Immunostimulants connecting innate and adaptive immunity in Atlantic salmon (*Salmo salar*)

by

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"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka' but 'That's funny…" (Isaac Asimov)

Summary

The use of immunostimulants in fish farming has received increased attention due to the discovery of Toll-like receptors (TLRs) and pattern recognition receptors (PRRs). These receptors have been found to bind to highly conserved motifs on the surface of pathogens, known as pathogen associated molecular patterns. Most of the studied PAMPs activate antigen presenting cells (APCs) together with naïve T-cells, and may induce T_H1 and T_H2 responses with production of signature molecules such as IFN-γ and IL-4 respectively (Aderem and Ulevitch, 2000). A T_H1 response is important for the elimination of intracellular pathogens, while a T_H2 response activates a humoral immune response.

In this study, different immunostimulants acting as ligands for TLRs, and combinations of these stimulants, were administered to Atlantic salmon by intraperitoneal injection. At different time points (1-14 days) post injection, samples of spleen, head kidney and liver were obtained from the different treatment groups. Gene transcripts for the T_H1 signature genes IFN-γ and TNF-α, T_H2 transcription factor GATA-3 and regulatory IL-10 as well as the surface receptors CD8 and CD4, were subjected to analysis by Q-RT-PCR. All the applied immunostimulants induced expression of TNF-α in both the spleen and kidney. Induction of IFN-γ gene transcription was performed by β-glucan, LPS, pDNA and imiquimod. Only β-glucan induced GATA-3 expression in both the spleen and head kidney. Interleukin-10 gene expression was induced by all immunostimulants except LPS. Transcription of CD8 and CD4 was induced by LPS and pDNA. β-glucan also induced CD8 gene expression. Adjustment of the expression levels according to their respective control group values showed that upregulation of T_H1 expression was induced by β-glucan, imiquimod and pDNA + LPS. Upregulation of GATA-3 gene transcription was induced by β-glucan and imiquimod, while pDNA, imiquimod and pDNA + LPS up-regulated transcription of IL-10. These results may pinpoint the use of selected immunostimulants in order to drive the adaptive immune response into either T_H1, T_H2, or both.

Keywords: Cytokines; Immunostimulants; Innate immunity; Toll-like receptors.

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1. Introduction

1.1. Innate immunity in fish

1.1.1. Immune system morphology in teleosts

The morphology of the immune system is quite different between fish and mammals. Most obvious is the fact that fish lack bone marrow and lymph nodes. Instead, the head kidney serves as a major lymphoid organ, in addition to the thymus and spleen (Press and Evensen, 1999). Gut associated lymphoid tissues are also known lymphoid organs, and have been shown to function in eliciting immune responses in carp (Joosten *et al.*, 1996). Some teleosts, such as plaice, have been shown to possess a lymphatic system that is differentiated from the blood vascular system (Wardle, 1971), though the existence of such a system has been challenged in other species. A secondary vascular system has therefore also been described, constituting a separate, parallel circulatory system. This system, which has been observed in species like rainbow trout and cod, originates from systemic arteries and forms secondary arteries that supply their own capillary network and then return to the systemic venous system (reviewed by Vogel and Claviez, 1981).

The foremost part of the teleost kidney lacks excretory tissue and is commonly referred to as the head kidney. It is an important haematopoietic organ, with morphological similarities to the bone marrow in higher vertebrates (Meseguer *et al.*, 1995). The head kidney is also a major producer of antibodies and contains sinusoidal macrophages that act together with the endothelial cell-lining in the sinusoids to trap particles and substances from the bloodstream (Dannevig *et al.*, 1994; Brattgjerd and Evensen, 1996). Trapped antigens may then be retained for a long period of time by aggregations of pigment-containing melanomacrophages, also called melanomacrophage centra (Agius and Roberts, 2003). This makes the head kidney serve not only as a primary, but also as a secondary lymphoid organ (Kaattari and Irwin, 1985). Aggregations of melano-macrophages have been suggested, both functionally and structurally, to be primitive analogues to the germinal centres of lymph nodes in mammals (Ferguson, 1976; Ellis, 1980; Poppe, 2002).

The spleen may be divided into a red and white pulp, though the white pulp is often poorly developed in teleosts (Press and Evensen, 1999). It can still be divided into a melano-macrophage accumulation compartment and ellipsoids, though in salmonids the accumulations of melanomacrophages are not as well confined as in other species, and are rather more spread throughout the organ (Press *et al.*, 1994). Ellipsoids are terminations of

arterioles and appear to function in plasma filtration and trapping of blood borne substances (Ellis, 1980; Espenes *et al.*, 1995). Migration of macrophages to melanomacrophage centra have also been described (Ellis, 1980). The red pulp consists mostly of cellular reticulum, haemato-poietic tissue and blood sinuses (Van Muiswinkel *et al.*, 1991).

Mucosa associated lymphoid tissues in fish include the gut, skin and gills and form an initial barrier to invasion by pathogens (Dalmo *et al.*, 1997). The epithelia of the gut has been subject to the most studies so far, and it has been shown that both the anterior and posterior intestine are able to absorb macromolecules and deliver them to circulation (Dorin *et al.*, 1994; Dalmo and Bøgwald, 1996). This ability varies between species, as does the amount of lymphoid cells found in the different gut segments.

1.1.2. Immune system components

All multicellular organisms possess a selection of cells and molecules that interact in order to ensure protection from pathogens (Abbas and Lichtmann, 2006). This collection of highly specialised components (table 1.1) makes up the immune system, and poses a physiological defence against microbe invasion. In vertebrates, one distinguishes between the innate immune system and the adaptive immune system. As the name suggests, the adaptive immune system is not active at birth, but has to be stimulated by exposure of the system to antigens after its development. The recombination activating genes (RAG1 and RAG2) generate a great diversity in B- and T-cells, giving the adaptive immune system an impressive capacity to recognize and respond to very specific structures on pathogens (Agrawal *et al.*, 1998). Fish belong to the earliest evolutionary branch possessing an adaptive immune system (Schluter *et al.*, 1999), and both cartilaginous and teleost fish have immune mechanisms comparable to those known from mammals (Iliev *et al.*, 2005a; Dooley and Flajnik, 2006). Activation of innate components through processes such as phagocyte stimulation, cytokine production and activation of cell receptors, will in turn stimulate B- and T-cells as well as antigen presenting cells (APCs, Lo *et al.*, 1999). It is believed that even though the innate response to antigens generally precedes the adaptive response, this form of interaction takes place also in teleosts (Fearon and Locksley, 1996; Fearon, 1997; Dixon and Stet, 2001). There are several mechanisms by which innate immunity may convey biological information on pathogens to the adaptive immunity. One is the secretion of cytokines by macrophages and natural killer (NK) cells (Unanue, 1997). Others are for instance the attachment of complement protein to an antigen (Abbas and Lichtmann, 2006), and the preferential uptake of microbial antigen by lectin receptors specialized in presenting these to lymphocytes (Jiang *et al.*, 1995). Both

innate and adaptive immunity in fish is affected by water temperature. Within the physiological range, lower temperatures tend to inhibit immune responses, while higher temperatures enhance them (reviewed by Bly and Clem, 1992). Alcorn *et al*. (2002) showed that sockeye salmon (*Oncorhynchus nerka*) seem to rely mostly on innate immune responses at low water temperatures, while higher temperatures increase the reliance on adaptive immune responses (both referring to temperatures within the physiological normal range). Because of this, it has been suggested that fish rely more on their innate defence mechanisms than do endothermic vertebrates (Ellis, 2001).

Table 1.1 – An overview of innate immunity components in teleosts and their mode of action (Based highly on Abbas and Lichtman (2006) and Magnadóttir (2006), additional authors are shown in the authors column).

Physical components	**Mode of action**	**Authors**
Fish scales	Physical barrier	
Mucous surfaces	Physical obstacle as well as chemical barrier.	(Alexander and Ingram, 1992; Dalmo *et al.*, 1997)
Cellular components		
Neutrophils	Phagocytosis, secretion and phagocyte activation.	
Monocytes/Macrophages	Phagocytosis and phagocyte activation. Cytokine production. Secretion of growth factors and enzymes to remodel injured tissue. T-lymphocyte stimulation.	
Natural killer (NK) cells	Induce apoptosis of infected cells. Synthesize and secrete IFN-γ	(Hamerman *et al.*, 2005)
Humoral components		
Complement system	Promote binding of microbes to phagocytes. Promote inflammation at the site of complement activation. Cause osmotic lysis or apoptotic death.	(Ellis, 2001)
Interferons/Mx-proteins	Inhibit virus replication	(Alexander and Ingram, 1992; Ellis, 2001)
Transferrin	Iron binding. Acts as growth inhibitor of bacteria. Activates macrophages.	(Bayne and Gerwick, 2001)
Lytic enzymes	Change the surface charge of microbes to facilitate phagocytosis.	(Galindo-Villegas and Hosokawa, 2004)
Antiproteases	Restricts the ability of bacteria to invade and grow *in vivo*	(Ellis, 2001)
Antibacterial peptides		(Smith *et al.*, 2000)
Lectins	Induce precipitation and agglutination reactions. Activate complement.	(Galindo-Villegas and Hosokawa, 2004)
C-reactive protein (CRP)	Coats microbes for phagocytosis by macrophages. Activate complement. Induce cytokine release.	(Baldo and Fletcher, 1973; Nakanishi *et al.*, 1991)

1.2. Toll-like receptors and their agonists

Cells of the innate immune system act through the recognition of pathogen associated molecular patterns (PAMPs), which are regions of highly conserved motifs displayed on the surface of most pathogens (Elward and Gasque, 2003; Werling and Jungi, 2003). Upon invasion of a host, these motifs are recognized by pattern recognition receptors (PRRs). Among these are a class of molecules known as toll-like receptors or TLRs (Kawai and Akira, 2005). These receptors are expressed on a variety of immune cells, such as macrophages, dendritic cells (DCs), B cells, specific T cells and even on some non-immune cells such as fibroblasts and epithelial cells (Akira *et al.*, 2006). A phylogenetic analysis conducted by Purcell *et al*. (2006) supports the evidence that fish are able to respond to many known mammalian TLR agonists, and also to produce a variety of cytokines in response to stimulation. So far, a total of 11 TLRs have been identified in mammals (Kawai and Akira, 2005; Meylan and Tschopp, 2006), and several more have been found in fish (Meijer *et al.*, 2003; Jault *et al.*, 2004). The TLRs are highly specific, as each responds to different TLR agonists (table 1.2). These may be either live pathogens, or simply isolated PAMPs acting as immunostimulants and activating cells of the immune system. Bricknell and Dalmo (2005) define an immunostimulant as *"…a naturally occurring compound that modulates the immune system by increasing the host's resistance…"*. However, synthetic compounds such as imiquimod are known to possess immunostimulatory properties (Hemmi *et al.*, 2002; Gorden *et al.*, 2005). It is important to note the use of the term "modulate", as a substance with potential immunostimulatory properties may lead to a down regulation of the immune response if administered in excess amounts (Sakai, 1999).

Table 1.2 – A selection of toll-like receptors and their agonists. Agonists written in italic have been explored in this thesis and will be further described later in the text. Toll-like receptors 3, 7, 8 and 9 all recognize PAMPs in endosomal/lyzosomal compartments, while the rest are expressed on the cell surface.

	Properties of the receptor	**Agonists**
TLR 2	The TLR2 receptor is dependent on the formation of dimers with TLR1 or TLR6 (Wetzler, 2003; Bricknell and Dalmo, 2005). Due to this heterodimerization, the TLR2 is able to recognize a great variety of ligands.	Microbial lipopeptide *β-glucan (Laminaran)* Atypical lipopolysaccharide (LPS) Bacterial lipoprotein Heat shock protein (HSP) (Sioud, 2006)
TLR 3	Binding of agonists to the receptor will induce synthesis of type I interferons (IFNα and IFNβ), which then exert antiviral and immunostimulatory activities (Kaisho, 2005).	Double stranded RNA Polyinosinic poly-cytidylic acid (poly I:C) (Eaton, 1990; Lockhart *et al.*, 2004)
TLR 4	TLR4 is unique in the TLR family in that it requires the molecule MD-2 in addition to LPS in order to initiate signaling. This molecule is indispensable for TLR4 but will not affect the response to other bacterial components such as peptidoglycans or CpG DNA (Viriyakosol *et al.*, 2001; Nagai *et al.*, 2002).	*LPS*
TLR 5	The TLR5 receptor recognizes bacterial flagellin (Hayashi *et al.*, 2001). The receptor is expressed on the basolateral surface of intestinal epithelia, and will therefore activate pro-inflammatory gene expression only if flagellin crosses the epithelium (Gewirtz *et al.*, 2001; Kaisho and Akira, 2002).	*Flagellin*
TLR 7/8	Along with TLR3 and 9, TLR7 and 8 recognize PAMPs in endosomal/lysozomal compartments, though the natural ligand for the receptors is still not known (Hemmi *et al.*, 2002; Lee *et al.*, 2003; Kaisho, 2005).	Single stranded RNA Imidazoquinoline compounds - *Imiquimod* - Resiquimod
TLR 9	Despite a variety of effects concerning this receptor, they are all dependent on the TLR9-MyD88-mediated pathway (Hemmi *et al.*, 2003).	*Bacterial and viral CpG (pDNA)*

1.2.1. Signaling pathways of toll-like receptors

The ability of TLRs to recognize different PAMPs comes down to two domains that are characteristic for the TLR family. One is an extracellular domain of leucine-rich repeats, the other is a cytoplasmic Toll-IL-1 receptor (TIR) domain which is similar to that found in the interleukin 1 family (Akira, 2003; Akira and Hemmi, 2003). In order to initiate a signaling pathway, the TLRs depend upon the recruitment of TIR containing adapter proteins such as MyD88, TIRAP, TRIF and TRAM, in addition to the recognized PAMP (Barton and Medzhitov, 2003; O'Neill *et al.*, 2003). These adapters also contain TIR domains and are essential for activation of MAP (mitogen activated protein) kinases and NF-κB (nuclear factor κB) translocation (Medzhitov *et al.*, 1997; Li *et al.*, 2004). This will in turn stimulate gene expression, especially of those that promote immune and inflammatory responses (Baeuerle and Baltimore, 1996; Baldwin Jr, 1996). Figure 1.1 shows how TLR signaling may be divided into MyD88-dependent and MyD88-independent pathways (Yamamoto and Akira, 2005). Signaling through TLR4 is both MyD88-dependent and independent. For TLR2 and 4, TIRAP activation elicits MyD88 activation downstream of the receptors. Only TLR3 signaling is independent of the MyD88 adapter, and depends instead on the binding of TRIF (Yamamoto *et al.*, 2002; Oshiumi *et al.*, 2003). Recruitment of TRIF activates the MyD88-independent pathway, and leads to activation of NF-κB, and the transcription factor IRF3. IRF3 activation is necessary for induction of type 1 interferons (IFNs), especially IFN-β (Kawai and Akira, 2007). Type 1 IFNs in turn activate genes for production of defense-mediating molecules, such as cytokines (Stark *et al.*, 1998). For TLR4, TRAM participates in activation of the pathway downstream of the receptor. Bonizzi and Karin (2004) also demonstrated a distinction between two different NF-κB pathways, where one is thought to be mostly involved in innate immunity and the other in adaptive immunity. This will not be adressed any further here.

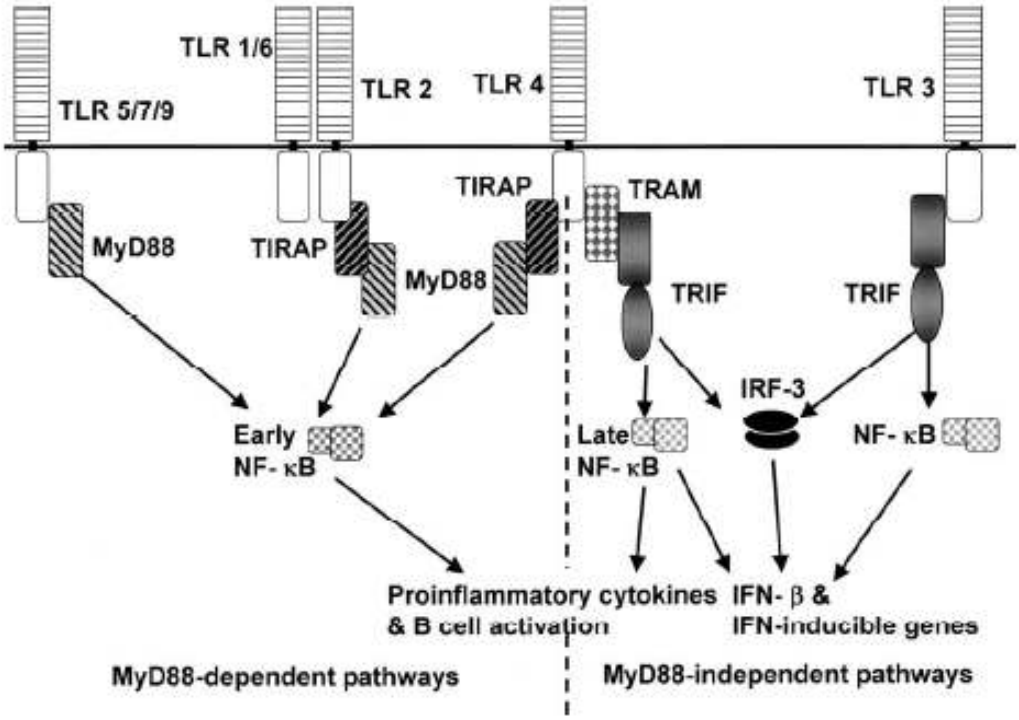

Figure 1.1 – The MyD88-dependent and MyD88-independent pathways and the participation of TIR-domain containing adaptors in eliciting TLR signaling (from Yamamoto and Akira, 2005).

Different endogenous signals can be induced through TLR activation, depending on the type of PAMP that is bound. These signals can be grouped into three categories (Medzhitov and Janeway, 1997):

1. Signals that mediate an inflammatory response. These include type 1 interleukins (ILs), tumor necrosis factor α (TNF-α), IL-6, type 1 interferons and also various chemokines. 2. Signals functioning as co-stimulators for T-cell activation, though few have been recognized so far. 3. Signals that control the induction of effector functions. These include IL-4, IL-10, IL-5, IL-12 and IFN-γ, and help regulate the differentiation of naïve helper T-cells into either T-helper 1 (T_H1) or T-helper 2 (T_H2). The different signals will be described further in the following sections.

1.3. Cytokines in activation of immune responses

1.3.1. Inflammation

Cytokines are regulators of the immune system and control cell-to-cell communication on a variety of target cells responsible for immune responses. Most cytokines are pleiotropic, meaning they have multiple sources, targets and also multiple functions (Gabay and Kushner,

1999), and they are usually products of TLR mediated signaling and NF-κB activation (Bonizzi and Karin, 2004). Feghali and Wright (1997) differentiate between two groups of inflammatory cytokines based on whether they are involved in acute inflammation or a more chronic inflammation. Interleukin-1 and TNF-α are the most potent cytokines in mediating inflammatory reactions, as well as playing a part in chronic inflammation and activation of the adaptive immune system (Bayne and Gerwick, 2001; Pasare and Medzhitov, 2004).

So far, IL-1β is the only type 1 interleukin to have been cloned and characterized in fish (reviewed by Savan and Sakai, 2006), and two IL-1β genes have been cloned in carp and rainbow trout (Pleguezuelos *et al.*, 2000; Engelsma *et al.*, 2003). Other known type 1 interleukin is IL-18. The first report of IL-18 in fish was from rainbow trout, in a study performed by Zou *et al*. (2004a). Like IL-1, the IL-18 cytokine is known to have pro-inflammatory properties. The inflammatory properties of the IL-1 cytokines lie in the ability to, amongst other functions, stimulate the expression of genes associated with inflammatory diseases, as well as increase the expression of adhesion molecules on endothelial cells (Thomson and Lotze, 2003). IL-1 also induces secondary inflammatory effects by stimulating IL-6 synthesis, a property it shares with TNF-α (Feghali and Wright, 1997). IL-6 is then involved in T-cell activation and differentiation, and also acts as a growth factor for B-cells. In addition to being a pro-inflammatory cytokine, TNF-α is also a mediator of other key functions such as apoptosis and immunological regulation. It is known to be LPS induced, and displays mRNA kinetics similar to those found in mammalian systems (MacKenzie *et al.*, 2002; MacKenzie *et al.*, 2003). After TLR activation, the transcription factor NF-κB may bind the promoter regions of IL-1, IL-6 and TNF-α and induce gene transcription.

