown-185	4.41E-15
2.900	Unknown-275	3.27E-10
2.891	Unknown-124	3.00E-08
2.828	Unknown-230	4.58E-07
2.785	Unknown-171	3.57E-08
2.770	Unknown-174	2.14E-12
2.705	Unknown-157	3.25E-13
2.645	Unknown-148	1.31E-09
2.608	Unknown-184	4.26E-15
2.557	Unknown-119	3.91E-11

		HYPOTHETICAL
1.09E-05	2.472	Hypothetical-fish 31
0.006470	2.070	Hypothetical-fish 17
8.39E-07	2.039	Hypothetical-fish 44

2.517	Unknown-175	5.50E-12
2.504	Unknown-28	1.05E-06
2.485	Unknown-232	2.38E-10
2.443	Unknown-202	2.13E-14
2.437	Unknown-198	1.08E-12
2.436	Unknown-231	1.22E-11
2.426	Unknown-247	1.94E-06
2.408	Unknown-121	2.94E-10
2.403	Unknown-189	4.39E-12
2.402	Unknown-44	0.000217
2.395	Unknown-117	2.20E-10
2.392	Unknown-235	4.33E-11
2.389	Unknown-146	5.39E-12
2.216	Unknown-115	4.16E-06
2.164	Unknown-179	1.14E-11
2.156	Unknown-200	2.80E-13
2.143	Unknown-110	1.06E-05
2.078	Unknown-161	0.002205
2.077	Unknown-165	7.76E-05
2.070	Unknown-176	2.19E-09
2.042	Unknown-112	2.00E-06
-17.002	Unknown-77	0.000314
-13.605	Unknown-242	0.0007
-12.061	Unknown-223	3.29E-05
-3.722	Unknown-255	0.005604
-2.254	Unknown-100	0.002281
-2.090	Unknown-163	7.92E-06
-2.083	Unknown-109	2.19E-09

	HYPOTHETICAL	
2.950	Hypothetical-fish 9	3.03E-14
2.748	Hypothetical-fish 10	7.81E-12
2.442	Hypothetical-fish 27	6.53E-08
2.409	Hypothetical-fish 18	1.30E-12
2.315	Hypothetical-fish 28	0.002639
2.132	Hypothetical-fish 17	0.001626
2.110	Hypothetical-fish 4	2.58E-11
-2.922	Hypothetical-fish 42	9.29E-07

8 General discussion

Overview and applications

The basic aim of this thesis was to further explore PAMP-driven immune responses in fish in a background of comparative immunology, and furthermore provide an insight in potential applications related to the aquaculture industry, using a broad range of molecular and functional genomic tools.

The aquaculture industry is probably the fastest growing food-producing sector and accounts for nearly 50 percent of the world's food fish [1]. Despite these factors, signicant economic losses derived from troubles such as stress or infectious diseases continue to be serious problems in the aquaculture sector. The use of antibiotics is known to induce resistance in both pathogenic and non-pathogenic micro-organisms, causing serious damages to the environment, animal health and human safety. Therefore the use of low-cost and effective programmes of vaccination or prophylactic treatment is essential in any fish farm. However, cost effective vaccines for many diseases have not yet been produced and there are still a number of important bacterial, viral and parasitic diseases for which there is no prophylactic treatment. Moreover, recently it has been discovered that commonly used oil-adjuvanted vaccines in salmon farming provoke autoimmunity reactions similar to oil-induced lupus in mice [2]. Research on subjects involved in improving aquaculture practices is essential for the sustainable development of this industry.

Currently, the view of the immune system is as a sophisticated, complex, highly redundant, and multilevel network of various mechanisms, ranging from pathways common to every cell in the body to cells specialized in immune regulation and responses at the level of the whole organism. Therefore, we tried to analyze different immune responses at different levels. Using from traditional molecular techniques, including PCR analysis or cloning, to functional genomic techniques, such as high-throughput *in situ* hybridization and microarray analysis, we studied two teleost species, sea bream and rainbow trout, which show a high genomic plasticity and can

be used as an outstanding model to analyze a multitude of questions related to adaptation and evolution. *In vitro* analyses were performed to investigate primary cultures of differentiated macrophages of both species, in parallel with the evaluation of the effect of immunostimulant diets in different organs of rainbow trout, at a functional, transcriptional and proteomic level.

The cloning and characterization of CD83 in sea bream together with the comparison at a transcriptional level of the immune response of sea bream and rainbow trout macrophages contribute to amplify the information available of the fish immunity and evolution of the immune system. The analysis of the transcriptomic and proteomic regulation of trout macrophages in response to bacterial LPS or synthetic dsRNA challenges provides novel insights into the knowledge of the molecular mechanisms modulated by fish macrophages when coping with inflammatory processes. The evaluation of the consequences of dietary acclimation of rainbow trout to immunostimulant diets is the first contribution of genomics tools into the understanding of the effect of such diets, which are commonly used in the aquaculture sector.

Characterization of sea bream macrophages. CD83

The first objective of this thesis was to *isolate and characterize sea bream macrophage population differentiated in vitro, through their morphologic changes, phagocytic capacity, activation and ability to response to LPS, together with the cloning and expression analysis of the myeloid marker CD83*.

