Immunostimulatory potential of outer membrane vesicles derived from *Aeromonas salmonicida* - possible use in vaccines?

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Sven Ostermann

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Dekan*in: Prof. Dr. Gerald Kerth

1. Gutachter*in: Prof. Dr. Dörte Becher

2. Gutachter*in: Prof. Dr. Dieter Steinhagen

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Aeromonas salmonicida subspecies salmonicida	*A. salmonicida*
Analysis of variance	ANOVA
Base pair	bp
Cell mediated cytotoxicity	CMC
Colony forming units	CFU
Cytotoxic T-lymphocyte-associated protein 4	CTLA4
Cycle threshold	Ct
Ethylenediaminetetraacetic acid	EDTA
Edwardsiella tarda	*E. tarda*
Elongation Factor-1α	EF1α
Escherichia coli	*E. coli*
Enzyme-linked immunosorbent assay	ELISA
Fluorescence Activated Cell Sorting	FACS
Forward scatter	FSC
Fragment crystallisable	Fc
Francisella noatunensis	*F. noatunensis*
Gill- associated lymphoid tissue	GIALT
Gut-associated lymphoid tissue	GALT
(Heavy + Light chains)	(H+L)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	HEPES
Immunoglobulin	Ig
Immunoglobulin heavy	IgH
Immunoglobulin light	IgL
Inducible nitric oxide synthases	iNOS
Infectious haematopoietic necrosis virus	IHNV
Infectious pancreatic necrosis virus	IPNV
Infectious salmon anaemia virus	ISAV
Interferon γ	IFNγ
Interleukin	IL

Interleukin-1 receptor-associated kinase 1	IRAK1
Intra muscular	i.m.
Intraperitoneal	i.p.
Koloniebildende Einheiten	KBE
Lipopolysaccharide	LPS
Luria Broth	LB
Major histocompatibility complex	MHC
Modified complete Freund's adjuvant	MFCA
Monoclonal antibody	mab
Myeloid differentiation primary response 88	Myd88
Nasopharynx- associated lymphoid tissue	NALT
Neisseria meningitidis	*N. meningitidis*
Nitric oxide	NO
Nonspecific cytotoxic cells	NCC
Nucleotide-binding oligomerization domain-containing protein 1	NOD1
Oncorhynchus mykiss	*O. mykiss*
Optical density	OD
Outer membrane vesicle	OMV
Paralichthys olivaceus	*P. olivaceus*
Pathogen associated molecular pattern	PAMP
Pattern recognition receptors	PRR
Piscirickettsia salmonis	*P. salmonis*
Polyethylene glycol	PEG
Recombination-activating gene	RAG
Retinoic acid-inducible gene I	RIG-I
Reverse transcription-quantitative polymerase chain reaction	RT-qPCR
Salmonella typhimurium	*S. typhimurium*
Salmonid alphavirus	SAV
Sideward scatter	SSC
Skin- associated lymphoid tissue	SALT

Spring viremia of carp virus	SVCV
T helper	Th
Terminal deoxynucleotidyl transferase	TdT
Tissue culture infection dose$_{50}$	TCID$_{50}$
Toll-like receptor	TLR
Tumor necrosis factor α	TNFα
Vibrio anguillarum	*V. anguillarum*
Viral hemorrhagic septicemia virus	VHSV
Viral nervous necrosis virus	VNNV
Whole genome duplication	WGD
Yersinia ruckeri	*Y. ruckeri*

1. Abstract

Gram-negative bacteria are known to naturally produce outer membrane vesicles (OMVs), which are closed nanoparticles (10 to 450 nm) containing virulence factors and pathogen associated molecular patterns (PAMPs) (1, 2). For over 20 years, OMVs of *Neisseria meningitidis* (*N. meningitidis*), in combination with three purified outer membrane proteins, have been successfully used as parts of human vaccines which illustrates the safety and potential of OMV based vaccines (1, 3-5). So far only little is known about the OMVs of fish pathogenic bacteria (6-9). The production of OMVs has been described for the fish pathogenic gram-negative bacterium *Aeromonas salmonicida (A. salmonicida)* (10) which is the causative agent of furunculosis resulting in high morbidity and mortality of salmonid fish (11).

