Characterization of Atlantic salmon Toll-like receptor 3

Poly I:C – a potential new adjuvant for vaccines against viral diseases in aquaculture

Stine Dalsbø Antonsen

Master thesis for the degree Master of Pharmacy
45 credits

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Department of Microbiology
The Faculty of Mathematics and Natural Sciences

UNIVERSITY OF OSLO

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http://www.duo.uio.no/

Trykk: Reprosentralen, Universitetet i Oslo
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Blindern, May 2017

Stine Dalsbø Antonsen
Summary of thesis

Aquaculture is a rapidly growing industry and is the second largest export trade in Norway after oil and gas. However, each year 10-20 breeding farms are confirmed infected with Infectious Salmon Anemic disease (ISA), the virus originates from orthomyxo viruses and is highly contagious. Breeding farms infected with ISA must consequently slaughter all fish resulting in huge financial losses. Making ISA the second largest viral threat to salmon farming.

Since vaccination of farmed fish against bacterial infections has been so effective, is it also desirable to produce better vaccines against viral diseases. The intention of this thesis was to investigate the possibility for using new adjuvants in already existing vaccines. A good adjuvant is a substance that activates and increases the immune response without causing serious side effects. Examples of adjuvants are mineral oils, LPS, viral capsids and Poly I:C.

In this thesis a biochemical assay for measuring binding of a TLR3 ligand to the salmon TLR3 was established. The assay is based on transcriptional activation of a gene for secretory embryonic alkaline phosphatase (SEAP) activity controlled by an Nf-κB promotor. The SEAP assay was verified using commercially available HEK Blue hsTLR2 and TLR3 cells with known binding activity for TLR2 and TLR3 ligands respectively. The HEK Blue hsTLR2 cell line was transfected with plasmids encoding salmon TLR3 and selected with antibiotics to obtain a stable ssTLR3 expressing cell line. The expression of the salmon receptor was verified with QPRC, western blotting, immunofluorescence and SEAP activity assays. The results confirmed protein expression and showed specific stimulation of the cells with Poly I:C, a TLR3 ligand. A comparison of the different cell lines (that expressed human and salmon receptors) showed significant differences in their stimulation, and indicated that Poly I:C was a good activator for both the human and salmon TLR3. A fish cell line (EPC) expressing salmonTLR3 was also established, and these cells displayed an increased sensitivity for Poly I:C compared to non-transfected cells. This was analyzed by QPCR of immune genes. Some of these genes were significantly upregulated, suggesting that transfection with ssTLR3 made the cell line more responsive to dsRNA and that Poly I:C is a good agonist for salmon TLR3. Future in vivo studies, for example in zebrafish, may further assess the possibility of Poly I:C as an adjuvant in fish vaccines.
Sammendrag

Akvakultur er en raskt voksende industri og er per dags dato den nest største eksportvaren Norge har etter olje og gass. Hvert år påvises 10-20 nye tilfeller av Infeksiøs Lakse anemi (ILA) på norske oppdrettsanlegg, noe som gjør sykdommen til en av de største truslene mot næringen. ILA-viruset tilhører familien Orthomyxo virus og det er svært smittsomt, noe som gjør at alle anlegg med påvist smitte pålegges å slakte ned all laks. Dette medfører store økonomiske tap.

Vaksinering av oppdrettsfisk mot bakterielle infeksjoner har vist seg å være svært effektivt, derfor ønsker man å finne vaksiner også mot virale sykdommer. Oppgavens hensikt har vært å se på muligheten for å løse dette ved hjelp av å tilføre nye adjuvanser til allerede eksisterende vaksiner. En adjuvan er en substans som vil kunne aktivere og forbedre immunresponsen som oppstår ved vaksinering, uten å føre til sykdom selv. Eksempler på adjuvanser er mineral oljer, LPS, virus kapsider og Poly I:C.


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<tr>
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<tr>
<td>Ab</td>
<td>Antibodies</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
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<tr>
<td>ASK</td>
<td>Atlantic salmon kidney cells</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
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<td>BSA-PBS</td>
<td>Bovine serum albumin-PBS</td>
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<td>CARD</td>
<td>Caspase recruitment domain</td>
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<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
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<tr>
<td>CSV</td>
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<tr>
<td>Ct</td>
<td>Cycle threshold</td>
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<tr>
<td>CTD</td>
<td>C-terminal Domain</td>
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<td>DAMP</td>
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<tr>
<td>Δ OD</td>
<td>Δ Optical Density</td>
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<tr>
<td>DMEM</td>
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<td>ELISA</td>
<td>The enzyme-linked immunosorbent assay</td>
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<td>EPC</td>
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<td>ER</td>
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<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>VIII</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>FBS</td>
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<td>gp96</td>
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<td>Human epithelial kidney cells expressing SEAP</td>
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<td>Heat Killed <em>Listeria monogenocyes</em></td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>hsTLR2</td>
<td><em>Homo sapiens</em> TLR2</td>
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<td>hsTLR3</td>
<td><em>Homo sapiens</em> TLR3</td>
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<td>IFIT</td>
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<td>Inhibitor of κB kinase</td>
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<td>Infectious pancreatic necrosis virus</td>
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<td>IRF</td>
<td>Interferon regulatory factor</td>
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<td>c-Jun N-terminal kinase</td>
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<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
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<tr>
<td>LBP</td>
<td>Lipopolysaccharide binding protein</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>LGP2</td>
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<tr>
<td>MAVS</td>
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<td>MD-2</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MOI</td>
<td>Multiplicity of infection</td>
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<td>MQ</td>
<td>Milli Q</td>
</tr>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<td>MW</td>
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<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>Paraformaldehyde</td>
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<td></td>
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<td>PKR</td>
<td>RNA regulated protein kinase</td>
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<td>RLR</td>
<td>RIG-Like receptor</td>
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<td>Secretory enzyme alkali phosphatase</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SOC</td>
<td>Super Optimal broth with Catabolite repression</td>
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<td>TIR Domain Containing Adaptor Protein</td>
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<td>TIRCAM</td>
<td>TIR domain-containing adaptor molecule</td>
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<tr>
<td>TLR</td>
<td>Toll-Like receptor</td>
</tr>
<tr>
<td>TPR</td>
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<tr>
<td>TRAF</td>
<td>TNF receptor associated factors</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
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<tr>
<td>TRAK</td>
<td>Trafficking kinesin protein</td>
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<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
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<td>TN</td>
<td>Tris-Cl-NaCl</td>
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<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TPRs</td>
<td>Tetratricopeptide repeats</td>
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<tr>
<td>Tween</td>
<td>Polyoxetylen-sorbitan-monolaurat</td>
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<tr>
<td>VIG</td>
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1. Intro

1.1 The human immune system

The immune system for humans is a complex system divided into two main categories. The first category is the first line of defense, also known as innate immune response. This response concerns the immediate recognition of foreign, non-human substances such as pathogens. Cells in the innate immune system release signal mediators as a consequence of this recognition and activate a range of cellular defense mechanisms. Rapid reaction to a foreign intruder will often be enough to stop the development of infection, but in some cases needs reinforcements. The release of cytokines and other effector molecules cause the target cells to release more signal molecules, activating the complement system, opsonization, phagocytosis, inducing chemotaxis, etc. Most importantly, in addition to activating the first line of response, it activates the adaptive immune system. When the second line of defense is activated, a full-scale assault on the intruder is mounted. The specific cell defenses include activation, proliferation, expansion of B-cells and antibody production. Activation, proliferation, and production of the different T-cell types (T-helper cells, T-regulatory cells, T-cytotoxic cells, T-memory cells and T-effector cells) are also initiated. These specific cell types help to reinforce the defense against the pathogen, and will ensure that when reinfection with the same pathogen, a better and quicker specific response is mounted (Parham and Janeway, 2015).

1.1.1 Innate immune response during viral infection

Sensor molecules called pattern-recognition receptors (PRRs) are the first to initiate responses against viruses. These receptor types are specific for different classes of molecules and recognize pathogen associated molecular patterns (PAMPs) or endogenous stress signals, known as damage-associated molecular patterns (DAMPs). PRR are found in both intra- and extracellular compartments and they can be soluble or transmembrane receptors. PRRs are subdivided into different types; RIG-like receptors (RLR), Toll-like receptors (TLR) and NOD-like receptors (NLR). PRRs activate specific signaling cascades to induce gene expression of immune modulation substances such as proinflammatory cytokines and type 1-interferons (IFNs) (DeWitte-Orr and Mossman, 2010; Yoneyama and Fujita, 2010).
1.1.2 Pattern Recognition Receptors

**RIG-like receptors (RLR)**

The RIG family of receptors belongs to a group of helicase PRRs that is divided into three different types of receptors; Retinoic acid inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA5), and Laboratory of genetic and Physiology 2 (LGP2). All of these soluble receptors are sensors involved in activation of the innate immune system. LGP2 is located in cytoplasm and displays the highest affinity to dsRNA among the RLRs. This receptor contains a domain important for sensing PAMPs (CTD), but lacks a domain for triggering downstream signaling pathways (CARD). Because LGP2 lacks the CARD domain it has been suggested that LGP2 is a regulatory receptor. LGP2 can inhibit RIG-I by direct interaction through its C-terminal repressor domain, or by sequestration of RNA due to the greater affinity. LGP2 can also activate MDA5 by binding to the receptor and initiate MDA5 cascade (DeWitte-Orr and Mossman, 2010; Kato and Fujita, 2015; Yoneyama and Fujita, 2010). MDA5 and RIG-I are receptors that both contain CTD and CARD, located in cytoplasm. RIG-I and MDA5 normally exist in an inactive state, but upon ligand binding will the helicase domain unfold to expose its CARD domain, and bind to the adaptor proteins (Poynter et al., 2015). RIG-I recognizes dsRNA between 300 and 1000bp while MDA5 recognizes dsRNA molecules longer than 1000 base pairs. All three receptors activate MAVS and MAVS activate the replication of the Nf-κB or IRF, which are transcription factors for inflammatory cytokines and IFN type 1 (DeWitte-Orr and Mossman, 2010; Yoneyama and Fujita, 2010).

**NOD-like receptors (NLR)**

The NOD receptor family is much larger than the RLR family. 23 members of NLRs have been identified in humans, but only three of these are characterized with a PRR function. NLRP1, NLRP3 and NLRC4 are receptors when activated; induce the assembly of CARD containing inflammasomes responsible for caspase-1 activation and release of proinflammatory cytokines mainly IL-1β and IL-18 (DeWitte-Orr and Mossman, 2010; Yoneyama and Fujita, 2010).

**Toll-like receptors (TLR)**

The Toll-like receptor family is a large family of single-transmembrane proteins predominantly expressed in immune cells. In humans, the family consists of 10 members, from TLR1 to TLR10. TLR1, TLR2, TLR4, TLR5, TL6, TLR10 are all located on the cell
surface while TLR3, TLR7, TLR8 and TLR9 are located in the endosomal compartment (Yoneyama and Fujita, 2010). The tertiary molecular structure and signaling mechanisms are similar in all the TLR receptors with some main differences.

TLRs are type 1-membrane proteins that consist of a N-terminal ligand binding leucine-rich extracellular domain (ED), a transmembrane domain and a C-terminal intracellular signaling domain (TIR) (McCoy, 2016). The extracellular regions of the receptor (ectodomains) consist of several (16-28) leucine rich repeats (LRRs). LRRs originate from a family with seven subfamilies where each LRR contains 24-29 amino acids, and they consist of β-strands and α-helixes that are connected by loops (Chang, 2010). Due to these LRR conformations, a characteristic TLR horseshoe shape is formed. The ability of TLRs to bind a broad range of different ligands is due to the unique combinatorial code of LRR for each ectodomain (Carpenter and O'Neill, 2009; Chang, 2010). In contrast to the differences in the LRR domains, the intracellular TIR domain of the receptor is more conserved. This cytoplasmic domain consists of a five-stranded β-sheet surrounded by five α-helixes, where the key residues within the domain include: BB-loop essential for dimerization and subsequent adaptor recruitment, the DD-loop used to bind TLR1 and TLR2 together, and the α-C-helix also essential for dimerization. The TIR domain activates of downstream signaling after binding of PAMPs to the PRR, resulting in the activation of proteins like Nf-κB, IRFs and AP-1. Their activation results in transcription of a range of proinflammatory cytokines like IL1-β and TNF-α (Carpenter and O'Neill, 2009).
Recognition, activation and downstream signaling all start with ligand binding. As previously mentioned, type of ligand and binding location is unique for each TLR due to the LRR combinatorial codes. Initially the ligand will bind to the LRR domains and the TLR will dimerize. The majority of TLRs will form homodimers, but a selective few will form heterodimers. This type of dimerization is found for TLR2 that can create heterodimers together with either TLR1 or TLR6. TLR4 primarily form homodimers, but also forms a complex together with the co-receptor MD-2 known as lymphocyte antigen 96 and lipopolysaccharide binding protein (LBP). There is currently no evidence for other effects or functions of TLRs making heterodimers instead of homodimers (Chang, 2010; Leifer and Medvedev, 2016).

After ligand induced receptor dimerization, a signaling cascade is initiated along two main pathways. Four main adaptor proteins in these signaling pathways have been discovered that enhance downstream signaling. In addition, one main negative regulator/adaptor inhibiting the signaling has also been identified. The positive adaptor proteins are myeloid differentiation factor 88 (MyD88), TIR domain-containing protein (TIRAP) also known as MyD88 adaptor-like protein (Mal), TIR domain-containing adaptor molecule-1 (TIRCAM-1) also known as TRIF and TIR domain-containing adaptor molecule-2 (TIRCAM-2) also known as TRAM.

Figure 1: Different TLRs with a schematic overview of the signaling pathways (Baxevanis et al., 2013).
The negative adaptor protein is Sterile-α and Armadillo-motif-containing protein (SARM) (Baxevanis et al., 2013; Chang, 2010; Verma and Bharti, 2017).

After activation of the TIR domain, the first main signaling event requires the adaptor proteins MyD88 and Mal. The downstream activation starts with TIR binding to MyD88 and ends in phosphorylation of the transcription factor MAPK, which result in a quick and early activation of Nf-κB. The activation involves MyD88, recruiting a range of kinases like IRAK4, which trigger the activation of the TRAK6/IKK complex and further activates MAPKs (ERK, JNK, p88) as well as transcription of Nf-κB. It has also been seen that the adaptor protein TIRAP can participate in the activation of MyD88-dependent pathway (Baxevanis et al., 2013; Carpenter and O'Neill, 2009; Chang, 2010; Leifer and Medvedev, 2016).

Through the second signaling pathway following TLR3 dimerization, late activation of Nf-κB and IRF-3 are induced, resulting in a release of IFN-1α and -β much more specific than for the first signaling pathway. Adaptor proteins important for this downstream signaling are TRIF, TRAM, TICAM-1 and TICAM-2. They induce activation of the transcription factors Nf-κB and IRF3/7 through other kinase pathways than MAPKs. TRIF has also been known to interact with receptor-interacting protein (RIP-1), which activates the enzyme TAK-1, inducing the IKK complex and MAPK activation (Baxevanis et al., 2013; Carpenter and O'Neill, 2009; Leifer and Medvedev, 2016).
Most of the TLRs require MyD88 for downstream signaling, with the exception of TLR3. TLR3 exclusively uses TRIF as an adaptor protein unlike TLR4 that can recruit TIRAP, TRAM, MyD88 and TRIF. The TLR1/TLR2 and TLR2/TLR6 complexes mainly recruit MyD88, but can also recruit TIRAP (Baxevanis et al., 2013; Chang, 2010).

Measurement of ligand binding to TLR3 has proved to be a challenge. Normally with extracellular receptors the measurement are done with radioactive labeled ligands. Cells are cooled to stop intracellular response happening, ligand is added, and when equilibrium is reached, cells are washed, and bound radioactivity is measured. The quantity of radioactivity will then be proportional to the amount of bound receptors. The isotope method is not suited for measurement of intracellular receptors because inhibition of intracellular response will prohibit the uptake of isotopes and the isotopes will not be able to bind to TLR3, hence no signal. Luciferase and phosphatase reporter systems are two alternative methods used for intracellular receptors. These methods involve measuring the signaling response to ligand binding instead of the binding itself. Luciferase and phosphatase are reporter molecules transcribed, translated and released to the culture media when TLR3 are activated, and signals are measured through detection of light (luciferase) or changes in optical density (phosphatase acting on chromogenic substrates like DNP-phosphate) of the media (Tombacz et al., 2017).

Contrary to the radioactive isotopes methods is the signal from the reporter system based on quantity of light and color detected from luciferase or phosphatase is not directly proportional to the amount of ligand bound to TLR3. The reason for this is that the signaling pathways are so complex and branched that downstream components can be a limiting step.
Figure 3: 3-D model of the TLR3 structure (PDB).

For the TLRs to be activated, start downstream signaling and induce the production of signal molecules (like IFNs, IL1-β and TNF-α), they have to be folded properly and transported to their final destination. As previously mentioned, most TLRs travel to the cell surface, but some have their final destination in the endosomal compartment. Some TLRs can travel through the secretory pathway alone, but the majority needs accessory proteins called chaperones helping them to fold correctly and guide the receptors to their final destination. Examples are UNC93b for the TLR3, TLR5, TLR7 and TLR9, where the UNC93b directs these specific TLRs to their correct cellular destination. In the absence or with mutated forms of UNC93b, TLRs do not traffic to endosomes or the cell surface (TLR3 and TLR5, respectively). Another example is gp96, which is a chaperone for TLR2, TLR4 and integrins. It has been difficult to establish their exact mechanism of action, but it has lately been
discovered that gp96 can stabilize several TLR ligands and serve as TLR2 and TLR4 agonist (Leifer and Medvedev, 2016).

**TLR3**

TLR3 is expressed in the endosomal compartment of B-cells, T-cells, macrophages, natural killer cells and dendritic cells. It recognizes viral dsRNA and synthetic ligands like polyinosinic-polylidylic acid (Poly I:C). Optimal size for binding with Poly I:C seems to be about 46bp, and in line with the other PRRs, TLR3 triggers several intracellular downstream signaling pathways upon ligand binding (Baxevanis et al., 2013; Yoneyama and Fujita, 2010).

The crystal structure of the extracellular domain of TLR3 displays a heavily glycosylated horseshoe-shaped solenoid structure. The ectodomain consist of 23 canonical LRRs and two irregular LRRs. These LRRs contain both hydrophilic and hydrophobic residues, and because of this the seven conserved hydrophobic residues of the LRRs make a tightly packed hydrophobic core by pointing inwards. Around this core, the concave surface of the LRR domain forms a chain of 25 parallel \( \beta \)-strands, which again forms a parallel \( \beta \)-sheet where 23 of the \( \beta \)-strands belong to LRRs. The last two \( \beta \)-stands come from the N- and C-terminal cap regions. The N- and C-terminal capping is necessary for the solenoid structure because of the exposed edges of the hydrophobic core. The LRR domain of the TLR3 also contains several putative glycosylation sites. The effect of glycosylation on the receptor function is unknown, but glycosylated sites in the concave side may inhibit binding of ligand, due to their proximity to the ligand-binding region (concave side of the horseshoe). However, not the whole surface of the LRR domain have glycosylation sites, which make it possible for the receptor to bind ligand independently of glycosylation (Carpenter and O'Neill, 2009; Chang, 2010; Choe et al., 2005; Leifer and Medvedev, 2016).
Figure 4: 3-D model showing known binding sites for RNA on TLR3 (Davies, 2017).

Figure 5: TLR3 with RNA (grey) showing possible binding sites (Bell, 2017; Sahoo et al., 2015).

Residues forming the TLR3 ligand-binding sites are not 100% accounted for, but different regions that may function as binding sites have been identified through analysis of several LRR proteins. One possible binding site is located on the concave side of the horseshoe, which means that when the receptors start to dimerize both of the horseshoes can bind a copy of dsRNA each. Another binding site is on the side of the horseshoe. This potential binding site lacks glycosylation sites, giving rise to the opportunity for the ligand to bind. This suggestion has also been shown to possibly confer more stability to the dsRNA-TLR3 complex (Bell et al., 2005; Carpenter and O'Neill, 2009; Choe et al., 2005). Once recognition and binding of ligand happens, the receptor starts to dimerize. Binding between the horseshoes takes place in the C-terminal of the ectodomain, stabilizing the dimer and initiates the TIR domain activation, to allow the downstream signaling cascade to take place (Carpenter and O'Neill, 2009).
The downstream signaling cascade from TLR3 exclusively uses TRIF as an adaptor molecule. Stimulation with dsRNA or Poly I:C will either directly in the endosomes or in the endoplasmatic reticulum (ER) where TLR3 binds to UNC93b and translocate from ER to the endosomes. In the endosome, TLR3s dimerize and creates a stable signaling complex through binding in the C-terminal of the ectodomain. The dimerization activates the downstream signaling by recruiting TRIF and directly interacts via TIR-TIR homotopic interaction. The complex then recruits a set of signaling molecules including TNF receptor-associated factors 3 (TRAF) that activate Nf-κB-activating kinase-associated protein-1 (NAP-1), leading to the activation of IKK complex and thereby IRF3 and IRF7 controlled transcription. To activate Nf-κB and AP-1, the TLR3-TRIF complex need to recruit TRAF6 to activate RIP-1 that activates TAK-1 which leads to IKK complex activation and Nf-κB and AP-1 transcription. Both cascade pathways result in activation of antiviral and proinflammatory responses, which arise from the induction of IFN type 1 and other cytokines (Verma and Bharti, 2017; Yoneyama and Fujita, 2010).