Globally, macrophages are versatile cells that play many roles. They act as scavengers, as antigen presenting cells or as secretory cells. They have a crucial role in initiating the immune response, and are vital to the regulation of immune responses and the development of inflammation; they can produce a wide array of cytokines, chemokines, enzymes, complement proteins and regulatory factors. It is also well described that macrophages are one of the innate immune cells involved in the identification of infectious pathogens and the subsequent modulation of the immune response. Macrophage can be induced by pathogens to produce inflammatory cytokines, reactive oxygen and nitrogen species and to activate the adaptive immune

system through antigen presentation [3]. In mammals, cultured monocytes undergo a variety of modifications in morphology, function and gene expression. *In vivo*, such changes in cellular activity could result from changes in the transcriptional profile of the cell possibly as a consequence of migration into tissue from the peripheral blood. Cultured human macrophage-monocytes are believed to resemble human resident tissue macrophages, and therefore they are an interesting tool that provides a deeper understanding of cellular processes during immune activation [4]. The cell culture characterized here was similar to that of mammals, thus the culture of gilthead sea bream monocytes over several days resulted in their differentiation into the mature macrophage phenotype, resembling resident tissue cells.

As described for other fish species [5, 6], head kidney myeloid cells of gilthead sea bream incubated for various days in primary culture became adherent to the plastic culture dish surface and spontaneously differentiate into mature macrophages, reaching the typical phenotype: elongated, significant branched extensions and rounded morphology. These terminally differentiated macrophages possess a high phagocytic capacity, since they phagocytosed a significant number of Zymosan (from *S. cerevisie*) and *E. coli* particles during 1h of incubation, and are highly responsive to LPS stimulation, therefore they increase mRNA expression of CSF-1R (marker for macrophage cells), TNFα and IL-1β in dose response experiments, in a similar fashion to results reported in mammals and in rainbow trout macrophages.

To further investigate gilthead sea bream macrophages, the next step was to try to determine a good cell marker for the activation of these cells. Because CD83, which has been commonly used as a standard surface marker for dendritic cells, had been recently cloned in rainbow trout, nurse shark, atlantic salmon and japanese flounder, but on the other hand, little is known about the existence of DC in fish, the objective was to clone and characterize CD83 as a myeloid surface marker. Thus, a full-length CD83, a cell surface membrane glycoprotein member of the Ig superfamily with important immune functions, was cloned and characterized from these gilthead sea bream macrophages cultured *in vitro*. The 222 amino acid putative peptide showed the conservation of some features shared by vertebrate CD83. Multiple alignment with other fish CD83 sequences revealed high homology with other fish sequences and with human CD83.

CD83 mRNA expression was significantly up regulated after stimulation with LPS, reaching maximal levels with 50μg/ml of LPS from 4h to 12h of incubation.

These results are consistent with the dynamic observed for mammalian dendritic cells. In mammals, CD83 regulates B cell function, thymic T cell maturation, and peripheral T cell activation, and confers immunosuppressive function to CD4(+) T cells [7]. In addition to the important functions for CD83 during immune responses, several studies have reported that CD83 blockade using soluble receptor constructs inhibits T cell responses in vitro and in vivo, can affect autoimmune disease development and progression, and can inhibit transplant rejection, and therefore CD83 has been proposed as a potential therapeutic target by using recombinant proteins against CD83 function [8]. The observed up-regulation of CD83 in fish macrophages suggests that CD83 may also play an important role in the regulation of immune responses in fish and this cell type may represent an important source of soluble CD83 in fish. On the other hand, CD83 in mammals is mainly expressed on mature DCs, the most efficient of antigen presenting cells, which are not characterized in fish. Therefore, it is interesting to see whether fish possess this cell type, their possible role in therapeutic treatments and the function of CD83 in the immune system. However, although a CD83 homolog has been cloned in nurse shark (*Ginglymostoma cirratum*) [9], suggesting that the role of CD83 has been conserved over 450 million years of vertebrate evolution, several unsuccessful attempts have been made to isolate and characterize dendritic cells in fish. For the time being, CD83 has been found in gilthead sea bream macrophages [10], in the long-term *S. salar* leukocyte cell line SHK-1 [11] and in the *S. salar* highly phagocytic *S. salar* cell line TO [12]. In addition, CD83 has been described as a good marker for activation of macrophage cells in fish [10]. All these data together led us to hypothesize that a macrophage phenotype, present in all vertebrates, specialized during evolution and gave rise to dendritic cell phenotype and a macrophage cell phenotype in higher vertebrates.

Anti-viral signalling in response to LPS in fish macrophages. Presence of contaminants in LPS preparations

Our second objective was to *address the ability of the two species to enhance the expression of antiviral-related genes upon LPS stimulation, using primary cell cultures of differentiated macrophage-like cells and to evaluate the contribution of contaminants in commercial LPS preparations in the activation of gene expression.*

The utility of the macrophage *in vitro* system for elucidating immune mechanisms has been widely demonstrated in both mammals and fish. Once that we had characterized at a molecular and functional level primary cultures of sea bream macrophages, the next goal was to compare macrophage cells from two evolutionary distant species.