Binding of pathogens or PAMPs to tissue resident macrophages induces the production and secretion of pro-inflammatory cytokines as well as different chemokines. Interleukin-1 and TNF-α stimulate endothelial cells to increase their expression of adhesion molecules such as selectins and ligands for integrins, and also to secrete chemokines (Luster, 2002; Abbas and Lichtmann, 2006). Selectin ligands on the surface of blood leukocytes bind loosely to the selectins, causing the leukocytes to roll along the endothelial surface (Abbas and Lichtmann, 2006). The leukocytes also express integrins, which will bind firmly to the integrin ligands. Chemokines secreted from the endothelial cells activate the leukocytes and stimulate a migration through the endothelium to the site of infection. While IL-8 induces the recruitment of neutrophils to the tissue, other chemokines will rather create an influx of macrophages, NK cells, immature DCs or activated T-cells.

1.3.2. Cytokines and chemokines linking innate and adaptive immunity

Cytokines produced during the acute inflammatory response are essential in shaping the following response in the tissue, and their nature depend upon the discrimination of the pathogen by TLRs (Luster, 2002). Binding of antigens to PRRs generates the (T_H1) subtype of $CD4^+$ T-cells by stimulating the transcription and secretion of particularly IL-12 (reviewed by Jankovic *et al.*, 2001). This interleukin is the predominant cytokine driving the differentiation of naïve T helper cells into T_H1 cells capable of producing IFN-γ (Ho and Glimcher, 2002). Interferon-γ is a type 2 interferon, and receptors for IFN-γ are expressed on nearly all cell types (Farrar and Schreiber, 1993). The cytokine is also produced by NK cells, and functions as a positive feedback by stimulating APCs into producing even more IL-12, thereby inducing the differentiation of even more T_H1 cells (Trinchieri, 1995; Stark *et al.*, 1998). This makes for some of the difference of IFN-γ from the type 1 interferons (IFNα/β), that are mainly involved in providing the adaptive immune responses necessary to resist viral infection. Interferon-γ also exhibits macrophage activating activity, an ability that is not shared by type 1 interferons (Stark *et al.*, 1998).

T helper 2 cells result from stimulation of naïve T-cells in the absence of PRR mediated signaling cytokines such as IL-12 (Jankovic *et al.*, 2001), and produce cytokines such as IL-4, IL-10 and IL-13. Interleukin-4 is the cytokine most potent in driving the differentiation of naïve T helper cells into T_H2, and it also acts inhibitory on macrophage activity (Ho and Glimcher, 2002; Heine and Lien, 2003). Interleukin-10 is believed to act as an important feedback regulator of the immune response, suppressing the function of the inflammatory T_H1 cells in order to protect the host from potential immunopathology (Moore *et al.*, 2001). It is produced not only by T_H2 cells, but also by other T-cells, B-cells, macrophages and DCs (Moore *et al.*, 2001; O'Garra and Vieira, 2004; Pengal *et al.*, 2006). Initially the cytokine was thought to be the product of T_H2 cells only, produced specifically to inhibit T_H1 cell responses (Fiorentino *et al.*, 1989). Later studies revealed that this was not the case. Del Prete *et al.* (1993) managed to clone human IL-10 from a T-cell which also secreted, among others, IFN-γ, indicating that IL-10 could not be produced exclusively by T_H2 cells. The differentiation of T_H2 cells is also regulated by the transcription factor gata binding protein 3 (GATA-3) (Chakir *et al.*, 2003). This transcription factor exerts its activity by increasing the expression of T_H2 selective cytokines and inhibiting T_H1 development (Ouyang *et al.*, 1998). Many of the signature cytokines of T_H1 and T_H2 have been cloned and sequenced, but rarely for a single species of fish (Zou *et al.*, 2004b; Li *et al.*, 2007). Salmon IFN-γ and TNF-α, both central in driving T_H1 responses, have been cloned and sequenced (Robertsen, 2006;

Haugland *et al.*, 2007). The transcription factor GATA-3 which binds the promoter region of the IL-4 gene has also been cloned, its sequence is currently being published (own work). The regulatory cytokine IL-10 has not yet been cloned, but EST sequence is available in the gene bank.

1.3.3. T-cell receptors CD8 and CD4

Two of the most important receptors of cytotoxic and helper T cells are CD8 and CD4 respectively, both of which have been reported in teleosts (Hansen and Strassburger, 2000; Suetake *et al.*, 2004). Their function is to stabilize the interaction between the T cell receptor complex (TCR) and the major histocompatibility complex (MHC) (reviewed in Moore *et al.*, 2005). While $CD4^+$ T cells induce immune responses by producing cytokines, $CD8^+$ cells target and kill infected cells (Suetake *et al.*, 2006). Moore *et al*. (2005) found that salmon that had been raised in an environment free of pathogens expressed CD8 mostly in thymus, though significant expression was also seen in spleen. Hansen and Strassburger (2000) also found expressions of CD8 in kidney. Gene sequences for salmonid CD4 and CD8 have been found (Moore *et al.*, 2005; Dijkstra *et al.*, 2006; Laing *et al.*, 2006).

1.4. TLR agonists explored in this thesis

Laminaran is a brown algae polysaccharide composed of (1,3)-β-D-glucan, with β(1,6) branching (Nelson and Lewis, 1974; Zvyagintseva *et al.*, 1999), and is known to have a low molecular weight of about 5000 Da (Patier *et al.*, 1993). Injection of Atlantic salmon with β-glucan has previously been shown to enhance resistance to different pathogens (Robertsen *et al.*, 1990). Also, intraperitoneal injection of β-glucan in combination with LPS induced protection in carp against the pathogen *Aeromonas hydrophila*, and it was suggested that the enhanced protection was due to the adjuvant effect of β-glucan with LPS (Selvaraj *et al.*, 2006). It has been suggested that β-glucans may bind the TLR2/TLR6 heterodimer, but the binding of β-glucans to dectin-1 has been proposed to be the most important in the recognition of β-glucans (Gantner *et al.*, 2003).

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria. It is generally considered to be the most potent immunostimulant among cell-wall components, and consists of polysaccharides extending outward from the cell surface and a lipid portion which is embedded in the membrane. This portion is known as Lipid A and is responsible for provoking immunostimulatory responses such as production of pro-

inflammatory cytokines and inflammatory effector substances such as nitric oxide (Kaisho and Akira, 2002; Akira and Hemmi, 2003). While LPS stimulation of TLR4 may trigger septic shock in mammals, it has long been known that fish and other lower vertebrates are resistant to these toxic effects (Berczi *et al.*, 1966). In addition to the high concentrations of LPS generally required to induce immune responses, it seems that other PRRs such as beta-2 integrins may play a vital role in LPS recognition by piscine immunity (MacKenzie *et al.*, 2003; Iliev *et al.*, 2005b). Studies with intraperitoneally injected LPS have shown a tendency towards high distribution in organs such as head-kidney and spleen (Dalmo and Bøgwald, 1996). Stimulation of macrophages by LPS will lead to the production of cytokines such as TNF-α, IL-1, IL-6 and IL-10, to mention a few (Akira and Hemmi, 2003).

Plasmid DNA contains unmethylated deoxycytidyl-deoxyguanosine (CpG) motifs that are common in bacterial DNA but suppressed in vertebrate DNA (Bird, 1987). These motifs stimulate the proliferation of B cells and also activate macrophages and DCs (Hemmi *et al.*, 2000). Like other immunostimulatory components, CpG DNA activates intra-cytoplasmic signaling molecules. However, unlike LPS which can activate TLR4 at the cell surface, uptake of CpG DNA and following endosomal maturation is required for the immunostimulatory activity (Ahmad-Nejad *et al.*, 2002). There have been identified three different classes of immunostimulatory CpG DNAs (class A, B and C), based on structural and biological differences (Verthelyi *et al.*, 2001; Vollmer *et al.*, 2004). Strandskog *et al.* (2007) showed that Atlantic salmon leukocytes respond to these classes in a manner comparable to those seen in mammals. Class A CpG was found to induce higher amounts of type 1 interferons than class B CpG, stimulating the production of IFN-γ by NK cells. On the other hand, B CpG proved to have a higher stimulatory effect on leukocyte proliferation as well as induction of IL-6 and IL-12 production by human monocytes. This suggests a requirement for higher concentrations of class A CpG to produce amounts of IL-12 comparable to those induced by B CpG (Kaisho, 2005). Class C resembled both A and B by stimulating interferon production as well as leukocyte proliferation. Yi *et al.* (2001) showed that combinations of CpG DNA and LPS synergized for TNF-α as well as later cytokines in murine monocytic cells. Substimulatory doses of CpG DNA synergistically enhanced LPS-mediated TNF-α production, and *vice versa*.

Flagellin is the major constituent of bacterial flagella. It is recognized as a potent activator of innate immune responses and induces TNF-α production. It contains a constant domain, D1, which is relatively conserved among different species of bacteria. This specific domain is recognized by TLR5 (Hayashi *et al.*, 2001). Studies suggest that the agonist is required for activation of NF-κB in intestinal epithelial cells (Tallant *et al.*, 2004), though only when it is present on the basolateral surface of the epithelial cells. This finding is consistent with the idea of TLR5 being a cell surface receptor of intestinal epithelia (Gewirtz *et al.*, 2001; Kaisho and Akira, 2002). An experiment conducted by Bilodeau and Waldbieser (2005) showed peaks in TLR5 mRNA expression in both spleen and kidney of channel catfish around five days post injection of the gram negative bacteria *Edwardsiella ictaluri*. The observations were suggested to indicate a cooperation of TLR5 in the initiation of an adaptive immune response.

Imiquimod belongs to a group of imidazoquinolinamines, low molecular weight compounds that display antiviral and antitumor properties (Perry and Lamb, 1999). Immunostimulation with imiquimod is known to induce TNF-α and also to upregulate the production of IFN-α and IL-12 cytokines in monocytes and macrophages, thereby enhancing a T_H1 cytokine profile including IFN-γ (Hemmi *et al.*, 2002; Akira, 2003; Gorden *et al.*, 2005). As a result, lymphocytes proliferate and B-lymphocytes are activated and start producing immunoglobulins. Dockrell and Kinghorn (2001) suggest that this link between the innate and adaptive immune responses makes imiquimod a potentially useful agent in enhancing vaccine responses. Work done by Kileng et al. (2007) shows that intraperitoneal injection of the imiquimod derivate S-27609 induces both IFN-α/β and IFN-γ expression in Atlantic salmon. Synergistic effects have been observed after simultaneous stimulation of DCs by the imidazoquinoline resiquimod and LPS (Napolitani *et al.*, 2005), suggesting the same observations might be made for imiquimod and LPS combinations.

1.5. Aim of study

- To measure the expression of various gene transcripts after intraperitoneal injection of Atlantic salmon with immunostimulants.
- Investigate the ability of the various stimulants to induce a differential T_H1, T_H2 and T_{reg} response.

2. Materials and methods

Tables for chemicals, solutions and equipment are shown in the appendix (page 59-67).

2.1. Fish

Unvaccinated Atlantic salmon (*Salmo salar*) with a weight range of 70-100 g were obtained from AquaGen AS Norway and kept at Tromsø Aquaculture Research Station (Norwegian Institute of Fisheries and Aquaculture Ltd. and University of Tromsø, Kårvika, Tromsø, Norway). The fish were kept in fresh water in circular, flow-through plastic tanks (~8 °C, flow rate 1 l/min) at 12/12 h illumination. They were fed a commercial salmon diet (Skretting NutraParr, 3 mm, Skretting AS, Norway) at rates and frequencies recommended by the manufacturer.

2.2. Immunostimulant fabrication

2.2.1. Isolation of plasmid R70pRomiLuc from Escherichia coli

Escherichia coli (strain DH5α) was used for amplification of the plasmid R70pRomiLuc. This plasmid contains a cytomegalovirus immediate early promoter (CMV-IEP) and genes coding for luciferase and ampicillin resistance. The bacteria were first grown at 37 °C on luria bertani (LB) agar plates containing ampicillin (amp^+). Separate colonies were transferred to and grown in test tubes with LB medium (5 ml) containing 100 µg ml^{-1} ampicillin, at 37 °C on a platform shaker (~300 rpm) until OD_{600} ~ 2.0. This value indicated that the bacteria had reached a phase of exponential growth. Cultures were then grown in larger volumes (750 ml) of LB amp^+ medium at 37 °C in an Innova 4300 Incubator shaker (~280 rpm). pDNA was isolated by use of Plasmid Giga Kit from QIAGEN® according to directions provided in the QIAfilter® Plasmid Purification Handbook (3^{rd} edition, 2005). A brief overview of the QIAfilter® purification process is shown in figure 2.1. Concentration and purity of the obtained pDNA was measured by NanoDrop® ND-1000 Spectrophotometer. The pDNA was considered pure when the A_{260}/A_{280} ratio was above 1.8. The restriction enzyme Hind III was used to linearize R70pRomiLuc prior to agarose (1 % ABgarose) gel-electrophoresis. The applied ladder for electrophoresis was a 1 kb Plus DNA ladder, applied in the amount of 5µl. A 6X DNA Loading Buffer was added with each DNA sample. Electrophoresis was conducted at room temperature at 80 V for one and a half hours. DNA bands were then visualized with GeneGenius (Syngene).

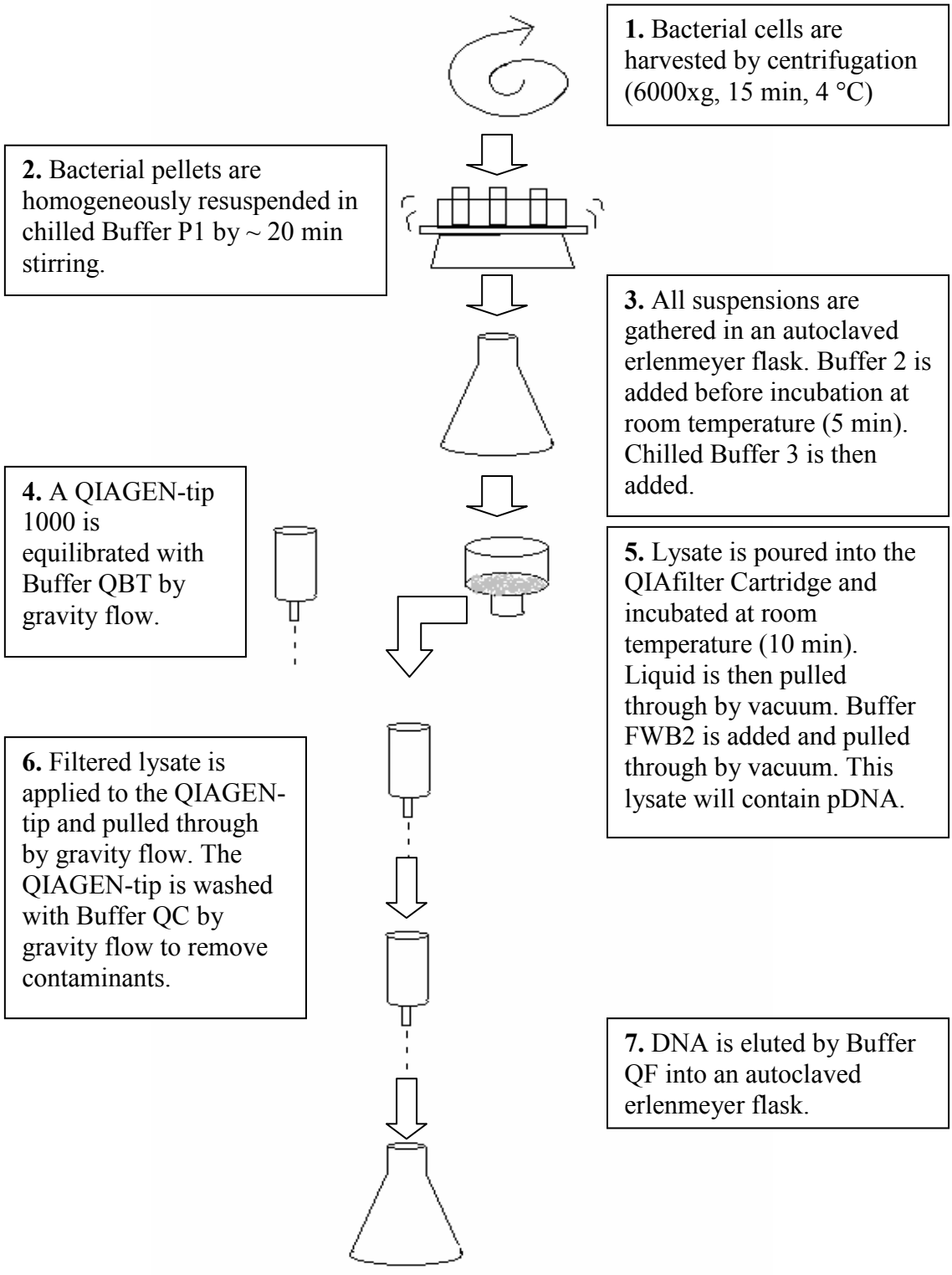

Figure 2.1 – Procedure for pDNA isolation with the Plasmid Giga Kit from QIAGEN®. Buffer P1 is a resuspension buffer containing RNase for removal of potential RNA contamination. It also contains the color indicator LyseBlue. Buffer P2 is a lysis buffer containing sodium dodecyl sulfate (SDS). It will react with LyseBlue to create a colored solution, allowing for a visual identification of the optimal buffer mixing. This ensures an efficient cell lysis. Buffer P3 acts as a neutralizer and precipitates SDS, causing the solution to turn colorless.

2.2.2. Isolation of lipopolysaccharide (LPS) from Aeromonas salmonicida

Aeromonas salmonicida bacteria were grown on blood agar in petri dishes at 12 °C until colonies were visible. Separate colonies were transferred to brain-heart infusion (BHI, 1 l) medium (2 % NaCl) and grown at 12 °C on a platform shaker until OD_{600} ~ 2.00. This corresponded to roughly 10^9 bacteria (Appendix Fig. A.1 and A.2). Cells were then washed twice by centrifugation with MilliQ water (3000 x g, 20 min) before they were dissolved in 225 ml of MilliQ water. The bacterial suspension was sonicated for four minutes before addition of lysozyme (0.1 % w/v) (Johnson and Perry, 1976). Incubation with stirring was performed overnight at 4 °C. The mixture was again sonicated, and the temperature raised to 37 °C. Magnesium chloride ($MgCl_2$) was added for a final concentration of 10 mM, and RNase (1 mg) and DNase (1 mg) was added for removal of RNA/DNA contamination. Incubation was performed for four hours at 37 °C before sonication. The bacterial suspension was transferred to dialysis tubes (Spectrum laboratories Inc.) cut to a length of ~ 20 cm, and set for dialysis for two days. LPS was isolated much in accordance with the phenol-water procedure described by Westphal and Jann (1965). Isolated LPS was set for dialysis and the MilliQ water changed a total of 14 times. The dialyzed solution was freeze-dried (Heto FD3 freeze dryer) before resuspension in MilliQ water (200 ml). The solution was ultracentrifuged (Sorvall Ultra pro® 80, 105000 x g, 3 h) and the pellets resuspended in MilliQ water (80 ml) before freeze-drying. Protein content in the isolated product was determined by the RC DC™ Protein Assay Kit from BIORAD, based on the Lowry assay (Lowry *et al.*, 1951). The absorbance for protein determination was read at OD_{650} in a 96 well reader (Beckman DU® 640 spectrophotometer). Protein contamination was measured to be ~0.25 µg/mg, or roughly 0.03%.

2.3. Stimulation and sampling

A total of 168 fish were divided into eight groups of 21 individuals, one of which was a control group injected only with phosphate buffered saline (PBS, 0.02 M, isoosmotic, pH 7.75). The other seven groups received intraperitoneal injections of different immunostimulants (table 2.1).

Table 2.1 – An overview over the applied immunostimulants and the dose given pr. kg body weight.

Group number	Immunostimulant	Concentration
1 (PBS injected control group)		
2	β-glucan (*Laminaria hyperborea*)	1 mg/kg
3	LPS (*A. salmonicida*)	1 mg/kg
4	LPS + pDNA	1 mg/kg of each
5	pDNA	1 mg/kg
6	LPS + Imiquimod	1 mg+0.1mg/kg
7	Flagellin (recombinant *Borrelia*)	0.5 mg/kg
8	Imiquimod	0.1 mg/kg

The immunostimulants were dissolved in PBS prior to injection. All fish were anaesthetized with benzokain (5 % in ethanol stock solution, 1 ml benzokain to 1 l water) prior to injection. Each fish was given a dose of 0.1 ml, with minor variations according to fish size.

Sampling was performed at 1, 2, 4, 7 and 14 days after injection. Three fish from each group were killed by an overdose of benzokain (2 ml benzokain to 1 l water) before samples were taken of spleen, kidney and liver. Tissue samples were preserved on RNA*later*™ in cryotubes and kept at room temperature overnight before being stored at -20 °C.