### 1.1.3 Interferons

IFNs are a family of cytokines with a broad range of action. They can display antiviral, antiproliferative and antitumor activities as well as immune modulatory effects on the immune system (Lopez de Padilla and Niewold, 2016). Three classes of IFNs have been identified in humans and classified according to the receptor signal complex they use for transcriptional activation. Type 2 IFNs (like IFN-γ) modulate immune responses to pathogens other than viruses and type 3 IFNs (interferon lamda) are not well characterized. Type 1 IFNs (interferon α and β) on the other hand plays an essential role in the host response against viral infections (Sadler and Williams, 2008). When released bind IFN-1 to the IFN receptor on the surface of the target cell, activates the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway, which start the producing more effector molecules (Schneider et al., 2014). The primary actions of a stimulation of IFN-1α are usually the production of more specific IFN1-β subtypes. IFN-1 also brings upon other actions like activation of autoreactive B-cell differentiation into Ig-secreting plasma cells, activation of a broad range of different T-cells, and participate in producing substances and components that help infected cells to protect themselves against incorporation of viral nucleic acid in their genome (Parham and Janeway, 2015).
1.1.4 Other effector molecules

When pathogens or their molecules activate PRR, the cell often respond by production of both signaling proteins (interferon and cytokines) and a group of cytosolic or secretory proteins known as effector proteins. These proteins are an important part of the innate immunity against those pathogens. Examples of such effector molecules are myxovirus resistance protein (Mx), protein kinase RNA-activated (PKR), interferon stimulated gene 15 (ISG-15), interferon-induced protein with tetratricopeptide repeats-1 (IFIT-1) and others (Parham and Janeway, 2015; Yoneyama and Fujita, 2010).

**Myxovirus resistance protein (Mx)** was one of the first effector molecules described regarding viral infection. Mx belongs to a small family of dynamin-like large guanosine triphosphatases (GTPases), and is induced by IFN stimulation. Two types of Mx have been identified in humans, Mx-1 and Mx-2 also known as MxA and MxB. The function of Mx-1 has not been fully described yet, but some suggestions have been made. Evidence suggests that Mx-1 prevents viral nucleocapsides from reaching their cellular destination by entrapment and redirecting the nucleocapsides to sites of degradation. Mx-2 has recently been characterized as being more specific, as an antiretroviral effector protein. Mx-2 keeps the reverse transcribed genome from reaching its nuclear destination, thereby preventing chromosomal incorporation of the viral genome. It has been suggested that Mx-2 also acts by inhibiting the nucleocapsid transport, and thereby prevents nuclear entry resulting in no incorporation in the human genome (Pillai et al., 2016; Sadler and Williams, 2008; Schneider et al., 2014).

**RNA regulated protein kinase (PKR)** is a protein kinase, being characterized as a regulator involved in maintaining the innate immune response against offensive viral infections. PKR can be activated by dsRNA, intracellular stress, environmental stress (such as temperature or chemicals), cytokines and PACT (cellular protein). When activated, PKR autophosphorylates itself. Next, PKR phosphorylate a range of other proteins, like elongation factor 2α (eIF-2α), a key protein in modulation of protein synthesis in the cell. During infection, viral dsRNA acts as a ligand to activate PKR. Activation will initiate phosphorylation of eIF-2α causing inhibition of cellular and viral protein synthesis, inhibiting viral replication. In addition to its role in viral defense, it has been suggested that PKR is also involved in regulation of several other physiological and pathological functions such as cell growth, apoptosis, stress, and transcription (Kalra and Dhar, 2016; Sen and Peters, 2007).
**Interferon stimulated gene 15 (ISG-15)** is an ubiquitin-like protein involved in nonspecific mechanisms in host defense against a range of different viruses. The effector molecule is bound covalently to target proteins and forms conjugates with one or multiple ISGs attached. This conjugation is called ISGylation and will occur through a series of enzymatic reactions similar to the ubiquitin conjugation pathway. The involvement of ISG-15 in many molecule functions branches out into two main categories: the intracellular role, which is mainly a consequence due to IFN signaling; involves the JAK-STAT pathway and RIG-I pathway, but also modification of the adaptor molecules like Mx-1 and PKR, the extracellular function is to act as a cytokine when secreted from immune cells; modulating the immune response. During viral infections ISG-15 inhibits budding and ubiquitination of the virus, disrupting the release of virus as well as ISGylate viral proteins. ISGylation of viral capsid proteins has been shown to decrease both the amount of released virus and also the infectivity of the virus produced. In addition to the above, ISGylation has been shown to enhance IFN stimulation of host proteins. The conjugation results in reduced degradation of IFN transcription factors, activation of JAK-STAT and RIG-I pathways, mediating antiviral effect on adaptor molecules and activation of PKR. This helps the cell to protect itself against viral intrusion (Morales and Lenschow, 2013; Sadler and Williams, 2008; Schneider et al., 2014).

ISGs contain several different proteins; including interferon-induced proteins with tetratricopeptide repeats (IFITs). These cytoplasmic effector molecules are a major component in antiviral protection; it works through inhibiting viral replication by binding and regulating the function of cellular and viral proteins. IFIT function through inhibition of viral replication by binding, and regulation of cellular and viral proteins function. The activation is initiated upon viral infection and mainly by the signal molecule IFN-1α and transcription factor ISGF3. The activation is initiated by a broad spectrum of different viruses, and in the absence of infection the basal levels of IFITs in the host are low. When activated IFIT recognizes ssRNA and binds to the 5’end of the viral mRNA, if the virus is not capped with 2’-O-metylation (viral protection mechanism). The IFIT will compete with elongation factor (eIF4E) for binding and prevents the translation of the viral mRNA because the eIF4E is not able to bind to the virus. Binding to virus depends on the structure of the IFIT, for example the IFIT1 is a monomer shaped like a clamp. It possesses a positively charged pocket where the RNA can bind. In contrast IFIT2 is a heterodimer that possesses a positively charged cavity, forming a channel where binding of the RNA occurs. The primary structure of all IFITs contains between 8 and 12 tetratricopeptide repeats (TPRs) interacting together by
protein-protein interactions. The relative orientation of the TPRs facilitates conformation changes and is extremely important for binding to the different types of viruses (Fensterl and Sen, 2015; Leung and Amarasinghe, 2016).

1.2 The immune system of fish

Fish is the largest class of vertebrates, further divided into jawless and jawed fish; and jawed fish can be divided into cartilaginous fish (e.g. sharks) and bony fish (teleost). It is in this last group, teleost, that Atlantic salmon has its origin together with zebrafish and rainbow trout (Plouffe et al., 2005). Research on the immunity of fish in general, is at an early stage. Genome sequencing has identified several immune genes to be homologues resembling immune genes from other vertebrates, like mammals. Many genes of the mammalian immune system, such as their ligands, adaptor molecules and effector proteins, are well characterized. Studies on fish immunity suggest that there are more similarities than differences between the mammalian and fish immunity, although fish in general display reduced capacity for generation of high affinity antibodies through affinity maturation (Secombes and Pilstrom, 2000).

The most important part of the immune system in fish is the innate immune system, in contrast to mammals where the adaptive immune system plays a more prominent role. One reason for this difference may be early life: mammals develop inside the female and are therefore protected the first weeks/months against pathogens by the maternal immune system. In contrast, fish are free-living organisms from the embryonic stage and therefore dependent on their innate immune system from the moment of fertilization, before development of specialized tissues and organs (Uribe et al., 2011).

Teleosts have a good first line defense with physical barriers and adaptor molecules like PRRs that recognizes foreign components in- and outside the cells. These receptors start downstream cascade pathways that produce signal molecules like TNF, IFN, and IL-1. The extracellular signal molecules can activate production of more cytokines, stimulate phagocytosis, chemotaxis, and induce production of other intracellular effector molecules (as Mx-1 and ISG-15) that will result in cell protection against pathogens (Collet, 2014; Uribe et al., 2011).
1.1.2 Sensor proteins in fish

Most of the sensor molecules described above are found in fish (RLRs, NLRs and TLRs). The NLRs together with RLR are cytoplasmic receptors. NLRs (NOD1, NOD2 and NLRP3) are most likely involved in detection of bacterial cell wall components and antiviral defense (Zhu et al., 2013). While the RLRs (RIG-I and MDA5) appears to be involved in the same antiviral immune responses as the ones described for mammals (Hansen et al., 2011).

**TLR**

The TLRs in fish are found located in the same areas as for the human TLRs (mainly lymphoid tissues). The receptors and factors involved in their downstream signaling cascade have high sequence and structural similarity to the mammalian TLR proteins, for example the receptor are divided into three; an extracellular part, transmembrane part, and an intracellular part (Lee et al., 2014). Fish LRR domain show less sequence similarity to the corresponding domains in mammals, but the TIR domain of the TLRs is more conserved. Upon TLR receptor activation, TIR signals mainly through the same adaptor molecules as for the human TLRs (mainly MyD88 and TRIF) (Rebl et al., 2010). Downstream cascade signaling has shown to result in the upregulation of many of the same genes (AP-1, IRFs, IFNs, Nf-κB and ISGs), and is also in need of the chaperone protein UNC93b. UNC93b has been identified in Atlantic salmon, rainbow trout and zebrafish, and is probably important for the endosomal trafficking of TLRs in fish (Gay et al., 2014; Rauta et al., 2014; Rebl et al., 2010).

The fish TLRs contain structural similarities to the vertebrates TLR2, but they also contain distinct features and differences. The largest difference is probably the number of TLRs: at least 20 TLR types (TLR1, 2, 3, 4, 5M, 5S, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, 23, 24, 25 and 26) have been identified in fish versus 10 TLRs identified in mammals. The fish TLRs found in Atlantic salmon are TLR 1, 2, 3, 5, 7, 8, 9, 19, 21 and 22 (Altmann et al., 2016; Lee et al., 2014; Palti, 2011; Rauta et al., 2014; Roach et al., 2005). The distinct feature separating the different TLRs is the varying number of LRR motifs in the extracellular LRR domain. For example TLR20a has two more LRRs than the usual amount of 26. Such variation in the number of LRR motifs may have a functional implication in terms of recognition of different types of ligands (Lee et al., 2014). In addition to membrane-bound TLRs, soluble forms have also been identified in fish. TLR5 is such an example, where a TLR5 membrane-bound receptor (TLR5M) and a TLR5 soluble receptor (TLR5S) have been identified. These soluble TLRs lack the TIR domain, and are believed to relay negative feedback signals on the
corresponding membrane-bound TLR. This negative feedback signaling is possibly induced to prevent overstimulation and tissue destructive reactions to pathogens (Rauta et al., 2014).

Specificity of ligand binding to TLR receptors in fish has yet to be fully clarified. Because of the different amount of fish TLRs and their possible structural differences in the LRR domain, it has been difficult to establish a unified list of ligands binding to each TLR. From the at least 20 TLRs identified in teleost, ligand specificity has only been determined for TLR3, TLR5M, TLR5S and TLR22 (Rauta et al., 2014). The experimental survey of the different ligands and their target TLRs is at an early stage, but research may have found some alternatives. For example TLR6 is not found in fish therefore creates TLR1 a heterodimer with TLR2, this heterodimer is upregulated by bacterial infections caused by for example with *M. marinum* (Lee et al., 2014). TLR3, 7, 8, and 22 are upregulated following an infection by virus or stimulation with Poly I:C this most likely indicates binding to dsRNA and ssRNA and involvement in antiviral immunity (Matsuo et al., 2008; Pietretti and Wiegertjes, 2013), it has also been done in silico modeling supporting this statement (Sahoo et al., 2015). However, receptor upregulation in the presence of a possible ligand does not necessarily mean that the ligand binds to the same receptor. The ligand may bind to different PRRs and initiate downstream cascades resulting in upregulation of other PRRs.

**Effector proteins**

Interferons in mammals are divided into three classes. IFN-1 is the primarily antiviral cytokine produced as a first line response to a viral infection. The evidence suggesting that fish IFN-1 genes work in a similar way to those found in mammals, and comes from observations of mRNA expressions during and after viral infection (Plouffe et al., 2005). Even so, it has been shown that the sequence similarity is low (25-30% identity); fish IFNs contain several introns whereas mammalian IFNs are intronless (Boudinot et al., 2016). Like in humans are IFN-1 (α and β) found to be released as a response to binding of viral ligands (ssRNA, dsRNA, glycoproteins), to one or more PRRs (Sadler and Williams, 2008). IFN-1α is released first and binds to intracellular or extracellular IFN receptors, which leads to activation of JAK-STAT signaling. This results in induction of several antiviral effector genes like Mx-1, IFN-1β, ISG-1, PKR, and development of resistance to viral infection (Boudinot et al., 2016; Uribe et al., 2011).
1.2.2 The adaptive immune system

As in mammals, the adaptive immune system in fish can be divided into cell-mediated and humeral immunity. The cell-mediated immunity in mainly concerns the different types of T-cells, such as regulatory T-cells, helper T-cells, memory T-cells, cytotoxic T-cells, and effector T-cells. Teleosts seem to have subpopulations of T-cells similar to mammals, and therefore possess several important adaptive immune response genes like MHC class I, MHC class II, T-cell receptors, CD4, CD8, and others (Nakanishi et al., 2011; Parham and Janeway, 2015). B-cells on the other hand, produce key elements of the human immune response known as immunoglobulins (Ig). These immunoglobulins can be plasma membrane anchored Ig attached to the surface of B- and T-cells or may be secreted, as antibodies. Antibodies are, as in mammals, very specific for the target pathogen and its production is activated by the innate immune system or by binding to antigen epitope (Mashoof and Criscitiello, 2016; Parham and Janeway, 2015). However, few differences between mammals and teleosts concerning immunoglobulins have been reported. The primary immunoglobulin in teleosts is IgM, while in mammals the primary immunoglobulin is IgG. Two other classes of Ig have been identified in teleost in addition to IgM: IgD and IgT/IgZ (IgT in trout and IgZ in zebrafish), in contrast to mammals where five different Igs (IgG, IgM, IgE, IgA, IgD) have been identified. Regardless of the similarity between teleosts and mammals, are the adaptive immune system in teleost less developed. Fish have a limited repertoire of antibodies with slow response, low affinity and have shown to be temperature dependent, in addition to slow proliferation, maturation and memory of their B- and T-cells (Mashoof and Criscitiello, 2016; Parham and Janeway, 2015; Uribe et al., 2011).

1.2.3 Antiviral response in teleosts

Antiviral immune responses in fish are as mentioned not well characterized. Previous experiments and observations have made it possible to draw red lines from the well-characterized immune response of humans. Most likely are the first antiviral defense mediated by interferon and interferon-induced genes (like PKR, ISG-1 and Mx) induced by downstream signaling starting by binding of ligand to PRRs. Examples of the PRRs in teleosts are RLRs (RIG-I and MDA5), NLRs (NOD1, NOD2 and NLRP3) and TLRs (TLR3, 7, 8, 9, 21 and 22). It seems that no other receptors have been detected for the RLRs and NLRs in fish, but for the TLRs it has been identified several other receptors. In the teleost
TLR family have the TLRs 3, 7, 8, 9 and 21 been located in the endosomal compartments. TLR3 sense viral dsRNA or Poly I:C, TLR7 and 8 sense viral ssRNA and TLR9 and 21 sense viral and bacterial unmethylated CpG DNA. The fish specific TLR22 is located exclusively on the cell surface, and it recognizes dsRNA and Poly I:C signaling through TRIF, inducing cytokine release in the same way as for TLR3. The downstream signaling is today totally unknown, but most likely activates downstream signaling cascades through TRIF, MyD88, MAVS and caspase-1 resulting in an activation of for example Nf-κB, AP-1, IRF-3 and IRF-7 for RLRs and TLRs, while IL-1 is transcribed from activation of caspase-1 very much alike the activation seen in humans and mice (Denyer and Hugo, 2011; Haller and Kochs, 2002; Matsuo et al., 2008; Poynter et al., 2015; Varela et al., 2017).

1.3 Vaccines

Vaccination is probably the most successful immunological intervention to improve quality of life in humans and animals. The principle of vaccination is to exposure to a pathogen in a dosage large enough to activate the immune system, but small enough not to cause illness (Denyer and Hugo, 2011; Kim and Jang, 2017). The idea of vaccines for humans began more than 200 years ago with scientists like Edward Jenner and Benjamin Jesty when they discovered that milkmaids who had caught cowpox were subsequently protected against smallpox. After this discovery, major advances have occurred in vaccine development. The next phases in vaccine development was based on Louis Pasteur’s principles from the late 1800s using inactivated toxins or live attenuated pathogens against several infectious diseases. After the 1950s, many new and more effective vaccines have been developed as a result of new knowledge in the fields of microbiology, immunology and gene technology (Plotkin, 2005; Rappuoli, 2007; Riedel, 2005).

Immunotherapy is the category of vaccines, where a recipient is exposed to an antigen and subsequently mounts a protective immune response. Such exposure can be an infection from multiplication of attenuated vaccine strains or associated with the direct introduction of non-viable antigenic material into the body, with a non-living or inactivated vaccine, where the route of the exposure will affect the subsequent immune response. In passive immune therapy, the patient is given preformed antibodies (from other individuals), usually to very recent infections (Denyer and Hugo, 2011).
We subdivide vaccines into live vaccines, killed and component vaccines, and DNA/RNA vaccines. Live vaccines are vaccines that contain live, infective microorganisms, attenuated in a way that makes them able to infect without causing disease in healthy individuals. This type of vaccine is favorable because immunizations induce a more natural sequence of responses and only one exposure is required to establish an appropriate immune response and immunity to that particular disease (Denyer and Hugo, 2011).

The killed and component vaccines (containing toxins, viral capsule or surface proteins from the pathogens) with no replicative capacity are unable to evoke a natural infection profile, and must be administered on several occasions to achieve an optimal antigen exposure and immunity. The lack of replication makes these vaccines more favorable for people without optimal immune systems, since there is lesser potential for a disease to occur. Component vaccines often possess adjuvants for a better immune response. Adjuvants are a class of substances with a capacity to increase the immunogenicity and the efficacy of vaccines. The use of these biological components have many benefits for the producer and recipient; increased antibody titer, increased protective immunity, dose sparing, increased immunological memory and increased effect in populations with low response (like for the elderly) (Denyer and Hugo, 2011).

The last type of vaccine is DNA/RNA vaccine. They contain strands of nucleic acids encoding specific antigens/virulence factors that will be expressed in the host after injection leading to immune responses. These vaccine types are able to present antigens in a way that resembles a natural infection (like expression of viral glycoproteins on the surface of cells) and are therefore very specific (Denyer and Hugo, 2011; Martins et al., 2015).

1.3.1 Aquaculture

Fish farming is a rapidly growing industry and is the second largest export trade after oil and gas in Norway. Norway is currently the largest global producer and exporter of Atlantic salmon, followed by Chile, the United Kingdom and Canada. In 2014, the Norwegian production of Atlantic salmon contributed to 50% of the total world production of farmed salmon. With the rapid growing production and expansion of the industry have the challenges also increased. One of the major challenges is the difficulty in overcoming infectious diseases that may occur in the netpens. Bacteria, viruses and parasites cause infectious diseases and lead to great production losses, unacceptable animal welfare situations and the spread of
disease to wild fish in the area. In contrast to viral diseases, control of bacterial diseases have been achieved with good vaccines, and today less than 1% of all Norwegian farmed Atlantic salmon have been in treated with antibiotics. Viral diseases has been difficult to control due to a lack of antiviral therapeutics and insufficient knowledge about pathogens and natural resistance mechanisms to viral infections. Vaccines currently used in fish farming are administered by injection or immersion and mainly protect against bacterial diseases. These vaccines are cheap to produce (bacteria fixed with formaldehyde and adjuvanted with oils), effective and safe. Vaccines against viral disease in fish have proven to be more difficult to develop and there are mixed opinions about their effectiveness.