In fish, gram-positive bacteria, like *Streptococcus,* and acid-fast bacteria, which includes *Mycobacterium* species, cause disease in cultured animals. However, most bacteria that infect fish are gram-negative, including *Aeromonas hydrophila, Aeromonas salmonicida, Flavobacterium columnare, Vibrio* and *Pseudomonas* species. LPS is the major constituent of the external layer of the membrane of Gram-negative bacteria, and has been found to induce a similar gene expression profile as whole bacteria, illustrating the major role LPS plays in stimulating the early response to bacterial infection [13]. Therefore is a typical PAMP widely used to study the immune mechanisms that are activated in response to bacteria recognition. In mammals LPS is recognized by TLR4, leading the expression of a wide array of genes which include both proinflammatory cytokines and genes specifically involved in the antiviral response [13, 14]. In both rainbow trout and chicken has been suggested that TLR4 receptor complex that mediates the robust cellular reactions to LPS may be restricted to mammals, as trout macrophages did not up-regulate the antiviral genes IFN-α and IP-10-like protein when the cells were stimulated with ultra-pure LPS [15] and LPS failed to activate the MyD88-independent induction of IFN-β in chicken cells [16]. Therefore activation of genes involved in the antiviral response by commercial LPS may have been driven by contaminants in the preparation [15]. To investigate whether this hypothesis can be applied to all teleost species, sea bream macrophages and rainbow trout macrophages were stimulated with both normal LPS (nLPS) and ultra-pure LPS (pLPS). Expression of typical anti-viral genes was analyzed and the results were compared between the two species. Re-purified LPS, free of other bacterial components, enhanced the expression of antiviral genes in sea bream macrophages, nevertheless, pLPS did not regulate the expression in rainbow trout macrophages. It is tempting to speculate that during 200 millions years of evolution between both species species-specific mechanisms in the recognition-response to bacteria may have been developed. However, we should take into account that culture conditions and differentiation of

sea bream macrophage cells may be different to previous studies resulting in different cell activation pathways, more similar to mammalian macrophage cells.

Furthermore, results clearly indicate that in other studies in fish the activation of genes involved in the antiviral response by non-repurified LPS may have been driven by "contaminants", as it is has been reported in mammals. However, these preliminary findings do not exactly mean that all previous works may have wrong results. Undoubtedly, the "contaminants" present in LPS preparation are present in the context of whole bacteria and are important in the inflammatory response to Gram-negative bacterial infection. Thus, discrimination between nLPS or pLPS is important for the cases that investigation is performed to address questions referred to specific TLR activation and signalling, and the subsequent inflammatory events. In fish, utilization of nLPS or pLPS may be important in projects related to the identification of elements involved in the TLR4-mediated signalling. Concerning this issue, the results presented here contribute with novel insights. While TLR4 may be involved in the response to LPS in non-mammalian vertebrates, the ability of TLR4 receptor complex to mediate ultra-sensitive and exceptionally robust cellular reactions to LPS appears to be restricted to mammals. Therefore much higher LPS concentrations are needed to induce a similar response than that of mammalian cells, and both fish and chicken seem to lack a functional LPS-specific TRAM-TRIF signalling pathway which culminate in antiviral-IFN cascade, suggesting that it arose late in the evolution of vertebrates and may be important only in higher vertebrates [15, 16]. Nevertheless, although our results in sea bream confirm the low sensitivity of fish macrophage to LPS, they suggest, in contrast to chicken and other fish species, a parallel activation of antiviral pathways in response to LPS. More detailed studies on non-mammalian vertebrates are necessary for an adequate understanding, although as both gene loss and gene gain within TLRs has been common processes during evolution [17], macrophage response to pLPS may suggest that sea bream mechanisms, belonging to the most evolved group of fish, converge with mammalian signalling response to LPS. An overall picture of TLR evolution is shown in figure 1.