2.4. Total RNA isolation – TRIzol® method

Tissue samples were thawed, and small samples (~30 mg tissue) added to TRIzol® (1 ml) in Greiner tubes before homogenization with Ultra-Thurax T25 basic crusher. The homogenizer was cleaned with chloroform, 70 % ethanol/DEPC, MilliQ and DEPC (diethylpyrocarbonate) water prior to each sample. The solution was then transferred to Eppendorf® tubes for centrifugation (12000 x g, 4 °C, 10 min). The supernatants were collected and chloroform

(200 µl) added for each ml of TRIzol®. After vortexing (15 s), the mixtures were incubated (5 min at room temperature) and centrifuged (12000 x g, 4 °C, 15 min). The supernatants were transferred to new Eppendorf® tubes before addition of TRIzol® (500 µl) and chloroform (100 µl). Vortex, incubation and centrifugation was performed as described in the previous step. Again, the resulting supernatants were transferred to new Eppendorf® tubes. Addition of isopropanol (500 µl) was followed by incubation (10 min at room temperature) and centrifugation (12000 x g, 4 °C, 10 min). The supernatants were carefully removed, and the pellets washed in ethanol/DEPC (70 %, 1 ml) by centrifugation (7500 x g, 4 °C, 5 min).

The supernatants were again removed, and the pellets dissolved in nuclease free water (NFW) once all ethanol had evaporated. The tubes were then heated at 60 °C for 10 minutes before the samples were put on ice. RNA concentrations were measured by NanoDrop®. The A_{260}/A_{280} ratio had to be above 1.8 for the sample to be considered pure. Quality was tested on agarose gels (1 % ABgarose), where samples of 4 µl NFW, 2 µl RNA and 3 µl formaldehyde Load Dye were run for 12 minutes at 250 V. Bands were visualized with GeneGenius Bio Imagine System. With two prominent bands marking 18S and 28S RNA, the quality of the RNA was considered satisfying.

The isolated RNA was DNase treated with TURBO DNase Treatment and Removal Reagents (Ambion®) for removal of genomic DNA contamination. This was done according to the protocol provided by the manufacturer (Cat #1907). RNA concentration was then measured by NanoDrop®.

2.5. cDNA synthesis by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

cDNA was synthesized according to producer protocol using TaqMan RT-reagents (Appendix table A.6) in a GeneAmp PCR system 2700. Reagents were mixed with RNA samples in 8-tube thermo strips (0.2 ml) to a total volume of 50 µl, and set at the thermal parameters shown in table 2.2. The cDNA product was then kept at -20 °C.

Table 2.2 – Thermal parameters for cDNA synthesis by reverse trancriptase PCR

Step	**Hexamer incubation**	**Reverse transcription**	**Reverse transcriptase inactivation**
Temperature	25 °C	37 °C	95 °C
Duration	10 min	60 min	5 min

2.6. Quantitative RT Polymerase Chain Reaction

For Q-RT-PCR the use of duplicates was applied. Each PCR reaction consisted of a 25 µl PCR-mix volume (table A.7, appendix) which included cDNA, TaqMan PCR mastermix, water and also probe and forward and reverse primers for IL-10, TNF-α1, IFN-γ, CD4, CD8 and GATA-3 (table 2.4). Set parameters for the reaction are shown in table 2.3.

Table 2.3 – Thermal cycler profile for Q-RT-Polymerase Chain Reaction.

Stage	Repetitions	Temperature	Time (mm:ss)
1	1	95 °C	0:20
2	40	95 °C	0:03
		60 °C	0:30

Table 2.4 – Probe (P) and forward (F) and reverse (R) primer sequences for the selected genes.

Target mRNA	Primer/Probe	Sequence
IL-10	Salmon IL10 F	5’ CCTGTTGGACGAAGGCATTCTAC 3’
	Salmon IL10 R	5’ AACTTCAGGATGCTGTCCATAGC 3’
	Salmon IL10 P	6FAM5’ CCACCGGGCTCTTCA 3’-MGBNFQ
TNF-α1	Salmon TNFa1 F	5’ CGTGGTGTCAGCATGGAAGA 3’
	Salmon TNFa1 R	5’ AGTATCTCCAGTTGAGGCTCCATT 3’
	Salmon TNFa1 P	6FAM5’ TTGTCCTGCATCATTGCCA 3’-MGBNFQ
IFN-γ	Salmon IFNg F	5’ CGTGTATCGGAGTATCTTCAACCA 3’
	Salmon IFNg R	5’ CTCCTGAACCTTCCCCTTGAC 3’
	Salmon IFNg P	6FAM5’ CTGGTCCAGCCTCTCC 3’-MGBNFQ
CD4	Salmon CD4 F	5’ TGACACCCTGAAGAGAAGTATTCGT 3’
	Salmon CD4 R	5’ GTTGACCTCCTGACCTACAAAGG 3’
	Salmon CD4 P	6FAM5’ AAAACACCTGTAGCACCTCC 3’-MGBNFQ
CD8	As CD8β F	5’ GGAGGCCAGGAGTTCTTCTC 3’
	As CD8β R	5’ GGCTTGGGCTTCGTGACA 3’
	As CD8β P	6FAM5’ ACCCGGAGAAACTC 3’-MGBNFQ
GATA-3	As GATA-3 F	5’ CCCAAGCGACGACTGTCT 3’
	As GATA-3 R	5’ TCGTTTGACAGTTTGCACATGATG 3’
	As GATA-3 P	6FAM5’ TTCCTGCCCGTCTTGC 3’-MGBNFQ

2.6.1. Standard curves and reference gene

In order to quantify the obtained results, standard curves were made for each of the genes (Fig. 2.2). The applied reference gene was 18S (table 2.5), where a 10^5 x dilution of the RNA was made before performing Q-RT-PCR.

Table 2.5 – Sequences of probe (P) and forward (F) and reverse (R) primers for 18S.

Target mRNA	Primer/Probe	Sequence
18S	As RT18S F	5' GATCCATTGGAGGGCAAGTCT 3'
	As RT18S R	5' CGAGCTTTTTAACTGCAGCAATTT 3'
	As RT18S P	6FAM5' TTGGAGCTGGAATTAC 3'-MGBNFQ

Efficiency calculations was performed by using the formula: $E = 10^{\frac{-1}{slope}}$

Calculation of the relative ratio of gene expression was based on the primer efficiency, Ct-values of unknown sample and the respective sample of the reference gene (Pfaffl, 2001).

$$R = \frac{E_{target}^{\Delta Ct,target(control-sample)}}{E_{reference}^{\Delta Ct,reference(control-sample)}}$$

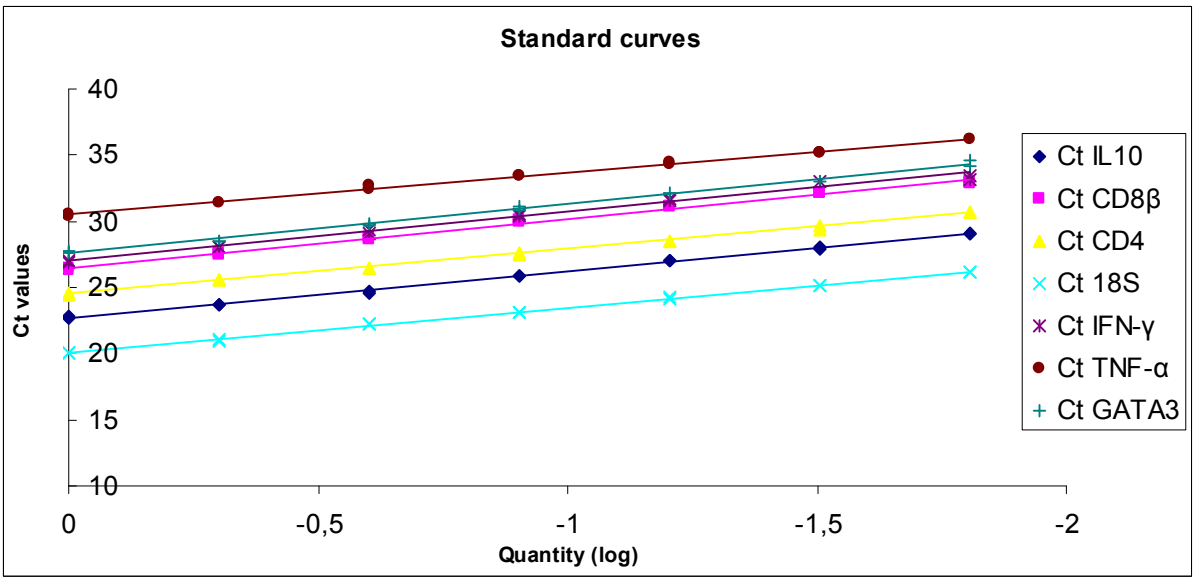

Figure 2.2 – Standard curves for each of the different genes. Plotting the log quantities of cDNA against Ct cycles gives the calculation numbers for primer efficiency. These are listed in table 2.6.

Table 2.6 – Shows slope, r^2 and efficiency values for each of the primers, calculated from the standard curves shown in figure 2.2.

Target gene	Slope	R^2	% E
IL-10	-3.3597	0.9977	0.9845
TNF-α	-3.1273	0.9964	1.0882
IFN-γ	-3.6849	0.9891	0.8680
CD4	-3.3769	0.9987	0.9776
CD8	-3.6875	0.9959	0.8672
GATA-3	-3.7133	0.9951	0.8591
18S	-3.3958	0.9990	0.9701

2.7. TaqMan Q-Real-Time PCR

There exist different applications for DNA synthesis by quantitative real time PCR. The SYBR Green method applies a fluorescent dye which binds to any double stranded DNA in the sample. In this thesis however, I have applied the TaqMan method which is more accurate and reliable than SYBR Green, though it is also more expensive. This method uses a fluorigenic probe designed to bind only to the DNA sequence between two specific PCR primers. Because of this, only specific PCR products will generate a fluorescent signal. The TaqMan method is more sensitive and therefore able to detect lower transcript levels than SYBR Green. Identification of the first cycle that gives a signal and following calculation of this threshold cycle (C_t) makes Q-RT-PCR a very precise method of analysis.

3. Results

3.1. Macroscopic observations

A mild swelling of the peritoneum was observed for several individuals as the experiment progressed, probably due to gathering of intraperitoneal fluids. Several fish also developed petecchia ("point bleeding") both in skin and at the base of pectoral, pelvic and anal fins. Most common was bleeding at the base of the pectoral fins. Some fish also showed damage to fins that may have been caused by handling or through interaction with other individuals. There were no systematic occurrences of these macroscopic changes with regard to different treatment groups receiving different immunostimulants. Whether the fish were infected by fungi, bacteria or virus was not addressed in this study.

3.2. Relative gene expression and chart explanation

Gel electrophoresis showed that all isolated RNA samples were of good quality, appearing as one distinct band, and therefore suitable for Q-RT-PCR. The charts display the relative expression levels as average values of the three fish sampled for each immunostimulant at the various time-points. I chose a baseline of ~2.0 (Ct value) for significant expression relative to 18S, because high individual variations in expression levels were often observed within a treatment group. In several cases the average value of expression was greatly affected by the expression level of one individual within that treatment group at each time-point. For most of these groups, this one individual caused the average value of the group to breach the baseline value, despite a lack of significant gene expression in any of the other individuals. In the charts these groups are shown with white-dotted bars, and standard deviation is not displayed. There were also cases of "high to extreme" values in groups where gene expression was high in the other parallels as well. The one extreme value caused a standard deviation significantly larger than the average value, and standard deviation was therefore not calculated or displayed in the charts. Extreme expression levels were observed only in immunostimulated fish, and not in fish that had received PBS injection. There were also cases where two of the samples from one treatment group at one specific time-point showed an increased level of gene expression, while the third sample possessed an expression value low enough to pull the average value beneath the baseline. In the charts these groups have been shown with checked bars. As expected, there was generally no significant increase in mRNA expression observed for the groups injected only with PBS.

3.2.1. Expression of the T_H1 cytokines IFN-γ and TNF-α

3.2.1.1. Expression of TNF-α in spleen

Fish injected with imiquimod showed the strongest induction of TNF-α expression in the spleen. The expression ratio was significantly higher than for 18S at the three first samplings. Rapid and high responses were also observed for the other groups, except group three (LPS) and the control group. Groups injected with β-glucan and imiquimod+LPS both showed increased expression of spleen TNF-α at day one and four post injection. Injection of pDNA caused elevated ratios at day one, two and four, like imiquimod, though the values were much lower. Flagellin induced high increase in expression ratios at day one, four and seven post injection. An unexpected increase of expression was observed for the day seven samples obtained from the control group (Fig. 3.1).

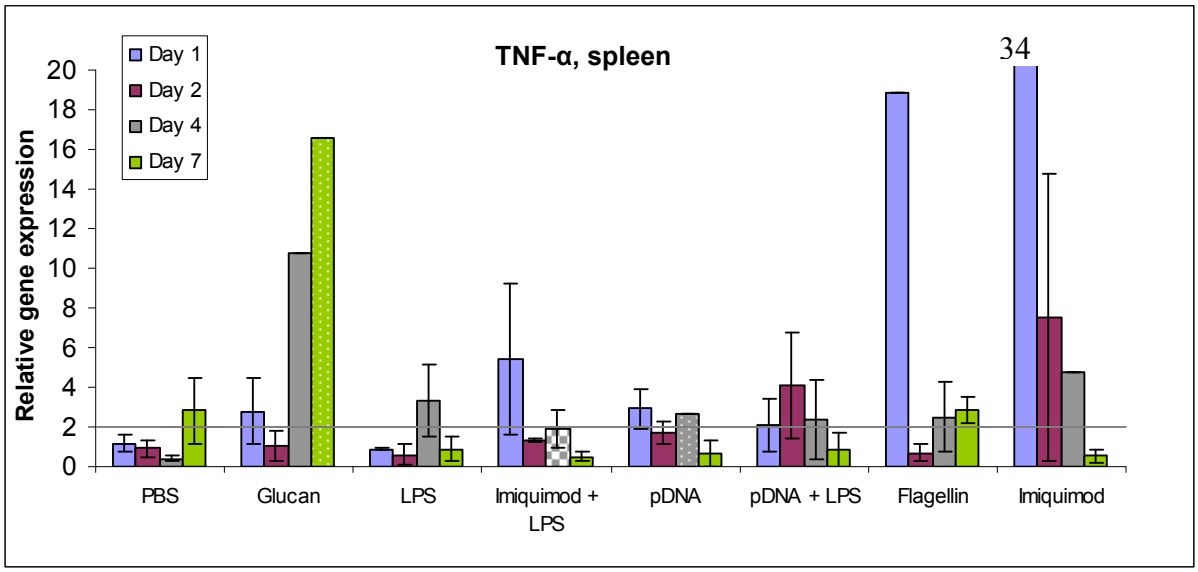

Figure 3.1 – Relative expression of TNF-α in spleen after injection of different immunostimulants. The variations between the different days of sample collection are represented by differently coloured bars. Dotted bars indicate that even though baseline is breached, there was generally no real increase in gene expression. Checked bars represent samples where relevant expression was evident, despite the average value not breaching baseline.

3.2.1.2. Expression of TNF-α in head kidney

All groups demonstrated elevated gene expression of TNF-α at some point during the sampling period. Fish injected with β-glucan, imiquimod and flagellin all displayed a rapid and short lived expression. At four days post injection no significant increases were observed. Injection with LPS and imiquimod + LPS both induced overall high expression values, with the exception of day two and four respectively. Plasmid DNA induced a bi-phased response,

where the average values for day one and four were both well above the baseline value. Injection of the pDNA + LPS combination gave significantly increased TNF-α gene expression for the three first timepoints (Fig. 3.2).

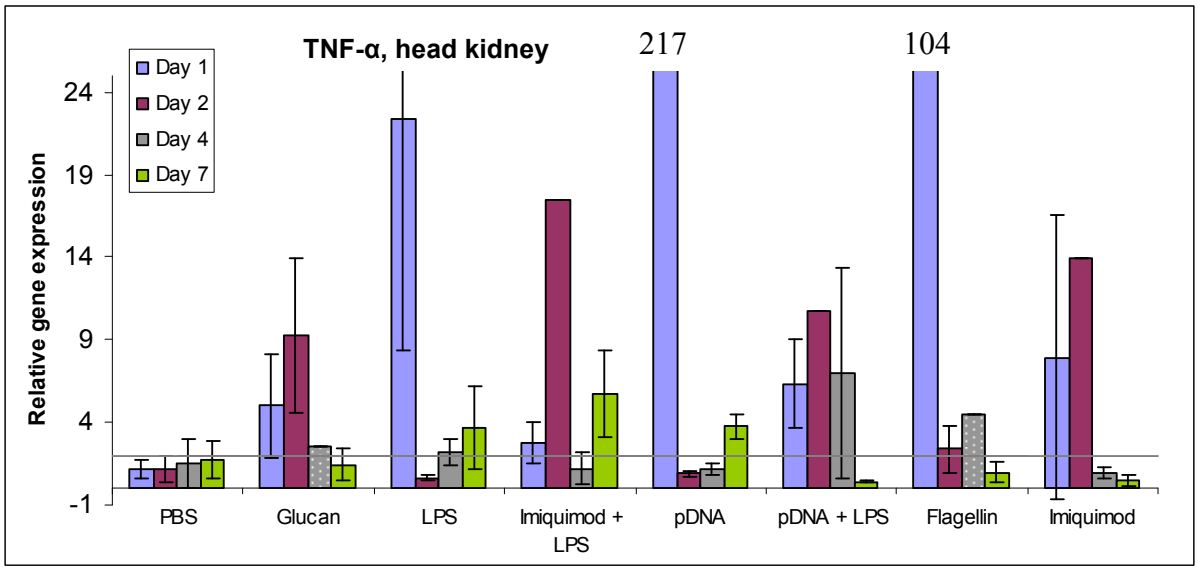

Figure 3.2 – Relative expression of TNF-α in head kidney after injection of different immunostimulants. The variations between the different days of sample collection are represented by differently coloured bars. Dotted bars indicate that even though baseline is breached, there was generally no real increase in gene expression.

Each of the administered immunostimulants and combinations managed to induce increased expression ratios of TNF-α in both spleen and kidney (Fig. 3.1-3.2).

3.2.1.3. Expression of IFN-γ in spleen

For IFN-γ, samples from day 14 were included to study the long-term expression of this cytokine. The expression of IFN-γ was generally low in all spleen samples. The only immunostimulants to cause relatively high gene expression at some time were β-glucan (day one and four), LPS (day four only), pDNA (day two, four and seven) and imiquimod (day four and seven). For both groups injected with immunostimulant combinations, gene expression levels were relatively stable throughout the sampling period, though none of the average values breached the baseline value (Fig. 3.3).

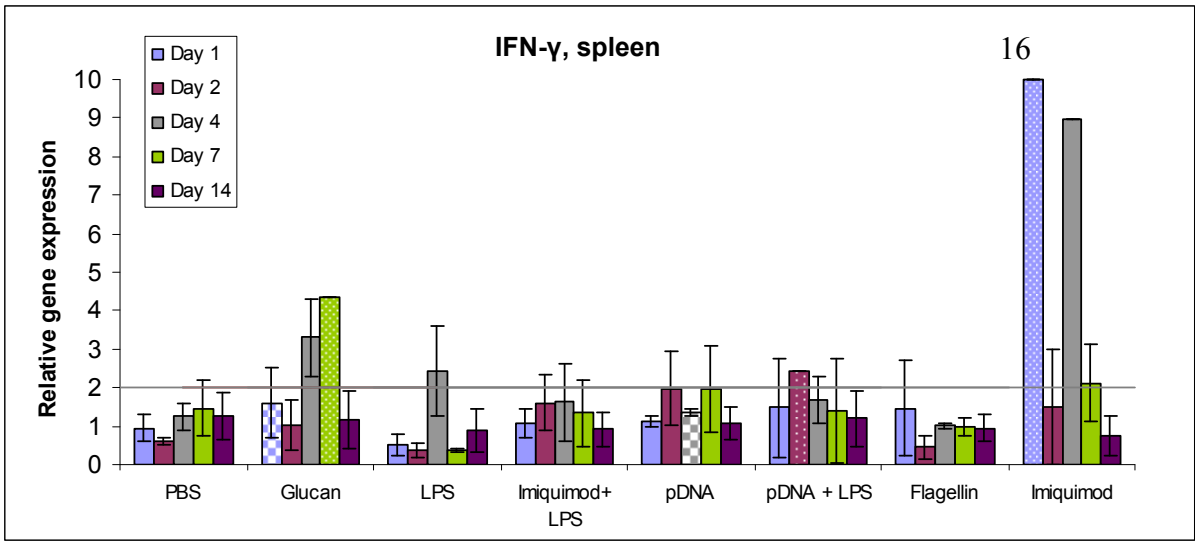

Figure 3.3 – Relative expression of IFN-γ in spleen after injection of different immunostimulants. The variations between the different days of sample collection are represented by differently coloured bars. Dotted bars indicate that even though baseline is breached, there was generally no real increase in gene expression. Checked bars represent samples where relevant expression was evident, despite the average value not breaching baseline.