Live attenuated vaccines are, to this day, the best way to induce a strong and sustained immune response against viral disease in both mammals and fish. However, there are environmental and regulatory concerns regarding the use of live attenuated vaccines. Because of these concerns, as well as the cost of development, component vaccines with adjuvants have been tried without much success. Viral vaccines against ISA and infectious pancreatic necrosis virus (IPNV) are available in Canada, but these are not approved in Europe or in the USA due to lack of published reports and the continued occurrences of viral outbreaks. The need for new viral vaccines in fish farming is without question a necessity, not only from an economic point of view but also from an ethical animal welfare standpoint (Brudeseth et al., 2013; Chang et al., 2015; Dhar et al., 2014; Evensen and Leong, 2013; Kibenge et al., 2012; Levine, 2010; Martins et al., 2015; Nodland E., 2016; Regjeringen, 2015; Yajie Liu, 2010).

1.3.2 Infectious Salmon Anemia disease (ISA)

ISA is a viral disease that originates from the family of aquatic orthomyxo viruses, a distant relative to the influenza viruses that causes disease in humans. The virus is only pathogenic to Atlantic salmon, rainbow trout and sea trout. The microbiota of farmed salmon contains non-virulent ISA (ISAV-HPR0), this type of ISAV is not pathogenic to the salmon, but mutations may lead to the pathogenic high-virulent ISAV, which is the cause of the deadly ISA. The virus is relatively contagious causing severe damage to the organs resulting in mortalities up to approximately 90% of all infected individuals (dependent on strain and season). The virus is mainly spread by contact with infected individuals, but it has been seen several cases of waterborne infections, as well as a few cases of vertical transmission. The disease affects the epithelial cells in the blood vessels and heart, resulting in internal bleeding and anemia in
several organs. Random sampling sets the diagnosis where clinical alterations in the organs (such as circulatory disturbance, swollen kidneys and blood accumulation in the intestines) are an indication of infection. Histopathological and histochemical techniques (like QPCR and growth in cell culture) are used to confirm a diagnosis together with the physical findings. Symptoms are not shown on the fish until a large amount in the netpen are infected and probably the relating netpens as well. The law imposes the breeding farms to slaughter down all the salmon when ISAV is detected, resulting in great financial losses. Each year are 10 breeding farms in average infected with ISA, making the disease one of the largest threats to the aquaculture industry (Hjeltnes, 2016; Veterinærinstituttet, 2017).
2. Aims of the thesis

ISAV is causing a serious infectious disease in salmon farms. The disease affects the blood vessels causing internal bleeding and anemia, resulting in high mortality in the farmed salmon (Hjeltnes, 2016).

In 2014, one outbreak of ISA alone resulted in slaughtering of almost 10 million Atlantic salmon, of which 75% was destructed resulting in a potential financial loss of almost 1.4 billion NOK. Although sanitary precautions have been implemented and reduced the number of outbreaks, are still 10-20 fish farming facilities detected with ISA every year. The ISA virus is the second most prevalent cause for viral outbreaks in netpens after pancreatic disease (PD). Because of the high number of cases with ISA there is a need for new vaccines (Hjeltnes, 2016; Nodland, 2015).

To make a fish vaccine cost-effective is it necessary to include antigens from several different pathogens into one vaccine, and improving the efficacy of those existing vaccines adding new adjuvants to them. The use of TLR ligands like Poly I:C as a adjuvant has shown promising results in vaccines for mammals, and because of this may it be worth testing Poly I:C as a potential new adjuvant in fish vaccines (Steinhagen et al., 2011; Toussi and Massari, 2014).

Main objective:

Investigate the TLR3 ligand Poly I:C as a potential new adjuvant in fish vaccines.

Sub-objectives:

- Establish an experimental system for analysis of salmon TLR activity based on SEAP secretion
- Analyze expression of salmon TLR3 in a mammalian cell line (HEK Blue)
- Compare binding characteristics of Poly I:C to different cell lines expressing human or salmon TLR3
- Analyze the effect of Poly I:C in a fish cell line expressing salmonTLR3
3. Materials

3.1 Chemicals and biological products

<table>
<thead>
<tr>
<th>Product</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-mercaptoetanol</td>
<td>Sigma Chemical, USA and Invitrogen, USA</td>
</tr>
<tr>
<td>10×TGS</td>
<td>Bio-Rad Laboratories USA</td>
</tr>
<tr>
<td>Aceton</td>
<td>Merck Germany</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Sigma-Aldrich USA</td>
</tr>
<tr>
<td>Blasticidin</td>
<td>Invitrogen USA</td>
</tr>
<tr>
<td>Complete Protease Inhibitor Cocktail</td>
<td>Roche Switzerland</td>
</tr>
<tr>
<td>Crystal violet</td>
<td>Sigma-Aldrich USA</td>
</tr>
<tr>
<td>Dry milk</td>
<td>Normilk AS Norway</td>
</tr>
<tr>
<td>DMEM</td>
<td>Bio Whittaker USA</td>
</tr>
<tr>
<td>Etanol</td>
<td>Arcus Kjemi AS Norway</td>
</tr>
<tr>
<td>Fetal Calf serum</td>
<td>GIBCO BRL England</td>
</tr>
<tr>
<td>Fluorsave™, Molecular Probes®</td>
<td>Thermo fisher Scientific USA</td>
</tr>
<tr>
<td>Geneticin (G418)</td>
<td>Invitrogen USA</td>
</tr>
<tr>
<td>Gentamicin-sulphate</td>
<td>Bio Whittaker USA</td>
</tr>
<tr>
<td>Laemmli lysis buffer</td>
<td>Bio-Rad Laboratories USA</td>
</tr>
<tr>
<td>Leibovitz L-15 medium</td>
<td>Bio Whittaker USA</td>
</tr>
<tr>
<td>L-Glutamin</td>
<td>Sigma-Aldrich USA</td>
</tr>
<tr>
<td>Luminata™ Classico Western HRP substrate</td>
<td>Merck USA</td>
</tr>
<tr>
<td>Luminata™ Forte Western HRP substrate</td>
<td>Merck USA</td>
</tr>
<tr>
<td>Mini-PROTEAN® TGX™ Gels (10 and 15 wells)</td>
<td>Bio-Rad Laboratories USA</td>
</tr>
<tr>
<td>Ponceu S solution</td>
<td>Sigma-Aldrich USA</td>
</tr>
</tbody>
</table>
3.2 Solutions

Table 3.2.1 Media for cell culturing

<table>
<thead>
<tr>
<th>Media</th>
<th>Ingredients</th>
<th>Amount</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% Leibovitz L-15 medium (complete L-15)</td>
<td>Leibovitz L-15 medium FBS L-glutamin G418 (50 mg/ml) 2-mercaptoethanol</td>
<td>500 ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 ml</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 ml</td>
<td>4 mM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500 µl</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400 µl</td>
<td>40 µM</td>
</tr>
<tr>
<td>1% Leibovitz L-15 medium</td>
<td>Leibovitz L-15 medium FBS L-glutamin G418 (50 mg/ml) 2-mercaptoethanol</td>
<td>500 ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 ml</td>
<td>1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 ml</td>
<td>4 mM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500 µl</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400 µl</td>
<td>40 µM</td>
</tr>
<tr>
<td>Leibovitz L-15 medium without FBS</td>
<td>Leibovitz L-15 medium L-glutamin G418 (50 mg/ml) 2-mercaptoethanol</td>
<td>500 ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 ml</td>
<td>4 mM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500 µl</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400 µl</td>
<td>40 µM</td>
</tr>
<tr>
<td>10% DMEM (complete)</td>
<td>DMEM medium FBS</td>
<td>500 ml</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glutamin pen/strep</td>
<td>5 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-------------------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>1% DMEM</td>
<td>DMEM medium</td>
<td>500 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td></td>
<td>FBS</td>
<td>5 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td></td>
<td>Glutamin pen/strep</td>
<td>5 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>DMEM without FBS</td>
<td>DMEM medium</td>
<td>500 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td></td>
<td>Glutamin pen/strep</td>
<td>5 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>Selection media for</td>
<td>Leibovitz L-15</td>
<td>100 ml</td>
<td></td>
</tr>
<tr>
<td>ASK/EPC cells transfected with ssTLR3</td>
<td>medium G418</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth media for</td>
<td>DMEM complete</td>
<td>100 ml</td>
<td></td>
</tr>
<tr>
<td>HEK Blue TLR2 cells</td>
<td>medium G418</td>
<td>1.6 ml</td>
<td></td>
</tr>
<tr>
<td>transfected with ssTLR3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selection media for</td>
<td>DMEM complete</td>
<td>250 ml</td>
<td></td>
</tr>
<tr>
<td>HEK Blue TLR2</td>
<td>medium Selection mix</td>
<td>1 ml</td>
<td></td>
</tr>
<tr>
<td>Selection media for</td>
<td>DMEM selection</td>
<td>100 ml</td>
<td></td>
</tr>
<tr>
<td>HEK Blue TLR2</td>
<td>mix medium medium G418</td>
<td>1.6 ml</td>
<td></td>
</tr>
<tr>
<td>Selection media for</td>
<td>DMEM complete</td>
<td>100 ml</td>
<td></td>
</tr>
<tr>
<td>HEK Blue hsTLR3</td>
<td>medium Blasticidin</td>
<td>60 µl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zeocin</td>
<td>100 µl</td>
<td></td>
</tr>
</tbody>
</table>

**Tablet 3.2.2 Phosphate buffered saline (PBS) pH 7.4 (4 Liters)**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>32 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.8 g</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>1.08 g</td>
</tr>
<tr>
<td>Na2HP=4×2H2O</td>
<td>7.12 g</td>
</tr>
<tr>
<td>MQ-water</td>
<td>3950 ml</td>
</tr>
<tr>
<td>HCl</td>
<td>pH adjust to 7.4</td>
</tr>
</tbody>
</table>

**Tablet 3.2.3 Phosphate buffered saline with Magnesium and Calcium**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solution A</strong></td>
<td></td>
</tr>
<tr>
<td>Na2HPO4</td>
<td>46.86 g</td>
</tr>
<tr>
<td>NaH2PO4</td>
<td>9.66 g</td>
</tr>
</tbody>
</table>
### Table 3.2.4 Tris-EDTA (TE) pH 7.6 (for solution of primers)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris pH 7.5</td>
<td>1 ml</td>
<td>10 mM</td>
</tr>
<tr>
<td>0.5 M EDTA pH 8.0</td>
<td>0.2 ml</td>
<td>1 mM</td>
</tr>
<tr>
<td>MQ-Water</td>
<td>8.8 ml</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3.2.5 Solutions and compounds for western blotting

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Ingredients</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Running buffer</td>
<td>10×TGS</td>
<td>100 ml</td>
</tr>
<tr>
<td></td>
<td>MQ-Water</td>
<td>900 ml</td>
</tr>
<tr>
<td>Laemmli buffer</td>
<td>Laemmeli buffer</td>
<td>500 µl</td>
</tr>
<tr>
<td></td>
<td>MQ-Water</td>
<td>450 µl</td>
</tr>
<tr>
<td></td>
<td>2-mercaptoetanol</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

### 3.3 Kits

- High Capacity cDNA Reverse Transcription Kit™ (Applied Biosystems, USA)
- LightCycler® 480 DNA SYBRgreen® Master (Roche, Switzerland)
- RNase free DNase set™ (Qiagen, Germany)
- RNase Mini Kit™ (Qiagen, Germany)
- High-speed plasmid Midi Kit™(25)(Qiagen, Germany)
- AMAXA Nucleofector II® (Lonza, Switzerland)

### 3.4 Antibodies

<table>
<thead>
<tr>
<th>Primary Ab</th>
<th>Dilution</th>
<th>Secondary Ab</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-FLAG (32)</td>
<td>1:5000</td>
<td>Anti-Mouse (313)</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-TLR3 (321)</td>
<td>1:1000</td>
<td>Anti-Rabbit (314)</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-Actin</td>
<td>1:100</td>
<td>Anti-Rabbit (304)</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-FLAG (32)</td>
<td>1:5000</td>
<td>Anti-Mouse (303)</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-TLR3 (321)</td>
<td>1:1000</td>
<td>Anti-Rabbit (304)</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-FLAG (32)</td>
<td>1:5000</td>
<td>Anti-Mouse (315)</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti-TLR3 (321)</td>
<td>1:1000</td>
<td>Anti-Rabbit (316)</td>
<td>1:500</td>
</tr>
</tbody>
</table>
### 3.4.1 Antibodies

- Anti-FLAG (32): Sigma-Aldrich, USA
- Anti-TLR3 (321): Anaspec, USA
- Anti-Actin: Sigma-Aldrich, USA
- Anti-Mouse IgG Alexa 680 (313): Jackson ImmunoResearch Laboratories, USA
- Anti-Rabbit IgG Alexa 680 (314): Jackson ImmunoResearch Laboratories, USA
- Anti-Mice IgG HRP (303): Bio-Rad Laboratories, USA
- Anti-Rabbit IgG HRP (304): Bio-Rad Laboratories, USA
- Anti-Mouse IgG (315) Alexa 488: Molecular Probes, USA
- Anti-Rabbit IgG (316) Alexa 546: Molecular Probes, USA

### 3.5 Cells and virus

- ASK (Atlantic salmon kidney) cells: Gift from the institute of fish and marine biology, University of Bergen, Norway
- EPC (fathead minnow) Cells: Gift from the veterinary institute at NMBU, Oslo, Norway
- HEK Blue TLR2 (Human embryonic kidney) Cells: Invivogen, USA
- HEK Blue hsTLR3 (Human embryonic kidney) Cells: Invivogen, USA
- ISA virus (strain Glesvær2/90): Gift from the Veterinary Institute, Oslo, Norway

### 3.6 Primers to QPCR

List of primers used in the present study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence</th>
<th>Acc.nr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pp18s</td>
<td>F</td>
<td>AACGGCTACCACATCCAAGG</td>
<td>AY855349</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CCCGAGATCCAACCTACGAGC</td>
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</tr>
<tr>
<td>ppTNF-α</td>
<td>F</td>
<td>TTACCGCTGGTGATGGTGTC</td>
<td>JN412133</td>
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<tr>
<td></td>
<td>R</td>
<td>TTAGCGTGAGACAAACAGGC</td>
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</tr>
<tr>
<td>ppISG-15</td>
<td>F</td>
<td>AATGCCACAGTCCGTGAAC</td>
<td>KM099174</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CCGTAACTGCTGAGGCTTCT</td>
<td></td>
</tr>
<tr>
<td>ppIRF-7</td>
<td>F</td>
<td>CAGGAGATACCTAGCCCGA</td>
<td>KF844251</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TGCAATTTCCCTCCACTGGCT</td>
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</tr>
<tr>
<td>ppIRF-3</td>
<td>F</td>
<td>CTTCCTCTCACCTGCTGGGTG</td>
<td>KJ027520</td>
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<tr>
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<td>R</td>
<td>TCCTCCAGCATGTGTGGAC</td>
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</tr>
<tr>
<td>ppIFN-1</td>
<td>F</td>
<td>TGTGCTTCTTGTGCTGGGTG</td>
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<td></td>
<td>R</td>
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</tr>
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<td>ppMX-1</td>
<td>F</td>
<td>TTGACATCGCCACCACAGAA</td>
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<tr>
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<td>R</td>
<td>TCTTTGTGCCTCCCTGCACCT</td>
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</tr>
<tr>
<td>ppVIG</td>
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<td>TAAGAGCGCCAAGTGTCTGG</td>
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</tr>
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<td></td>
<td>R</td>
<td>CCAGAAACGGACTTTGACGGGA</td>
<td></td>
</tr>
<tr>
<td>ppActin</td>
<td>F</td>
<td>CATCTACGAGGTTAGCACC</td>
<td>KF844250</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GCCCATCTCTGCTCAAGT</td>
<td></td>
</tr>
<tr>
<td>Ligand</td>
<td>Main receptor</td>
<td>Structure</td>
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</tr>
<tr>
<td>----------</td>
<td>---------------</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td>ppTLR3</td>
<td>F</td>
<td>GGGGAAAAAGCCTAAACG</td>
<td>DT128710</td>
</tr>
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<td>R</td>
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<td>R</td>
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<td>R</td>
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<td></td>
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<td>hsActin</td>
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<tr>
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### 3.7 Structures of ligands and substrate used

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<thead>
<tr>
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<th>Main receptor</th>
<th>Structure</th>
</tr>
</thead>
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<td>FSL-1</td>
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<tr>
<td>Ligand</td>
<td>Cell type</td>
<td>Picture</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
<td>---------</td>
</tr>
<tr>
<td>PAMC</td>
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<tr>
<td>Poly I:C</td>
<td>TLR3</td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>SEAP-substrate</td>
<td></td>
<td><img src="image3.png" alt="Image" /></td>
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**Biological**
<table>
<thead>
<tr>
<th>Heat killed <em>Listeria monogenocytes</em></th>
<th>TLR2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycobacterium marinum</em></td>
<td>TLR2</td>
</tr>
</tbody>
</table>
4. Methods

4.1 Cell cultures

The cell types used included human embryonic kidney cell line (HEK Blue), Atlantic salmon kidney cell line (ASK) and fathead minnow cell line (EPC). ASK and EPC cells where routinely cultured at 20°C in Leibovitz complete L-15 medium with 10% FBS. The EPC and ASK cells were split in a 1:2 dilution with trypsin twice a week. The HEK Blue cells where routinely cultured at 37°C in DMEM with 10% FBS and split twice a week at 1:10.

![Figure 6: Left picture: HEK Blue Cells. Middle picture: ASK Cells. Right picture: EPC Cells.](image)

4.2 Western blot

Cultured cells were lysed in laemmlı lysis buffer. The solution was then centrifuged at 2500×g for 10 minutes to get rid of the insoluble material and bubbles created during lysis. Precasted polyacrylamide gels with 10 wells (max 50 µl per well) or 15 wells (max 15 µl per well) were used, depending on the number of samples to be analyzed. For positive control of the FLAG antibody, samples from HEK Blue cells transfected with a hsFLAG-Alfy construct (65-70kD) were used. Negative controls were samples from the same cell type without the transfected plasmid. In addition, a Molecular Weight (MW) standard was used to estimate the MW of positive bands. The gels were run at 75 volts through the stacking gel, after this the gel was run at 150 volts until the indicator dye had reached the bottom. The gel was then placed in a blotting apparatus (Trans-Blot® Turbo™ Transfer System, Bio-Rad Laboratories Inc., USA) between filters and buffer. After blotting, the filter was washed with distilled...
water and stained with Ponceu S. The membrane was blocked with 500 mg dry milk in 10 ml PBS; this was incubated at room temperature on a rolling device for one hour. After incubation, five ml was removed from the dry milk suspension and primary antibody at the appropriate dilution and added to the remaining suspension. Incubation was carried at 4°C overnight on a rolling device. The next day the blot was washed for 10 min with 3×10 ml PBS containing 0.5% tween (PBS-T). Five ml of the PBS-T was removed again and an appropriate dilution of the secondary antibody was applied. This was incubated on a rolling device for one hour at room temperature. After the incubation time the blot was washed for 10min with 3×10 ml PBS-T. Because of difference in detection limits and sensitivity both Odyssey CLx® (Near-Infrared Imaging System, Li-Cor Biosience, USA) and Chemi Genius2® (Bio Imaging System, Frederic, USA) were used. Odyssey used the Alexa Ab for staining of the primary Ab, while for the Chemi Genius2 the IgG HRP Ab is used and it is followed by HRP substrate (Luminata™ Forte or Classico) depending on the expected protein detection signal.

4.3 Transformation of bacteria

The amount of plasmid used for transformation was generally between 10 pg and 100 ng. The bacterial cells to be transformed were kept on ice at all times, except during the heat shock. 10 ng plasmid was added to 50 µl bacteria. For every transformation, three different vials containing positive control, negative control and our sample were used. After incubation on ice for 30 minutes the samples were heat shocked in a water bath at 42°C for 30 seconds, followed by incubation on ice for two minutes. Then a preheated (37°C) Super Optimal broth with Catabolite repression (SOC) medium was added and the mixture was incubated on 37°C for one hour on a shaker. 100 µl bacterial cells transformed with different concentration of the plasmids were spread on agar plates and incubated overnight at 37°C. All of which was done according to the transformation protocol. The negative control only contained bacteria without plasmids, the positive control contained pUC19 (plasmid which confers antibiotic resistance) and our sample contained our plasmid.