The immunostimulatory potential of OMVs derived from *A. salmonicida* as well as the possibility of establishing an oral vaccine model in *Oncorhynchus mykiss* (*O. mykiss*) (Rainbow trout) has been investigated in this study by conducting *in vitro* and *in vivo* experiments. Innate immune cells such as macrophages are one of the first cells to respond to pathogens once they breach the skin barrier (12), therefore the monocyte/macrophage cell line RTS-11 as well as leukocytes from the head kidney, consisting of a high percentage of phagocytic cells (13) have been investigated. Additionally, leukocytes isolated from the peritoneal cavity as the main target for injectable vaccines (14) have been studied in the *in vitro* experiments. These experiments indicate that OMVs derived from *A. salmonicida* are recognized by the monocyte/macrophage cell line RTS-11 as well as by leukocytes from the head kidney resulting in significant changes of the mRNA expression pattern of early inflammatory markers (IL-1β, IL-6, IL-8, IL-10, TGFβ). Having used the established peritoneal inflammation model of rainbow trout (15) it could be shown that intraperitoneal (i.p.) vaccination of rainbow trout with OMVs results in a similar local immune response, especially in the recruitment of myeloid cells, compared to the injection of inactivated bacteria. The systemic cellular immune response differed between the two vaccine groups, even though a similar humoral immune response could be observed. Interestingly, i.p. vaccination with 10 µg of OMVs resulted in similar antibody titers as observed for fish, that were i.p. vaccinated with 10^8 CFU of inactivated *A. salmonicida*. The similar antibody titers after vaccination with OMVs might be explained by a stronger activation of $CD8^-$ T cells (likely $CD4^+$ T cells (16)) in the head kidney as well as in the blood in the OMV vaccinated group alone, which might result in an increased stimulation of B cells to produce antibodies.

Oral vaccination has been described as the ideal vaccination method for fish, but only few vaccines for oral application are licensed (17, 18). Therefore, the established oral model for vaccination of rainbow trout with attenuated viral hemorrhagic septicemia virus (VHSV) (19) was adapted to be used for inactivated *A. salmonicida*, even though initial trials indicated great similarities in the cellular response after i.p. and oral vaccination with inactivated strains of *A. salmonicida*, particularly in the response of the myeloid cells and lymphocytes in the target organs as well as the thrombocytes in the spleen. This could not be confirmed in a second oral vaccination trial. These results show how challenging the development of oral vaccines for fish is. The main challenge is the reproducibility of reliable results (20), since this is influenced by the difference in uptake of vaccine pellets or antigen degradation in the gut (21). Future oral vaccine trials should investigate different vaccination regimes, e.g., consecutive feeding, or a different composition of vaccine pellets, in order to further investigate the possibility of establishing an oral vaccine model for trout and so that future vaccine candidates, like OMVs, can be reliably tested in fish.

1. Zusammenfassung

Es ist bekannt, dass Gram-negative Bakterien äußere Membran Vesikel (OMVs) produzieren, welches geschlossene Nanopartikel sind (10 bis 450 nm) die Virulenzfaktoren sowie pathogen-assoziierter molekulare Strukturen beinhalten (PAMPs) (1, 2). Seit über 20 Jahren werden die OMVs von *Neisseria meningitidis* in Verbindung mit drei aufgereinigten Proteinen der äußeren Membran erfolgreich als Impfstoff eingesetzt, dies zeigt die Sicherheit und das Potenzial von Impfstoffen auf Basis von OMVs (1, 3-5). Bisher ist nur wenig über die OMVs von pathogenen Fischbakterien bekannt (6-9). Die Produktion von OMVs wurde für das Gram-negative Fischbakterium *Aeromonas salmonicida* beschrieben (10), welches für die Erkrankung Furunkulose verantwortlich ist, wodurch eine hohe Krankheits- und Sterblichkeitsrate in Salmoniden hervorgerufen wird (11).