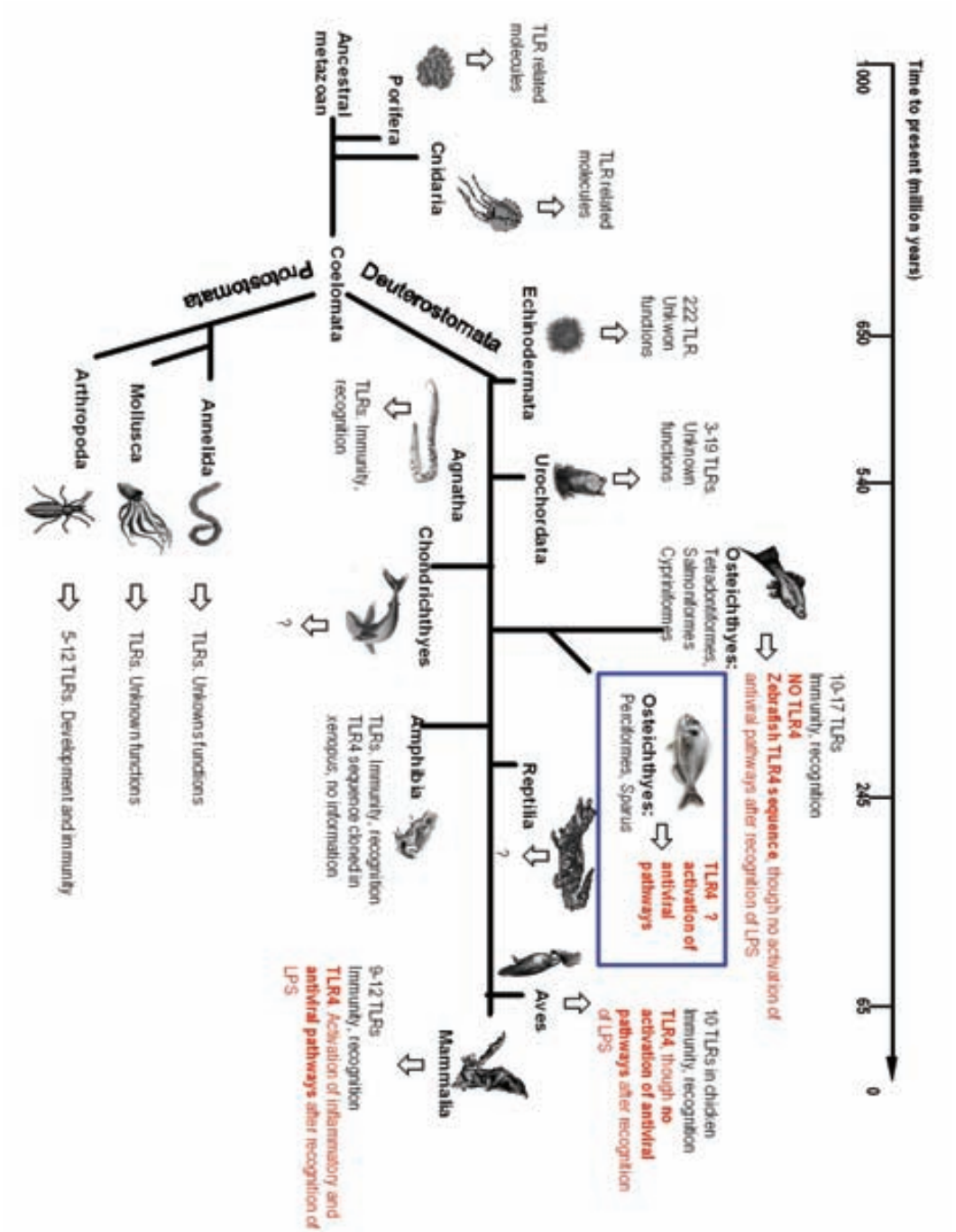


Figure 1: TLR responses in evolution

Transcriptomic responses of rainbow trout macrophages to bacterial LPS and viral ds(RNA) analog

The third objective was to *compare the differential gene expression patterns of rainbow trout macrophage cells in response to the bacteria component E.coli LPS and the virus analog poly(I:C), assessed by microarray analysis and TNFa western blot analysis.*

As previously explained, among the microorganisms recognized by macrophages, bacteria are an important and highly diverse class of pathogens. Moreover, viral diseases are also a very important problem in aquaculture as are responsible for high mortalities in farmed fish, causing significant economic losses. Viruses affecting fish are Infectious Pancreatic Necrosis Virus (IPNV), Viral Hemorrhagic Septicemia virus (VHSV), Infectious Salmon Anemia virus (ISAV) or Novirhabdovirus infectious hematopoietic necrosis virus (IHNV). Despite of the substantial amount of research done on fish viruses affecting species in aquaculture, both in commercial companies and in academic organizations, few viral vaccines are licensed [18].

The understanding of the host response to bacteria and virus provides a foundation to elucidate microbial tactics for evading the immune system of fish and thus further investigate disease prevention and treatment. To that end, microarray analyses were performed, using the salmonic-specific cDNA microarray SFA 2.0, containing 1800 clones [20]. Although it has a relatively small number of genes, compared with other platforms, each clone was selected by functional category and has six replicates spots, than together with the dye-swap design of experiment provide robust normalization of gene expression values and robust statistical analysis, allowing the differentiation of small changes in the expression of the genes. Those differentially expressed genes are grouped by functional classes, by the Gene Ontology (GO) system. GO is the standard method to classify genes by their biological function, allowing a useful interpretation of the microarray data. Thus, the results are processed and analyzed, obtaining a list of ranked differentially expressed genes and differentially expressed GO categories, revealing key information of regulated biological systems and processes.

Many conclusions can be extracted from the analysis of rainbow trout macrophages differentiated in vitro and stimulated with LPS or Poly (I:C). Firstly, the

list of differentially expressed genes (575 genes for LPS, of those 401 with a FC>2, and 537 for Poly (I:C), 219 with a FC>2) indicates that LPS induces a more intense response, though genes regulated by Poly (I:C) showed FC which are much higher than LPS. From the total differentially expressed genes, 226 transcripts were common for both treatments of which 75 showed a reverse pattern of expression for LPS and Poly (I:C), thus 41 genes were down-regulated in LPS and up-regulated in Poly (I:C) and 34 were increased in LPS and decreased in Poly (I:C). The GO categories that grouped these genes, which are classes mainly involved in defence response, communication and signal cascades, showed same profile as that of the genes. Among the genes induced by Poly (I:C) and repressed by LPS are specific anti-viral proteins, a number of genes related to cell activation and differentiation and genes involved in inflammatory response at the level of cytokinesis and cytoskeleton. On the other hand, acute phase response genes were enhanced by LPS and decreased by Poly (I:C). Transcriptomic profiles shared for both treatments were also found, such as the regulation of genes involved in extracellular matrix proteolysis and several cytokines, chemokines, receptors and signalling proteins. Furthermore, whereas LPS declined expression of both MHC I and MHC II, the viral agent clearly switched regulation towards MHC I pathway, and at the protein level, western blot analysis in stimulated macrophage supernatants showed that LPS induced a faster TNFα release than Poly (I:C). Collectively, the results reveal the transcriptomic regulation when fish must cope with bacterial or viral components, emphasizing the different mechanisms activated by fish macrophages in response to bacterial or viral agents.