4.4 Preparation of plasmids

A buffer with high pH was used to lyse the bacterial cells. Thereafter plasmids were separated from the bacterial lysate using anion-exchange-based plasmid DNA preparation columns using High-speed plasmid Midi Kit™ (25) (Qiagen, Germany).
4.5 Enzymatic measurement of SEAP activity

The HEK-Blue cell lines stably transfected with a reporter plasmid encoding a secretory form of embryonic alkaline phosphatase (SEAP) and various human TLRs (hsTLRs) were developed for assays of TLR ligand binding by monitoring the activation of Nf-κB (Invivogen, USA). The SEAP reporter gene is placed under the control of five Nf-κB binding IFN-1β promoter sites and one AP-1 site, controlling the SEAP transcription. Upon activation of TLR, the production of SEAP will start. The amount of secreted SEAP is measured by adding a chromogenic substrate (dinitrophenylphosphate) to the medium. All laboratory tests were standardized with $2 \times 10^4$ cells/well seeded out on 96-well plates (blank) and incubated overnight at 20° or 37°C, depending on the cell type. The medium was changed the next day from 10% FBS medium to 1% FBS medium with ligand, 100 µl in each well. The cells were incubated for an additional 24 hours with TLR ligands to allow receptor activation and induction of SEAP synthesis/secretion. On the third day SEAP substrate was prepared: two tablets dinitrophenylphosphate were mixed together with 5.5 ml buffer (TN) to a final concentration of 1.8 mM (this mixture was always used the same day as prepared). 50 µl substrate was carefully added to each well to avoid bubbles that could interfere with the absorbance readings. The plate was inserted in the plate reader (CLARIOstar®, BMG LabTech, USA) and absorbance read every minute at 405 nm for one and a half to two hours for some ligands whereas others were measured each hour for four hours at 405 nm (details described in the results section).

4.6 Protein measurement

Protein concentration was analyzed using the BCA Protein measurement kit™ (Pierce, USA). When measuring directly in cell culture plates, the wells were washed with $2 \times 10$ ml of PBS before adding a 50 µl protein dye. A standard curve was made from human serum albumin. The plate was incubated at 37°C for one to three hours until visible coloring of the samples were observed. Plate reader was used to measure the absorbance at 570 nm.
4.7 Quantitative polymerase Chain Reaction (QPCR)

QPCR is a method used to amplify genomic DNA directly (copy number estimation) or cDNA made from RNA for quantitation of transcription. The method is based on interactive polymerization of a specific stretch of DNA; defined by complementary oligonucleotide primers coupled to denatured mRNA, and annealing of new primers to the template. Theoretically, the amount of DNA doubles by every cycle. RNA was extracted from the samples using the RNeasy Midi Kit™ (Qiagen, Germany). Routinely, 200 ng RNA was used for cDNA synthesis. A QPCR master mix containing buffer, thermostable DNA polymerase, 10 µl fluorescent stain (SYBRgreen®), two µl forward primer, two µl reverse primer and one µl MQ-water was mixed with five µl cDNA in each well. Finally, the plate was covered with a plastic sheet and it was centrifuged for two minutes at 1000×g to sediment the sample. The plate was placed in the Roche Lightcycler® 480 cycler and analyzed for 40 cycles (preincubation: 95°C for five minutes; the cycle: 95°C for 10 seconds, 60°C for 10 seconds and 72°C for 10 seconds; melting curve: 95°C for five seconds and 65°C for one minute; cooling for 30 seconds).

4.8 Transfection of cells with AMAXA®

Transfection by nucleoporation requires a large amount of cells in order to achieve workable results. This is due to the fact that the electric shock used to “open” the cell membrane kills about 50% of the cells in the procedure.

To be sure to have enough cells, a T175 cm² bottle approximately 90% confluent was used. Further steps will depend on which cells are used (if you use trypsin or not).

The amount of cells needed was counted and collected, and then centrifuged at 1000×g for 10 minutes. The supernatant was discarded. After this step everything had to be done on ice. The pellet was resuspended with 200 µl of buffer T. The suspension was then split in two eppendorff tubes. In one tube four µl of turboGFP plasmid were added, as a positive control. TurboGFP is a well-expressed plasmid that labels transfected cells with the green fluorescent protein from the jellyfish Aequorea victoria. This can be used to assess the transfection frequency. If the cells turn green in the fluorescence microscope the transfection is successful. In the other tube 10 µl containing one µg plasmid was added.
The nucleofection was carried out by program T-20 in AMAXA® (Lonza, Germany). Suspensions were put in two different cuvettes and placed in the AMAXA®.

Following nucleofection three ml of medium were added to each cuvette. Seeded out three six-well plates. The first plate contained $2 \times 10^5$ cells/well, with untransfected cells; and this plate was used as a control. The second plate seeded out contained transfected cells with concentration $7.5 \times 10^5$ cells/well. The third plate seeded out GFP with concentration $7.5 \times 10^5$ cells/well, but on this plate only two wells were seeded out.

To each well with cells, 2.5 ml with media were added, left to adhere and incubated overnight at 20°C. After the overnight incubation the media was changed to selection media containing gentamycin (G418). When the control cells were dead, the remaining cells in the transfected samples had most likely acquired resistance from the transgene.

### 4.9 Growth of ISA virus

ASK cells where used as a host, and were first grown in two three-layer cell flasks until approximately 80% confluency. When the flasks were 80% confluent the media was changed from 10% FBS to 2% FBS, and the flask infected with one ml of ISA virus per flask (at an MOI of approximately 0.01). They were then incubated at 15°C for approximately seven days, until almost all of the ASK cells were killed by the virus by cytopathic effect (CPE). The supernatant was centrifuged to pellet dead cells and debris (6000×g for 15 minutes). The supernatant was then alicotted and frozen at -80°C.

#### 4.9.1 Titration of virus

Titration was used to estimate the amount of virus grown in the ASK cells. Approximately 20,000 cells per well were seeded out on a 96-well plate. The plates were then incubated overnight at 20°C. The next day virus were added to the plate where the first row had undiluted virus and the next rows were serially diluted 10 times for each row all up to a $10^{11}$ dilution in a final volume of 200 µl. To prevent the plates from drying out during incubation they were covered with a plastic sheet.

Plates were incubated at 15°C until CPE was observed in multiple wells. The number of wells with positive CPE can be used as a measurement to calculate the concentration of the virus.
To facilitate identification of CPE, cells were stained. The wells were washed three times with 50 µl of PBS and added 100 µl of PFA 4% to fix the cells. The fixation with PFA 4% took about an hour before the cells were washed again with PBS once and stained with 0.05% crystal violet. The crystal violet will stain the wells with adherent cells. The staining lasted for about one hour before the wells were washed again with water. The plate was dried, and the number of wells with CPE counted. The titer was calculated using the formula of Reed-Muench (L.J. Reed, 1938).

4.10 Immunofluorescence microscopy

Immunofluorescence microscopy (IF) is a method used to detect a specific protein, and to determine where in the cell this protein is located. Different HEK Blue cells expressing different proteins (hsTLR2, hsTLR3, ssTLR3, hsAlfy) were seeded out at 2×10⁴ cell/well in 200 µl medium (DMEM 10% FBS), and incubated overnight at 37°C. The next day, the medium was removed and cells were washed with PBS containing magnesium and calcium (to reduce loss of cells). Cells were fixed with 4% PFA for one hour and washed twice with PBS (Mg⁺ and Ca²⁺). Cells were permeabilized with 0.1% Triton for five minutes and washed again with PBS (Mg⁺ and Ca²⁺) twice for five minutes. To reduce non-specific binding, the cells were blocked with 10% BSA for 30 minutes at room temperature. The cells were then incubated with a primary antibody in 3% BSA-PBS for two hours at room temperature. The antibodies used were Anti-FLAG and Anti-TLR3 diluted to 1:500. After incubation, the cells were washed again twice for five minutes with PBS (Mg⁺ and Ca²⁺). Next, staining with secondary antibodies (donkey Anti-Mouse IgG Alexa 488 and mouse Anti-Rabbit IgG Alexa546 both diluted to 1:500) in 3% BSA for 45 minutes. Then the cells were finally washed three times with PBS for five minutes each time, and mounted for microscopy using five µl Fluorsave™.

4.11 Data analysis and statistical methods

Raw data from instruments (plate reader, Lightcycler®) were exported as comma separated files (CSV) directly or via import to Microsoft® Excel® for Mac 2011 version 14.6.4. Ct values from QPCR were converted to relative expression using the 2-ΔΔ Ct method described by (Livak and Schmittgen, 2001). Differences between samples were analyzed with two-sample t-test (Student, 1908). Data from the plate reader (absorbance values or rate of change
values (Δ OD/h)) were imported to R Studio for Mac Version 1.0.136©2009-2016 RStudio, Inc (Team, 2017) and processed for graphic display or calculation of binding kinetics parameters using the drc-package (Cedergreen et al., 2005; Ritz C., 2015). Time series data were fitted by linear regression whereas Δ OD/hour data were fitted to a four-parameter logistic model (the most widely used non-linear regression model for modeling dose-response bioassay data) for calculation of effective dose 50 (ED$_{50}$) values. The equation for the model is:

$$f(x) = c + \frac{d - c}{1 + \exp\left(b \left(\log(x) - \log(e)\right)\right)}$$

Where $x$ = the independent variable (dose) and $f(x)$ = the dependent variable (response Δ OD/h). The four estimated parameters consist of the following:

- $c$ = the minimum value that can be obtained (i.e. what happens at dose zero)
- $d$ = the maximum value that can be obtained (i.e. what happens at infinite dose)
- $e$ = the point of inflection (i.e. the ED$_{50}$ value)
- $b$ = Hill’s slope of the curve (i.e. this is related to the steepness of the curve at point $e$).

The mean ED$_{50}$ calculated from at least eight replicates were compared between samples using two-sampled t-test and considered different when $p<0.05$. The drc-package was also used to draw sigmoidal dose-response curves based on the calculated parameters.

Using R studio for data analysis based on scripting increases the reproducibility of the data analysis (Peng, 2009). The calculations and plots can be repeated on any computer with installed software. The script used for analysis and generation of plots is therefore included in the thesis (Appendix 1).
5. Results

5.1 Effect of ligand concentration on SEAP secretion from HEK hsTLR2 - Evaluation of alternative substrate.

The HEK Blue hsTLR2 cell line came with a substrate and a protocol for the measurement of the SEAP activity. For economical reasons we used a different substrate (dinitrophenylphosphate tablets) than the one recommended in Invivogen’s protocol, and therefore the suitability needed to be confirmed. We started with an SEAP activity measurement following the protocol from Invivogen, stimulated with various concentrations of a well-known TLR2 ligand (FSL-1).

![Figure 7](image)

**Figure 7:** Effect of ligand concentration on FSL-1 induced SEAP activity in HEK Blue hsTLR2 using SEAP substrate at a concentration of 50 µg/ml. Plot shows individual measurements from eight wells per concentration and a linear fit curve (least squares regression).

Figure 7 shows time series of SEAP measurements on HEK Blue cells stimulated overnight with FSL-1. The data shows detection of SEAP activity above background down to concentrations 1 ng/ml of FSL-1. This was comparable to results obtained with proprietary substrate provided by Invivogen. The data also suggested that absorbance measurements at regular intervals (every 10 minutes) for 80 minutes were sufficient to estimate enzyme activity.
5.2 Establishing and optimizing of the system

In order to measure and compare the different cells lines and ligands of TLR2 and TLR3, the SEAP system was optimized. Total SEAP activity was quantified by spectrophotometry to characterize the effect of cell number and substrate concentration.

5.2.1 Effect of cell number

First the establishment of the minimum cell count/well acquired for good SEAP activity signal was measured. HEK Blue hsTLR2 cells were seeded at different densities and stimulated with FSL-1 as ligand with a concentration of 10 µg/ml in all wells. The amount of cells in each well ranged from 0 to 20,000 cells/well by 1:2 dilutions.

![Figure 8: Effect of cell number on FSL-1 induced SEAP activity in HEK Blue hsTLR2 cells. Vertical bars show mean of eight measurements ± SD.](image)

Figure 8 show the effect of cell number/well on SEAP activity induced by incubation of HEK Blue hsTLR2 cells overnight with FSL-1. When the cell count was less than 10,000 cells/well was there no specific SEAP activity in the medium (same as background). This result suggested that a cell count over 20,000 cells/well was preferable to obtain a robust signal.

5.2.2 Effect of substrate concentration

Second, an optimal concentration of the SEAP substrate had to be established, and compare the result to the concentration suggested by the Invivogen protocol. HEK Blue hsTLR2 cells were seeded out in concentration 20,000 cells/well. The cells were stimulated with FSL-1
overnight at concentration 10 µg/ml in each well. The substrate was added at a maximal concentration of 100 µg/ml and further diluted 1:2.

Figure 9: Effect of substrate concentration on FSL-1 induced SEAP activity in HEK blue hsTLR2 cells. Vertical bars show mean of eight measurements ± SD. Line is four-parameter logistic fit curve; the vertical line crosses x-axis at estimated ED₅₀.

Figure 9 show a sigmoidal fit to the data of SEAP activity induced by HEK blue hsTLR2 cells + FSL-1 incubated overnight, stimulated with diluted substrate concentrations. From the curve is it possible to estimate ED₅₀ under 50 µg/ml for the substrate (Appendix 1). ED₅₀ is defined as the concentration of the substrate, in this case at 50 µg/ml, where 50% of the SEAP enzymes will be saturated with substrate and the other half will not be metabolizing at all.

5.3 The TLR2 cells

Transfection of fish cells using standard transfection agents has been demonstrated to be very difficult. Before transfecting ssTLR3 into different fish cell lines (ASK or EPC) we used a well-known cell line (HEK Blue) as a positive control. The HEK Blue cells are known to transfect easily, and are also much used to establish stable cell lines. The HEK Blue cells have already, as mentioned above, been transfected with TLR2, which made it possible to use TLR2 ligands for the positive control of signaling pathways and reporter enzyme secretion.

5.3.1 Effect of TLR2 ligands on HEK Blue hsTLR2 cells

After optimizing the protocol for FSL-1 stimulation, three additional types of TLR2 ligands were tested to explore which ligand at which concentration produced the best SEAP activity
signal. Two soluble, chemical low molecular weight ligands (PAMC and FSL-1) and two particulate ligands (formaldehyde fixed *Mycobacterium marinum* and Heat Killed *Listeria monogenocytes*) were compared.

### 5.3.1.1 Soluble chemical ligands

We tested the chemical ligands following the established protocol for the HEK Blue hsTLR2. The cells were stimulated with FSL-1 at concentrations from 1µg/ml and PAMC at concentrations from 10 µg/ml serially diluted 1:10.

![Figure 10](image)

*Figure 10: Effects of FSL-1 (left panel) and PAMC (Right panel) induced SEAP activity in HEK Blue hsTLR2 cells. Vertical bars show mean of eight measurements ± SD. Line is four-parameter logistic fit curve; the vertical line crosses x-axis at estimated ED$_{50}$."

Figure 10 compares the effects of the two chemical TLR2 ligands on SEAP activity in HEK Blue hsTLR2 cells. Both ligands displayed potent activity with ED$_{50}$ values 2 ng/ml for FSL-1 and 3 ng/ml for PAMC (p=0.038) (Table 1).

### 5.3.1.2 Particulate ligands

The biological TLR2 ligands were also tested following the new established protocol for the HEK Blue hsTLR2 cell line. The particulate ligands used to stimulate were *L.
monogenocytotes and *M. marinum* serially diluted 1:10 from 100,000,000 cells/ml and 50,000,000 cells/ml, respectively.

**Figure 11:** Effect of HKLM (left panel) and *M. marinum* (right panel) induced SEAP activity in HEK Blue hsTLR2 cells. Vertical bars show mean of eight measurements ± SD. Line is four-parameter logistic fit curve; the vertical line crosses x-axis at estimated ED$_{50}$.

Figure 11 shows the effect of the biological ligands on the HEK Blue hsTLR2 cells when incubated overnight. The graph shows a good signal from both bacteria species, with ED$_{50}$ values at approximately 1.58×10$^6$ cells/ml for HKLM and approximately 2.3×10$^7$ cells/ml for *M. marinum* (p=8.2×10$^{-5}$).

### 5.3.2 Effect of TLR3 ligands on HEK Blue TLR2 cells

HEK Blue hsTLR2 cells express low amounts of endogenous hsTLR3. When the sensitivity to several TLR2 ligands were established, we investigated the effects of a synthetic dsRNA TLR3 ligand (Poly I:C) on HEK Blue hsTLR2 cells. This was mandatory to establish the background activity in the cells before transfecting them with Atlantic salmon TLR3 (ssTLR3). HEK Blue hsTLR2 was seeded out following the standard protocol, and stimulated with Poly I:C at 1:10 serial dilutions starting at 100 µg/ml.
Figure 12: Effect of Poly I:C induced SEAP activity in HEK Blue hsTLR2 cells. Vertical bars show mean of eight measurements ± SD. Line is four-parameter logistic fit curve, and an ED$_{50}$ value could not be estimated from these data.

Figure 12 show the SEAP activity when HEK Blue hsTLR2 cells are incubated with Poly I:C overnight. As can be seen from the graph the maximal value of Δ OD reached approximately 0.3 Δ OD/hour, a level much lower than observed with proper TLR2 ligands (see figure 13 for comparison).

Figure 13: Comparing SEAP activity in HEK Blue hsTLR2 cells stimulated with Poly I:C (left panel) and FSL-1 (right panel). Vertical bars show mean of eight measurements ± SD. Line is four-parameter logistic fit curve; the vertical line crosses x-axis at estimated ED$_{50}$ for the right panel (an ED$_{50}$ value could not be calculated for Poly I:C (left panel)).
Shown above is the effect of a TLR3 ligand compared with the TLR2 ligand FSL-1. As expected, these cells were not very sensitive to Poly I:C since the HEK hsTLR2 express only low amounts of endogenous hsTLR3.

5.4 The hsTLR3 cells

When we started to analyze the HEK Blue hsTLR3 cells it became necessary to do some changes in the protocol developed for HEK Blue hsTLR2 cells. Even high concentrations of Poly I:C analyzed under the same conditions as for the HEK Blue hsTLR2 cells failed to induce a robust SEAP activity signal in HEK Blue hsTLR3 cells. The differences in SEAP activity between the TLR2 and TLR3 ligands were significant, and therefore some adjustments had to be made. As shown in Figure 10 with the stimulation of HEK Blue hsTLR2 cell for 24 hours with a TLR2 ligand, followed by adding of the substrate, it was possible to measure a significant absorbance increase within 90 minutes. TLR3 is an intracellular receptor, which may delay the response, allowing for endocytosis of the ligands before signaling occur.

5.4.1 Effect of SEAP assay duration with TLR3 ligand

First we increased the SEAP assay duration and measured the SEAP activity at time zero hours, 30 minutes, one hour, three hours and six hours, on HEK Blue hsTLR3 cells. Cells were seeded out following the new protocol, and stimulated for 24 hours with Poly I:C at concentration 10 µg/ml (+ non-stimulated control).

![Figure 14: Effect of exposure duration with TLR3 ligand. Plot shows individual measurements from eight (control) and 32 (Poly I:C) wells per concentration and a linear fit curve (least squares regression).]
Figure 14 shows that by increasing the assay duration, measurement of Poly I:C stimulated HEK hsTLR3 cells could produce a robust signal. The graph shows that the increase in SEAP signal for HEK Blue hsTLR3 stimulated with Poly I:C was slower than the HEK Blue hsTLR2 stimulated with a TLR2 ligand. A sufficient signal (OD value above 1.0) for the TLR3 ligand was reached after three hours.

5.4.2 Effect of exposure time of Poly I:C

Next we tested the effect of ligand exposure time of Poly I:C on hsTLR3 expressing HEK Blue cells, to check if exposure time of Poly I:C would affect the SEAP activity signaling. HEK Blue hsTLR3 cells were seeded out following the new protocol, stimulated with Poly I:C at concentration 10 µg/ml for one, two and three days. The SEAP activity measurement took place three hours after adding of the SEAP substrate, the measurement was measured over 80 minutes.

Figure 15 display the effect of different exposure times with ligand Poly I:C in HEK Blue HsTLR3 cells. As the graphs show, the best signal from SEAP activity was measured in cells exposed for Poly I:C for 24 hours. Longer exposure times had a detrimental effect on the level of SEAP in these cultures.
5.4.3 Effect of FBS concentration on SEAP activity

One drawback of prolonging the SEAP measurements up to three hours was a concomitant increase in the background signal (Figure 14). This SEAP activity most likely came from the FBS (10%) used in the cell culture media. To test this hypothesis, the SEAP activity in the medium was measured without any ligand and cells, only changing the concentration of FBS. Activity measurements with cell culture media and FBS concentration started at 10%, and was serially diluted 1:2.

Figure 16: Effect of FBS concentrations on SEAP activity. Vertical bars show mean of eight measurements ± SD.

Figure 16 shows the effect of serum concentration on SEAP activity without any cells in the wells (background). As the graph shows, the SEAP activity was higher in the samples with serum concentrations over 1%. The remaining experiments with TLR3 expressing cells were therefore performed with a FBS concentration of 1% in the medium to reduce background.

5.4.4 Effect of TLR3 ligand on HEK Blue hsTLR3 cells

After the new protocol was made, HEK blue hsTLR3 cells were seeded out and stimulated with serial dilutions of Poly I:C starting at 100 µg/ml. The SEAP assay duration time was measured every hour for four hours.
Figure 17: Effect of Poly I:C on HEK Blue hsTLR3 cells. Vertical bars show mean of eight measurements ± SD. Line is four-parameter logistic fit curve; the vertical line crosses x-axis at estimated ED$_{50}$.