3.2.1.4. Expression of IFN-γ in head kidney

Expression ratios were, in general, slightly higher in head kidney samples compared to the spleen samples. Again, β-glucan, LPS, pDNA and imiquimod were the stimulants that seemed to induce IFN-γ expression at a higher level than in controls and the other treatment groups. β-glucan yielded increased responses at two and 14 days post injection, though the expression ratios were generally lower than in spleen. LPS induced a bi-phased response, with significant increase one day after injection, and then again at day four. Both groups injected with immunostimulant combinations showed a somewhat delayed response. As with flagellin, imiquimod + LPS induced responses at day seven, while pDNA + LPS induced gene expression four days post injection. The head kidney response to pDNA seemed to be the

opposite of that observed in spleen. Whereas the spleen samples from fish injected with pDNA showed increased expression at two, four and seven days post injection, the ratios in the head kidney samples were highest at the first and last day of the sampling period. The expression ratios for the imiquimod samples were relatively stable, with day seven and 14 breaching baseline. Unexpected increases in gene expression were observed for the control group at four and 14 days after PBS injection (Fig. 3.4).

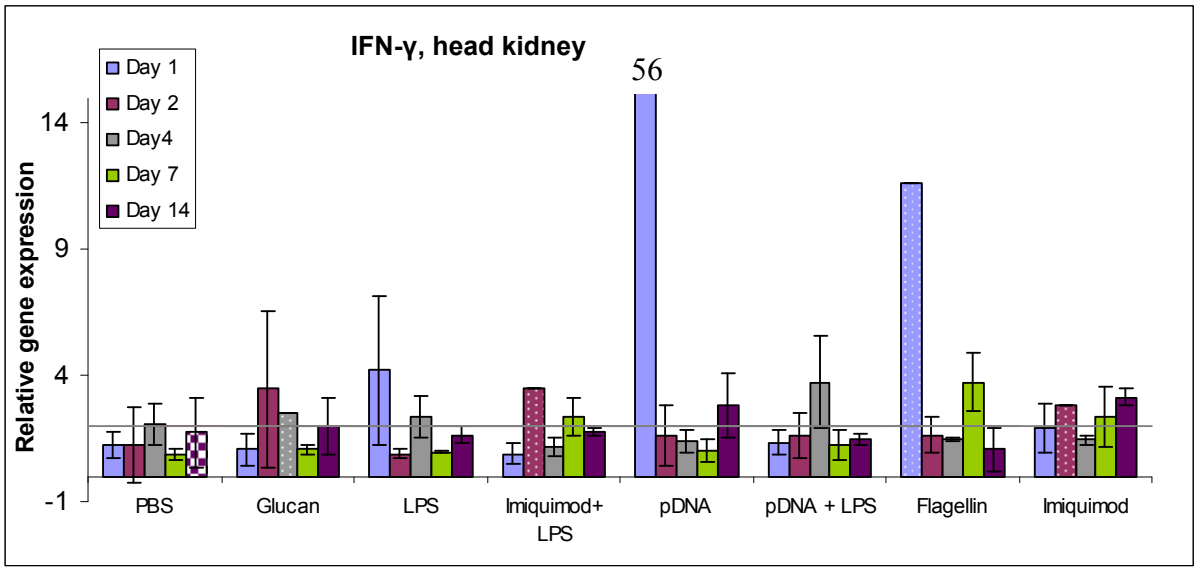

Figure 3.4 – Relative expression of IFN-γ in head kidney after injection of different immunostimulants. The variations between the different days of sample collection are represented by differently coloured bars. Dotted bars indicate that even though baseline is breached, there was generally no real increase in gene expression. Checked bars represent samples where relevant expression was evident, despite the average value not breaching baseline.

Overall, β-glucan, pDNA and imiquimod seemed to be the most effective inducers of IFN-γ gene expression, though the expression ratios were generally close to the set baseline value.

3.2.2. Expression of the T_H2 promoting transcription factor GATA-3

The expression of GATA-3, the main transcription factor driving a T_H2 response, was low in both spleen and head kidney. In spleen, β-glucan increased GATA-3 expression at day one and four, pDNA at day seven, and the pDNA + LPS combination at day one and two (Fig 3.5). Only β-glucan induced GATA-3 in both tissues (Fig. 3.5 and 3.6).

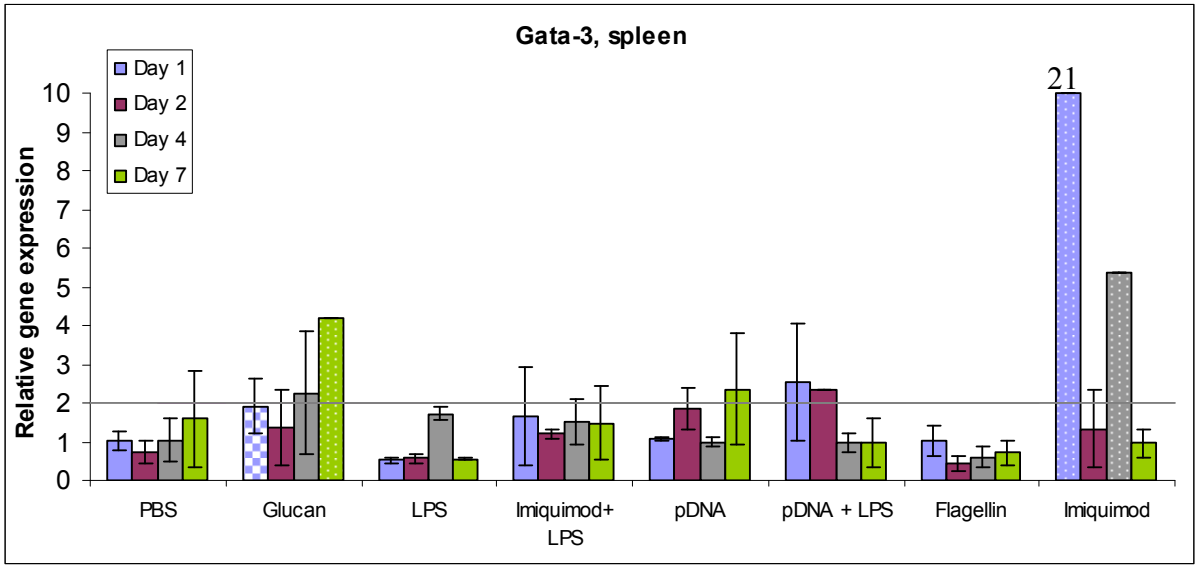

Figure 3.5 – Relative expression of GATA-3 in spleen after injection of different immunostimulants. The variations between the different days of sample collection are represented by differently coloured bars. Dotted bars indicate that even though baseline is breached, there was generally no real increase in gene expression. Checked bars represent samples where relevant expression was evident, despite the average value not breaching baseline.

In the head kidney, the highest values induced by injection of β-glucan appeared two days post injection. Imiquimod and LPS both induced increased gene expression at day one, while flagellin induced expression of GATA-3 at day seven only (Fig. 3.6).

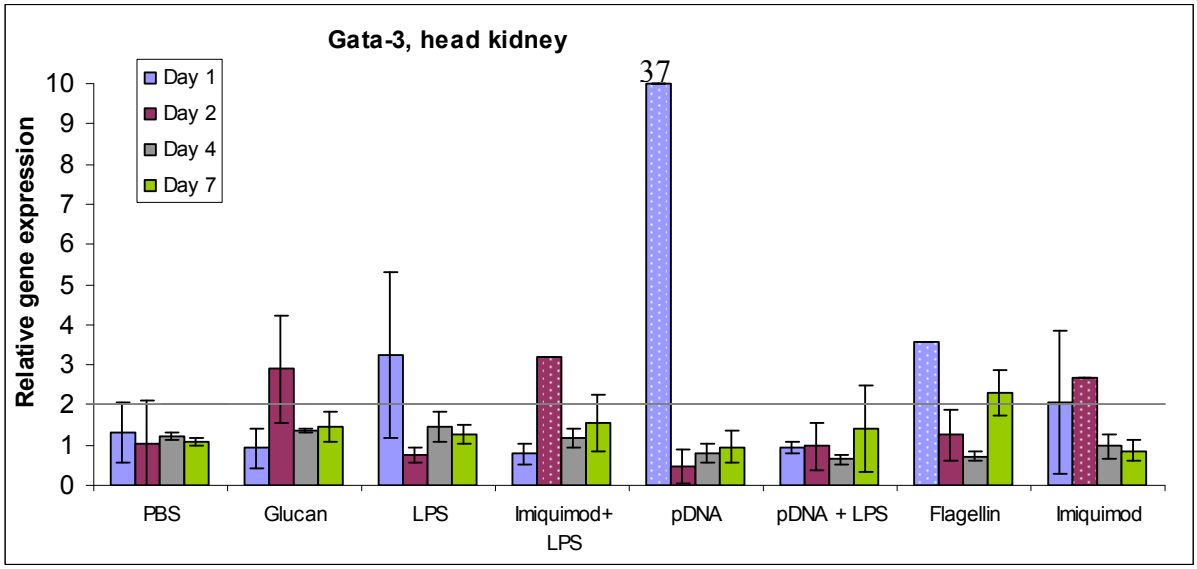

Figure 3.6 – Relative expression of GATA-3 in head kidney after injection of different immunostimulants. The variations between the different days of sample collection are represented by differently coloured bars. Dotted bars indicate that even though baseline is breached, there was generally no real increase in gene expression.

3.2.3. Expression of the regulatory IL-10

3.2.3.1. Expression of IL-10 in spleen

High increases in expression of IL-10, a cytokine possessing regulatory features, were found at some time-point for most groups, exceptions being the control group and the group receiving LPS injection. Injection with β-glucan induced a bi-phased response, with significant values at day one and seven. Groups injected with imiquimod + LPS combination and flagellin showed increased expression of IL-10 on the first day after injection only. For the pDNA + LPS combination, expression ratios dropped to low values within the fourth day post injection. Both pDNA and imiquimod induced bi-phased responses, with significant expression ratios at day one, two and seven (Fig. 3.7).

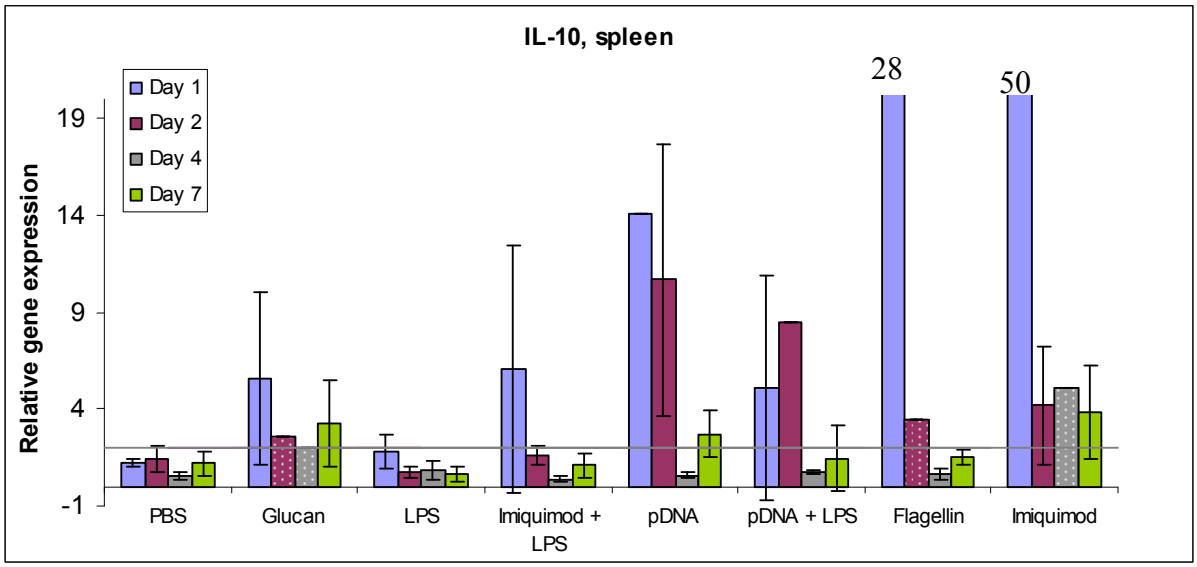

Figure 3.7 – Relative expression of IL-10 in spleen after injection of different immunostimulants. The variations between the different days of sample collection are represented by differently coloured bars. Dotted bars indicate that even though baseline is breached, there was generally no real increase in gene expression.

3.2.3.2. IL-10 expression in head kidney

Early responses to IL-10 were not as prominent in head kidney as in the spleen. β-glucan and pDNA + LPS both induced high increases in gene expression on the second day post injection, while imiquimod + LPS induced highest IL-10 expression four days post injection. Injection of LPS induced a bi-phased response with high values at one and four days post injection. Plasmid DNA induced a strong and early response, which dropped to low values within seven days post injection. Injection with imiquimod caused high IL-10 gene

expression, as evaluated by Q-RT-PCR, in samples from fish obtained at the three last samplings. Expression ratios of IL-10 in head kidney samples obtained from flagellin injected fish were high throughout the sampling period. The control group showed an unexpected increase of expression four days after PBS injection (Fig. 3.8).

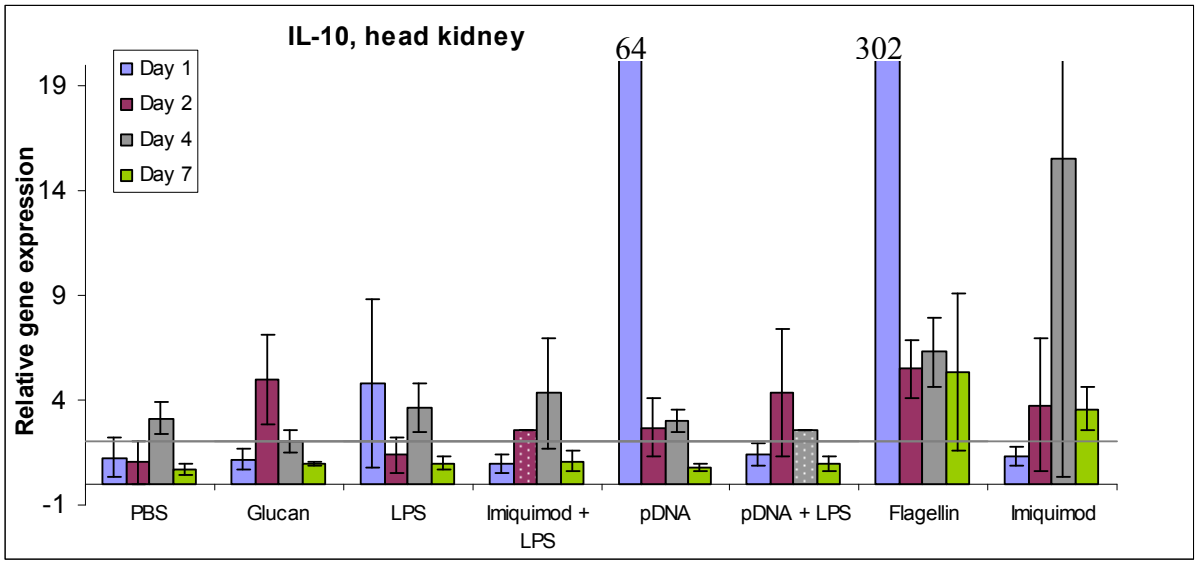

Figure 3.8 – Relative expression of IL-10 in head kidney after injection of different immunostimulants. The variations between the different days of sample collection are represented by differently coloured bars. Dotted bars indicate that even though baseline is breached, there was generally no real increase in gene expression.

3.2.3.3. IL-10 expression in liver

In addition to the spleen and head kidney, it was decided to study the expression of IL-10 in liver samples for groups five (pDNA) and seven (flagellin), as these immunostimulants seemed to be potent inducers of IL-10 expression (Fig. 3.7-3.8). In addition, we speculated that IL-10 may have been an acute phase response product, often produced by liver cells. This was after evaluating the early gene expression found in kidney. Both pDNA and flagellin induced bi-phased responses with values for the second day of sampling being low. As in spleen and head kidney, flagellin induced the highest response on the first day post injection (Fig. 3.9).

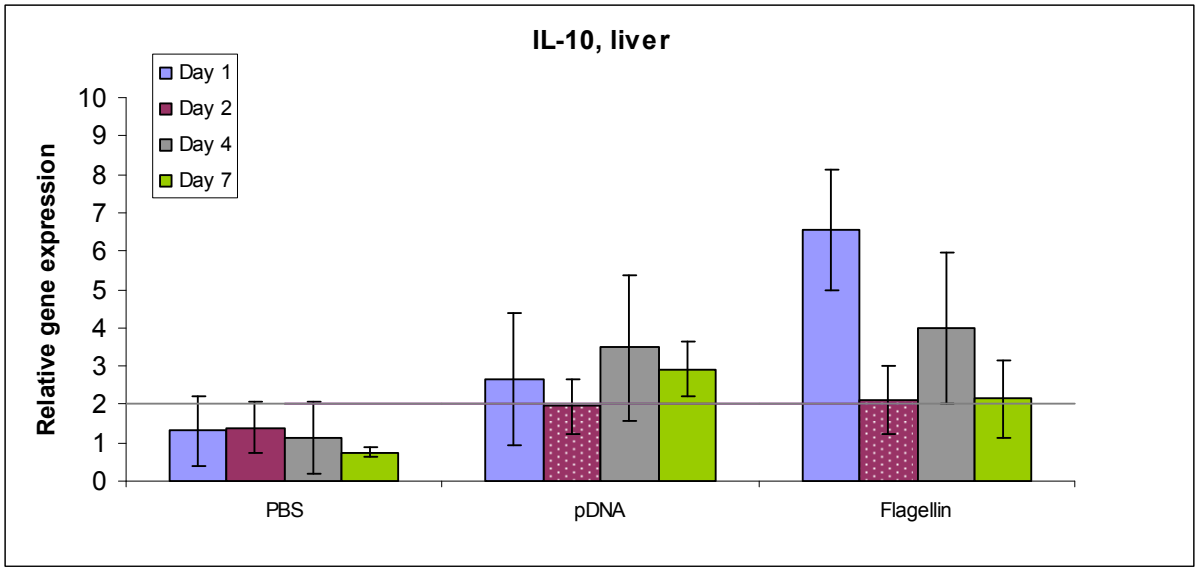

Figure 3.9 – Relative expression of IL-10 in liver of groups injected with PBS, pDNA and flagellin. The variations between the different days of sample collection are represented by differently coloured bars. Dotted bars indicate that even though baseline is breached, there was generally no real increase in gene expression.

The highest IL-10 gene expression values were observed in groups that had been injected with β-glucan, pDNA, flagellin and imiquimod. Stimulation with pDNA caused elevated gene expressions in each of the sampled tissues. Imiquimod induced high levels of expression in both spleen and kidney. Flagellin was the most potent immunostimulant in head kidney, and also caused elevated gene expression in liver. β-glucan induced significant expression ratios in both spleen and head kidney (Fig. 3.8 - 3.9).

3.2.4. Expression of the T-cell receptors CD8 and CD4

3.2.4.1. Expression of CD8 in spleen

Expression of CD8 was low throughout the time-points in the spleen samples, and significant increases in gene expression were found only in groups injected with β-glucan, LPS and pDNA. β-glucan and pDNA both induced elevated expression levels at day seven post injection, while the highest values for LPS was measured at day four. An unexpected increase in CD8 expression was observed at day seven of the control group (Fig. 3.10).

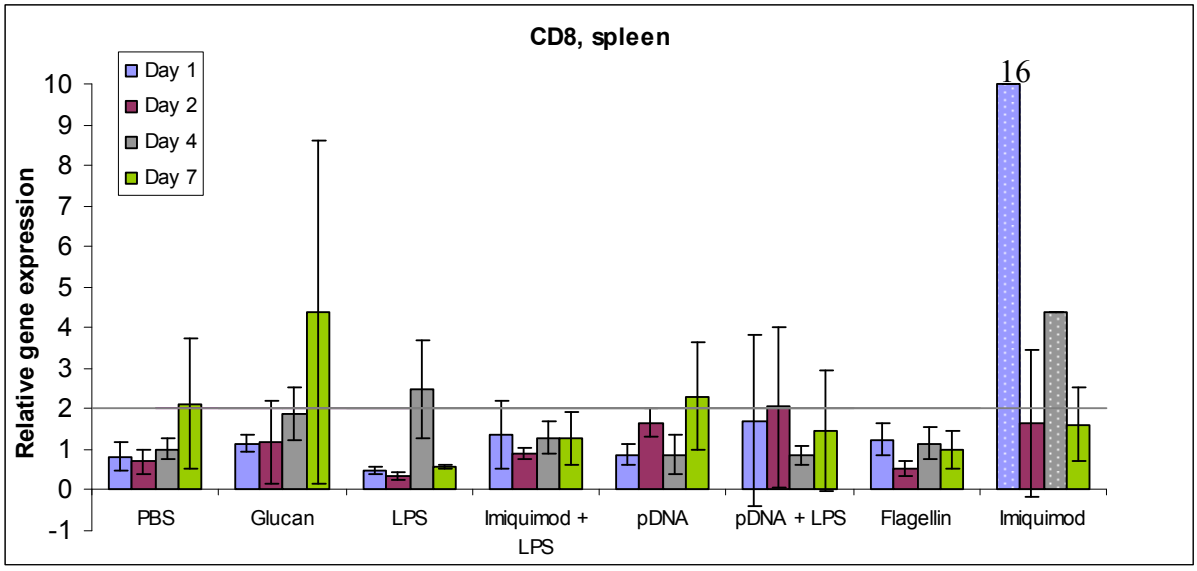

Figure 3.10 – Relative expression of CD8 in spleen after injection of different immunostimulants. The variations between the different days of sample collection are represented by differently coloured bars. Dotted bars indicate that even though baseline is breached, there was generally no real increase in gene expression.