Die Analyse des immunstimulatorischen Potenzials von OMVs, isoliert von *A. salmonicida*, sowie die Möglichkeit, ein Model für die Testung von oralen Impfstoffen in *Oncorhynchus mykiss* (Regenbogenforelle) zu etablieren, waren die Hauptthemen dieser Arbeit. Zellen des angeborenen Immunsystems, wie z.B. Makrophagen, gehören zu den ersten Zellen, die auf Pathogene reagieren, welche den natürlichen Schutz z.B. der Haut durchbrochen haben (12). Um die Reaktion der Zellen des angeborenen Immunsystems auf OMVs isoliert von *A. salmonicida* zu

untersuchen, wurde die Monozyten/Makrophagen-Zelllinie RTS-11 sowie Leukozyten der Kopfniere, welche zu großen Teilen aus phagozytierenden Zellen bestehen (13), und Leukozyten aus der Bauchhöhle, welche das Hauptziel für injizierbare Impfstoffe ist (14), in *in vitro*-Experimenten untersucht. Diese Experimente haben gezeigt, dass OMVs, isoliert von *A. salmonicida*, von der Monozyten/Makrophagen Zelllinie-RTS-11 sowie von Leukozyten der Kopfniere erkannt werden und zu signifikanten Unterschieden in der mRNA-Expression von frühen Entzündungsmarkern führen (IL-1β, IL-6, IL-8, IL-10, TGFβ). Durch die Nutzung des etablierten Entzündungsmodells der Peritonealhöhle in *Oncorhynchus mykiss* (Regenbogenforellen) (15) konnte gezeigt werden, dass die intra-peritoneale (i.p.) Impfung von Regenbogenforellen mit OMVs zu einer ähnlichen zellulären Immunantwort führt, insbesondere dem Einstrom von myeloiden Zellen, wie nach einer i.p. Impfung mit inaktivierten Bakterien beobachtet wurde. Die systemische zelluläre Immunantwort zeigte hingegen Unterschiede zwischen den beiden Gruppen, obwohl ein ähnlicher Verlauf der humoralen Immunantwort beobachtet werden konnte. Interessanterweise hat die i.p. Impfung mit 10 µg OMVs zu einem vergleichbar hohen Antikörpertiter geführt wie bei Fischen, die mit 10^8 Koloniebildende Einheiten (KBE) inaktivierter *A. salmonicida* geimpft wurden. Dies könnte vielleicht durch eine frühzeitige und stärkere Aktivierung von $CD8^-$ T-Zellen (wahrschein $CD4^+$ T-Zellen (16)) in der Kopfniere sowie im Blut durch die Impfung mit OMVs erklärt werden, was zum Beispiel in dieser Gruppe allein zu einer erhöhten Stimulation von B-Zellen zur Antikörperproduktion führen könnte.

Die orale Impfung wurde als die ideale Strategie zur Impfung von Fischen beschrieben, aber nur wenige oral applizierbare Impfstoffe sind kommerziell erhältlich (17, 18). Um ein Modell für die Analyse oraler Impfstoff zu entwickeln wurde das bereits etablierte Modell zur oralen Impfung von Regenbogenforellen mit attenuiertem VHSV (19) für die Nutzung mit inaktivierten *A. salmonicida* abgeändert. Die ersten Tierversuche zeigten eine große Ähnlichkeit in der zellulären Immunantwort zwischen i.p. und oraler Immunisierung, insbesondere bei der Zellmigration der myeloiden Zellen und der Lymphozyten im Zielorgan, sowie den Thrombozyten in der Milz. Weitere Tierversuche konnten dies nicht bestätigen, wodurch wieder einmal die Schwierigkeiten in der Entwicklung eines oralen Impfstoffes verdeutlicht werden. Das größte Problem in der Entwicklung oraler Impfstoffe liegt in der Reproduzierbarkeit der Ergebnisse (20), bedingt durch zum einen die unterschiedliche Anzahl an aufgenommenen Impfpellets und zum anderen durch die Zersetzung von Antigen im Magen der Fische (21).

Zukünftige orale Impfversuche sollten auch die Möglichkeit von unterschiedlichen Fütterungsregimen, wie zum Beispiel aufeinanderfolgenden Fütterungen, oder die Zusammensetzung der Impfpellets betrachten, um die Etablierung eines oralen Impfstoffmodells für die Analyse von möglichen Impfstoffkandidaten, wie zum Beispiel OMVs, in Regenbogenforellen zu etablieren.