Figure 17 shows the effect of Poly I:C on SEAP activity in HEK Blue hsTLR3 cells incubated overnight with Poly I:C. Δ OD value increased from approximately 0.1/hour in control to a max Δ OD value approximately at 0.8/hour at concentration 100 µg/ml Poly I:C. The sigmoidal fit curve estimated an ED$_{50}$ of Poly I:C at 0.02 µg/ml, which is 10 times higher than the ED$_{50}$ value measured in HEK hsTLR2 cells stimulated with a TLR2 ligand.

5.4.5 Effect of TLR3 ligand on HEK Blue hsTLR2 cells

To be certain that the data for previous experiments matched the new adjustment in the protocol, it was necessary to try the measurement of SEAP activity again. HEK Blue hsTLR2 was seeded out now following the new protocol, and stimulated again with Poly I:C at concentration100 µg/ml, diluted 1:10. The SEAP activity was measured at zero, one hour, two hours, three and four hours.
Figure 18 show the effect of SEAP activity in HEK hsTLR2 cells incubated overnight with Poly I:C, following the new protocol (lower FBS concentration and longer assay time). The graph shows a good signal on SEAP activity for these cells all the way down to a Poly I:C concentration approximately right over 100 µg/ml, even though they possess very little of TLR3. The estimated ED\textsubscript{50} value show a 1000 times higher ED\textsubscript{50} values for the Poly I:C stimulated cells than the same cells stimulated with FSL-1. Effect of FSL-1 on HEK Blue hsTLR3 was also tested but a signal was not shown, indicating no cross binding to receptors.

5.5 The HEK Blue ssTLR3 transfected cells

After the establishment of the protocols for the different cell lines, the HEK Blue hsTLR2 cells was transfected with a plasmid encoding the Atlantic salmon TLR3 (ssTLR3). The transfected cells were grown in selection medium from day one after the transfection. After 10 weeks of selection, the cells were tested for presence of ssTLR3 activity.

5.5.1 Effect of TLR3 ligand on ssTLR3 transfected HEK Blue cells

Now we wanted to know if ssTLR3 could give a robust SEAP activity signal in HEK Blue hsTLR2 cells. After growing the cells in selection media for 10 weeks (suggesting stable expression of the construct), HEK Blue hsTLR2 + ssTLR3 cell line were seeded out
following the last protocol, and stimulated with Poly I:C at concentration 100 µg/ml and dilution of 1:10.

Figure 19: Effect of Poly I:C on HEK Blue hsTLR2 + ssTLR3. Vertical bars show mean of eight measurements ± SD. Line is four-parameter logistic fit curve; the vertical line crosses x-axes at estimated ED\(_{50}\).

Figure 19 shows the SEAP activity in HEK Blue hsTLR2 cells + ssTLR3 incubated overnight with Poly I:C. The graph show a range on the Δ OD value from 0.1 at Poly I:C concentration 0 µg/ml to approximately max Δ OD value at 0.6 for Poly I:C concentration 100 µg/ml. This Δ OD range is 0.2 values lower than the max Δ OD values of both HEK Blue hsTLR2 and HEK Blue hsTLR3 stimulated with TLR2 and TLR3 ligands respectively. On the contrary to the max Δ OD value the ED\(_{50}\) value is lower for the transfected HEK Blue hsTLR2 + ssTLR3 cells (0.27 µg/ml) compared to 1.88 µg/ml for the HEK Blue hsTLR2 cells. The HEK Blue hsTLR3 have an ED\(_{50}\) value at 0.02 µg/ml, providing the lowest ED\(_{50}\) value for all the three cell lines.

This is better described in figure 20.
Figure 20: Comparing response to Poly I:C in three cell lines: Upper panel: hsTLR2 + Poly I:C. Middle panel: hsTLR3 + Poly I:C. Lower panel: hsTLR2 + ssTLR3 + Poly I:C. Vertical bars shows mean of eight measurements ± SD. Line is four-parameter logistic fit curve; the vertical line cross x-axis at estimated ED$_{50}$. 
Compared, the three cell lines show a significant difference in ED$_{50}$ values when stimulated with Poly I:C. The significant difference were between the HEK Blue hsTLR2 cells compared to the HEK Blue cell lines expressing both hsTLR3 and ssTLR3. The t-test also revealed a significant difference in ED$_{50}$ values between the HEK Blue cell lines expressing TLR3, where the HEK Blue hsTLR3 was shown to be the cell line with the best ED$_{50}$ value out of the three different cell lines (Appendix 1).

**Table 1: ED$_{50}$ values for the different HEK Blue cell lines.**

<table>
<thead>
<tr>
<th></th>
<th>HEK Blue hsTLR2</th>
<th>HEK Blue hsTLR3</th>
<th>HEK Blue hsTLR2+ssTLR3</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSL-1</td>
<td>0.002 ± 0.004 µg/ml</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>PAMC</td>
<td>0.003 ± 0.001 µg/ml</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>HKLM</td>
<td>1.58×10$^6$ Cells/well ± 4×10$^5$ Cells/well</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>M.mariunum</td>
<td>2.3×10$^7$ Cells/well ± 7.5×10$^6$ Cells/well</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>1.88 ± 1.4 µg/ml</td>
<td>0.02 ± 0.005 µg/ml</td>
<td>0.27 ± 0.09 µg/ml</td>
</tr>
</tbody>
</table>

### 5.5.2 Analysis of TLR expression by QPCR

Although results with HEK Blue cells transfected with ssTLR3 suggested that the ssTLR3 protein was expressed, we performed QPCR to estimate the expression level of the various transgenes (hsTLR2, hsTLR3 and ssTLR3). Using QPCR we analyzed the levels of mRNA for the three receptors in the three cell lines.
Figure 21: TLR expression levels in HEK Blue Cells. cDNA from hsTLR2-, hsTLR3- and ssTLR3 expressing HEK Blue cells analyzed by QPCR using TLR specific primers (and two housekeeping genes). Values are relative expression (RE) to β2-microglobulin and levels of β-actin are included for reference. Mean of two technical replicates.

Figure 21 Show the relative levels of transcripts (including β-actin for reference) in the different cell lines. The analysis failed to detect hsTLR2 transcript in any of the cell lines suggesting that these primers were nonfunctional. Both human and salmon TLR3s were robustly expressed (same order of magnitude as β-actin) in their respective cell lines. The analysis also suggested that the ssTLR3 primers might hybridize with the human TLR3 transcript since they produced a signal in the hsTLR3 cells but not in hsTLR2 cells.

5.5.3 Analysis of TLR expression by western blotting

A signal on SEAP activity may not be sufficient evidence to claim ssTLR3 activity from the transfected protein. We have to be able to prove that we have successfully transfected ssTLR3 plasmid into the HEK Blue hsTLR2 cells and that the protein is synthesized. Using a selection medium will partly do this; the cells that survive for weeks have most likely incorporated the plasmid encoding antibiotic resistance and the gene coding for ssTLR3 into their genome. However, expression of antibiotic resistance without TLR expression is possible, so we used western blot to analyze if the receptor protein was present in the cells.
HEK Blue hsTLR2, HEK Blue hsTLR3 and HEK Blue hsTLR2 + ssTLR3 were grown in 6-well plates and samples prepared following the protocol for Western blot. After blotting to membrane we used Anti-FLAG antibody to detect ssTLR3 protein using FLAG-tagged hsAlfy protein as a positive control. Negative controls were HEK Blue hsTLR2 and HEK Blue hsTLR3.

Figure 22: Western blot of cell lysates from HEK Blue hsTLR2, HEK Blue hsTLR2 + ssTLR3, HEK Blue hsTLR3 and hsAlfy using Anti-FLAG antibody. Left lane is MW standard.

Figure 22 shows that the Anti-FLAG antibody bound to a protein with MW around 30 kDa present in all cells. In addition, a protein band with higher MW in HEK Blue hsTLR2 transfected with ssTLR3 was detected. The band had a molecular weight corresponding to a protein size around 140 kDa. TLRs are supposed to be located in this range and it is therefore likely ssTLR3, since there was no detection of the same band in any of the other cell lines. The weak signaling can be caused by low amount of the protein or because of a weak antibody binding. The positive Alfy control was has also detected (MW between 65-70 kDa) suggesting that the method was successful.

5.5.4 Analysis of TLR expression by immunofluorescence

Another way to detect protein expression in cells is by immunofluorescence microscopy (IF). Using IF it is possible to determine the location of the protein in question (here TLR3) in the cell, and expression level. HEK Blue hsTLR2, HEK Blue hsTLR3 and HEK Blue hsTLR2 +ssTLR3 were all seeded out following the protocol of immunofluorescence. The antibodies
used were Anti-FLAG to detect ssTLR3 expressing cells and Anti-TLR3 to hsTLR3 expressing cells.

Figure 23: ssTLR3 (green) in HEK Blue hsTLR2 + ssTLR3 cells stained with Anti-FLAG Ab (left panel). hsTLR3 (green) in HEK Blue hsTLR3 cells stained with Anti-hsTLR3 Ab (right panel). The blue color marks the nucleus of the cells (DAPI).

Figure 23 shows the IF signals in the HEK Blue hsTLR2 + ssTLR3 cells and HEK Blue hsTLR3 cells. From the pictures is it possible to see that only a small fraction of cells, either from HEK Blue hsTLR2 + ssTLR3 or HEK Blue hsTLR3, were stained by this procedure even though they both are stable cell lines. The low resolution in these micrographs did not reveal information about subcellular localization of the antigen (except from being excluded from the nucleus).

5.6 Analysis of ssTLR3 signaling in a fish cell line (EPC cells)

When the data from the HEK Blue cell lines were analyzed and found promising (ssTLR3 dependent signaling), the next step was to try the similar experiments in a fish cell line. The optimal cell line would be to use ASK cells from Atlantic salmon, but because of difficulties concerning the transfection of these cells we decided to use EPC cells, a cell line shown to be more accessible to transfection than ASK (Schiotz et al., 2011). The EPC cells do not contain the reporter gene construct responsible for the signal in the HEK Blue cells (secreted SEAP). The Poly I:C stimulated activity in EPC cells was therefore quantified by QPCR analysis of cytokines and interferon response genes (IRGs).
First, we analyzed if the cells were expressing the ssTLR3 mRNA after transient transfection. The presence of ssTLR3 protein in EPC cells was tested in the same way as for the HEK Blue cells (western blot and IF using Anti-FLAG antibody). However, we were not able to detect the ssTLR3 protein neither in western blot nor in the IF using the same protocols as for HEK Blue cells. This therefore suggested that if ssTLR3 were expressed in EPC, the level was below our protein detection limits.

Next we investigated what effect the stimulation of transfected EPC cells with poly I:C would have on cytokine and IRG expression. Control EPC cells and EPC ssTLR3 were seeded out into two 12-well plates following the protocol for QPCR. When cells were adherent, 50 µg/ml Poly I:C were added and the cells incubated for zero (control), one, two, and three days.

**Figure 24:** Expression kinetics of innate immune genes in EPC cells and EPC + ssTLR3 cells incubated with Poly I:C for three days. Vertical bars show mean of three measurements ±SD, relative expression (RE) levels calculated relative to β-Actin used as a housekeeping gene.

Figure 24 show the effects of incubating EPC cells and EPC + ssTLR3 cells with Poly I:C over three days on cytokine and IRG expression. In general, the EPC + ssTLR3 cells
displayed increased sensitivity to Poly I:C induced cytokine and IRG expression. Both endogenous ppTLR3 and transgene ssTLR3 were significantly upregulated in the EPC + ssTLR3 cells in contrast to the EPC cells not expressing ssTLR3, which showed no upregulation. The cytokine and signaling transcripts IRF-3 and IRF-7 were also significantly upregulated in the EPC + ssTLR3 cells relative to the EPC cells. The IRG genes Mx-1, PKR, TNF-α and ISG-15 were not significantly different between cell lines (t-test Appendix 1). The EPC cell line also displayed upregulation of PKR, Mx-1 and ISG-15, but to a lower degree. VIG showed an upregulation in both the EPC + ssTLR3 and EPC cells, the difference was not significant between cells. The IFN-1 gene showed upregulation after day three; different from the EPC cells not expressing ssTLR3 that showed no upregulation. IFN-1 shows after day one a significant difference to the EPC cells not expressing ssTLR3, the reason for this is probably due to the great variation in the data (Appendix 1).

Figure 25: Correlation matrix plot displaying the correlation between the expression levels of antiviral genes all samples (EPC cells with or without ssTLR3), when stimulated with Poly I:C over time. Lower left panel visualize data using the “ellipse” method. Upper right panel display r squared values ($r^2$).

Figure 25 shows the correlation matrix between the different genes, visualizing the degree of co-regulation. Best correlation was found between IRF-7 and the ppTLR3 (0.93). The correlation between ssTLR3 and IRF-7 (0.84) and between ssLTR3 and ppTLR3 (0.85) was
also high. ISG-15 seems to have a good correlation with Mx-1 and IRF-3, both close to 0.9, while the correlation between IRF-3 and Mx-1 was closer to 0.83. IRF-3 is also well correlated to both PKR (0.81) and IRF-7 (0.83). Mx-1 has a good correlation with TNF-α at 0.84. IFN-1 on the other hand has no correlation with any gene present, the values range from -0.17 as the lowest to 0.26 as the highest value.
6. Discussion

The development of efficient and cheap vaccines against aquatic viruses is important for a sustainable salmon farming industry. The significant losses of salmon during farming are mainly due to infections caused by bacteria, virus and parasites, related to high densities of fish in netpens (Hjeltnes, 2016; Nodland E., 2017). In the 1980s the greatest concern for fish farming arose from bacterial infections. Bacterial disease furunculosis, started the “vaccination-revolution” in fish farming, and by 1994 all fish farms in Norway had switched from antibiotics to prophylactic vaccines. This switch has made it possible to reach today’s results where the current use of antibiotics is limited to less than 1% of all farmed salmon produced in Norway (Hjeltnes, 2016; WHO., 2015). Since then, fish-farming industry has evolved even further and new challenges concerning diseases have occurred. Sea lice and viral outbreaks of PD and ISA cause some of the largest health concerns involving breeding farms (Hjeltnes, 2016; WHO., 2015). Because of the positive results using vaccines against bacterial disease, an urge to develop vaccines against both parasites and viruses has evolved. The aim of this study was to investigate new adjuvants in viral vaccines for Atlantic salmon, using TLR3 as a target molecule and the ligand Poly I:C as an adjuvant component.

6.1 Discussion of the methods

6.1.1 The SEAP assay

The main bulk of data in this thesis was provided by the measurement of SEAP activity with CLARIOstar® plate reader. This method was chosen because of its fast and reproducible measurements of TLR activity when stimulated in vitro. The variation in the SEAP reporter assay may occur due to cell culture variability or sample handling. Replication of the experiments with four to eight parallels in each assay, and background controls were used to correct for these variations in the system. The human TLR3 is an intracellular receptor located in the ER and endosomes (Akira, 2003). Because of its location, it may be harder to demonstrate a direct correlation between ligand- or pathogen binding and upregulation of reporter gene (SEAP) for this receptor. A more direct pathway would be to use TLRs located extracellularly on the plasma membrane to relay on the signal (like TLR2 or TLR4). The Nf-κB plasmid transfected into the cell line (HEK Blue) made it possible to measure SEAP
activity to verify binding of Poly I:C to TLR3, and the effectiveness of the assay made it possible to measure up to 96 samples for every experiment.

6.1.2 Western blot

Western blot is a semi-quantitative method used to separate and identify specific proteins. It is often used together with assays from SEAP or luciferase; confirmation of the result with western will only strengthen the data from the SEAP/luciferase assay. It is difficult to get good antibodies (Ab) for *Salmon salar* TLR3 (Trede et al., 2004), which may be a source of error when using western to identify this particular protein. The method produces some variations in the data due to differences in the quantity of the sample or uneven transferring of the protein from the cell to the membrane (Mahmood and Yang, 2012). These variations can be corrected fore with a positive control, like Alfy.

6.1.3 Immunofluorescence

Immunofluorescence is a method used to identify cellular structures like cytoskeleton and organelles, visualizing a biochemical or a protein component and the anatomy of a structure. It can also be used to demonstrate heterogeneity of protein expression in mixed cultures. In this thesis the immunofluorescence was used to identify the frequency of TLR3 expression in different cell lines (Matsuo et al., 2008; Toscano et al., 2013). As previously mentioned, the localization of human TLR3 is mainly in the ER and endosomes. The variations present in the method may be due to the Ab, where the Ab can be host species specific (Buchwalow and Böcker, 2010), resulting in no staining of the protein studied. The Ab used here for ssTLR3 (Anti-FLAG) and hsTLR3 (Anti-TLR3) staining did not produce strong signals and it is possible that the protocol needed further optimization (like Ab dilutions, fixation method and blocking method). Our pictures may therefore underestimate the amount of TLR3 present in the cell. Another problem with Ab is that the secondary Ab may bind to other organelles giving a false fluorescence signal and an overestimation of the receptor present. This was corrected for by adding a second control consisting of cells stained with only secondary Ab, which produced no signal. The fixation step may cause the cells to loosen from the well making the staining unsuccessful due to no cells present. We tried to prevent this by using room temperature PBS with Mg$^+$ and Ca$^{2+}$ to help the surface proteins adhering stronger to the surface of the wells (Celis, 2006).
6.1.4 QPCR

QPCR is a quantitative method normally used for mRNA determination because of its sensitivity, specificity, reproducibility and wide dynamic quantification range. Even though QPCR has all the characteristics above it is also a method with pitfalls. The method has tremendous sensitivity, and can therefore divide the causes of variability into three main categories: biological variability (the experiments will never yield identical results), technically variability (concerning contamination, primers and technical faults) and inappropriate experimental design. There are many different variables in each of these three main categories, but I have chosen to highlight those variables that could affect my results the most. First to be mentioned is sample contamination, when working with DNA and RNA samples, especially from human sources can hair, saliva or skin cells contaminate the sample resulting in incorrect Ct values. Because the RNA samples on this project mainly originate from fish (HEK Blue cells is an exception) this was probably not a big problem. Gloves and glasses minimize the risk of contamination from lab personnel and water controls were always used to be sure that other reagents were not contaminated. Another source of contamination is DNA contamination from the host cell, when producing mRNA the sample is treated with DNase to correct for the possibility that some DNA are still in the sample (Bustin, 2010).

The second highlighted issue is the primers used for the different genes. When primers are designed based on previously published articles uncertainties may arise connected to the chemistry and analytical instrumentation used for QPCR. It is also possible that the primers designed are, to some degree, complimentary to other genes in the genome and thereby amplify more than the gene studied. To correct for this variable, primers are tested on cells known to give good signaling, and using housekeeping genes that will provide good indication on whether the method is working or not. The housekeeping genes are also used as data normalizers, where housekeeping gene expression level is set to be 1 and the other genes studied calculated relative to this (Bustin, 2010; Logan, 2009).

Third, variations caused by inappropriate experimental design are most likely due to insufficient sample size. If the sample size is small is it a possibility that differences between the samples are not detected and therefore do not give accurate signals. If small-scale differences in data are of interest it is preferable to use more than three samples as used in this thesis (Bustin, 2010).
6.2 Discussion of the results

6.2.1 Establishing the experimental system with HEK Blue hsTLR2 cells

HEK Blue cells are cell lines produced by Invivogen. These cell lines are stably transfected with a plasmid containing one or two different TLR-related genes originating from either humans or mice. They can be used to determine TLR activation when stimulated with a TLR ligand by assessing IL-8 secretion or Nf-κB driven SEAP transcription (Invivogen website). The HEK Blue hsTLR2 cells were transfected with ssTLR3, and ligand binding to the different TLRs were assessed by Nf-κB transcription. The HEK Blue cells express low levels of endogenous TLRs (Geiger et al., 2012); HEK Blue hsTLR2 cells express a hundred times more of hsTLR2 compared to the parent cell line (Invivogen website). The QPCR analysis of TLR expression in these cells confirmed expression of hsTLR3 and ssTLR3 in the respective cell lines, but hsTLR2 expression could not be confirmed by QPCR. The reason for this is not clear, but nonfunctional primers are a possible explanation.

In an attempt to be cost-effective, dinitrophenylphosphate tablets were chosen instead of the substrate described in Invivogen's protocol. Experiment 5.2.2 confirmed that the SEAP substrate from the lab could be used at a concentration of 50 µg/ml providing comparable results with substrate from the Invivogen protocol (Invivogen website).