Microarray studies are a high-throughput method that offers the possibility to address the regulation of thousand of single genes, sets of genes and functional categories, comparing cellular mechanisms, signal elements, and metabolic and regulatory pathways in a single assay, thus analyzing a wide context in the study of the immune responses to virus and bacteria. Set of genes with similar expression patterns can be connected, and as more microarray analysis are conducted using different pathogens, host response profiles begin to highlight a number of genes with conserved expression patterns following infection, many of which may play important roles in teleost immunity [19]. The microarray used for the analysis, as well as the analysis of the data, can considerably influence the quality of the results obtained from global expression studies. The major goal of this study was to provide a quantitative

description of cellular transcriptomic regulation in response to bacterial and viral components. Careful experimental designs as well as correct data mining approaches were necessary, though it is also important to keep in mind that microarray technology has some limitations. For instance, the cut-off for minimal expression applied, which can exclude small but biologically relevant regulations, the selected transcript enrichment of the array, or the fact that mRNA levels may not reflect the abundance of the proteins they encode since post-transcriptional regulation and post-translational modifications frequently influence the level of biologically active protein. In the present study, protein levels of the proinflammatory cytokine TNFα are in agreement with transcriptomic profiles. Some efforts have been made to elucidate the global relationship between mRNA modulation and protein abundance. In mammals, the human macrophage response to *M. tuberculosis* has been compared by transcriptome and proteomic analysis, and differential expression of a greater number of genes was detected by microarray analysis than by 2D gel electrophoresis, although overlap between the results was observed [21]. Other report studying single-cell proteomic analysis of *S. cerevisiae* indicated that changes in mRNA levels are largely captured by changes in protein levels, though in a number of cases protein changes are not mirrored by mRNA regulation [22]. The knowledge of transcriptional changes that lead to tissue responses is necessary to understand immune responses, and despite its limitations, microarray analysis is a very useful tool that has helped to further understand the mechanisms activated by fish macrophages in response to viral or bacterial components.

Effects of immunostimulation through dietary manipulation. Evaluation of mucosal immunity

The fourth objective was to *investigate in rainbow trout the changes in the transcriptome induced by a commercial immunostimulant diet at two major sites of mucosal immunity; the gills and the intestine, analyzed by microarray analysis and in situ hybridization.*

Diets containing immunostimulants are a common element in aquaculture, used to enhance the resistance of cultured fish to disease and stress. Surprisingly, although several studies have addressed the efficacy of such diets analyzing classical

immune activation parameters, very little is known about the transcriptome regulation induced by immunostimulants. From our point of view, to fully understand effects and/or efficacy of IS-diets, changes in gene expression induced by the acclimation to the diet need to be elucidated. In fact, research in crustaceans has paid more attention to this approach and some stimulatory effects of immunostimulants at the level of mRNA regulation have been evaluated [23]. Furthermore, despite the key role of the portals of entry of the animal, their function after this immunostimulation through dietary manipulation remains unknown. Portals of entry are gills, skin and intestine, the organs in contact with the medium, therefore potential site of infections, where a strong local immune response is important to avoid disease [24]. For these reasons the investigation performed throughout chapters 5, 6 and 7 seemed necessary to increase knowledge in the molecular mechanisms regulated by IS-diets and its efficacy. Microarray analysis, ISH, real-time Q-PCR and R.I.A were the tools used to address the questions relative to the effects of IS-diets in rainbow trout.

Transcriptomic profiles obtained show that dietary acclimation to a IS-diet for 4 week significantly alters gene expression at portals of entry in a tissue-specific manner, thus gills show a stronger transcriptomic regulation although the magnitude of the response is higher in the intestine. Strikingly, the global tendency in both tissues analyzed shows an overall decrease. Among the profiles shared by both organs, genes and categories related to cell communication and signal transduction were decreased, and classes involved in structural constituent of ribosome and synthesis of proteins were increased. As in the response of macrophages to the PAMPs LPS or Poly (I:C), many transcripts involved in migration of myeloid cells and remodelling of the extracellular matrix were modulated through β-glucan PAMP-activation in both organs. Within the tissue-specific responses, gills increased genes associated to processing and antigen presenting capacity, probably due to the recruitment of antigen presenting cells (APCs) to the outermost cellular layer in contact with the external milieu together with the up-regulation induced in the resident APCs. In intestine, groups including inflammatory response, response to bacteria or to biotic stimulus were reduced, and a clear regulation of chemotaxis and cellular differentiation was observed. This tissue-specific effect of the diet in the fish likely represents direct contact of the PAMP with the intestine and indirect, cytokine-mediated, effects in the gill.

Moreover, several differentially expressed transcripts were novel genes with unknown function. In fact, in all the microarray experiments performed in this thesis, described in the chapters 4-7, many unknown genes were found as highly regulated. Gene expression profiling using microarray analysis is a useful tool for the discovery of important genes and their roles, providing the bases for future studies. The modulation of unknown transcripts in macrophage cells in response to LPS and Poly (I:C), or in different organs in response to the administration of IS-diets, suggest an important role for these novel transcripts. The increase of the number of fish EST sequences and the efforts in identification, annotation and assembling of sequences in fish genomic databases may drive to the determination of important genes.

On the other hand, *in situ* hybridization (ISH) studies provide the possibility to visualize the localization of target genes. The morphology, including the structure and form of the immune system, is different between fish and mammals [25]. The distributions observed by ISH analysis in gills and intestine of genes involved in tissue remodelling, cell differentiation, antigen presenting capacity and chemotaxis are useful information for the deeper understanding of the organisation at a tissue level of the immune system of fish.