3.2.4.2. Expression of CD8 in the head kidney

The expression of CD8 was slightly higher in head kidney than in the spleen. Injection with LPS and pDNA induced rapid and short lived responses measured by Q-RT-PCR. Ratios for day two, four and seven were all below the baseline value. Bi-phased responses were observed in groups injected with β-glucan and flagellin. While β-glucan induced the strongest expression at day two and seven post injection, the highest ratios for CD8 gene expression were observed at day one and seven after injection of flagellin. The Imiquimod + LPS combination gave a delayed response for CD8 expression. Only samples taken seven days after injection of the immunostimulant combination showed a high increase in gene expression (Fig. 3.11).

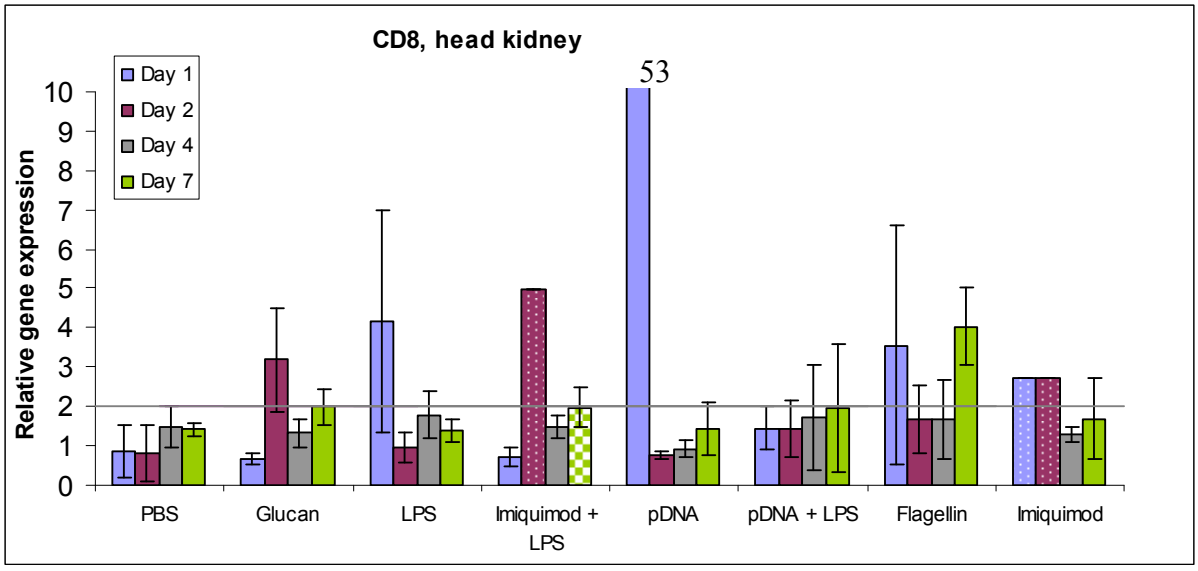

Figure 3.11 – Relative expression of CD8 in head kidney after injection of different immunostimulants. The variations between the different days of sample collection are represented by differently coloured bars. Dotted bars indicate that even though baseline is breached, there was generally no real increase in gene expression. Checked bars represent samples where relevant expression was evident, despite the average value not breaching baseline.

The only immunostimulants to induce relatively high CD8 expression in both tissues were β-glucan, LPS and pDNA, of which β-glucan seemed the most potent. Flagellin proved to be a strong inducer of CD8 in head kidney, though no response was found in spleen (Fig. 3.10-3.11).

3.2.4.3. Expression of CD4 in spleen and head kidney

Expression of CD4 was generally low in spleen samples. Only β-glucan, LPS and pDNA induced increase in ratio at some time-points post injection. This was for day one, four and two respectively (Fig. 3.12). In the head kidney, significant expression ratios were observed in samples obtained from fish at the first day after injection of PBS, LPS and pDNA. Injection of flagellin induced a bi-phased response, with elevated gene expressions at two and four days post injection (Fig. 3.13).

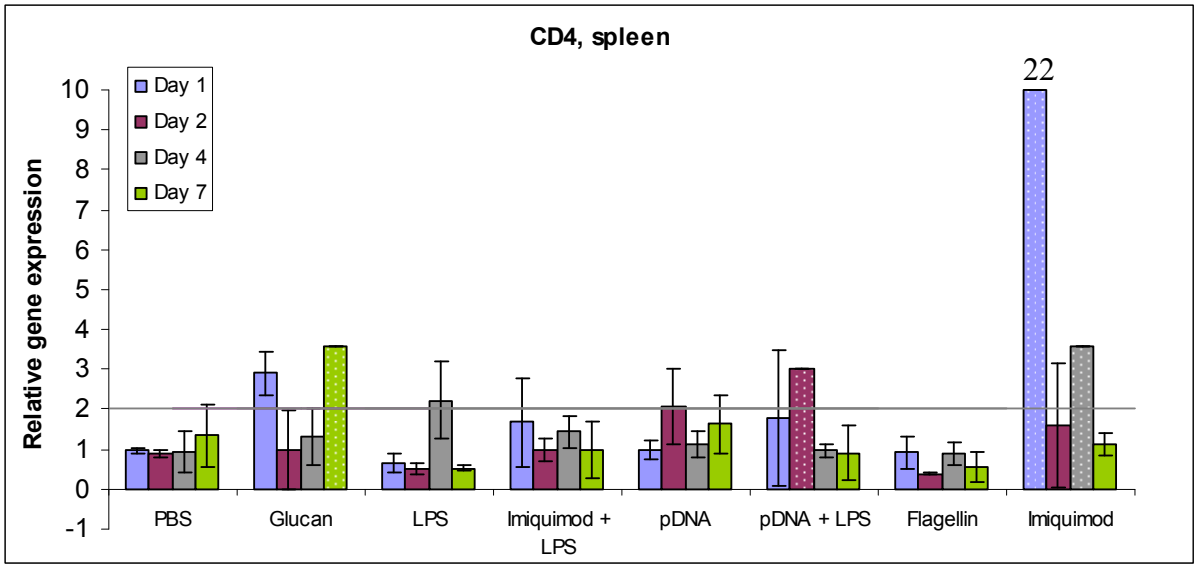

Figure 3.12 – Relative expression of CD4 in spleen after injection of different immunostimulants. The variations between the different days of sample collection are represented by differently coloured bars. Dotted bars indicate that even though baseline is breached, there was generally no real increase in gene expression.

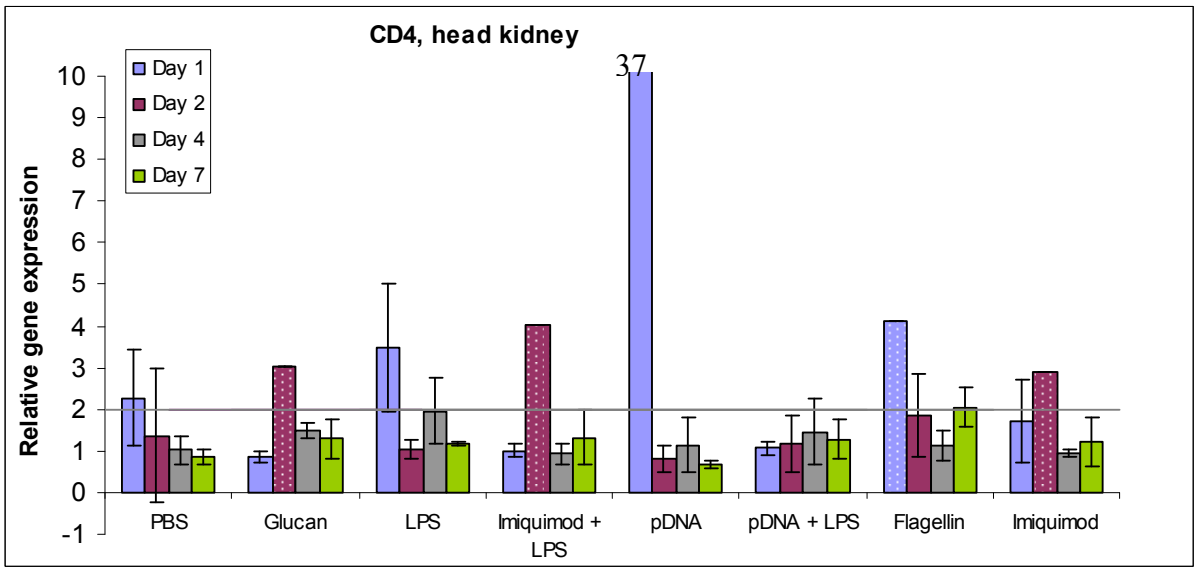

Figure 3.13 – Relative expression of CD4 in head kidney after injection of different immunostimulants. The variations between the different days of sample collection are represented by differently coloured bars. Dotted bars indicate that even though baseline is breached, there was generally no real increase in gene expression.

None of the administered immunostimulants proved to be very effective inducers of CD4 gene expression. Only LPS and pDNA caused elevated expression in both tissues (Fig. 3.12 and 3.13).

3.3. Actual quantities of administered immunostimulants

The responses of the T_H1 cytokines IFN-γ and TNF-α and also the regulatory IL-10 after injection of imiquimod were generally much higher than those induced by any of the other immunostimulants. As such, it was decided to investigate this observation by calculating how many moles that were actually administered of each stimulant (table 3.1).

Table 3.1 – Overview of the different immunostimulants together with molecular weight, injected amount in mg and the corresponding dose given as µmol kg^{-1} body weight. Sources of the molecular weights are also mentioned in the table.

Immunostimulant	**Molecular weight (kDa)**	**Injected amount (mg)**	**Injected amount (µmol)**	**µmol pr. kg body weight**
β-glucan	Approx. 5 (Patier *et al.*, 1993)	0.1	0.02	0.2
LPS	Approx. 30 (Personal communication, Roy Dalmo)	0.1	0.003	0.03
pDNA	5870 (Personal communication, Tom Tonheim)	0.1	1.7×10^{-5}	1.7×10^{-4}
Flagellin	41 (Product information)	0.05	0.001	0.01
Imiquimod	0.24 (Product information)	0.01	0.04	0.4

The calculations clearly showed that even though the milligram amount of administered imiquimod was lower than for the other immunostimulants, the dose in terms of µmol kg^{-1} body weight was much higher than for any of the others. An interesting observance was that pDNA and flagellin, which in term of mole amounts had been administered at the lowest doses, turned out to be very potent at inducing IL-10 gene expression.

3.4. Regulation of T_H1, T_H2 and T_{reg} post stimulation

GATA-3 is a transcription factor that binds the promoter region for IL-4, thereby activating IL-4 transcription. This will lead to a biased T_H2 response, whereas IL-10 functions as a regulatory cytokine inhibiting T_H1 responses. I wanted to compare the expression of these with the expression of the T_H1 cytokines IFN-γ and TNF-α, and see whether the different immunostimulants polarized the expression into T_H1, T_H2 or T_{reg}. I did so by calculating the differences in expression values between immunostimulated fish and the corresponding control groups. By plotting the values in box-plots (Fig. 3.14 – 3.21), an overall up- or down-regulation of gene expression could easily be detected. As a box-plot is meant to be representative of the trends within a group, extreme values (outsiders) have been left out. That is; values which were significantly larger and often more than ten times higher than the other values within that data set (e.g. within a treatment group). Box-plots including these extreme values within the data-set have been moved to the appendix of this thesis (Fig. A.3-A.10).

3.4.1. Regulation of the T_H1 cytokines IFN-γ and TNF-α

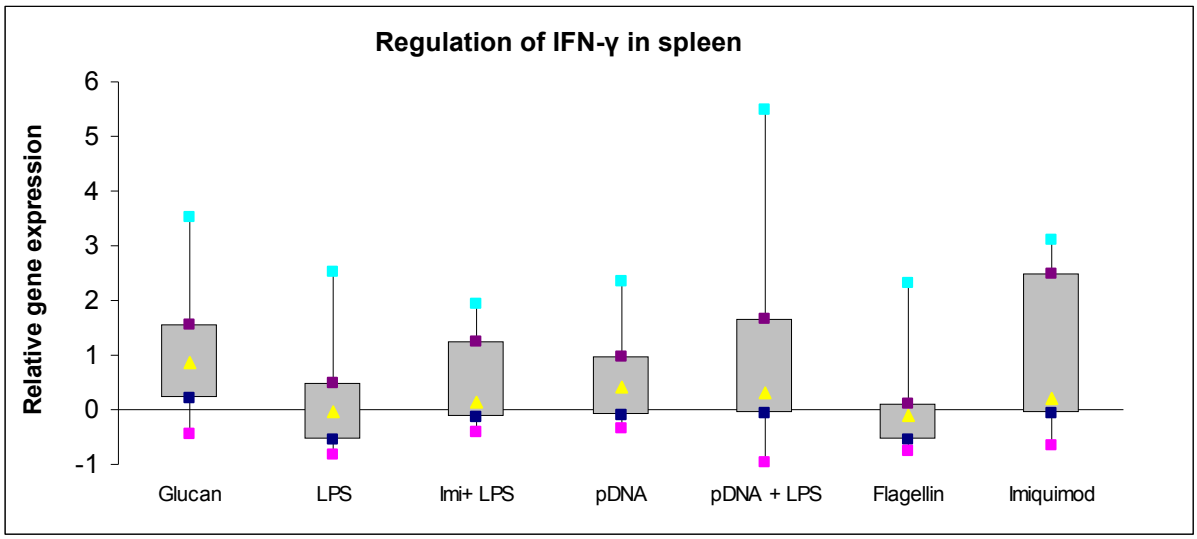

Figure 3.14 – Expression of IFN-γ in spleen, relative to 18S and adjusted to the respective values of the control groups. Turquoise and pink boxes indicate maximum and minimum values respectively. Purple boxes represent the upper quartile (75 %) and the blue boxes the lower quartile (25 %). The yellow triangles mark the median for the values. A down-regulation of gene expression is represented by median, lower quartile and minimum values being below zero. In case of an up-regulation of expression, median, the higher quartile and the maximum value are all above zero.

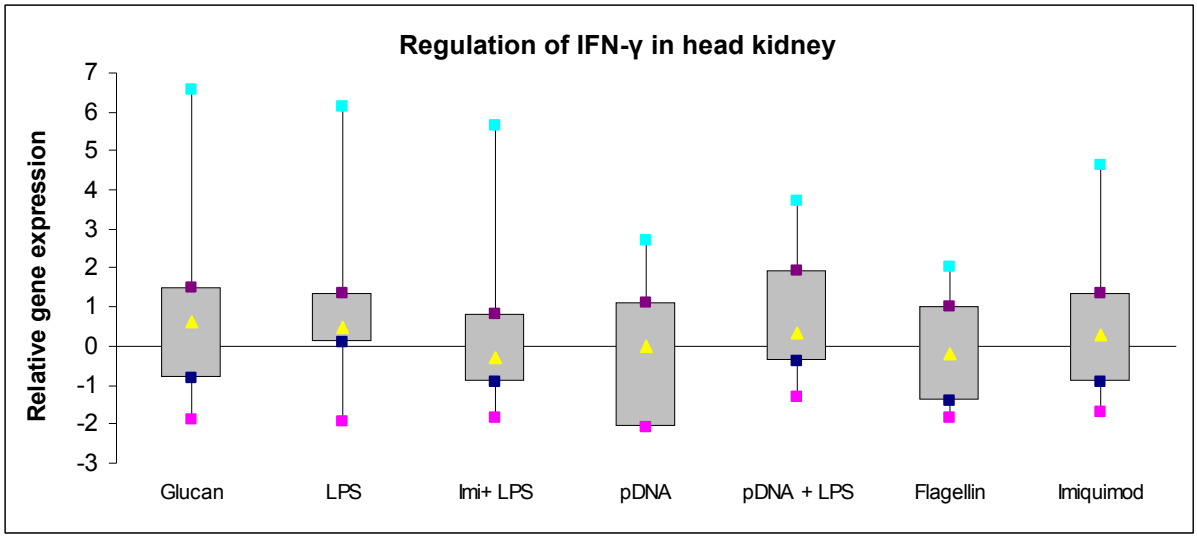

Figure 3.15 – Expression of IFN-γ in head kidney, relative to 18S and adjusted to the respective values of the control groups. Turquoise and pink boxes indicate maximum and minimum values respectively. Purple boxes represent the upper quartile (75 %) and the blue boxes the lower quartile (25 %). The yellow triangles mark the median for the values. A down-regulation of gene expression is represented by median, lower quartile and minimum values being below zero. In case of an up-regulation of expression, median, the higher quartile and the maximum value are all above zero.

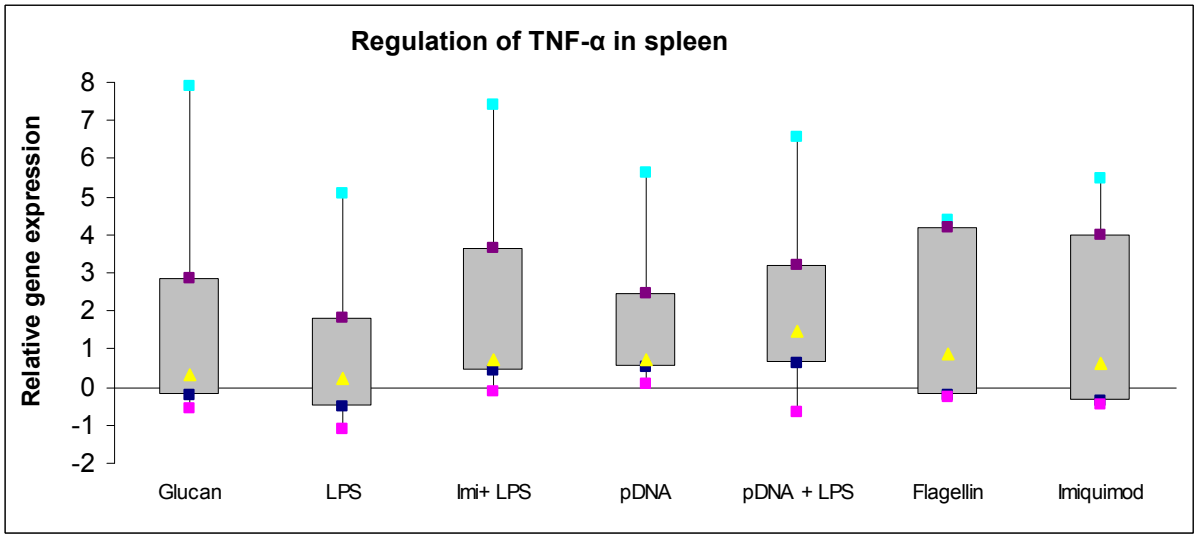

Figure 3.16 – Expression of TNF-α in spleen, relative to 18S and adjusted to the respective values of the control groups. Turquoise and pink boxes indicate maximum and minimum values respectively. Purple boxes represent the upper quartile (75 %) and the blue boxes the lower quartile (25 %). The yellow triangles mark the median for the values. A down-regulation of gene expression is represented by median, lower quartile and minimum values being below zero. In case of an up-regulation of expression, median, the higher quartile and the maximum value are all above zero.