Experiment 5.2.1 test the effect of cell number on the SEAP assay. The Invivogen protocol suggested the use of 50,000 cells/well. Their protocol claimed just to add ligand and substrate, immediately followed by measurement. Because of the stimulation time with the ligands (seeding out on day one, add ligand on day two, then measuring the SEAP activity after 24 hours on day three), the cells were in the wells for approximately 72 hours before the measurement of SEAP activity started. If the initial amount of cells at day one was set at 50,000, there would be over 80% confluence on day three in the 96-well plates, affecting the OD value and the ED$_{50}$ value in a negative matter (Jäger et al., 2013). When the cells are in an exponential growth phase they produce different metabolites, proteins and other constituents necessary for cell growth. When adding the ligand during this phase it is more likely that the cells will be able to produce optimal amounts of SEAP. If the cell density is over 80% confluent the growth levels off and reduces the production of proteins and other constituents necessary for growth and thereby possibly reducing the production of SEAP. Downregulation of different proteins has been shown in other experiments, and supports the theory that a high-
density level in the well will affect the measurement and signaling in a negative matter (Hamburger et al., 1991; Singh et al., 1996). That is why the minimum cell count had to be clarified before measuring the SEAP activity. Experiment 5.2.1 showed that the cell count had to be over 20,000 cells/well to get a signal similar to the one described in the Invivogen protocol.

6.2.2 HEK Blue hsTLR2 ligands

FSL-1 and PAMC are characterized as chemical ligands. They have been described in several articles as good TLR2 agonists (Brietzke et al., 2016; Okusawa et al., 2004). FSL-1 is a synthetic lipopeptide derived from *Mycoplasma salivarium*, recognized by TLR2 and TLR6 homodimers. According to Invivogen, FSL-1 has a working concentration between 1-100 ng/ml. PAMC is a synthetic tripalmytoilated lipopeptide that mimics the acylated amino terminus of bacterial LPS. The ligand binds to TLR2/TLR1 heterodimer, and has a working concentration between 1-300 ng/ml (Invivogen website). The chemical ligands both induced a good signal when measuring SEAP activity. FSL-1 and PAMC gave ED$_{50}$ values between approximately 2 and 3 ng/ml (Table 1), respectively. FSL-1 gave a significantly higher Δ OD value than PAMC at ED$_{50}$, which suggests that FSL-1 has a higher affinity for the hsTLR2 receptor also confirmed by the American Chemical Society (American Chemical Society. Division of Medicinal Chemistry., 1965; Okusawa et al., 2004).

Previous attempts in the lab using fish-specific pathogens to stimulate fish TLRs in HEK Blue cells has failed. However, our findings indicated that both human (HKLM) and fish (*M. marinum*) pathogens activates human TLR2. It was expected to observe a good signal for the hsTLR2 cells stimulated with HKLM as it has been shown in previous articles published (Anand et al., 2011; Torres et al., 2004), in contrast to previous results shown for fish TLR2 stimulated with *M. marinum* leaving this ligand more uncertain (Brietzke et al., 2016). HKLM is heat-killed *Listeria monocytogens*, which is a gram-positive bacterium produced by Invivogen. The gram-positive bacteria is recognized by TLR2 and has a working concentration between $10^7$ - $10^8$ cells/ml. *M. marinum* is a gram-positive mycobacterium, which cause infection in fish. The bacterium was recognized by hsTLR2 and stimulated cells at concentration between $10^8$- $10^9$ cells/ml. The biological ligands induced SEAP activity in estimated working concentrations of approximately $10^6$-$10^8$ cells/well for HKLM with a ED$_{50}$ value at $1.58\times10^6$ cell /ml. For *M. marinum*, the estimated working concentration was
between $10^8$-$10^9$ cells/ml with an ED$_{50}$ value at $2.3 \times 10^7$ cells/ml (Table 1). HKLM gave a significant lower ED$_{50}$ value than *M. marinum* (p=$8.2 \times 10^{-5}$) (Appendix 1), and *M. marinum* gave a good signal in the HEK Blue hsTLR2 cells not matching the result in the scientific article of Brietzke, possibly due to poor expression of TLR2 in trout.

All TLR2 ligands tested produced good signals in the HEK Blue hsTLR2 cells. Some of these ligands are well characterized but to our knowledge, *M. marinum* has not been previously tested on cells expressing hsTLR2. Taking all the different measurements ($\Delta$ OD value and ED$_{50}$ value) into consideration as well as cost, FSL-1 was chosen as positive control for TLR2. The justification of this choice was that FSL-1 is well described in literature as a TLR2 ligand and it gave good signaling in the cell line used (American Chemical Society. Division of Medicinal Chemistry., 1965).

After the functionality of the HEK Blue hsTLR2 cells were established, the cells were stimulated with Poly I:C, a synthetic dsRNA ligand for TLR3. According to Invivogen, the working concentration is between 0.03-10 µg/ml in TLR3 expressing cells. The result from experiment 5.3.2 showed a no signal suggesting HEK Blue hsTLR2 cells as a good host cells, providing low background when transfected with ssTLR3. Repeated experiments with these cells were in general reproducible, but some fluctuation in the response was observed. One possible reason for these fluctuations may be alternations between selection media and normal media every week (to keep the transgenes in place). The different media vary in their antibiotic content and these antibiotics have different mechanisms of action, where the main mechanism is to inhibit protein synthesis in the cell. Because the cells were on and off the selection media every other week is it possible that this could have affected the result of the SEAP activity. If the cells were taken off the selection media when measured some amount of the antibiotic would still be inside the cell, and could most likely affect its SEAP production. The use of G418, which is the antibiotic mainly used on this project as a selection marker show stress responses also in (Buchanan et al., 1987; Qian et al., 2006). The HEK Blue cells grew so fast they had to be split two times a week; resulting in an increase in passage number over the time frame the experiments were done. For every time the cells were split they may reduce expression of their receptors. Invivogen and (Center, 2012) has shown that usage of HEK Blue hsTLR2 cells over passage 20 may result in reduced signal. Our experiments done over passage 20 have shown good signaling, but this may be the reason why some experiments did not give SEAP activity as expected.
6.2.3 Activation of the human TLR3 with Poly I:C

HEK Blue hsTLR3 was stimulated with Poly I:C to be sure that the SEAP activity measured in the transfected cells most likely came from the ssTLR3 rather than other receptors. The HEK Blue hsTLR3 cells were also purchased from Invivogen and allegedly expressed a hundred times more TLR3 than the parent HEK Blue. However, the stimulation gave a poor signal and resulted in further adjustments to the protocol.

The increase in SEAP activity following TLR activation is the result of a complex chain of events containing many levels and several possible limiting steps in the cascade (Akira, 2003; KEGG; Parham and Janeway, 2015). It was therefore important to develop protocols taking these complexities into account. When using HEK Blue hsTLR3 cells we observed that the optimal exposure time with Poly I:C was 24 hours, the same as for TLR2 ligands (Figure 15). When stimulation lasted over 24 hours the data showed reduced signaling. This was possibly due to the fact that Poly I:C can be toxic to the cells after prolonged exposure, resulting in apoptosis and low SEAP activity signals also shown by others (Hu et al., 2015; Kato et al., 2014). Other scientific articles have shown that activation of TLR3 is done by Poly I:C (Guo et al., 2012; Huh and Lee, 2013; Lai et al., 2011). The difference between the mentioned papers and this thesis is that they have an already well-prepared system, giving them the opportunity to measure the SEAP or luciferase activity only one time. For us this was not possible, our system was not well characterized leaving us with no assumption on when the exponential phase of the SEAP activity would take place. Consequently measurements had to be taken every hour for 24 hours, and after experiment 5.4.1 the measurement time was set to every hour, from zero to four hours, after adding the substrate. An explanation for the expanded measurement time in contrast to the experiments concerning TLR2 is perhaps the location of the receptor. Human TLR3s are located in the ER or in the endosomes and therefore the time from adding the ligand to the ligand binds on to the receptor takes longer (Akira, 2003).

An unexpected problem arose when the protocol was adjusted to the TLR3 ligand (by extending assay time from 90 minutes to four hours). The medium started to give higher background, caused by the serum in the medium. This is also shown in experiments done with bone marrow mesenchymal stem cells, where the background were larger for higher concentrations with FBS (over 8%) (Bian et al., 2015). The FBS concentration is normally 10%, large enough to reveal phosphatase activity in the four-hour assay. This was not a
surprise as SEAP is used as a diagnostic marker in clinical biochemistry (increased in cholestatic liver disease) and may therefore also be present in calf serum (Withold et al., 1995). In experiments carried out by others, 10% FBS have been used (Huh and Lee, 2013; Lai et al., 2011), but these measurements of SEAP activity were endpoint analysis, not kinetic. Another variation we had account for may be that background SEAP activity in calf serum may be batch sensitive. We therefore optimized the concentration of FBS for the long duration assay and concluded that FBS concentrations under 1% in the media would not affect the SEAP activity and new adjustments were made to the protocol, corresponding with the findings found by Bian and collaborators (Bian et al., 2015).

New adjustments to the previous protocol lead to repetition of experiment 5.3.2 (HEK Blue hsTLR2 stimulated with Poly I:C). Results with the new protocol (Experiment 5.4.5) showed a higher response compared to experiment 5.3.2. Low serum and long assay time resulted in max Δ OD values resembling the max Δ OD value of HEK Blue hsTLR3 cells stimulated with Poly I:C. On the contrary to the ED50 value which was higher for the HEK Blue hsTLR2 than for the HEK Blue hsTLR3 cells. It is shown that MDA5 and RIG-I do not possess the same ability to be activated by Poly I:C as TLR3 in some cell lines, and it is a possibility that this is accurate for HEK Blue cells as well. However is it possible that the signal from the HEK Blue hsTLR2 originates from RIG-I and MDA5 (Geiger et al., 2012; Zhou et al., 2013). RIG-I and MDA5 activate transcription of Nf-κB in a downstream cascade way as TLR3, therefore providing an SEAP activity signal (Yin et al., 2015). The higher ED50 value can be explained by affinity to the receptor, and that the amount of receptors present is lower than for HEK Blue hsTLR3. This is a logical conclusion given that HEK Blue hsTLR3 cells most likely contain the same amount of RIG-I and MDA5 as HEK Blue hsTLR2 cells (Geiger et al., 2012).

### 6.2.4 Does salmon TLR3 bind Poly I:C?

New adjustments to the protocol made it possible to better analyze the binding activity of salmon TLR3 (using transfected HEK Blue hsTLR2 + ssTLR3). Although Poly I:C is a well characterized activator of the salmon innate immune system, there is no direct proof demonstrating Poly I:C binding to ssTLR3 (Poly I:C may stimulate salmon cells via RIG-I and MDA5) (Kileng et al., 2009; Matsuo et al., 2008; Rodriguez et al., 2005). Previous scientific papers suggest that specific stimulation of TLR3 in different cell lines require
approximately 125 ng/ml with Poly I:C to initiate signaling (Alizadeh et al., 2014; Lai et al., 2011; Wu et al., 2017).

Our data suggests that most of the signal from the ssTLR3 expressing cells is caused by the new transgene ssTLR3. The justification of this is the significant difference in signal when comparing HEK Blue hsTLR2 cells with or without ssTLR3, it has also been shown in other scientific papers that when HEK-293 cells are transfected with TLR2 from goose, signaling of Nf-κB is upregulated (Yong et al., 2015). This supporting the theory that a transfection of genes coding for TLRs in other species also can stimulate Nf-κB signaling in human cell lines. The stimulation with Poly I:C produced a signal in HEK Blue hsTLR2 cells probably caused by RLRs naturally present in HEK Blue cells. The ED50 value for the HEK Blue hsTLR2 cells had 10 times difference compared to the HEK Blue hsTLR2 + ssTLR3 (Table 1). Because of this is it natural to assume that the signal provided in the HEK Blue ssTLR3 expressing cells originates from the ssTLR3. Results from the measurements of HEK Blue hsTLR3 cells, stimulated with Poly I:C, and previous articles supports the statement that presence of TLR3 will lower the ED50 value. In these articles and product sheets show different ED50 values for 100 μg/ml Poly I:C in HEK Blue hsTLR3 cells, some show ED50 values at 800 ng/ml, others show ED50 values under 30 μg/ml for (Biosciences, 2014; Devilder et al., 2009; PeproTech, 2013). Last but not least since binding to TLR3 by Pol I:C has been shown in humans and mice may also Poly I:C bind ssTLR3 as well (Akira, 2003; Alexopoulou et al., 2001; Yong et al., 2015), our experiments provides a lower ED50 value indicating better binding of the receptor than in the published papers.

Differences between the two TLR3 expressing cells (hsTLR3 and ssTLR3) were also shown, and some of which were significant. Figure 20 shows a 0.2 difference in max Δ OD value as well as a significant difference in ED50 values between the different TLR3 cells (Table 1). The differences are possibly explained by various parameters: receptor number, selection media, folding, and other substances necessary for production of proteins, or affinity to the receptor.

The two main factors controlling the response in this assay are receptor number and receptor agonist affinity. A large amount of receptors will, under most circumstances, give a higher signal. After transfecting ssTLR3 into the HEK Blue hsTLR2 cell line the frequency of cells expressing ssTLR3 was uncertain, but was probably increased by using selection media. In theory, all the cells not expressing ssTLR3 die during selection, but the possibility exists that some cells have incorporated the antibiotic resistance gene in their genome without
expressing ssTLR3 right. It has been shown that for some genes the site of incorporation in the genome can affect the transcription of the transgene. This will result in low levels of the receptor and thereby providing a poorer signal (Nakatake et al., 2013). The QPCR (Experiment 5.5.2) done on the different HEK Blue cell lines showed a good expression level for the mRNA coding for ssTLR3 in the transfected HEK Blue hsTLR2 cells. The hsTLR3 primers gave a good signal in HEK Blue hsTLR3 cells; the expression level was lower than measured for ssTLR3 in the transfected HEK Blue cells. The problem with QPCR like this is that it only tells us if the genome is transcribed, it does not verify that the receptor is translated and located in the right place. The result from the QPCR shows a good signal for mRNA coding for ssTLR3, but hsTLR3 were still more responsive to Poly I:C (Figure 20) possibly explained by (Nakatake et al., 2013).

If the ssTLR3 gene has been incorporated in the right place in the genome, it may not be properly translated or modified (like proteolytic processing) thereby not transported to the right cellular location. As mentioned earlier, TLR3 are produced in ER where it is folded, packed, and shipped to the endosomes upon activation. UNC93b, a chaperon protein, usually controls these steps (Leifer and Medvedev, 2016). If the HEK Blue cells do not express enough UNC93b it may explain for lower max Δ OD values and ED50 values in the HEK Blue hsTLR2 + ssTLR3 cells. Another possible reason may due to the downstream cascade pathway; this pathway is well characterized in mice and human cells, but for fish cells in general, and in this case for salmon, no information is confirmed yet (Secombes and Pilstrom, 2000). The downstream cascade may need other downstream components resulting in a not adequate SEAP activity when they are not present.

The effect of selection media on cells was not expected, but considering stress provided on transfected cells is this not illogical (Buchanan et al., 1987). The HEK Blue hsTLR2 + ssTLR3 is a stable cell line, but to remain stable the cell line was grown for longer periods in selection media compared to the other two cell lines that did not express ssTLR3. This can affect the SEAP activity because of antibiotics inhibit protein synthesis in cells, and can inhibit the production of SEAP, as mentioned before (Buchanan et al., 1987; Qian et al., 2006).

A final possible reason for the lower max Δ OD and higher ED50 value in figure 20 is the affinity to the receptors. It is most likely that the affinity for Poly I:C is higher for the TLR3 than for the RLRs (Zhou et al., 2013), but it is also possible that the affinity for Poly I:C is
lower for ssTLR3 than for hsTLR3; providing a lower max Δ OD and ED₅₀ value in the HEK Blue cell line expressing hsTLR3. This has to my knowledge not yet been tested.

Figure 20 displays max Δ OD values and ED₅₀ values that did not correspond with the results from the QPCR (experiment 5.5.2). Western blot and immunofluorescence were used to clarify and support the data collected. Figure 22 shows a faint band with size corresponding to ssTLR3 (140 kDa); this may be due to low protein amount in the cells or weak antibody binding (Trede et al., 2004). Although weak, the band is most likely ssTLR3 and it matches the size of other western blot for TLR3 (130 kDa ± some due to glycosylation) (Garcia-Cattaneo et al., 2012; Toscano et al., 2013). The positive control (hsAlfy) and negative controls (hsTLR2 and hsTLR3) confirm that the method was successful. These results supports the SEAP assay data, and also support the hypothesis that ssTLR3 are not properly translated or modified in spite of high mRNA levels. This may result in reduced number of mature receptors and hence, signal.

Immunofluorescence (experiment 5.5.4 and figure 23) showed adequate signals in very few cells. The labeling of ssTLR3 showed only a small number of cells containing ssTLR3, in agreement with the faint western signal, and higher ED₅₀ values compared to the HEK Blue hsTLR3 cells. The labeling of hsTLR3 did not work properly either maybe due to a poor Ab, or/and by a high detection limit only capturing the cells with a high level of the receptor (Trede et al., 2004). The location of TLR3 in both cell lines matches the expectations and description in published work (Toscano et al., 2013), but we are not able to confirming that the endosomes in cytoplasm is the locations for TLR3 because we have not labeled the endosomes or ER; making it only possible to say that TLR3 is located in the cytoplasm.

### 6.2.5 Expression of salmon TLR3 in fish cells

The epithelioma papulosum cyprinid (EPC) cell line was originally deposited as a carp (Cyprinid carpio) cell line, but later found to be contaminated by cells from fathead minnow (Pimephales promelas) (Winton et al., 2010). This cell line was a gift from the Veterinary institute at NMBU. The EPC cells were chosen as the fish cell line because they are much easier to transfect than, for example, ASK cells, and they are stable (keeps the transgene) under the right conditions (Schiotz et al., 2011). The amount of endogenous TLR3 is also here close to none as for the HEK Blue cells, because it is not an immune cell line (Geiger et al., 2012).
EPC cells are not transfected with Nf-κB-SEAP plasmid (codes for the SEAP protein), making measurement of SEAP activity impossible. A solution to this problem could be to transfect EPC cells with the Nf-κB-SEAP plasmid and select for a stable cell line, but this can take months and was not feasible within the framework of this master thesis. ELISA analysis of secreted cytokines was also not an option due to lack of EPC or Atlantic salmon specific Abs to measure the specific amount of proteins, like interleukins and IFNs (Trede et al., 2004). This left us with the third best choice, QPCR. With QPCR several viral induced genes were measured as a response to stimulation with Poly I:C. We could also support the QPCR analysis by electrophoresis of amplicons confirming that the bands amplified have the correct size. Previous published articles have shown that an upregulation of receptors, antiviral proteins, and cytokines occurs when EPC or other fish cells are stimulated with viral pathogens (Holopainen et al., 2012; Kim et al., 2016; Qian et al., 2013). The upregulation shown in figure 24 was therefore not surprising. The differences observed between EPC cells with or without salmon TLR3 were supported by t-tests (Appendix 1). Significant differences were found between the EPC cell line and the EPC + ssTLR3 cell line (for example for the mRNA encoding ppTLR3, IRF-3 and IRF-7). Our findings together with the similar results found in the transfection of geese TLR2 into HEK-293 cells activating IL-1β (Yong et al., 2015), supports our hypothesis that the increased upregulation of antiviral genes is caused by the transfection of ssTLR3 into the EPC cells. From the data we also made a plot showing correlation between expression values providing information whether some genes were co-regulated. Values above 0.8 may indicate such a connection. The correlation plot showed that IRF-7 was correlated with both ppTLR3 and ssTLR3, while IRF-3 correlated well with IRF-7, Mx-1, ISG-15 and PKR. Some genes correlated better than others, but the plot show that several of the antiviral genes are probably co-regulated in the same way as Yeung have shown in his article (Yeung et al., 2004). Together with the QPCR it would be preferable to confirm that the ssTLR3 was in fact expressed as a whole protein and not only as mRNA, using western blot and immunofluorescence. It was unfortunately impossible to detect ssTLR3 in either of the methods due to limited amount of protein in the cells or a poor Ab.

6.2.6 Future plans

The results in experiment 5.6 showed a greater upregulation of different antiviral genes in cells transfected with ssTLR3. From this experiment and the binding assays with the HEK Blue cells, we may assume that ssTLR3 recognize Poly I:C and the increased upregulation of
antiviral genes in cells expressing the receptor. A natural continuation of these studies would be to express ssTLR3 in salmon cells, and test the binding characteristics in an endogenous environment. Furthermore, downstream signaling should also lead to synthesis of cytokines. The preferred assay would be to measure cytokines released by ssTLR3 expressing cells when stimulated with Poly I:C using ELISA. Unfortunately, general lack of good anti-fish-cytokine antibodies makes it difficult to develop specific ELISA for these proteins (Trede et al., 2004).

Research on nanoparticles has increased tremendously in recent years, especially concerning development of new drugs and vaccines. A natural next step would be to investigate whether nanoparticles could increase Poly I:C uptake into the cell and therefore engage TLR3 more efficiently (Kavaliauskis et al., 2015; Lee et al., 2010; Rahimian et al., 2015). Because of the location of the receptor it may be more difficult to induce an immune response with Poly I:C. This difficulty may be related to the fact that dsRNA is not entering cells as effectively.