Effects of immunostimulation through dietary manipulation. Evaluation of immune response

The fifth objective was to *evaluate in rainbow trout the changes in the transcriptome induced by a commercial immunostimulant diet at two important immune organs in fish, head kidney and spleen, assessed by microarray analysis and in situ hybridization.*

The head kidney of fish is a complex tissue containing cell populations similar to those found in lymphoid organs in mammals. In addition, it is the most important organ involved in haematopoiesis and serves as secondary lymphoid organ. A rich population of leukocytes, including lymphocytes and macrophages can be found, and furthermore, performs an important role removing damage cells and foreign or antigenic substances from the blood and contributes in the systemic immunity in teleosts [26]. The second organ analyzed, spleen, can be devided in white pulp

(lymphcoyte-rich) and red pulp (erythrocyte-rich portions), possess also melanomacrophage centers, major sites of erythrocyte destruction and centers in general have been considered to be metabolic dumps [25]. When invading pathogens are able to pass the initial barrier of the mucosal organs, mainly head kidney and spleen activate immune responses to protect the organisms. The role of these organs when fish are fed with diets containing IS remains unclear, thus throughout this chapter the effects of the IS-diets were studied in both head kidney and spleen.

β-glucans, in mammals, activate the production of cytokines and responses associated with Th1 immunity. Therefore, it is assumed that diets containing β-glucans provoke a priming effect in the immunity of fish. We wanted to investigate whether IS-diets increase hematopoietic proliferation, differentiation and survival events in the organs with important immune functions. Microarray technology has allowed us to conduct simultaneous expression analysis on 1800 genes, with functions such as immune response, cell communication, receptor activity, apoptosis or response to oxidative stress, to investigate the effects of such diets in head kidney and spleen.

In summary, the transcriptomic profiles showed that head kidney was not highly affected, and modulated a total of 32 differential expressed genes ($p<0.01$), whereas a higher regulation was observed in spleen which altered expression of 168 genes ($p<0.01$). As in the organs acting as portals of entry, a larger reduction of genes and GO categories rather than induction was obtained. The goal of this work was to address the trancriptomic changes induced by a commercial diet following manufacture's instructions (4 weeks of administration). The results show that administration of IS-diets for 4 weeks induce a global down-regulation in the immunity of the animals, and this effect could be due to the prolonged period of administration. Recently, it has been reported the importance of the period of administration of β-glucans for a successful protection [27]. The prolonged administration may result in the global decrease in transcriptomic activity.

As showed by microarray analysis and ISH, head kidney decreased expression of HSPs and hematopoietic genes that together also suggest a repression in the activation of cells by exterior stimulus, and increased genes involved in signalling and apoptosis. IS-diet suppresses hematopoietic activity in head kidney, coordinated with changes in cells and extracellular matrix components of the surrounding environment. On the other hand, spleen followed the same tendency as intestine and gills, and genes

and GO categories related to ribosome and synthesis of proteins were increased in spleen, though were decreased in head kidney. The haemoglobin complex was elevated in spleen, as well as MHC II complex, antigen-presenting related genes and some apoptosis transcripts, whereas several proteins involved in tissue remodelling and cytoskeleton, Immunoglobulin receptors and functional classes related to immune system were repressed. The differences in the expression patterns of spleen and head kidney support the hypothesis that these organs play different roles in the fish immune system. A summary of the effects of IS-diet in trout is shown in figure 2:

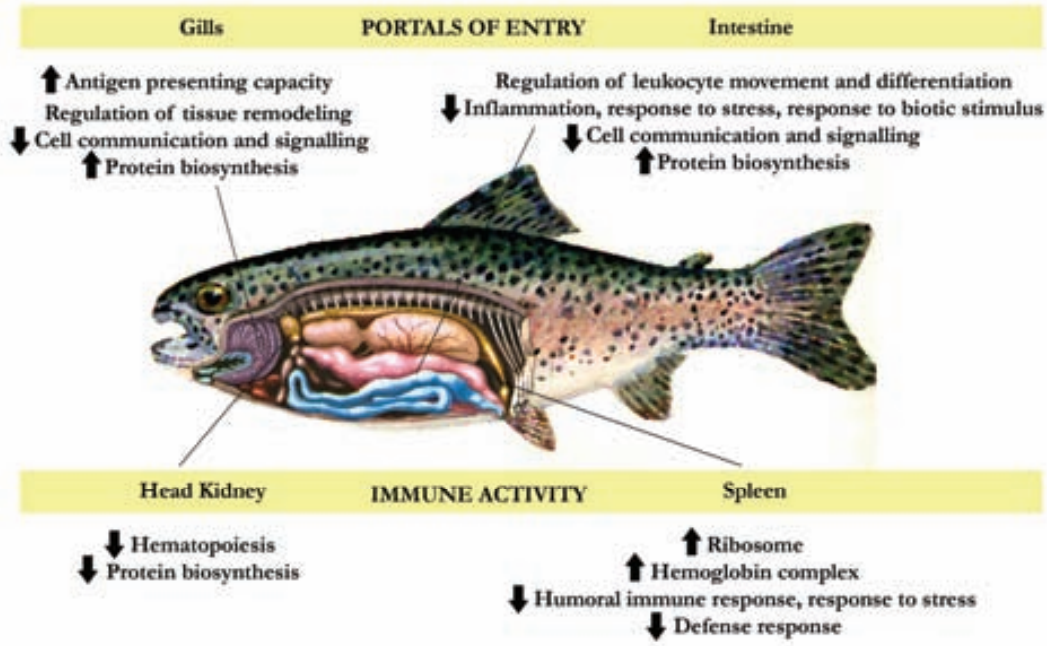


Figure 2: Effect of IS-diet in rainbow trout at the portals of entry and organs involved in immune function