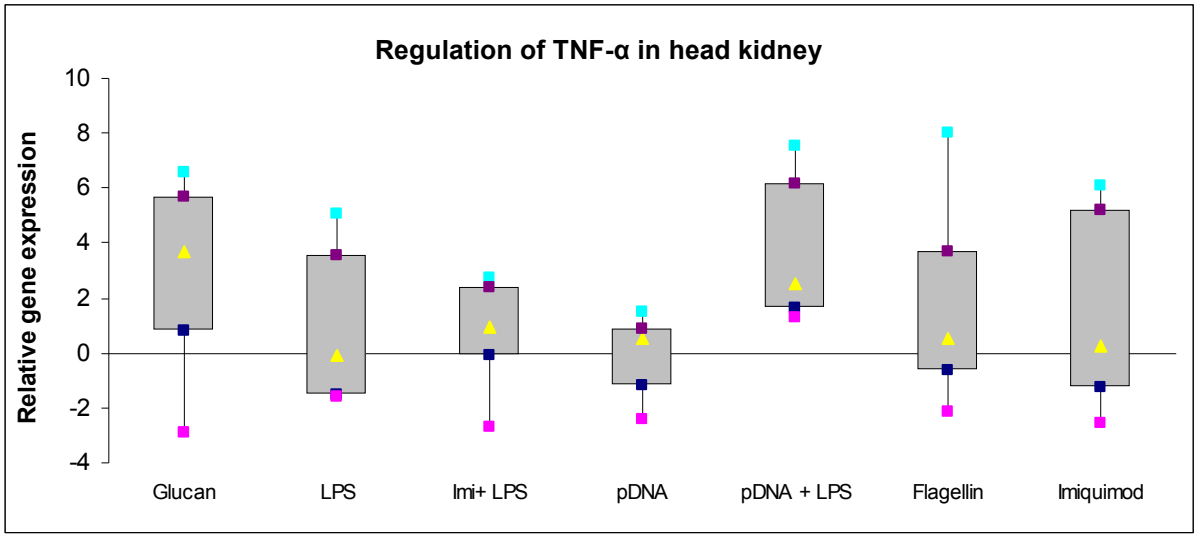

Figure 3.17 – Expression of TNF-α in head kidney, relative to 18S and adjusted to the respective values of the control groups. Turquoise and pink boxes indicate maximum and minimum values respectively. Purple boxes represent the upper quartile (75 %) and the blue boxes the lower quartile (25 %). The yellow triangles mark the median for the values. A down-regulation of gene expression is represented by median, lower quartile and minimum values being below zero. In case of an up-regulation of expression, median, the higher quartile and the maximum value are all above zero.

The plots showed that LPS and flagellin both induced a down-regulation of IFN-γ gene expression in the spleen (Fig. 3.14). The rest of the immunostimulants induced relatively clear up-regulations of IFN-γ expression. In the head kidney, down-regulation was induced by imiquimod + LPS, pDNA and flagellin (Fig. 3.15). The strongest up-regulation of gene expression was observed in samples obtained from LPS and pDNA + LPS injected fish.

For TNF-α, the general trend was for the immunostimulants to induce an up-regulation of gene expression in both the spleen and head kidney following immunostimulation. Increased expression of TNF-α was induced by the immunostimulant combinations, as well as pDNA (Fig. 3.16 and 3.17). Lipopolysaccharide may have induced a down-regulation of the cytokine in the head kidney, though this is somewhat uncertain (Fig. 3.17). In the present experiments, the strongest up-regulations were induced by β-glucan, flagellin, and both of the immunostimulant combinations.

3.4.2. Regulation of the TH2 transcription factor GATA-3 and T-regulatory IL-10

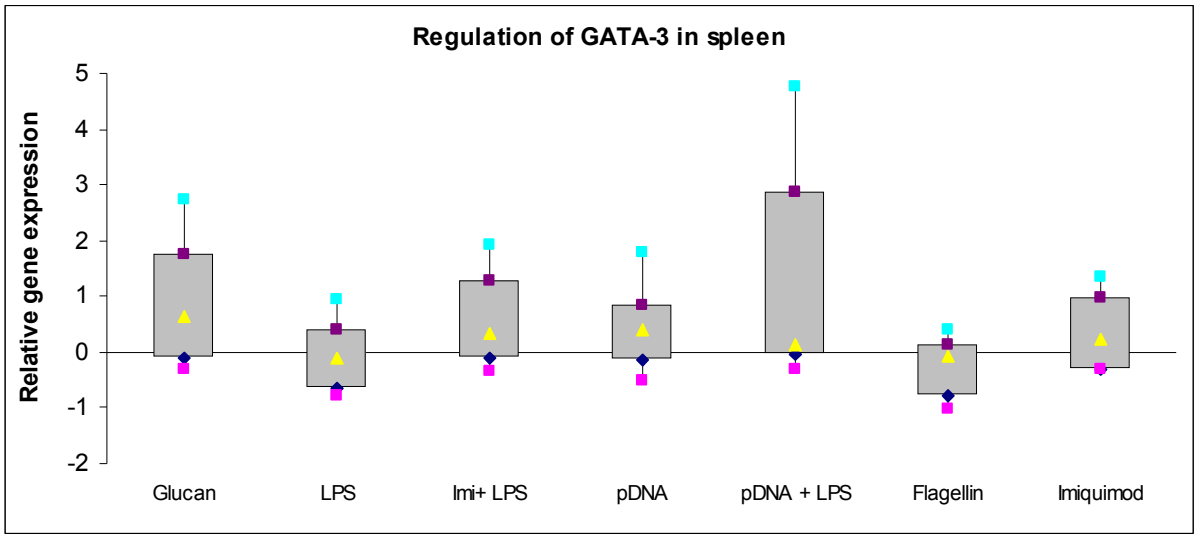

Figure 3.18 – Expression of GATA-3 in spleen, relative to 18S and adjusted to the respective values of the control groups. Turquoise and pink boxes indicate maximum and minimum values respectively. Purple boxes represent the upper quartile (75 %) and the blue boxes the lower quartile (25 %). The yellow triangles mark the median for the values. A down-regulation of gene expression is represented by median, lower quartile and minimum values being below zero. In case of an up-regulation of expression, median, the higher quartile and the maximum value are all above zero.

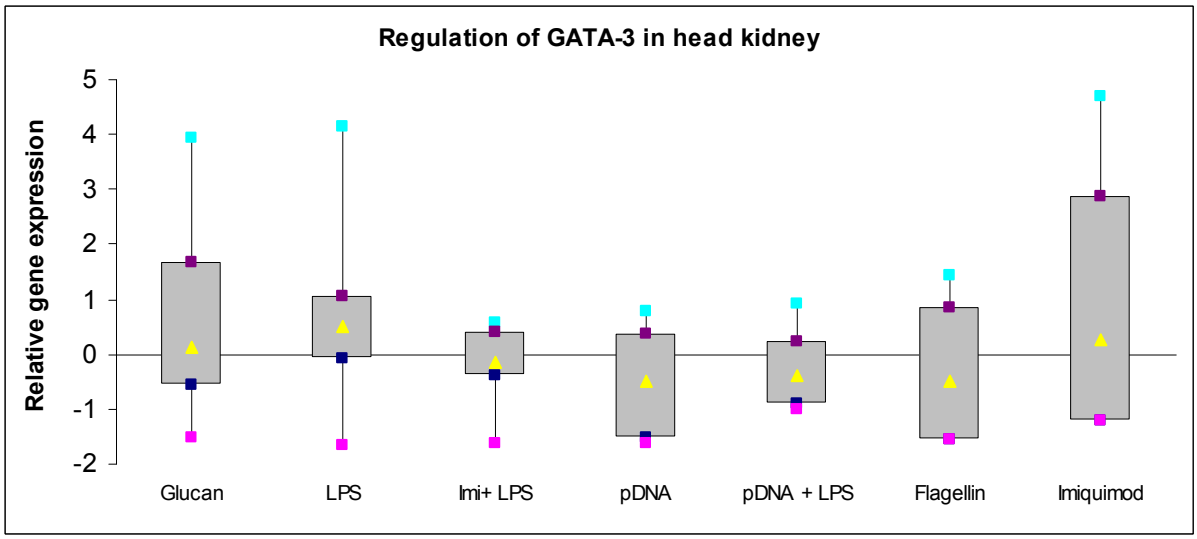

Figure 3.19 – Expression of GATA-3 in head kidney, relative to 18S and adjusted to the respective values of the control groups. Turquoise and pink boxes indicate maximum and minimum values respectively. Purple boxes represent the upper quartile (75 %) and the blue boxes the lower quartile (25 %). The yellow triangles mark the median for the values. A down-regulation of gene expression is represented by median, lower quartile and minimum values being below zero. In case of an up-regulation of expression, median, the higher quartile and the maximum value are all above zero.

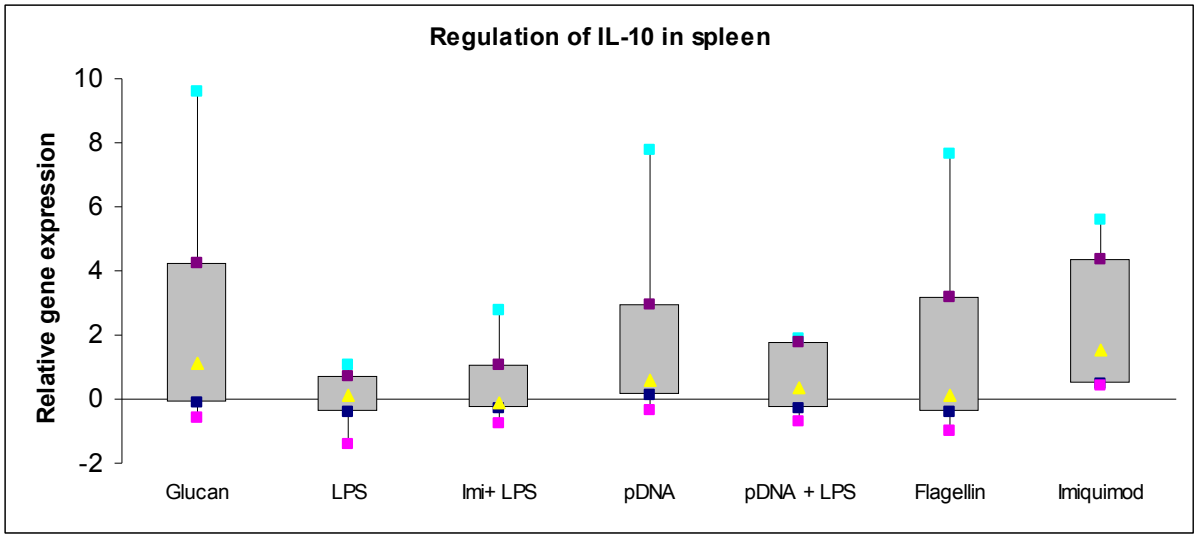

Figure 3.20 – Expression of IL-10 in spleen, relative to 18S and adjusted to the respective values of the control groups. Turquoise and pink boxes indicate maximum and minimum values respectively. Purple boxes represent the upper quartile (75 %) and the blue boxes the lower quartile (25 %). The yellow triangles mark the median for the values. A down-regulation of gene expression is represented by median, lower quartile and minimum values being below zero. In case of an up-regulation of expression, median, the higher quartile and the maximum value are all above zero.

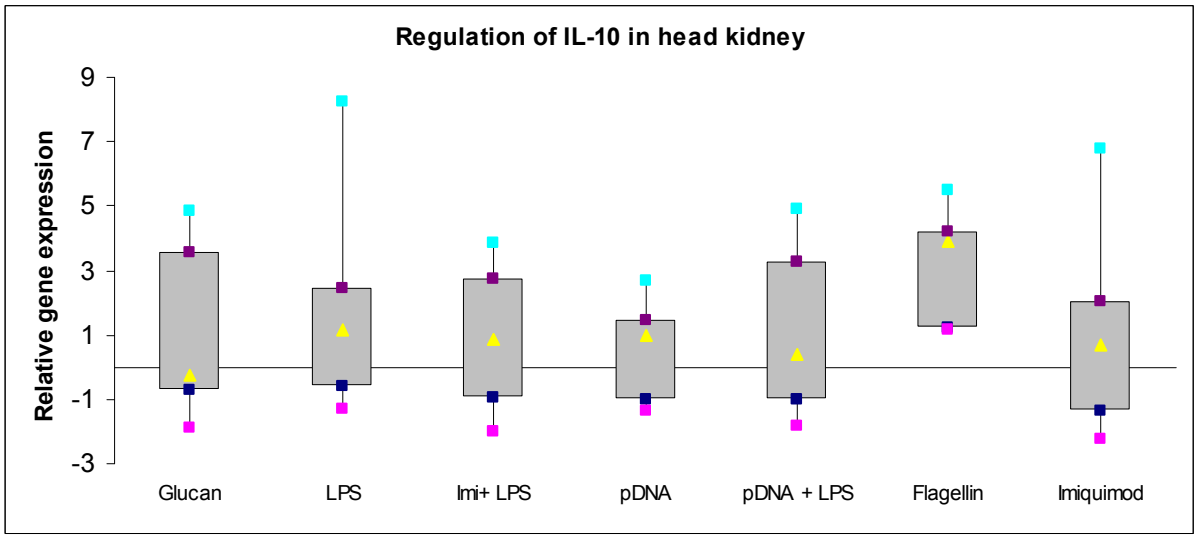

Figure 3.21 – Expression of IL-10 in head kidney, relative to 18S and adjusted to the respective values of the control groups. Turquoise and pink boxes indicate maximum and minimum values respectively. Purple boxes represent the upper quartile (75 %) and the blue boxes the lower quartile (25 %). The yellow triangles mark the median for the values. A down-regulation of gene expression is represented by median, lower quartile and minimum values being below zero. In case of an up-regulation of expression, median, the higher quartile and the maximum value are all above zero.

Lipopolysaccharide and flagellin induced a down-regulation of GATA-3 transcript expression in the spleen (Fig. 3.18). The strongest up-regulation of gene expression was induced by β-glucan and pDNA, though both immunostimulant combinations also exerted a up-regulatory effect with respect to GATA-3 gene expression. In the head kidney, down-regulation of GATA-3 gene expression was exerted by pDNA and flagellin, together with both immunostimulant combinations (Fig. 3.19). The rest of the stimulants all induced up-regulation of GATA-3 expression, and this trend was most consistent in the groups injected with LPS (Fig. 3.18 – 3.19).

Only the combination of imiquimod + LPS induced a down-regulation of IL-10 expression in spleen (Fig. 3.20), whereas β-glucan, pDNA and imiquimod all were strong inducers of IL-10 gene expression. For LPS and pDNA + LPS, the average values did not lead to a differential regulation with respect to IL-10. In the head kidney, the most prominent down-regulation was observed in samples from β-glucan injected fish (Fig. 3.21). The remaining immunostimulants induced relatively consistent up-regulation of IL-10 expression. For pDNA + LPS there was an equal number of samples showing Ct values that suggested down- and up-regulation, though the values for up-regulation were higher than those for the opposite response.

3.4.3. Immunostimulants inducing T_H2, T_H1 and T_{reg} responses

β-glucan, imiquimod and the combination of pDNA + LPS appeared to be the strongest inducers of T_H1 response in the spleen and head kidney, and induced the expression of both IFN-γ and TNF-α (Fig. 3.14-3.17). Plasmid DNA and the combination of imiquimod + LPS generally also up-regulated T_H1 responses, except in the spleen, where both treatments induced a down-regulation of IFN-γ gene expression post injection (Fig. 3.14). The best inducers of GATA-3 related T_H2 response in both the spleen and head kidney were β-glucan and imiquimod (Fig. 3.18-3.19), though the results for imiquimod were somewhat questionable due to the extreme values detected by Q-RT-PCR. None of the other immunostimulants induced a differential regulation with respect to GATA-3. The best inducers of IL-10 gene expression were pDNA, pDNA + LPS and imiquimod. All of these immunostimulants induced an up-regulation of IL-10 expression in both spleen and head kidney (Fig. 3.20 - 3.21). The head kidney samples obtained from fish injected with flagellin showed a high up-regulation of IL-10 gene expression (Fig. 3.21).

4. Discussion

The aim of this thesis was to study the expression of various genes in response to intraperitoneal injection of different immunostimulants and combinations of these. Also subject to investigation was the regulation of T_H1 and T_H2 responses post injection.

4.1. Experiment setup and materials

Sampling of spleen, liver and head-kidney are all simple procedures, as is the isolation of mRNA from the different tissues. The generally high quality of the isolated mRNA and the results obtained from Q-RT-PCR indicated that the applied methods were suitable to address the objectives of this study. One solution for improving the overall validity of the results would have been to include a higher number of parallels than the three fish that were applied in this study. As observed, results were greatly affected by the occurrence of extreme Ct values due to individual high responders. By sampling more fish at each time-point, one would gain a better understanding of the general impact the different immunostimulants exert. A greater number of samples would for instance allow for removal of minimum and maximum values, leaving a data set that would be more representative of the stimulatory capacities of each immunostimulant. According to the calculations in table 3.1, there was a great variation concerning the actual administered dose of immunostimulants. After calculating the dose as mol kg^{-1} body weight, the highest dose injected was for imiquimod, the dose being twice as high as for β-glucan. An interesting observation was that pDNA and flagellin, which were the immunostimulants administered in the lowest doses, both proved to be strong inducers of gene transcription. Increased expression levels detected by Q-RT-PCR in samples from fish injected with pDNA were observed at some time-point for all genes, except for GATA-3 in the head kidney (Fig. 3.1-3.13). These results are quite remarkable, when considering the very small amount, in terms of μmol pDNA, that was actually injected into the fish. However, the general belief is that the stimulatory effect of pDNAs lies within the unmethylated CpG motifs of the plasmids, and there also exist differences among different classes of CpGs in terms of their stimulatory effects (Verthelyi *et al.*, 2001; Vollmer *et al.*, 2004; Strandskog *et al.*, 2007). Had the content of such motifs been taken into account, the calculation of the administered μmol dose could have yielded a quite different result. Knowledge as to the nature of the CpG classes within R70pRomiLuc could also have shed some light as to the high stimulatory capacities that were observed. To analyse the content of

CpG motifs, the whole pDNA should be sequenced. Flagellin was especially effective for induction of TNF-α and IL-10 expression (Fig. 3.1-3.2 and 3.7-3.9). Extreme values were also frequently observed for samples obtained from fish injected with flagellin. Not much is known about the optimal doses for immunostimulants in fish, but results from this study suggest that this is certainly a field worthy of further investigation.

4.2. Relative gene expression

As mentioned, all observations of "high to extreme" expression levels were done in groups that had received immunostimulants, indicating that they were not results of errors performed during the processes of cDNA synthesis and further preparations for Q-RT-PCR. This was supported by the fact that "high to extreme" transcription levels in samples obtained from flagellin and β-glucan injected fish were only observed for a few of the investigated genes. For the immunostimulants pDNA and imiquimod, extreme transcription levels by Q-RT-PCR were observed at day one post injection for each of the investigated genes (Fig. 3.1-3.13). However, fish have been shown to exert large individual differences with regard to immune responses, and especially responses with regard to gene expression (Gerwick *et al.*, 2007; Løvoll *et al.*, 2007). I would expect this to be the case for these particular samples. This fact could also be applied with regard to the increased gene expression levels that were observed for some samples obtained from fish injected with PBS. However, this increased expression level could also be a result of an inflammatory response caused by the actual injection process. The fish were given their respective injections at the same time as they were moved to new tanks. This change in environment, combined with the handling stress and the injection process, could affect the observed transcription levels not only in fish receiving immunostimulants, but also in those injected with PBS.

It is also important to consider the fact that detected mRNA transcript levels are not necessarily representative of the actual protein levels and their respective activities (Gygi *et al.*, 1999). This is because gene expression is a combined process, comprising the transcription of a gene into mRNA, processing of that mRNA and the following translation of that particular mRNA into a protein (Berg *et al.*, 2002). As such, rapid and high increases in gene transcription levels may not be directly related to any of the physiological effects one would expect from matured proteins. Gene expression levels were at some occasions bi-phased, with two peaks within the experimental period. The appearing bi-phased responses, as observed for many genes following injection of immunostimulants, were likely caused by

high individual variations at some time-points, thus not reflecting true time-course responses. More parallels would have to be included in such a study to address this fully.

4.2.1. Expression of the T_H1 cytokines IFN-γ and TNF-α

The increases in expression of TNF-α observed in both spleen and head kidney at day one post injection, and for almost every administered immunostimulant, were in agreement with the knowledge of TNF-α being an acute, pro-inflammatory cytokine (Bayne and Gerwick, 2001; Pasare and Medzhitov, 2004). In comparison, expression of IFN-γ was generally observed a few days after the fish had been stimulated. This was to be expected, as production and secretion of IFN-γ is regulated by the initial inflammatory response (Feghali and Wright, 1997). Additional time would therefore be required for the IFN-γ gene transcripts to reach sufficient values for detection by Q-RT-PCR. The low observations of IFN-γ gene expression in both the spleen and head kidney (Fig. 3.3-3.4) would suggest that transcription of the cytokine was performed mainly by innate immune cells, such as NK cells (Trinchieri, 1995; Stark *et al.*, 1998). This is supported further by the observations made of GATA-3, CD8 and CD4 gene transcripts (discussed in sections 4.1.2 and 4.1.4), which suggest that an adaptive response had not truly been initiated. Differentiation of naïve T-helper cells into T_H1 would probably have led to increased IFN-γ gene transcription, resulting in the detection of higher expression levels by Q-RT-PCR. Lipopolysaccharide has been shown to induce TNF-α expression by monocytes and macrophages *in vitro* (MacKenzie *et al.*, 2002; MacKenzie *et al.*, 2003), and the current results were in accordance with those observations. Expression levels of TNF-α in fish injected with LPS were relatively high in the head kidney (Fig. 3.2), and could be a result of trapping and receptor binding of LPS by sinusoidal macrophages in this organ (Dannevig *et al.*, 1994; Brattgjerd and Evensen, 1996). Tumour necrosis factor-α turned out to be the cytokine that was most induced by the combinatory immunostimulant injections pDNA + LPS and imiquimod + LPS, which could be an indication of synergy amongst the combined stimulants. This was particularly striking in the samples taken from spleen (Fig. 3.1), where the administration of LPS alone induced relatively low expressions of TNF-α. When combined with imiquimod and pDNA respectively, there was a distinct increase in gene expression at almost every time-point (Fig. 3.1). Taking into account the high expression levels induced by injection of imiquimod alone, I would suggest that pDNA and imiquimod both acted to enhance the stimuli exerted by LPS. This suggestion agrees with the findings by Yi *et al*. (2001), which showed that combinations of CpG DNA and LPS synergized for, amongst others, increased TNF-α gene transcription. Napolitani *et al*. (2005)

also observed synergistic effects after stimulation of DCs with resiquimod and LPS. Further investigation would be needed in order to determine the nature of this potential synergy.