A further step would also be to test out Poly I:C as an adjuvant in vaccine experiments. Zebrafish have proven to be a good test organisms for immune research, making it possible to test out if Poly I:C could activate the fish immune system during vaccination. For this type of experiments it would be preferable to use knockout fish without TLR3 to see if this would affect the immune response, perhaps in the same way as has been done for mice (Alvarez et al., 2017; Kavaliauskis et al., 2015; Kavaliauskis et al., 2016; Takada et al., 2017). It would also be possible to use the TLR3 knockout fish as a control when infecting the fish with viruses.
Main conclusions

- The SEAP assay was established with an alternative substrate and gave reliable and reproducible results. Some adjustments had to be made according to type of cell lines studied.

- The HEK Blue hsTLR2 cell line showed to be a good host for expression of ssTLR3. Transgene expression was confirmed by QPCR, western blot and by immunofluorescence.

- Poly I:C was a good ligand for salmonTLR3, but with a lower affinity to the receptor compared to human TLR3.

- EPC cells transfected with ssTLR displayed increased responsiveness to Poly I:C with enhanced upregulation of several antiviral genes.
References


Bell, J.K. (2017). The molecular structure of the TLR3 ligand bidding domain (Proceedings of the National Academy of Sienes of the USA).


KEGG. The TLR3 receptor signaling pathway.


PDB. Picture of TLR3 receptor (RSCB Protein Data Bank: PDB).


Appendix 1
Stine D Anthonsen
15 5 2017

Raw data, figures and statistics for Stine D. Antonsens master thesis "Characterization of Atlantic salmon Toll-like receptor 3"

This is an R Markdown document. By running this computer code, plots and statistical analysis will be created from raw data in a reproducible way.

```r
library(reshape2)
library(ggplot2)
library(drc)
library(polynom)
library(sandwich)
library(lmtest)
library(tidyr)
library(plyr)
library(dplyr)
library(knitr)
library(corrplot)
library(readr)

# T-test on summary statistics

# m1, m2: the sample means
# s1, s2: the sample standard deviations
# n1, n2: the same sizes
# m0: the null value for the difference in means to be tested for. Default is 0.
# equal.variance: whether or not to assume equal variance. Default is FALSE.

t.test2 <- function(m1,m2,s1,s2,n1,n2,m0=0,equal.variance=FALSE)
{
  if( equal.variance==FALSE )
  {
    se <- sqrt( (s1^2/n1) + (s2^2/n2) )
    # welch-satterthwaite df
    df <- ( (s1^2/n1 + s2^2/n2)^2 )/( (s1^2/n1)^2/(n1-1) + (s2^2/n2)^2/(n2-1) )
  } else
  {
    # pooled standard deviation, scaled by the sample sizes
    se <- sqrt( (1/n1 + 1/n2) * ((n1-1)*s1^2 + (n2-1)*s2^2)/(n1+n2-2) )
    df <- n1+n2-2
  }
  t <- (m1-m2-m0)/se
  dat <- c(m1-m2, se, t, 2*pt(-abs(t),df))
  names(dat) <- c("Difference of means", "Std Error", "t", "p-value")
  return(dat)
}
```
```r
# Function to calculate the mean and the standard deviation
# for each group in a dataframe
#
# data : a data frame
# varname : the name of a column containing the variable
to be summarized
# groupnames : vector of column names to be used as
grouping variables

data_summary <- function(data, varname, groupnames){
  require(plyr)
  summary_func <- function(x, col){
    c(mean = mean(x[[col]], na.rm=TRUE),
      sd = sd(x[[col]], na.rm=TRUE))
  }
  data_sum<-ddply(data, groupnames, .fun=summary_func, varname)
  data_sum<-plyr::rename(data_sum, c(mean = varname))
  return(data_sum)
}

# Function to summarize data.
## Gives count, mean, standard deviation, standard error of the mean, and
## confidence interval (default 95%).
## data: a data frame.
## measurevar: the name of a column that contains the variable to be summa-
## rized
## groupvars: a vector containing names of columns that contain grouping v
## ariables
## na.rm: a boolean that indicates whether to ignore NA's
## conf.interval: the percent range of the confidence interval (default is
## 95%)

summarySE <- function(data=NULL, measurevar, groupvars=NULL, na.rm=FALSE, conf.interval=.95, .drop=TRUE) {

  # New version of length which can handle NA's: if na.rm==T, don't count
  # them
  length2 <- function (x, na.rm=FALSE) {
    if (na.rm) sum(!is.na(x))
    else length(x)
  }

  # This does the summary. For each group's data frame, return a vector wi-
  # th
  # N, mean, and sd
  datac <- ddply(data, groupvars, .drop=.drop,
```
fun = function(xx, col) {
  cN = length2(xx[[col]], na.rm=na.rm),
  mean = mean (xx[[col]], na.rm=na.rm),
  sd = sd (xx[[col]], na.rm=na.rm)
},
measurevar
}

# Rename the "mean" column

datac <- plyr::rename(datac, c("mean" = measurevar))

datac$se <- datac$sd / sqrt(datac$N)  # Calculate standard error of the mean

# Confidence interval multiplier for standard error
# Calculate t-statistic for confidence interval:
# e.g., if conf.interval is .95, use .975 (above/below), and use df=N-1

ciMult <- qt(conf.interval/2 + .5, datac$N-1)
datac$ci <- datac$se * ciMult

return(datac)
}

PLOTS

Plot 1, Exp. 5.1 Effect of ligand concentration on SEAP secretion from HEK Blue hsTLR2 - evaluation of alternative substrate (Figure 7)

# Add information about the experiment from the plate map. For each Well
# defined in both the reshaped data and the platemap, each resulting row
# will contain the absorbance measurement as well as the additional columns
# and values from the platemap.

setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV file")
rawdata1 <- read.csv2("TidskurverFSL1_plate.csv", dec = ",")
platemap1 <- read.csv("TidskurverFSL1_platemap.csv", sep=";", dec = ",")

# Reshape the data. Instead of rows containing the Time, Temperature,
# and readings for each Well, rows will contain the Time, Temperature, a
# Well ID, and the reading at that Well.

reshaped1 <- gather(rawdata1, Well,OD405,A1:D6)
annotated1 <- inner_join(reshaped1, platemap1, by="Well")

p1<-ggplot(annotated1, aes(x=Time, y= OD405)) + geom_point() + geom_smooth
  (se = TRUE,
   method = "lm", col = "red") +
   facet_grid(~Cons, margins = FALSE, scales = "fixed", space = "fixed",
             shrink = TRUE, labeller = "label_value", as.table = TRUE,
             ...)
```r
switch = NULL, drop = TRUE)

p1 + theme_bw() + xlab("Time (min)")
```

Plot 2, Exp. 5.2.1 Effect of cell number (Figure 8)

```r
setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV filer")
df2 <- read.csv2("Mengde_celler_stigninstall.csv")
df2
```

<table>
<thead>
<tr>
<th></th>
<th>Cons</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20000</td>
<td>0.793</td>
<td>0.766</td>
<td>0.677</td>
<td>0.928</td>
<td>0.714</td>
<td>0.916</td>
<td>0.750</td>
<td>0.665</td>
</tr>
<tr>
<td>2</td>
<td>10000</td>
<td>0.176</td>
<td>NA</td>
<td>0.154</td>
<td>0.210</td>
<td>0.204</td>
<td>0.154</td>
<td>0.189</td>
<td>0.216</td>
</tr>
<tr>
<td>3</td>
<td>5000</td>
<td>0.267</td>
<td>0.184</td>
<td>0.209</td>
<td>0.106</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.191</td>
</tr>
<tr>
<td>4</td>
<td>2500</td>
<td>0.150</td>
<td>0.163</td>
<td>0.140</td>
<td>0.681</td>
<td>0.231</td>
<td>0.189</td>
<td>0.222</td>
<td>0.209</td>
</tr>
<tr>
<td>5</td>
<td>1250</td>
<td>0.127</td>
<td>0.162</td>
<td>0.201</td>
<td>0.355</td>
<td>0.000</td>
<td>0.180</td>
<td>0.343</td>
<td>0.216</td>
</tr>
<tr>
<td>6</td>
<td>625</td>
<td>0.146</td>
<td>0.152</td>
<td>0.200</td>
<td>0.000</td>
<td>0.150</td>
<td>0.012</td>
<td>0.175</td>
<td>0.150</td>
</tr>
<tr>
<td>7</td>
<td>312</td>
<td>0.132</td>
<td>0.134</td>
<td>0.041</td>
<td>0.142</td>
<td>0.135</td>
<td>0.000</td>
<td>0.083</td>
<td>0.118</td>
</tr>
<tr>
<td>8</td>
<td>156</td>
<td>0.135</td>
<td>0.119</td>
<td>0.123</td>
<td>0.119</td>
<td>0.071</td>
<td>0.000</td>
<td>0.123</td>
<td>0.128</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0.115</td>
<td>0.096</td>
<td>0.094</td>
<td>0.096</td>
<td>0.091</td>
<td>0.101</td>
<td>0.114</td>
<td></td>
</tr>
</tbody>
</table>

```r
long2 <- melt(df2, id="Cons")
```

`#Summarize raw data with mean, SD and SEM`

```r
long2mean <- ddply(long2,"Cons", summarize, OD=mean(value, na.rm=TRUE),
```

83
\[
SD = \text{sd}(\text{value, na.rm=TRUE}), \quad SEM = SD / \sqrt{\text{length}(\text{value})}
\]

long2mean

<table>
<thead>
<tr>
<th></th>
<th>Cons</th>
<th>OD</th>
<th>SD</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.1007500</td>
<td>0.009003967</td>
<td>0.003183383</td>
</tr>
<tr>
<td>2</td>
<td>156</td>
<td>0.1022500</td>
<td>0.045653196</td>
<td>0.016140842</td>
</tr>
<tr>
<td>3</td>
<td>312</td>
<td>0.0981250</td>
<td>0.052419837</td>
<td>0.018533211</td>
</tr>
<tr>
<td>4</td>
<td>625</td>
<td>0.0981250</td>
<td>0.078522858</td>
<td>0.027762023</td>
</tr>
<tr>
<td>5</td>
<td>1250</td>
<td>0.1980000</td>
<td>0.114562522</td>
<td>0.040503968</td>
</tr>
<tr>
<td>6</td>
<td>2500</td>
<td>0.2481250</td>
<td>0.178064545</td>
<td>0.062955324</td>
</tr>
<tr>
<td>7</td>
<td>5000</td>
<td>0.1196250</td>
<td>0.108273381</td>
<td>0.038280421</td>
</tr>
<tr>
<td>8</td>
<td>10000</td>
<td>0.1861429</td>
<td>0.025680269</td>
<td>0.009079346</td>
</tr>
<tr>
<td>9</td>
<td>20000</td>
<td>0.7761250</td>
<td>0.099810445</td>
<td>0.035283321</td>
</tr>
</tbody>
</table>

\[\text{p2<-ggplot(long2mean, aes(x=log10(Cons+1), y= OD)) + geom_errorbar(aes(ymin=OD-SEM, ymax=OD+SEM), width=.1) + geom_line() + geom_point() + theme_bw() + labs(x="Log10 Cell number/well", y="Delta OD/hour")}\]

\[\text{p2}\]
Plot 3. Exp 5.2.2 Effect of substrate concentration (Figure 9)

```r
setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV filer")
df3 <- read.csv2("Substratmengde_stigningstall.csv")
df3
```

```
##    Cons    A    B    C    D    E    F    G    H
## 1  100 1.482 1.015 1.444 1.483 1.323 1.264 1.489 1.133
## 2   50 0.935 0.876 0.955 0.781 0.835 0.842 0.843 0.888
## 3   25 0.433 0.419 0.000 0.000 0.000 0.280 0.398
## 4   12 0.059 0.061 0.063 0.035 0.058 0.046 0.041 0.050
## 5    0 0.115 0.143 0.000 0.118 0.114 0.132 0.003
```

```r
long3<-melt(df3,id="Cons")
```

```r
fit3 <- drm(value ~ Cons, data = long3, fct = LL.4())
```

```r
summary(fit3)
```

```
## Model fitted: Log-logistic (ED50 as parameter) (4 parms)
## Parameter estimates:
## b:(Intercept) -3.994869 0.796414 -5.016069 0.000
## c:(Intercept) 0.070415 0.033768 2.085259 0.0442
## d:(Intercept) 1.379141 0.064908 21.247778 0.000
## e:(Intercept) 44.656592 2.153802 20.733842 0.000
## Residual standard error:
## 0.1280088 (36 degrees of freedom)
```

```r
ED50fit3<-ED(fit3,50)
```

```
## Estimated effective doses:
## e:1:50   44.6566 2.1538
```

```r
plot(fit3, broken = TRUE, type="bars", normal = FALSE, ylab="Delta OD/hour",
     xlab="Substrate concentration (ug/ml)"
)
```

```r
abline(v=ED50fit3[1,1], col="red")
```
Plot 4. Exp. 5.3.1 Effect of TLR2 ligand (FSL-1) on HEK Blue hsTLR2 cells [Figure 10]

```r
setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV filer")
df4 <- read.csv2("041016FSL_stigningstall.csv")
df4
```

<table>
<thead>
<tr>
<th></th>
<th>Cons</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0e+00</td>
<td>0.00</td>
<td>0.08</td>
<td>0.09</td>
<td>0.08</td>
</tr>
<tr>
<td>2</td>
<td>1e-05</td>
<td>0.08</td>
<td>0.10</td>
<td>0.06</td>
<td>0.23</td>
</tr>
<tr>
<td>3</td>
<td>1e-04</td>
<td>0.04</td>
<td>0.09</td>
<td>0.09</td>
<td>0.12</td>
</tr>
<tr>
<td>4</td>
<td>1e-03</td>
<td>0.79</td>
<td>0.41</td>
<td>0.61</td>
<td>0.65</td>
</tr>
<tr>
<td>5</td>
<td>1e-02</td>
<td>1.33</td>
<td>1.45</td>
<td>1.36</td>
<td>1.38</td>
</tr>
<tr>
<td>6</td>
<td>1e-01</td>
<td>1.46</td>
<td>1.67</td>
<td>1.61</td>
<td>1.53</td>
</tr>
<tr>
<td>7</td>
<td>1e+00</td>
<td>1.64</td>
<td>2.01</td>
<td>1.75</td>
<td>1.75</td>
</tr>
</tbody>
</table>

```r
long4<-melt(df4, id="Cons")
fit4 <- drm(value ~ Cons, data = long4, fct = LL.4())
summary(fit4)
```

```r
Model fitted: Log-logistic (ED50 as parameter) (4 parms)
```

```r
Parameter estimates:
```
##
##
##
##
##
##
##
##
##
## Estimate  Std. Error  t-value  p-value
## b:(Intercept) -0.89476752  0.12550898 -7.12911149  0.000
## c:(Intercept)  0.85733517  0.03766461  1.5225579  0.141
## d:(Intercept)  1.71518687  0.05070750 33.82511206  0.000
## e:(Intercept)  0.00226114  0.00041424  5.45851168  0.000
##
## Residual standard error:
## 0.1112782 (24 degrees of freedom)

ED50fit4 <- ED(fit4, 50)

##
## Estimated effective doses:
##
## e:1:50  0.00226114  0.00041424

plot(fit4, broken = TRUE, type="bars", normal = FALSE, ylim = c(0, 2.5), ylab="Delta OD/hour", xlab="Ligand concentration (ug/ml)")

abline(v=ED50fit4[1,1], col="red")
Plot 5. Exp. 5.3.1 Effect of TLR2 ligand (PAMC) on HEK Blue hsTLR2 cells (Figure 10)

```r
setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV filer")
df5 <- read.csv2("260916PAMC_stigningstall.csv")
df5

# Cons   A    B    C    D    E    F    G    H
# 1 0e+00 0.03 0.03 0.03 0.03 0.03 0.03 0.04
# 2 1e-08 0.13 0.11 0.11 0.11 0.04 0.11 0.10 0.14
# 3 1e-07 0.20 0.12 0.16 0.20 0.11 0.12 0.12 0.12
# 4 1e-06 0.18 0.14 0.14 0.14 0.16 0.15 0.14 0.21
# 5 1e-05 0.40 0.30 0.26 0.30 0.27 0.38 0.27 0.36
# 6 1e-04 0.43 0.41 0.28 0.49 0.43 0.49 0.49 0.52
# 7 1e-03 0.93 0.88 0.87 0.96 1.01 1.20 1.32
# 8 1e-02 1.08 1.69 1.36 1.29 1.34 1.26 1.29 1.80
# 9 1e-01 2.05 1.97 1.05 1.08 1.87 1.89 1.98 1.64
#10 1e+00 2.10 2.00 2.03 1.96 1.94 1.92 1.98 1.99
#11 1e+01 2.11 2.25 2.26 2.02 2.12 2.23 2.19 2.05

long5<-melt(df5, id="Cons")

fit5 <- drm(value ~ Cons, data = long5, fct = LL.4())
summary(fit5)

## Model fitted: Log-logistic (ED50 as parameter) (4 parms)
##
## Parameter estimates:
##
## Estimate Std. Error t-value p-value
## b:(Intercept) -0.3593640 0.0374265 -9.6018723 0.0000
## c:(Intercept)  0.0593535 0.0356089 1.6668171 0.0993
## d:(Intercept)  2.2428879 0.0863199 25.9834247 0.0000
## e:(Intercept)  0.0032552 0.0010815 3.0098082 0.0035

## Residual standard error:
##
## 0.1580773 (84 degrees of freedom)

ED50fit5<-ED(fit5,50)

## Estimated effective doses:
##
## Estimate Std. Error
## e:1:50 0.0032552 0.0010815

plot(fit5, broken = TRUE, type="bars", normal = FALSE, ylim = c(0,2.5), ylab="Delta OD/hour", xlab="Ligand concentration (ug/ml)")
abline(v=ED50fit5[1,1], col="red")
```

88
Plot 6. Exp. 5.3.1 Effect of TLR2 ligand (HKLM) on HEK Blue hsTLR2 cells (Figure 11)

```r
setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV filer")
df6 <- read.csv2("170117HKLM_stigningstall.csv")
df6
```

<table>
<thead>
<tr>
<th></th>
<th>Cons</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.01775</td>
<td>0.01775</td>
<td>0.01975</td>
<td>0.01942</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.14042</td>
<td>0.12208</td>
<td>0.10942</td>
<td>0.11908</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.15175</td>
<td>0.12575</td>
<td>0.15075</td>
<td>0.15408</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.38308</td>
<td>0.31975</td>
<td>0.32008</td>
<td>0.33742</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.10275</td>
<td>1.10275</td>
<td>1.10275</td>
<td>1.10275</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.10275</td>
<td>1.10275</td>
<td>1.10275</td>
<td>1.10275</td>
<td></td>
</tr>
</tbody>
</table>

```r
long6<-melt(df6,id="Cons")
fit6 <- drm(value ~ Cons, data = long6, fct = LL.4())
summary(fit6)
```

```
##
## Model fitted: Log-logistic (ED50 as parameter) (4 parms)
##
## Parameter estimates:
##
```
## Estimate  Std. Error  t-value  p-value
## b:(Intercept)  -2.4806e+00  1.3396e+00  -1.8518e+00  0.0789
## c:(Intercept)  9.5167e-02  1.3062e-02   7.2857e+00  0.0000
## d:(Intercept)  1.1079e+00  2.2374e-02   4.9517e+01  0.0000
## e:(Intercept)  1.5831e+06  4.0146e+05  3.9434e+00  0.0008
##
## Residual standard error:
## 0.0447847 (20 degrees of freedom)

ED50fit6<-ED(fit6,50)

## Estimated effective doses:
##
##      Estimate Std. Error
## e:1:50  1583104   401455

ED50fit6

##      Estimate Std. Error
## e:1:50  1583104   401455.1

plot(fit6, broken = TRUE, type="bars", normal = FALSE, ylim= c(0,1.5), ylab="Delta OD/hour",
     xlab="Ligand concentration (Cells/ml)"
     abline(v=ED50fit6[1,1], col="red")