2. Introduction

2.1. Fish aquaculture

Capture fisheries were the main source of seafood until the 1970s whereas only 3-6 % of the global fish were farmed in aquacultures at that time (22). Increasing demands for seafood and a growing human population led to excessive overfishing of natural fish stocks and resulted in aquacultures being the fastest growing livestock-producing sector worldwide (22), with approximately 46 % of the consumed fish produced in aquacultures nowadays (23). In 2014, 2.93 million tons of fish were produced in Europe alone, with 64 % of the produced fish belonging to the family *Salmonidae* (24). Most abundantly produced is the Atlantic salmon, *Salmo salar* (900 kt/year), followed by the rainbow trout, *Oncorhynchus mykiss* (320 kt/year) (24), whereas the sea bream *Sparus aurata*, sea bass *Dicentrarchus labrax*, carp *Cyprinus carpio* and turbot *Scophthalmus maximus* have a production volume ranging from 150 kt/year to 10 kt/year in European aquaculture (25). In Germany, the main fish produced in aquaculture are rainbow trout and carp (26).The dominating aquaculture systems used in Germany are recirculating systems such as earthen ponds or raceways (27), while worldwide around 50 % of aquaculture products were produced via large sea cages in the open sea and only 3 % in recirculating systems in 2010 (28). Regardless of which aquaculture production system is used, the high density of fish and the resulting stress increase the risk of disease transmission leading to high mortality rates (29). Since rainbow trout are the dominating aquaculture species in Germany it has been used as a model organism in different studies, as well as in this thesis in order to further examine the cellular immune response after vaccination with different antigens.

2.2. Rainbow trout

Rainbow trout belong to the superclass Osteichthyes (bony fish) (30), the family *Salmonidae* and the genus *Oncorhynchus* (31). They were originally located in the Pacific Ocean along the east coast of Russia as well as on the west coast of Canada and the USA, but nowadays rainbow trout have been introduced to all continents (32) due to their economic value in aquacultures (18). This fish species consists of two life strategies, resident rainbow trout spend their whole life in fresh water while anadromous, for example steelhead rainbow trout, migrate to the oceans for maturation and return to fresh water to reproduce (33). Rainbow trout are a

carnivorous fish species (34) and in aquacultures they are mainly fed with food pellets containing a mix of fishmeal and fish oil as well as terrestrial protein sources (35).

Like all vertebrate species, rainbow trout have undergone whole genome duplication (WGD) resulting in the duplication of the complete genome sequence (32). Their evolutionary position can be seen in figure 1. Unlike other vertebrates the *Salmonidae* have undergone 4 WGD resulting in up to 8 copies of each gene (36). Most copies of the genes have been lost but others are involved in new functional pathways which might be the reason for the biological diversity and evolutionary success of the *Teleostei* and *Salmonidae* (36). For example, it has been demonstrated that different paralogues of one gene have different functions in immune response and the general development of fish (37, 38).