4.2.2. Expression of the T_H2 promoting transcription factor GATA-3

The low expression values observed for GATA-3 in both spleen and head kidney (Fig. 3.5-3.6) could be explained by the short duration of the experiment. Expression of GATA-3 would be an indicator of an adaptive immune response, and the seven-day period during which samples were taken may have been too short for such a response to truly be initiated. This was supported by the observations made for expression of the T-cell receptors CD8 and CD4 (Fig. 3.10-3.13 and section 4.1.4.), which suggested that T-cell proliferation was initiated towards the end of the experiment.

4.2.3. Expression of the regulatory IL-10

When considering the low expression levels observed for the T_H2 promoting GATA-3, the high expressions of IL-10 observed for various immunostimulants were most likely the result of transcription by innate immunity cells such as macrophages (Akira and Hemmi, 2003; Pengal *et al.*, 2006). However, the expression levels in samples obtained from fish injected with LPS were among the lowest observed for all treatment groups, an unexpected result considering the known ability of LPS to induce IL-10 production in macrophages (Akira and Hemmi, 2003). An explanation for this might be that the administered dose of LPS was not the optimal for inducing IL-10 gene expression. Fish are known to require high doses of LPS in order to elicit innate immune responses (Berczi *et al.*, 1966), and this could very well apply to the current experiment.

4.2.4. Expression of the T-cell receptors CD8 and CD4

The slightly higher expression values of the investigated T-cell receptors as opposed to the T_H2 transcription factor GATA-3 may indicate an early adaptive immune response. As would be expected of an adaptive response, the majority of the increase in gene expressions were observed a few days post injection. The general trend of the expression ratios within each treatment group, was an increase toward the end of the experimental period. However, as with the other investigated mRNA transcripts, some observations indicated early responses. The investigations performed for this thesis had no means of determining whether the increase in receptor gene expression was a result of increased receptor gene transcription within the naïve T-cells, or rather an indication of increased T-cell proliferation.

4.3. Regulation of T_H1, T_H2 and T_{reg} post stimulation

As the data-sets for the box-plots (Fig. 3.14-3.21) where adjusted to not include the observations of high-to-extreme values, the plots were naturally not exact representations of the expression levels obtained from the Q-RT-PCR experiments. However, I considered the modified data sets to be more representative of the actual expression levels induced within each treatment group. Had a higher number of fish been used in each treatment group, and more parallels included, a larger data set would have been available. Then it may not have been necessary to exclude any of the values. As the samples included in the data sets were from the three first samplings post injection only, gene transcription related to innate immunity was easier to detect. As such, the T_H1 related gene transcripts were expected to show clearer indications of up- or downregulation than the T_H2 and regulatory gene transcripts, given that they are also connected to cells of the innate immune system.

4.3.1. Regulation of the T_H1 cytokines IFN-γ and TNF-α

The apparent down-regulation of IFN-γ gene expression observed in the spleen and head kidney samples obtained from flagellin injected fish (Fig. 3.14-3.15), may be due to the short duration of the experiment, as described in section 4.2.1. As the expression levels of TNF-α gene transcripts were somewhat upregulated (Fig. 3.16-3.17), I expect the levels of IFN-γ would have been higher if later time-points had been included. This assumption was supported by the observation that for TNF-α, the up-regulation of gene expressions was, generally, much more distinct than for IFN-γ. The maximum values for up-regulation of TNF-α gene transcription were also higher than those found for IFN-γ. This could be explained by the fact that TNF-α gene transcription was initiated somewhat earlier than the transcription of IFN-γ, as I have explained earlier. There was also a general trend that the regulation levels of TNF-α expression varied more than those observed for IFN-γ. In the box-plots (figure 3.14 – 3.21) this was indicated by the interval between the upper and the lower quartile. This could perhaps be explained by the individual differences that are known to be quite prominent in fish (Gerwick *et al.*, 2007; Løvoll *et al.*, 2007), and that these differences became more pronounced in cases where gene transcription levels were relatively high.

4.3.2. Regulation of the T_H2 transcription factor GATA-3 and T-regulatory IL-10

The low expression levels of GATA-3 relative to 18S that were shown in figure 3.5 and 3.6, were reflected by the box-plots that showed both an up- and down-regulation of the gene transcripts (Fig. 3.18 and 3.19). With the low expression levels relative to 18S that were found

earlier, the detection of differential regulation of gene transcription was somewhat difficult to interpret. However, the relatively high maximum levels that were observed for some of the samples could be an indication that even though transcription of GATA-3 had not been fully initiated, the stimulation necessary for such a transcription had been exerted. One way of determining if this was actually the case would have been to look at GATA-3 gene transcripts from samples obtained at later time-points post injection. Compared to GATA-3, the regulatory cytokine IL-10 showed a more distinct up-regulation of expression levels, and also higher maximum values (Fig. 3.20-3.21). As I suggested earlier, this could be because the IL-10 gene transcripts that were detected by Q-RT-PCR originated not necessarily from differentiated T-cells, but more likely from cells of the innate immunity, such as macrophages (Akira and Hemmi, 2003; Pengal *et al.*, 2006).

4.3.3. On down-regulations of gene expression

In general, the cases where the observation of low expression values suggested a down-regulation, relatively low individual variations were observed by assaying the transcript levels by Q-RT-PCR. In the box-plots (Fig. 3.14-3.21), this was shown by relatively small intervals between the upper and lower quartile. I would suggest these cases to be clear indications of an actual down-regulation, though the number of values in the applied data set would have to be higher to determine whether this trend was consistent.

4.4. Toll-like receptors and adaptive immunity

Despite the generally low gene transcript levels that were observed for GATA-3, CD8 and CD4, it could be assumed that the stimulation of TLRs by their respective agonists is a means of inducing adaptive immune responses. Current observations of up-regulated gene transcript levels for GATA-3, CD8 and CD4 (Fig. 3.5-3.6 and 3.10-3.13) support this assumption, and a review by Heine and Lien (2003) also states the role of innate pathogen recognition by TLRs in eliciting an adaptive response. The link between the innate and the adaptive immune responses is exerted by DCs, that express the full repertoire of TLRs (Muzio *et al.*, 2000). These cells act by presenting the antigens on major histocompatibility complexes (MHCs), and are thereby able to activate antigen specific, naïve T-cells (Banchereau and Steinman, 1998). Additional linkage between the innate and the adaptive immunity is also exerted by chemokines that are secreted as a response to TLR signaling mediated by pathogens. These chemokines assist in the recruitment of leukocytes to the infected tissues (Luster, 2002; Abbas

and Lichtmann, 2006). All current observations indicate the activation of innate immunity as a response to injection of immunostimulants. As such, the observed increase in gene expressions for GATA-3, CD8 and CD4 (Fig. 3.5-3.6 and 3.10-3.13) are most likely a result of the mentioned cooperation between innate and adaptive responses.

4.5. Further research

Intraperitoneal injection of immunostimulants seems to be an easy and effective way of inducing innate as well as adaptive immune responses in fish. However, further studies on long term expression should be performed in order to gain a better understanding of the effect on adaptive immune responses. More research should also be performed as to the optimal doses related to the different immunostimulants, as the determination and application of such could induce more effective immune responses post injection. The ability of immuno-stimulants to induce rapid immune responses in the host post injection make them applicable as adjuvants for vaccines. The vaccination process is known to induce stress in fish, perhaps making them more susceptible to disease in the time before the vaccine induces an effective response. The use of immunostimulants as adjuvants could be a way of reducing stress symptoms in vaccinated fish. However, no vaccine was applied during this study. As such, the effect of the immunostimulants when delivered as adjuvants is not known.

5. Conclusion

- Despite somewhat fluctuating results, all the applied immunostimulants exerted stimulatory effects on gene transcription detectable by Q-RT-PCR.
- The levels of gene transcription related exclusively to adaptive immune responses were generally low.
- Both up- and down-regulation of T_H1, T_H2 and T_{reg} responses was observed.
- Most effective at upregulating transcription of the T_H1 signature genes IFN-γ and TNF-α were β-glucan, imiquimod and the combination pDNA+LPS. Up-regulation of GATA-3 gene transcription was best induced by β-glucan and imiquimod. The T-regulatory IL-10 was up-regulated by pDNA, imiquimod and the combination pDNA + LPS

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6. References

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7. Appendix

7.1. Chemicals and reagents

Table A.1

Name	**Manufacturer**
Etidium bromide	Continental lab products
Hind III	Promega
6X DNA Loading Buffer	
Magnesium chloride ($MgCl_2$)	Merck
RNase (bovine pancreas)	Sigma Aldrich
DNase (bovine pancreas)	Sigma Aldrich
1 x PBS	Gibco, Invitrogen™
Benzokain (20 %)	ACD SA, Belgium
RNAlater	Ambion®
TRIzol®	Invitrogen AS, Carlsbad, California, USA
Chloroform	Merck
Isopropanol	Arcus kjemi AS, Norway
MilliQ water	
Nuclease free water	Invitrogen™
Formaldehyde loading buffer	Ambion®
10X Turbo DNase buffer	Ambion®
Turbo DNase	Ambion®
DNase Inactivation reagent	Ambion®
10X TaqMan RT Buffer	Applied Biosystems
25 mM $MgCl_2$	Applied Biosystems
dNTP mix (10 mM)	Applied Biosystems, Invitrogen™
Random Hexamere	Applied Biosystems
RNase inhibitor (20 U/µl)	Applied Biosystems
Multiscribe Reverse Transcriptase (50 U/µl)	Applied Biosystems
TaqMan® Fast Universal PCR Master Mix (2X)	Applied Biosystems
TaqMan® Primer & Probe, IL-10	Applied Biosystems
TaqMan® Primer & Probe, IFN-γ	Applied Biosystems
TaqMan® Primer & Probe, 18S	Applied Biosystems
TaqMan® Primer & Probe, Gata-3	Applied Biosystems
TaqMan® Primer & Probe, CD4	Applied Biosystems
TaqMan® Primer & Probe, TNF-a	Applied Biosystems
TaqMan® Primer & Probe, CD8	Applied Biosystems
Plasmid Giga Kit	QIAGEN®, GmbH, Hilden, Germany

7.2. Immunostimulants

Table A.2

PBS	
B-glucan (*Laminaria hyperborea*)	Pronova, Norway
LPS	Isolated from *A. salmonicida* at NCFS
pDNA	Isolated from *E. coli* at NCFS
Flagellin	Recombinant Borrelia p41 ProSpec – Tany TechnoGene LTD
Imiquimod	InvivoGen

7.3. Solutions

Table A.3

RNA/DNA quality check
1 % Agarose Gel: 4 g Multi ABgarose 400 ml TAE buffer 3.5 µl Etidium Bromide (added to a 100 ml volume at electrophoresis preparations)

Table A.4

***Aeromonas salmonicida* growth mediums**	
Blood agar (700 ml solution): 28 g Trypton Soy Agar 10.5 g NaCl 1 teaspoon agar 700 ml dH_2O 35 ml whole blood Autoclavation is performed before addition of fullblod. Filled petridishes are kept at 4 °C	Brain heart infusion (BHI) with 2 % NaCl (1000 ml solution): 1000 ml dH_2O 37 g Brain heart infusion 20 g NaCl The solution is autoclaved and kept at 4 °C

Table A.5

Total RNA isolation	
DEPC water: 0.5 ml Diethyl pyrocarbonat (DEPC) 500 ml distilled water (DEPC water is autoclaved before use)	70 % EtOH/DEPC: 70 ml Absolute Alcohol 30 ml DEPC water

Table A.6

cDNA synthesis			
RT mastermix:		Random hexamere	2.5 µl
10X RT buffer	5.0 µl	RNase inhibitor (20U/µl)	1.0 µl
$MgCl_2$ (25 mM)	11.0 µl	Multiscribe reverse	
dNTP mix (10 mM)	10.0 µl	transcriptase (50U/µl)	3.1 µl

Table A.7

Mastermix for Q-RT-PCR (1 sample)			
For 18S, IL-10, IFN-γ, CD4 and GATA-3:		For CD8 and TNF-α:	
TaqMan® Fast Universal		TaqMan® Fast Universal	
PCR Master Mix (2X)	12.5 µl	PCR Master Mix (2X)	12.5 µl
MilliQ water	8.75 µl	MilliQ water	4.25 µl
TaqMan® Primer & Probe (20X)	1.25 µl	Primer (F, 10 µM)	2.25 µl
cDNA	2.5 µl	Primer (R, 10 µM)	2.25 µl
		Probe (5µM)	1.25 µl
		cDNA	2.5 µl

7.4. Equipment

Table A.8

Immunostimulant injection and tissue sampling	
Plastipak sterile syringe	BD
Microlance needle (0.6x25 mm)	BD
Cryotubes (2 ml)	Nunc CryoTubes™ CryoLine™ System
Scissors	
Sterile, stainless surgical blades no. 3	
Tweezers	

Table A.9

pDNA isolation	
BioMate Thermo Spectronic spectrophotometer	Thermo Fisher Scientific Inc.
Powerpak 300 power supply	BIO-RAD
Innova 4300 Incubator shaker	New Brunswick Scientific, USA
Avanti Centrifuge J-26 XP	Beckman Coulter
Plasmid Giga Kit	QIAGEN®
VIBRAX VXR basic	IKA® WERKE
NanoDrop® ND-1000 Spectrophotometer	NanoDrop® Technologies, DE, USA
GeneGenius Bio Imagine System	Syngene

Table A.10

LPS isolation	
RC DC™ Protein Assay Kit	BIORAD
Ultracentrifuge	Sorvall, Ultra pro® 80, DuPont, Hertforshire, England
Heto FD3 freeze dryer	Heto labequipment, Denmark
Sonicator	Sonics VibraCell Amplitude
Dialysis tubes	Spectrum laboratories Inc.
Beckman DU® 640 spectrophotometer	

Table A.11

Total RNA isolation	
Ultra-Thurax T25 basic crusher	IKA®-WERKE
Eppendorf® Microcentrifuge tubes (1,5 ml)	Eppendorf North America
Eppendorf® Centrifuge 5417	Eppendorf North America
NanoDrop® ND-1000 Spectrophotometer	NanoDrop® Technologies, DE, USA
ND-1000 software	NanoDrop® Technologies, DE, USA
GeneGenius Bio Imagine System	Syngene, UK
Genesnap software	Syngene, UK
Greiner tubes	Greiner Bio-One International AG
TURBO DNA-free^{TM} kit	Ambion

Table A.12

RNA quality check	
Biorad power pack 300	Biorad, Norway
Biokey Screener 8 Gel chamber	Biokeystone, US
GeneGenius Bio imaging systems	Syngene, UK
Genesnap software	Syngene, UK
NanoDrop® ND-1000 Spectrophotometer	NanoDrop® Technologies, DE, USA
ND-1000 software	NanoDrop® Technologies, DE, USA

Table A.13

cDNA synthesis by RT-PCR	
GeneAmp® PCR system 2700	Applied Biosystems
Heating board	
ABgene 0.2 ml Thermo-strip (tubes and caps)	Abgene, UK
Biofuge pico	Heraeus, Germany

Table A.15

Quantitative PCR	
Microamp™ optical 96-well reaction plate w/barcode	Applied Biosystems
Microamp™ optical adhesive films	Applied Biosystems
7500 Fast Real-Time PCR System	Applied Biosystems
0.2 ml Thermo-strip, 8 tubes and caps	ABgene, UK
Centrifuge	

7.5. Growth curves for Aeromonas salmonicida

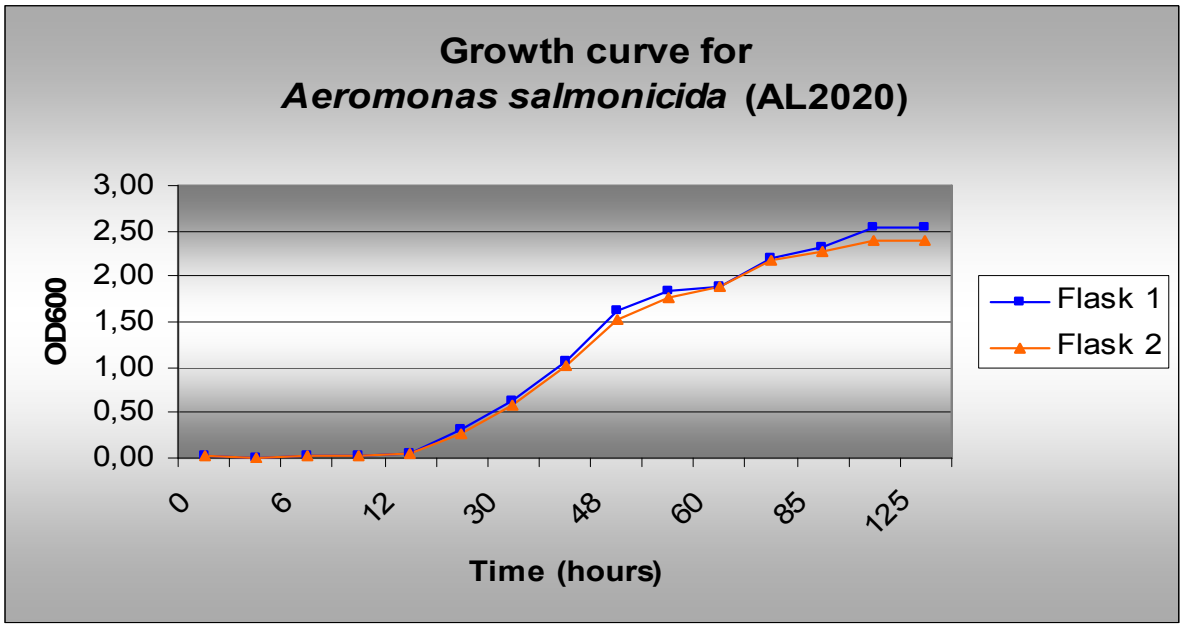

Figure A.1 – The growth of *Aeromonas salmonicida* presented as OD600 at a given time point (Graph obtained from Børge N. Fredriksen).

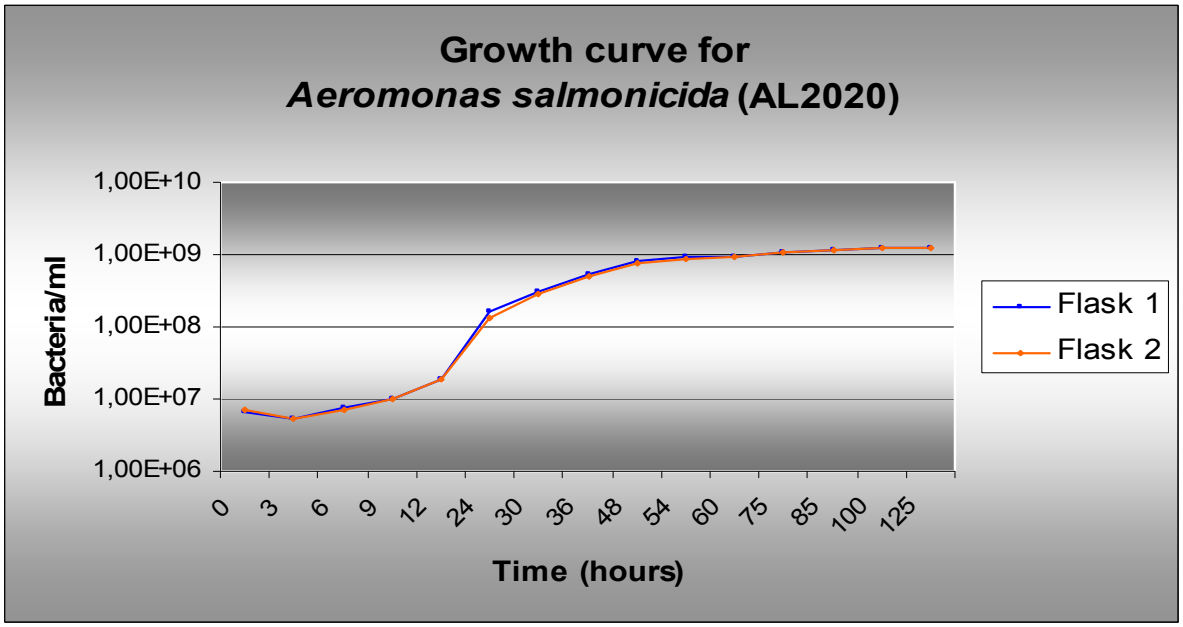

Figure A.2 – The number of bacteria in a culture of *Aeromonas salmonicida* at given time points (Graph obtained from Børge N. Fredriksen).