Microarray analysis provided a global vision of trasncriptomic regulation and cellular and tissular processes during acclimation to IS-diets, whilst ISH makes a great contribution to the understanding of the morphology of the tissues and the distribution of the selected mRNAs. The pooling of several individuals (eight) in each sample reduced biological variability, however, allows a deeper understanding of the transcriptomic response of a population of fish to a diet containing β-glucans. As chip

technology has disadvantages like that rarely expressed transcripts may not be detected or that false positives occur, the results of interest obtained by microarray analysis must be validated by other technique, such as Q-PCR. The ISH analysis performed in individual fish and the Q-PCR carried out in both individual and pooled samples supply significant information of the general action over the population of IS-diet. The low biological variability suggests a robust response induced by IS-diets in all fish.

Effects of immunostimulation through dietary manipulation. Challenge with LPS

Finally, our last objective was to *compare in rainbow trout differential gene expression profiles induced by a LPS challenge between fish fed with a basal diet and fish fed with a commercial immunostimulant diet, at the two of the organs which act as portals of entry in fish, gills and intestine, and to analyze effects of such immunostimulant diet in cortisol regulation.*

Finally, microarray analysis and R.I.A. were performed to fully evaluate the effects of IS-diets at the portals of entry of the fish in an immune-compromised situation. As we have explained throughout this report, under the intensive conditions of aquaculture some of the innate defences may be affected. Mucus and epidermal barriers can be damaged by physical abrasions, stress conditions or immunosuppressive events. Therefore it seems necessary to determine the role of the administration of β-glucans when fish cope with bacterial components. With this aim, changes in gene expression and in cortisol regulation were evaluated in fish injected with LPS which previously were fed either with a basal diet or with an IS-diet.

Gut and gills are central for the uptake of pathogens and/or antigens and the subsequent progression of immunity or disease. 24h after intraperitoneal LPS injection, fish fed with basal diet or IS-diet showed different transcriptomic activity in these organs. In control gills 63 genes displayed a FC>2, of which 81% were increased and 19% were decreased, whereas in IS-gills 77 genes possessed a FC>2, of which 23% were up-regulated and 77% were down-regulated. In control intestine, 66 genes had a FC>2, 48% enhanced and 52% were repressed, and interestingly, in IS-intestine

312 genes displayed a FC>2 of which 54% were up-regulated and 46% were down-regulated.

The response of gills in control fish to LPS was characterized by an increase in inflammatory response highlighted by the recruitment and differentiation of immune cells, regulation of immune response and cytokine production, unlike the gills of fish fed with IS-diet, which decreased these activities. In contrast, genes of the adaptive response involved in antigen processing and presentation and MHC I complex were up-regulated in fish fed with IS-diet. Furthermore, LPS enhanced regulation of tissue and muscle remodelling activity in gills of fish fed with both diets though provoked a large induction in intestine of control fish, a process repressed in intestine of fish which were previously primed in response to the administration of the IS-diet.

Other different transcriptional regulations induced by LPS involved the transposon activity in gills of control fish, that after immunostimulant dietary accommodation was reduced; the hemoglobin complex, which were increased in control fish after LPS challenge and decreased in IS-fish; the synthesis of proteins, repressed in gills of control fish and increased in IS-fish; the antioxidant activity, down-regulated in gills of control fish and increased in IS-fish; the more intense response in immune signalling and communication in intestine of fish fed with IS-diet; the elevation of complement related proteins only in gills of IS-fish, and the coordinated down-regulation in intestine of IS-fish in groups comprising genes involved in ATP biosynthesis, DNA repair, response to unfolded protein, cell proliferation or response to virus. In addition, highly increased plasma cortisol levels following LPS injection were observed only in control-fed fish.

In our knowledge, this is the first study that has allowed evaluating the effects of IS-diets in the transcriptomic regulation of fish. Nevertheless, the complete picture of the interactions between Immunostimulants, pathogens and immune system is not known (figure 3). The time of exposition to β-glucans has been showed to be a very important factor in order to provide protection for both fish and crustaceans [23, 27]. The formulation of IS-diets, the type of PAMP included, the concentration of the PAMP or the period of administration are other factors that deserves more investigation to elucidate the optimum procedure to incorporate immunostimulant diets in the aquaculture sector.

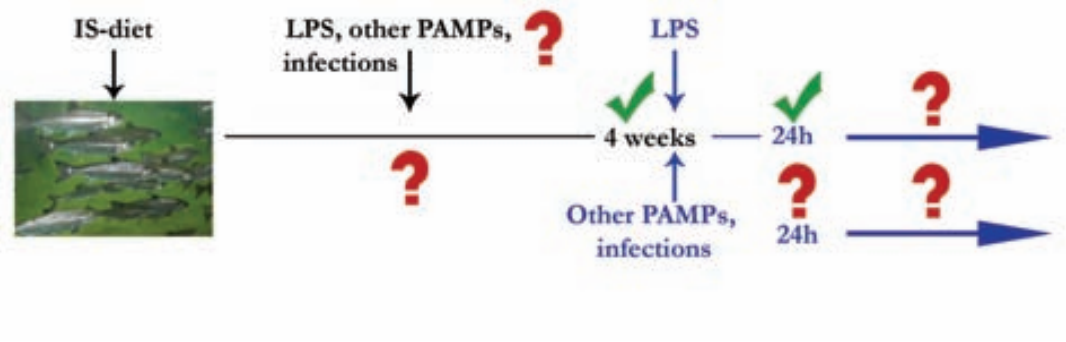


Figure 3: Overall picture of known ✓ and unknown ? transcriptome responses in fish fed with IS-diet