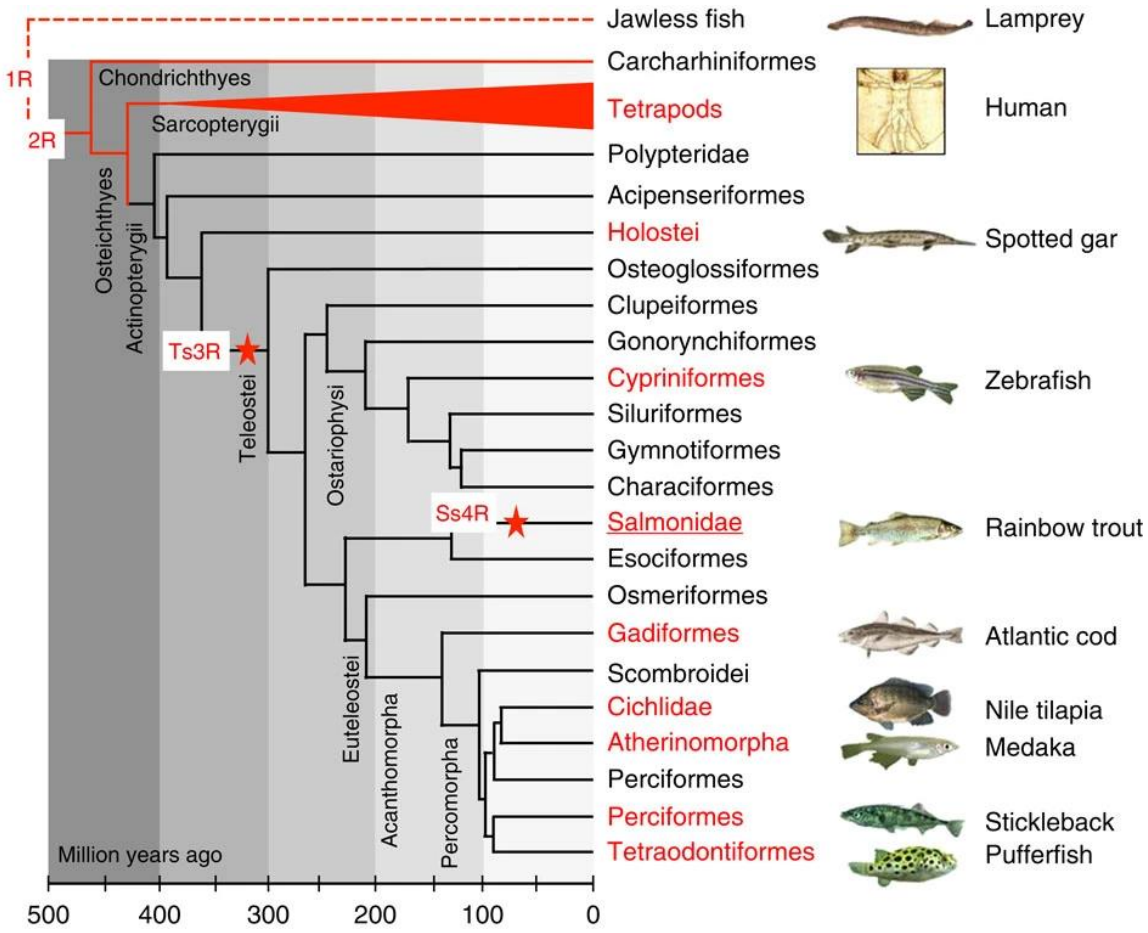

Figure 1: Evolutionary position of the rainbow trout.

"This tree is based on the time-calibrated phylogeny information (Near *et al.*, 2012[(39)]) except for the additional branches in red. The red stars show the position of the teleost-specific (Ts3R) and the salmonid-specific (Ss4R) whole-genome duplications. Groups of species in which a genome sequence is available are shown in red bold type, with one example in each group. "(32)[1]

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2.3. Diseases in aquaculture

Even though rainbow trout have existed since millions of years and are well adapted to life in the wild, the conditions created in aquaculture make fish more susceptible to different diseases. Apart from the stress caused by close contact of fish in aquaculture systems, the high organic content as well as the low levels of dissolved oxygen increase the disease susceptibility of aquaculture fish. The close proximity of the fish also enables easy spread of infections. (40) Over 50 % of fish diseases are associated with bacterial agents and over 22 % with viral pathogens (14), which can enter the aquaculture system via the water intake, infected fish stocks or contaminated feed (29). The major viral agents causing the highest impact in European aquaculture are RNA viruses such as viral nervous necrosis virus (VNNV), infectious pancreatic necrosis virus (IPNV), spring viremia of carp virus (SVCV), salmonid alphavirus (SAV), infectious haematopoietic necrosis virus (IHNV) and viral haemorrhagic septicemia virus (VHSV) (25). Among the bacterial agents mostly responsible for disease outbreaks in aquaculture systems are gram-negative bacteria such as the genera *Aeromonas*, *Edwardsiella*, *Piscirickettsia*, *Pseudomonas*, *Vibrio* and *Yersinia* as well as gram-positive bacteria such as the genera *Renibacterium* and *Streptococcus* (41). Both viral and bacterial diseases can be spread to the wild population by escaping fish and this again leads to devastating consequences for the local fish populations (29). Among the most frequent pathogens, *Aeromonas salmonicida* and VHSV have been used as model pathogens in the present thesis.