---

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Plot 7. Exp. 5.3.1 Effect of TLR2 ligand (\textit{M. marium}) on HEK Blue hsTLR2 cells (Figure 11)

```r
setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV filer")
df7 <- read.csv2("120916MMarinum_stigningstall.csv")
df7

##
##  Cons A  B  C  D  E  F  G  H
##
##   1  0  0  0  0  0  0  0  0
##   2 187500 0.11 0.00 0.00 0.02 0.07 0.07 0.06
##   3 3750000 0.12 0.00 0.09 0.00 0.07 0.09 0.06
##   4 750000 0.22 0.13 0.13 0.12 0.15 0.11 0.09 0.12
##   5 1500000 0.29 0.25 0.22 0.20 0.21 0.20 0.17 0.19
##   6 3000000 0.39 0.33 0.34 0.30 0.29 0.27 0.25 0.27
##   7 6000000 0.51 0.42 0.42 0.42 0.44 0.41 0.36 0.37
##   8 12000000 0.63 0.58 0.58 0.55 0.52 0.51 0.48 0.47
##   9 25000000 0.85 0.83 0.83 0.82 0.83 0.77 0.77 0.74
##  10 50000000 0.92 0.90 0.94 0.98 0.97 0.93 0.92 0.84

long7< melt(df7, id="Cons")

fit7 <- drm(value ~ Cons, data = long7, fct = LL.4())
summary(fit7)

##
## Model fitted: Log-logistic (ED50 as parameter) (4 parms)
##
## Parameter estimates:
##
## b:(Intercept) -6.8707e-01  5.9636e-02 -1.1521e+01  0.0000
## c:(Intercept) -9.0169e-04  1.3909e-02 -6.4826e-02  0.9485
## d:(Intercept)  1.4871e+00  1.6301e-01  9.1226e+00  0.0000
## e:(Intercept)  2.2954e+07  7.4647e+06  3.0751e+00  0.0029

## Residual standard error:
##
##  0.04525778 (76 degrees of freedom)

ED50fit7< - ED(fit7, 50)

## Estimated effective doses:
##
## e:1:50  22954458  7464704

ED50fit7

##
## e:1:50  22954458  7464704

# allplot< - par(mfcol = c(1,3))
# allplot
plot(fit7, broken = TRUE, type = "bars", normal = FALSE, ylim = c(0, 1.5),
```
Plot 8 Exp. 5.3.2 Effect of TLR3 ligand (Poly I:C) on HEK Blue hsTLR2 cells (Figure 12)

```r
setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV filer")
df8 <- read.csv2("FSL1_1time_PLCListingstall.csv")
df8

##    Cons    A    C    D    E    F    G    H
## 1 0e+00 0.03 0.03 0.03 0.02 0.02 0.05 0.08
## 2 2e-06 0.18 0.06 0.05 0.04 0.04 0.06 0.08
## 3 2e-05 0.05 0.05 0.05 0.05 0.06 0.07 0.09
## 4 2e-04 0.11 0.03 0.04 0.13 0.05 0.04 0.07
## 5 2e-03 0.05 0.03 0.03 0.02 0.00 0.04 0.07
## 6 2e-02 0.00 0.03 0.03 0.04 0.00 0.05 0.06
## 7 2e-01 0.01 0.06 0.07 0.07 0.07 0.07 0.16
## 8 2e+00 0.12 0.05 0.10 0.13 0.12 0.11 0.13
## 9 2e+01 0.21 0.21 0.19 0.22 0.00 0.24 0.27
##10 2e+02 0.38 0.31 0.30 0.31 0.00 0.33 0.48

long8<-melt(df8,id="Cons")

fit8 <- drm(value ~ Cons, data = long8, fct = LL.4())
summary(fit8)
```
## Model fitted: Log-logistic (ED50 as parameter) (4 parms)

## Parameter estimates:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Std. Error</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>b:(Intercept)</td>
<td>-3.1655e-01</td>
<td>4.7195e-02</td>
<td>-6.7073e+00</td>
<td>0.0000</td>
</tr>
<tr>
<td>c:(Intercept)</td>
<td>4.6899e-02</td>
<td>1.0139e-02</td>
<td>4.6257e+00</td>
<td>0.0000</td>
</tr>
<tr>
<td>d:(Intercept)</td>
<td>1.0905e+01</td>
<td>5.9627e+00</td>
<td>1.8288e+00</td>
<td>0.0719</td>
</tr>
<tr>
<td>e:(Intercept)</td>
<td>2.4254e+07</td>
<td>1.0000e+01</td>
<td>2.4254e+06</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

## Residual standard error:

0.06115369 (66 degrees of freedom)

ED50fit8<-ED(fit8,50)

## Estimated effective doses:

<table>
<thead>
<tr>
<th>Estimate</th>
<th>Std. Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>e:1:50</td>
<td>24253905</td>
</tr>
</tbody>
</table>

plot(fit8, broken = TRUE, type="bars", normal = FALSE, ylim = c(0,2.5), ylab="Delta OD/hour", xlab="Ligand concentration (ug/ml)")

abline(v=ED50fit8[1,1], col="red")
Plot 9 Exp. 5.4.1 Effect of SEAP assay duration with TLR3 ligand (Poly I:C) (Figure 14)

```r
setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV file")
rawdata9 <- read.csv2("TidskurverTLR3_plate.csv", dec = ",")
platemap9 <- read.csv("TidskurverTLR3_platemap.csv", sep=";", dec=";")

reshaped9 <- gather(rawdata9, Well,OD405,A8:H12)
annotated9 <- inner_join(reshaped9, platemap9, by="Well")

p9<-ggplot(annotated9, aes(x=Time, y=OD405)) + geom_point() + geom_smooth (se = TRUE, 
method = "lm", col = "red") + 
  facet_grid(~Cons, margins = FALSE, scales = "fixed", space = "fixed", 
  shrink = TRUE, labeller = "label_value", as.table = TRUE, 
  switch = NULL, drop = TRUE)

p9 + theme_bw() + xlab("Time (Hours)")
```

Plot 10 Exp. 5.4.2 Effect of exposure time of Poly I:C (Figure 15)

```r
setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV file")
rawdata10 <- read.csv2("TidskurverPLC_plate.csv", dec = ",")
platemap10 <- read.csv("TidskurverPLC_platemap.csv", sep=";", dec=";")
```
```r
reshaped10 <- melt(rawdata10, id=c("Time"), variable.name="Well", value.name="OD405")
reshaped10 <- gather(reshaped10, Well,OD405,A3:H12)
annotated10 <- inner_join(reshaped10, platemap10, by="Well")

p10 <- ggplot(annotated10, aes(x=Time, y= OD405)) + geom_point() + geom_smooth(se = TRUE, method = "lm", col = "red") + facet_grid(~Day, margins = FALSE, scales = "fixed", space = "fixed", shrink = TRUE, labeller = "label_value", as.table = TRUE, switch = NULL, drop = TRUE)

p10 + theme_bw() + xlab("Time (min)")
```

Plot 11 Exp. 5.4.3 Effect of FBS concentration on SEAP activity (Figure 16)
```r
setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV filer")
df11 <- read.csv2("Bare_mediet_stigningstall.csv")
df11
##
##    Cons     A     B     C     D
## 1  0.00 -0.00700 -0.00800 -0.00800 -0.00650
## 2  0.60  0.00700  0.00275  0.00500  0.00525
```
```r
#long11

long11mean<-ddply(long11,"Cons", summarize, OD=mean(value,na.rm=TRUE),
                   SD=sd(value,na.rm=TRUE), SEM=SD/sqrt(length(value)))
long11mean

p11<-ggplot(long11mean,aes(x=Cons, y=OD)) +
  geom_errorbar(aes(ymin=OD-SEM, ymax=OD+SEM), width=.1) +
  geom_line() +
  theme_point() +
  labs(x="Serum concentration (%)", y="Delta OD/hour")

p11
```
Plot 12 Exp. 5.4.4 Effect of TLR3 ligand (Poly I:C) on HEK Blue hsTLR3 cells (Figure 17)

```r
setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV file")
df12 <- read.csv2("071216hsTLR3_stigningstall.csv")
dl12
```

```r
#long12
```
fit12 <- drm(value ~ Cons, data = long12, fct = LL.4())
summary(fit12)

##
## Model fitted: Log-logistic (ED50 as parameter) (4 parms)
##
## Parameter estimates:
##
##                  Estimate Std. Error  t-value p-value
## b:(Intercept) -1.251930 0.2462585 -5.0838049 0
## c:(Intercept)  0.123202 0.0131300  9.3832191 0
## d:(Intercept)  0.778963 0.0203713 38.2381916 0
## e:(Intercept)  0.021824 0.0045958  4.7489191 0
##
## Residual standard error:
##
## 0.06758149 (44 degrees of freedom)

ED50fit12<-ED(fit12,50)

##
## Estimated effective doses:
##
## e:1:50 0.021824 0.0045958

plot(fit12, broken = TRUE, type="bars", normal = FALSE, ylab="Delta OD/hour",
     xlab="Ligand concentration (ug/ml)")
abline(v=ED50fit12[1,1], col="red")
Plot 13

Exp. 5.4.5 Effect of TLR3 ligand (Poly I:C) on HEK Blue hsTLR2 cells after four hours (Figure 18)

```r
setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV file")
df13 <- read.csv2("110117PLC_stigningstall.csv")
df13
```

```r
##
## Cons  A   B   C   D
## 1 0e+00 0.0515625 0.0508125 0.0508125
## 2 1e-07 0.0458125 0.0418125 0.0418125
## 3 1e-06 0.0595625 0.0478125 0.0430625
## 4 1e-05 0.0410625 0.0990625 0.0975625
## 5 1e-04 0.0515625 0.2535625 0.0415625
## 6 1e-03 0.0428125 0.0430625 0.0430625
## 7 1e-02 0.3445625 0.2213125 0.2695625
## 8 1e-01 0.2795625 0.2195625 0.2558125
## 9 1e+00 0.3658125 0.3663125 0.3548125
## 10 1e+01 0.8055625 0.8055625 0.8055625
## 11 1e+02 0.8055625 0.8055625 0.8055625

long13<-melt(df13,id="Cons")

fit13 <- drm(value ~ Cons, data = long13, fct = LL.4())
summary(fit13)
```
## Model fitted: Log-logistic (ED50 as parameter) (4 parms)

## Parameter estimates:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Std. Error</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>b:(Intercept)</td>
<td>0.401926</td>
<td>0.085860</td>
<td>-4.681206</td>
<td>0.0000</td>
</tr>
<tr>
<td>c:(Intercept)</td>
<td>0.068387</td>
<td>0.020549</td>
<td>3.327975</td>
<td>0.0019</td>
</tr>
<tr>
<td>d:(Intercept)</td>
<td>1.004316</td>
<td>0.115566</td>
<td>8.690432</td>
<td>0.0000</td>
</tr>
<tr>
<td>e:(Intercept)</td>
<td>1.884851</td>
<td>1.399981</td>
<td>1.346341</td>
<td>0.1858</td>
</tr>
</tbody>
</table>

## Residual standard error:

0.07822543 (40 degrees of freedom)

ED50fit13 <- ED(fit13, 50)

## Estimated effective doses:

<table>
<thead>
<tr>
<th>Estimate</th>
<th>Std. Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>e:1:50</td>
<td>1.8849</td>
</tr>
</tbody>
</table>

plot(fit13, broken = TRUE, type="bars", normal = FALSE, ylab="Delta OD/hour", xlab="Ligand concentration (ug/ml)")

abline(v=ED50fit13[1,1], col="red")
Plot 14 Exp. 5.5.1 Effect of TLR3 ligand (Poly I:C) on HEK Blue hsTLR2 + ssTLR3 cells (Figure 19)

```r
setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV filer")
df14 <- read.csv2("071216ssTLR3_stigningstall.csv")
df14
```

```
# Cons     A            B        C        D
# 1        0.02925     0.02725  0.02775
# 2        0.06275     0.06050  0.06500
# 3        0.07350     0.08200  0.07750
# 4        0.06200     0.05925  0.05650
# 5        0.06300     0.05575  0.05825
# 6        0.06650     0.05600  0.05425
# 7        0.07275     0.06175  0.05500
# 8        0.07425     0.07625  0.07700
# 9        0.19550     0.16225  0.27950
# 10       0.47925     0.35750  0.35850
# 11       0.50050     0.46600  0.49750
# 12       0.56050     0.54225  0.58525
long14<-melt(df14,id="Cons")
fit14 <- drm(value ~ Cons, data = long14, fct = LL.4())
summary(fit14)
```

```
## Model fitted: Log-logistic (ED50 as parameter) (4 parms)
## Parameter estimates:
##
## Estimate Std. Error    t-value  p-value
## b:(Intercept)         -0.601739  0.0897196 -6.7068835   0.0000
## c:(Intercept)         0.052004  0.0080471   6.4623711   0.0000
## d:(Intercept)         0.562357  0.0266081  21.1347896   0.0000
## e:(Intercept)         0.270761  0.0917478   2.9511476   0.0051
##
## Residual standard error:
## 0.04072125 (44 degrees of freedom)
ED50fit14<-ED(fit14,50)
```

```
## Estimated effective doses:
##
## Estimate Std. Error
## e:1:50     0.270761     0.091748
plot(fit14, broken = TRUE, type="bars", ylim = c(0,0.8), normal = FALSE)
    ylab="Delta OD/hour", xlab="Ligand concentration (ug/ml)"
abline(v=ED50fit14[1,1], col="red")
```
Plot 15 Exp. 5.5.2 Analysis of TLR expression by QPCR (Figure 21)

```r
setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV filer")
hek_pcr1 <- read.csv2("hek_pcr2.csv", header=TRUE)

long <- hek_pcr1 %>% gather(Gene, RE, hsActB:ssTLR3)

g <- ggplot(long, aes(x=Gene, y=RE)) + geom_bar(stat="identity") + facet_wrap(~Cell_line)

g + theme(axis.text.x=element_text(angle=45, hjust=1))
```
Plot 16 Exp. 5.6 Analysis of ssTLR3 signaling in a fish cell line (EPC cells) (Figure 24)

setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV filer")
df16<- read.csv2("EPC_QPCR.csv")

long16<-melt(df16, id.vars = c("Cell_type","Time"), variable.name = "Gene" ,
               value.name = "RE")
colnames(long16)<-c("Cell_type","Time","Gene","RE")

# Call summary function - tgc
tgc <- summarySE(long16, measurevar="RE", groupvars=c("Time","Gene","Cell_type"))

# Standard error of the mean
p16<-ggplot(tgc, aes(x=Time, y=RE, colour=Cell_type)) +
  geom_errorbar(aes(ymin=RE-se, ymax=RE+se), width=.1) +
  geom_line() +
  geom_point() +
  facet_wrap(~Gene, ncol = 2, scales ="free_y")

p16
#Test for difference in expression at each time point:
testresults16<-vector("list", 30)

for(j in seq(1,80,1)) {
  testresults16[[j]]<-t.test2(tgc[j+1,5],tgc[j,5],tgc[j+1,7],tgc[j,7],3,3)
}

#Convert list of t-test output to dataframe
res16<-do.call(rbind,testresults16)
colnames(res16)<-c("Diffeence_of_means","Std_error", "T","p_value")

#Delete every second row (wrong combinations)
delete<-seq(2,length(res16),2)
res16<-res16[-delete,]

#Combine dataframe with testresults with dataframe containing time point and gene name
test16<-tgc[,c(1,2)])
test16<-distinct(test16)
test16<-cbind(test16,res16)
test16<-filter(test16[complete.cases(res16),])

#Filter out significant values
significant16<-test16
significant16<-significant16[significant16$p_value <=0.05,]
# Round the numbers

```r
is.num <- sapply(significant16, is.numeric)
significant16[is.num] <- lapply(significant16[is.num], round, 3)
significant16
```

```
## Time Gene Difference_of_means Std_error T p_value
## 13 1 IRF7 2.756 0.338 8.155 0.013
## 14 1 IFN1 1.350 0.253 5.336 0.033
## 15 1 ssTLR3 4.238 0.601 7.055 0.019
## 16 1 IRF3 20.192 3.678 5.490 0.032
## 17 1 PKR 7.343 1.650 4.451 0.037
## 18 1 Mx1 80.555 14.875 5.415 0.031
## 19 2 TLR3 150.424 15.433 9.747 0.010
## 20 2 IRF7 24.489 2.454 9.978 0.010
## 21 2 IFN1 0.815 0.192 4.242 0.049
## 22 2 ssTLR3 39.148 5.143 7.612 0.017
## 23 2 IRF3 38.728 8.526 4.542 0.045
## 24 3 TNFa 42.469 2.890 14.694 0.005
## 25 3 TLR3 84.405 8.750 9.646 0.010
## 26 3 IRF7 16.771 1.019 16.460 0.004
## 27 3 ssTLR3 24.416 0.654 37.314 0.001
## 28 3 IRF3 18.998 3.085 6.158 0.025
```

Plot 17 Exp. 5.6 Analysis of ssTLR3 signaling in a fish cell line (EPC cells) (Figure 25)

```r
# Correlation matrix for expression in EPC cells
setwd("~/Documents/Farmasi 10. semester MASTER/Masterdeler/R-CSV filer")
df17<-dplyr::select(df16, 3:12)
df17<-slice(df17, c(2:10,12:20))
M<-cor(df17)
corrplot.mixed(M, lower = "ellipse", upper = "number", order="FPC", tl.col = "black")
```
#corrplot(M, order = "FPC", method = "ellipse", type = "lower")

**t-tests**

*Statistical testing of differences in calculated parameters:*

*Testing the difference in ED50 values*
*between FSL-1 and PAMC on HEK Blue hsTLR2 cells:*
```r
t.test2(ED50fit4[1,1],ED50fit5[1,1],ED50fit4[1,2],ED50fit5[1,2],8,8)
```

<table>
<thead>
<tr>
<th>Difference of means</th>
<th>Std Error</th>
<th>t</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.0009940352</td>
<td>0.0004094635</td>
<td>-2.4276523791</td>
</tr>
</tbody>
</table>

*Testing the difference in ED50 values*
*between M.marinum and HKLM on HEK Blue hsTLR2 cells:*
```r
t.test2(ED50fit6[1,1],ED50fit7[1,1],ED50fit6[1,2],ED50fit7[1,2],8,8)
```

<table>
<thead>
<tr>
<th>Difference of means</th>
<th>Std Error</th>
<th>t</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2.137135e+07</td>
<td>2.642985e+06</td>
<td>-8.086066e+00</td>
</tr>
</tbody>
</table>

*Testing the difference in ED50 values*
*between FSL-1 and Poly I:C in HEK Blue hsTLR2 cells stimulated for 1 hour*
# Testing the difference in ED50 values
# between HEK Blue hsTLR2 + ssTLR3 + HEK Blue hsTLR2 cells stimulated with Poly I:C:
t.test2(ED50fit13[1,1],ED50fit14[1,1],ED50fit13[1,2],ED50fit14[1,2],8,8)

## Difference of means   Std Error       t
##  -0.248936537    0.0324784331    -7.6646719137
## p-value
##  0.0001166508

# Testing the difference in ED50 values
# between HEK Blue hsTLR2 + ssTLR3 + HEK Blue hsTLR3 cells stimulated with Poly I:C:
t.test2(ED50fit12[1,1],ED50fit14[1,1],ED50fit12[1,2],ED50fit14[1,2],8,8)

## Difference of means   Std Error       t
##  -1.863026531    0.494970723    -3.763912577
## p-value
##  0.007038472

sessionInfo()

## R version 3.3.2 (2016-10-31)
## Platform: x86_64-apple-darwin13.4.0 (64-bit)
## Running under: OS X Yosemite 10.10.5
##
## locale:
## [1] C
##
## attached base packages:
## [1] stats graphics grDevices utils datasets methods base
##
## other attached packages:
## [1] readr_1.0.0    corrplot_0.77    knitr_1.15.1    dplyr_0.5.0
## [5] plyr_1.8.4      tidyr_0.6.1      lmtest_0.9-35    zoo_1.7-14
## [9] sandwich_2.3-4  polynom_1.3-9    drc_3.0-1         MASS_7.3-45
##[13] ggplot2_2.2.1    reshape2_1.4.2
## loaded via a namespace (and not attached):

- Rcpp_0.12.9
- nloptr_1.0.4
- tools_3.3.2
- digest_0.6.12
- lme4_1.1-12
- evaluate_0.10
- tibble_1.2
- gtable_0.2.0
- nlme_3.1-128
- lattice_0.20-34
- mgcv_1.8-17
- Matrix_1.2-8
- DBI_0.5-1
- yam1_2.1.14
- parallel_3.3.2
- mvtnorm_1.0-5
- SparseM_1.74
- stringr_1.2.0
- MatrixModels_0.4-1
- gtools_3.5.0
- rprojroot_1.2
- grid_3.3.2
- nnet_7.3-12
- R6_2.2.0
- plotrix_3.6-4
- survival_2.40-1
- rmarkdown_1.3
- multcomp_1.4-6
- TH.data_1.0-8
- minqa_1.2.4
- car_2.1-4
- magrittr_1.5
- codetools_0.2-15
- backports_1.0.5
- scales_0.4.1
- htmltools_0.3.5
- splines_3.3.2
- assertthat_0.1
- pbkrtest_0.4-6
- colorspace_1.3-2
- labeling_0.3
- quantreg_5.29
- stringi_1.1.2
- lazyeval_0.2.0
- munsell_0.4.3

# End of file