7.6. Regulation of T_H1, T_H2 and T_{reg} post stimulation

7.6.1. Regulation of the TH1 cytokines IFN-γ and TNF-α

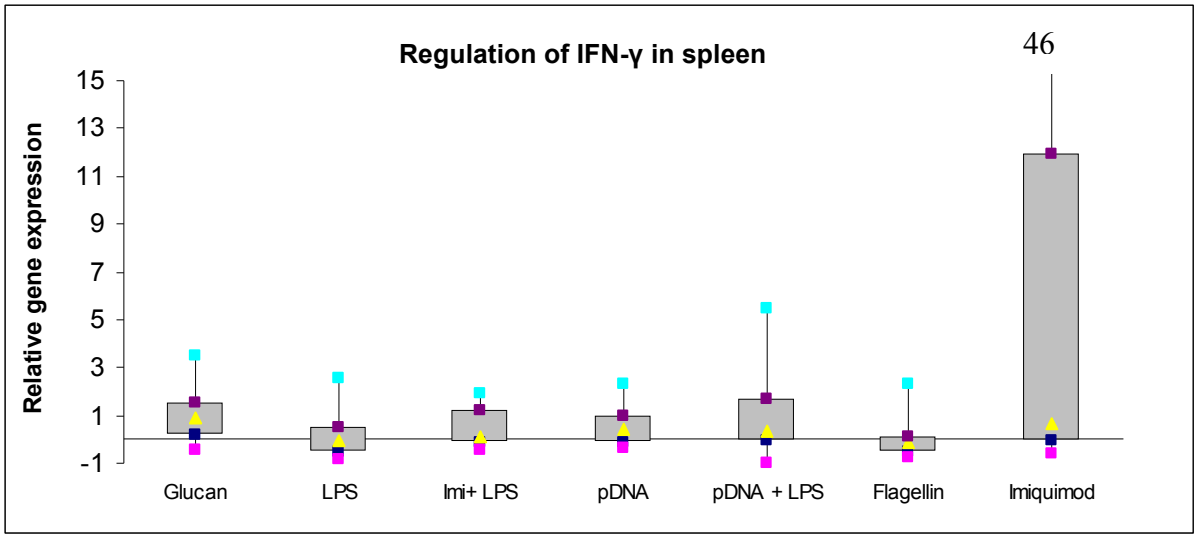

Figure A.3 – Expression of IFN-γ in spleen, relative to 18S and adjusted to the respective values of the control groups. Turquoise and pink boxes indicate maximum and minimum values respectively. Purple boxes represent the upper quartile (75 %) and the blue boxes the lower quartile (25 %). The yellow triangles mark the median for the values. A down-regulation of gene expression is represented by median, lower quartile and minimum values being below zero. In case of an up-regulation of expression, median, the higher quartile and the maximum value are all above zero.

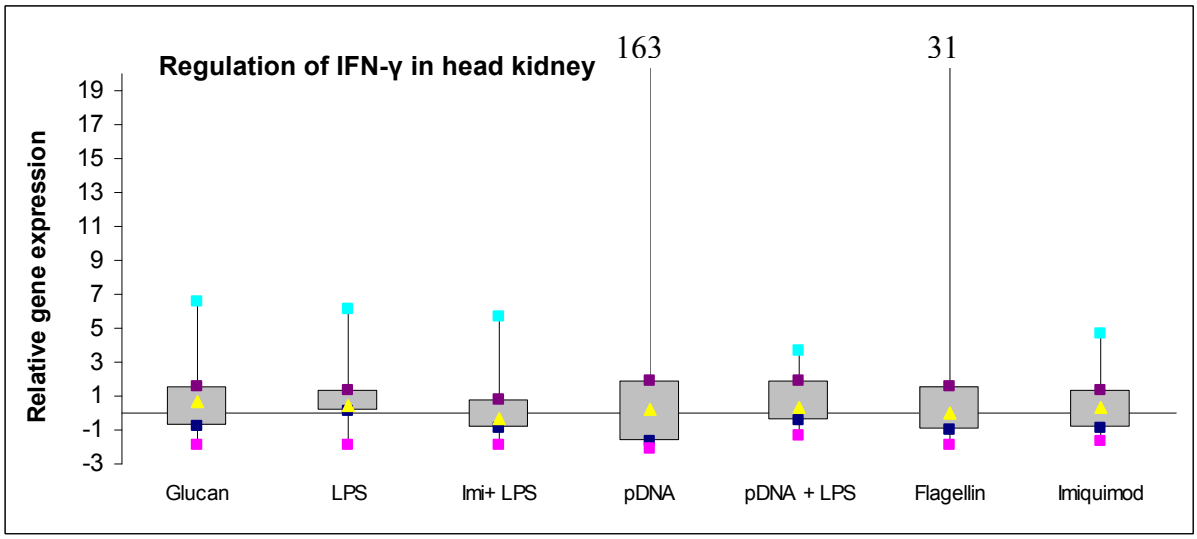

Figure A.4 – Expression of IFN-γ in head kidney, relative to 18S and adjusted to the respective values of the control groups. Turquoise and pink boxes indicate maximum and minimum values respectively. Purple boxes represent the upper quartile (75 %) and the blue boxes the lower quartile (25 %). The yellow triangles mark the median for the values. A down-regulation of gene expression is represented by median, lower quartile and minimum values being below zero. In case of an up-regulation of expression, median, the higher quartile and the maximum value are all above zero.

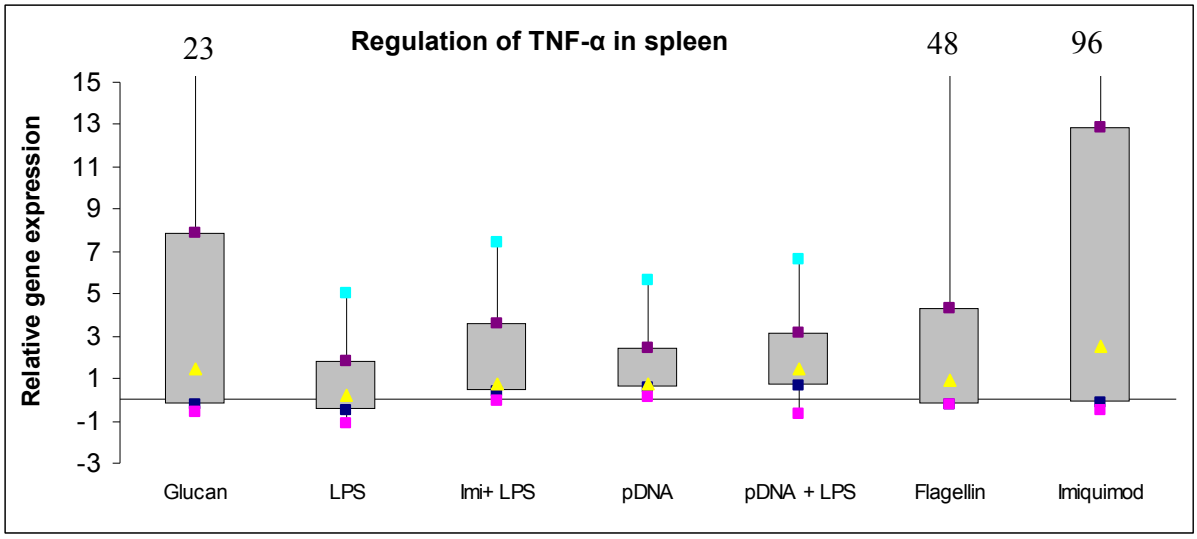

Figure A.5 – Expression of TNF-α in spleen, relative to 18S and adjusted to the respective values of the control groups. Turquoise and pink boxes indicate maximum and minimum values respectively. Purple boxes represent the upper quartile (75 %) and the blue boxes the lower quartile (25 %). The yellow triangles mark the median for the values. A down-regulation of gene expression is represented by median, lower quartile and minimum values being below zero. In case of an up-regulation of expression, median, the higher quartile and the maximum value are all above zero.

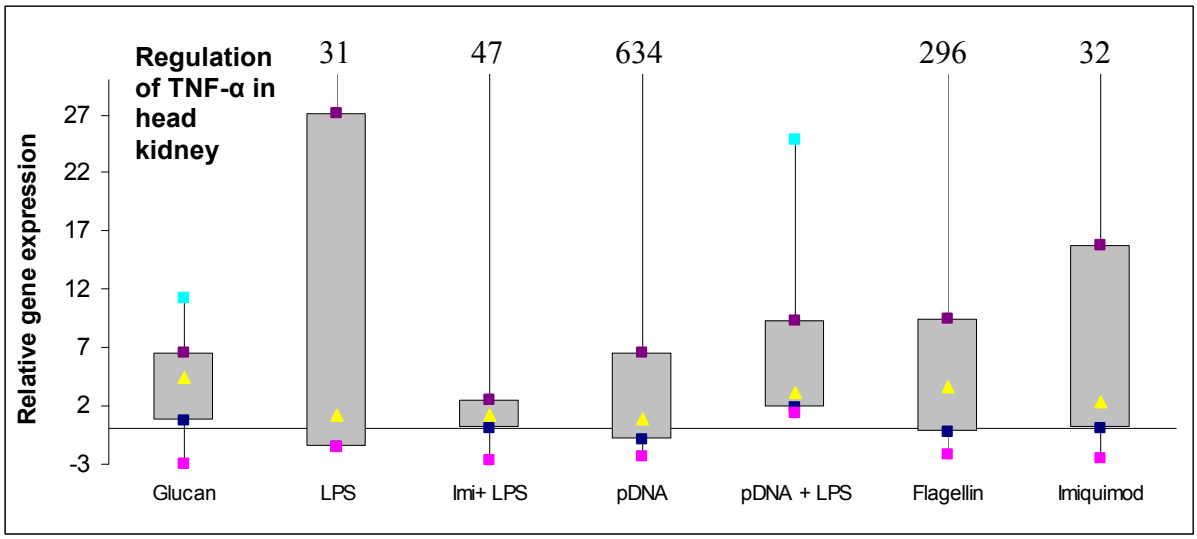

Figure A.6 – Expression of TNF-α in head kidney, relative to 18S and adjusted to the respective values of the control groups. Turquoise and pink boxes indicate maximum and minimum values respectively. Purple boxes represent the upper quartile (75 %) and the blue boxes the lower quartile (25 %). The yellow triangles mark the median for the values. A down-regulation of gene expression is represented by median, lower quartile and minimum values being below zero. In case of an up-regulation of expression, median, the higher quartile and the maximum value are all above zero.

7.6.2. Regulation of the TH2 transcription factor GATA-3 and T-regulatory IL-10

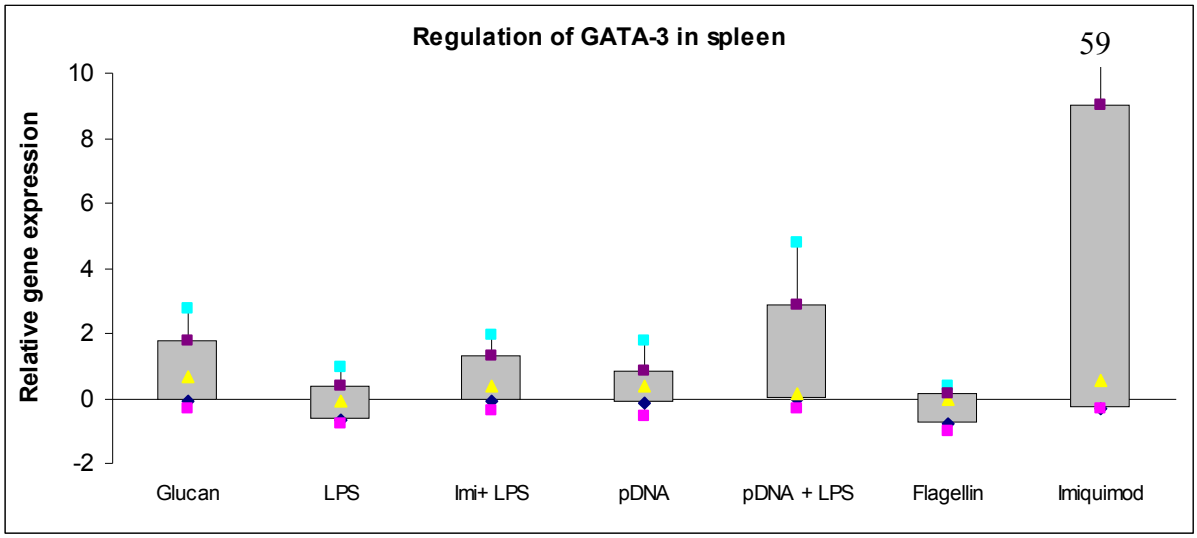

Figure A.7 – Expression of GATA-3 in spleen, relative to 18S and adjusted to the respective values of the control groups. Turquoise and pink boxes indicate maximum and minimum values respectively. Purple boxes represent the upper quartile (75 %) and the blue boxes the lower quartile (25 %). The yellow triangles mark the median for the values. A down-regulation of gene expression is represented by median, lower quartile and minimum values being below zero. In case of an up-regulation of expression, median, the higher quartile and the maximum value are all above zero.

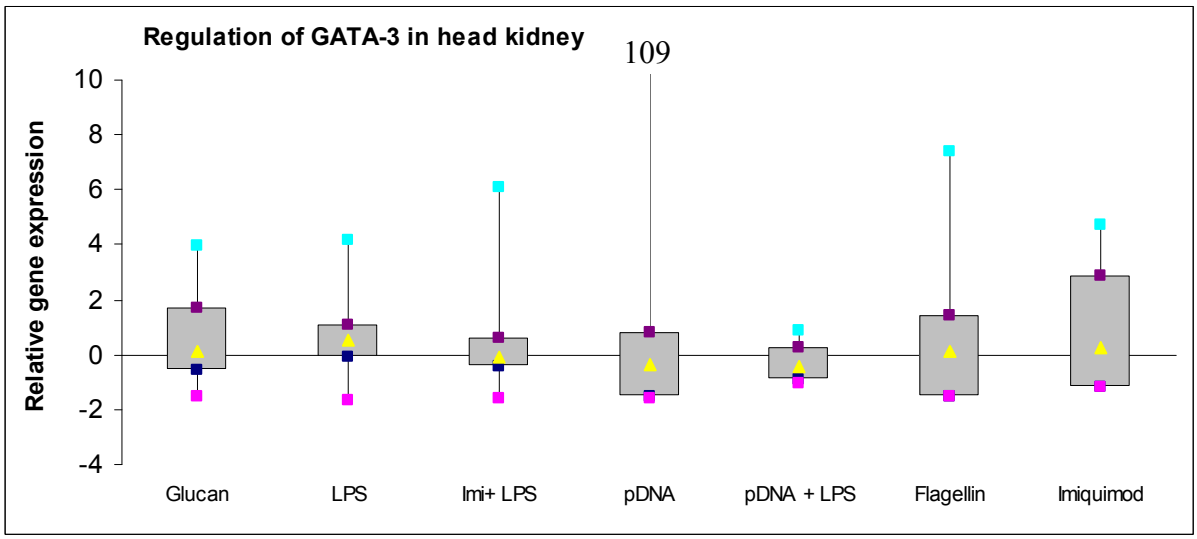

Figure A.8 – Expression of GATA-3 in head kidney, relative to 18S and adjusted to the respective values of the control groups. Turquoise and pink boxes indicate maximum and minimum values respectively. Purple boxes represent the upper quartile (75 %) and the blue boxes the lower quartile (25 %). The yellow triangles mark the median for the values. A down-regulation of gene expression is represented by median, lower quartile and minimum values being below zero. In case of an up-regulation of expression, median, the higher quartile and the maximum value are all above zero.

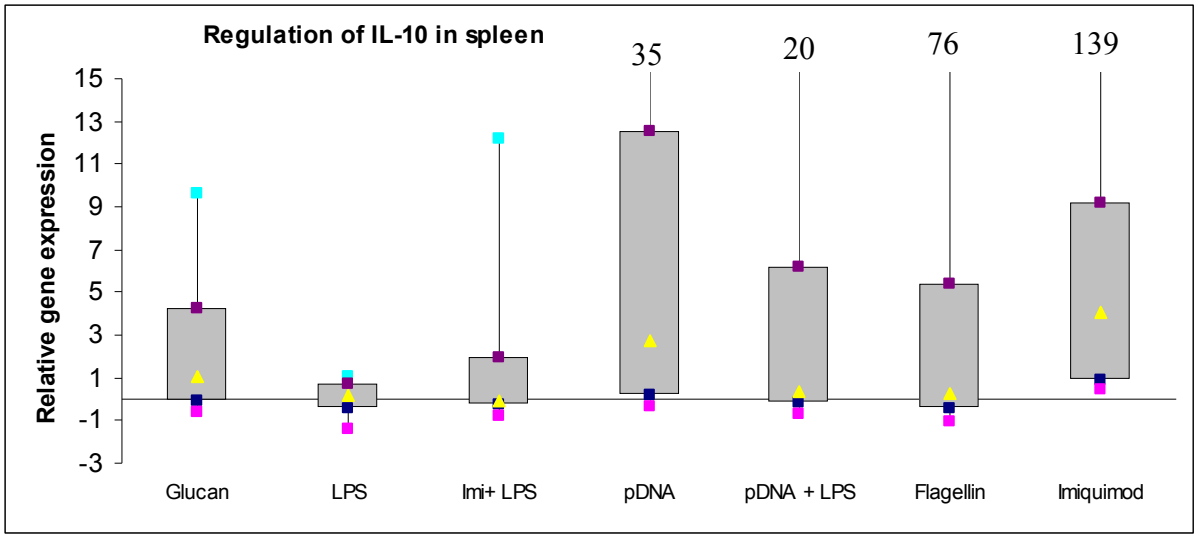

Figure A.9 – Expression of IL-10 in spleen, relative to 18S and adjusted to the respective values of the control groups. Turquoise and pink boxes indicate maximum and minimum values respectively. Purple boxes represent the upper quartile (75 %) and the blue boxes the lower quartile (25 %). The yellow triangles mark the median for the values. A down-regulation of gene expression is represented by median, lower quartile and minimum values being below zero. In case of an up-regulation of expression, median, the higher quartile and the maximum value are all above zero.

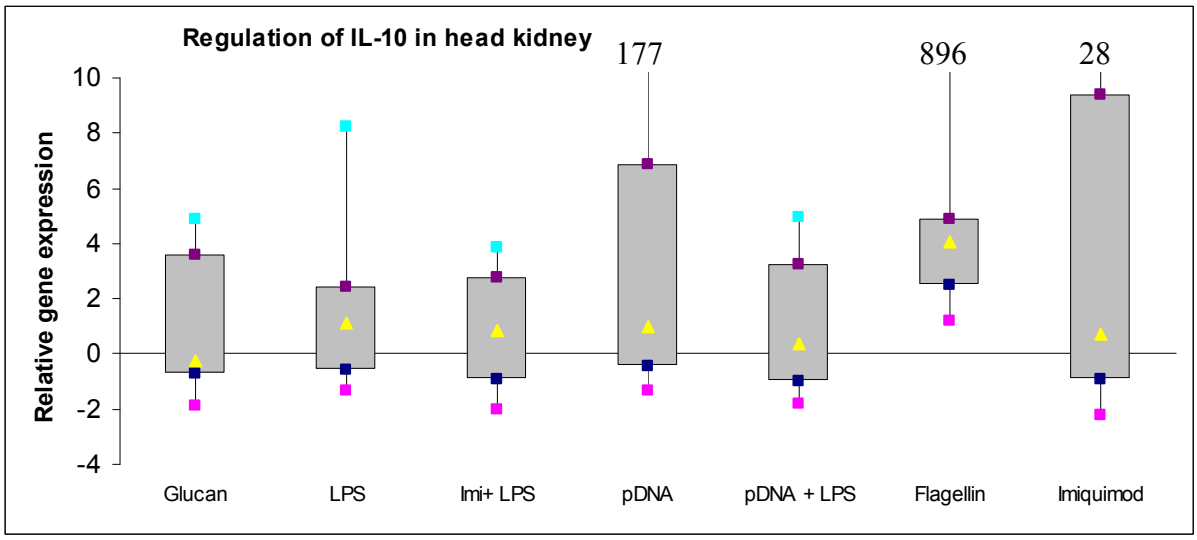

Figure A.10 – Expression of IL-10 in head kidney, relative to 18S and adjusted to the respective values of the control groups. Turquoise and pink boxes indicate maximum and minimum values respectively. Purple boxes represent the upper quartile (75 %) and the blue boxes the lower quartile (25 %). The yellow triangles mark the median for the values. A down-regulation of gene expression is represented by median, lower quartile and minimum values being below zero. In case of an up-regulation of expression, median, the higher quartile and the maximum value are all above zero.