Conclusions

1. Gilthead sea bream macrophages were isolated from head kidnay in primary culture through their capacity to become adherent to plastic dish surface. Myeloid type cells differentiated spontaneously, reaching macrophage-like morphology, size and phenotype, including the expression of macrophage markers. Gilthead sea bream differentiated macrophages possess a high phagocytic capacity and the ability to regulate immune-related genes in response to LPS.

2. CD83, a cell surface membrane glycoprotein member of the Ig superfamily which is commonly used as marker for dendritic cells, was cloned from gilthead sea bream macrophages. CD83 mRNA expression was significantly induced in macrophage primary cultures after LPS stimulation in dose- and time-dependent fashion, therefore can act as a marker for the activation of macrophage cells.

3. Gilthead sea bream possess the ability to enhance the expression of antiviral-related genes, such as Mx and IRF-1, upon ultrapure LPS stimulation, unlike rainbow trout macrophages, which did not up-regulate Mx1 and IFNγ expression. This difference suggest a specie-specific activity, that in the case of sea bream is parallel to mammalian pathways, supporting the evidence that evolutionary distant fish species may have distinct immune mechanisms.

4. Recognition of different PAMPs by rainbow trout macrophages induces specific immune-regulation. Thus, bacterial LPS repressed expression of some genes involved in signalling (including several immune cascades), defence and immune response, and cell activation and differentiation, whereas macrophages challenged with the viral analog Poly (I:C) increased expression of those genes. Coordinated and also specific changes in genes related to cell morphology and extracellular matrix are observed for both treatments, clearly

indicating the important role of the regulation of tissue remodelling processes after infection.

5. Acute phase response is enhanced in trout macrophages treated by LPS, whilst both MHC class I and MHC class II are down-regulated, suggesting that LPS trigger mainly the induction of innate immune mechanisms. On the other hand, macrophages stimulated with Poly (I:C) declined complement regulation and MHC class II activity favoring expression towards the adaptive response.

6. Production of TNFα is faster in trout macrophages stimulated with LPS (12h) than in cells induced with Poly (I:C) (24h), thus confirming at the protein level some differences observed in the transcriptomic profiles.

7. Commercial immunostimulant diets significantly alter gene expression at the major portals of entry in fish, gills and intestine, in a tissue-specific manner. Stronger intensity is observed in gills, in terms of genes with high fold changes, and intestine possess a greater magnitude in the response, measured in total number of differentially expressed genes. The global tendency in both organs is an overall repression.

8. Transcriptomic profiles in gills showed that genes and functional categories involved in signalling, cell communication, matrix remodelling and cell differentiation were decreased, and categories and genes related to antigen recognition, ribosome and protein biosynthesis and cytoskeleton were increased. Furthermore, genes involved in tissue remodelling, cell differentiation and antigen presenting capacity were localized by ISH analysis in gills, confirming that one of the main effects of IS-diets in trout is the increase of the expression of some genes involved in antigen recognition in the epithelial cells of the secondary lamella of gills.

9. Transcriptomic profiles in intestine revealed that this organ repressed, in response to dietary stimulation, genes and GO categories involved in inflammation, response to stress, cell communication, signalling and structural remodelling processes, and enhanced chemotaxis, leukocyte movement and cellular differentiation. Distribution in the expression of some of these genes was detected in the epithelium and the membrane, whilst the lamina propia and mucous cells lack reactivity.

10. Diet containing immunostimulants induced differential expression profiles in two important immune organs, head kidney and spleen. The global effect was similar to that observed for the portals of entry, thus a larger reduction of genes and GO categories rather than induction was detected, and was tissue-specific.

11. Gene expression profiles showed that head kidney was weakly affected by the IS-diets, though decreased the synthesis of proteins and hematopoietic activity. The reduction in the haematopoiesis was also observed by ISH analysis.

12. Major changes in gene expression were observed in spleen. IS-diet caused a strong effect in the immune activity of spleen, with an increase in apoptosis activity, haemoglobin complex and antigen presentation, and a decrease in tissue remodelling and genes and categories related to immunity. ISH analysis of genes related to antigen presentation, apoptosis and tissue remodelling confirmed the microarray results.

13. Dietary administration for 4 weeks previous to LPS challenge regulates cortisol concentrations in plasma and LPS-induced transcriptomic profiles at the mucosal immune barriers in the portals of entry of rainbow trout, gills and intestine.

14. In gills, the main effect of IS-diet in the LPS-reponse was the increase in the expression of genes and GO categories related to antigen processing-presentation and antioxidant activity, in an adaptive response context, maintaining tissue remodelling activity.

15. In intestine, the LPS-response of fish fed with IS-diet was a mixture of adaptive (MHC I, Ig) and innate (complement, lysozyme) immunity, together with the regulation in the response to stress (protein folding, response to oxidative stress), metabolism, apoptosis, immune signalling and cell activation, in a background of muscle-matrix remodelling.

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