2.3.1. Viral hemorrhagic septicemia virus

VHSV is described as "one of the most pathogenic viral diseases of finfish worldwide" (42) infecting over 70 fish species causing viral hemorrhagic septicemia (43). VHSV is a negative-sense single-stranded RNA virus (44) and part of the family *Rhabdoviridae*, genus *Novirhabdovirus* with four known genotypes I, II, III and IV (43). The subtype Ia is responsible for most of the disease outbreaks in rainbow trout aquacultures in Europe (45). The viral genome consists of six genes: N (nucleoprotein), P (polymerase-associated phosphoprotein), M (matrix protein), G (glycoprotein), NV (nonvirion protein) and L (large RNA-dependent RNA polymerase) (43).

The G-protein is responsible for VHSV attachment to, and entry into the host cells, and is also considered the most antigenic protein (45). The NV-protein is known to be responsible for modulating the host immune response and inhibiting cell apoptosis after viral infection (46),

enabling viral replication for example in macrophages (47). Additionally it was demonstrated that the NV-protein is necessary for *in vivo* pathogenicity (46).

The disease caused by VHSV was first described in 1938 in Danish rainbow trout and in 1963 the virus could be identified as the reason for the symptoms (42). These vary among fish species but range from hemorrhagic lesions in external and internal organs as well as in the lateral musculature to necrotic changes in visceral organs, while some fish species only show dermal lesions (48). Mortality rates after infection with VHSV in aquaculture can reach 90 %, which can result in losses of up to 44 million EUR per year in the European aquaculture (49). VHSV can for example be transmitted horizontally via urine and feces or via the uptake of infected prey fish or contaminated food pellets. It is also described that VHSV persists in wild fish which can introduce the virus into open aquaculture systems. (50)

Viral entry has been suggested to take place via the gills or wounds in the skin, for rainbow trout it was shown that VHSV infection occurs via the fins (51) before spreading to other organs (45) and results in most severe hemorrhagic lesions of the lateral musculature and internal organs (48).

2.3.2. *Aeromonas salmonicida*

Aeromonas salmonicida subspecies salmonicida (*A. salmonicida*) is a gram-negative bacterium and a member of the *Gammaproteobacteria* class, it is facultative anaerobic, rod-shaped, and non-motile (40). The genus *Aeromona*s was first described in 1943 (52) and contains 26 species (40). Bacteria of the genus *Aeromonas* are isolated from fresh and saltwater aquatic animal species, but some strains can also infect humans and other mammals (40). *A. salmonicida* is the causative agent of furunculosis in fish, mostly salmonids (53). The disease is named after the large skin lesions that form after infection, which contain necrotic tissue and blood (40). Other symptoms of furunculosis are lethargy, swelling of the eyes and hemorrhagic lesions in external and internal organs (54). *A. salmonicida* infections often lead to so called ''die-offs'' (52), with mortality rates of up to 100 % resulting in tremendous economic losses, for example 32 million EUR in the Indonesian aquaculture in 2002 (52). *A. salmonicida* possesses many virulence factors such as the A-layer or S-layer, which is a complex protein structure associated with lipopolysaccharides (LPS) resulting in attachment to macrophages and in resistance to clearance by macrophages, which enables replication in these cells (40, 55, 56). Next to the A-layer and S-layer there are also filamentous adhesions such as flagella and fimbriae (54).

External molecules include cytotoxic enterotoxins such as aerolysin, several haemolysins, proteases, phospholipases and DNases (54). Additionally, this species has a type 3 secretion system which enables it to translocate toxins and other effector molecules from the bacterial cytoplasm directly into the host cell cytoplasm (40). Furthermore, the ability to release outer membrane vesicles (OMVs) has been described for *A. salmonicida* (10), which is another method of delivering virulence factors and toxins into host cells (1).

2.3.2.1. Outer membrane vesicles

Gram-negative bacteria are known to naturally produce outer membrane vesicles (OMVs), which are closed nanoparticles (10 to 450 nm) containing virulence factors and pathogen associated molecular patterns (PAMPs) (1, 2). OMVs are formed from bulges of the outer membrane followed by separation of the vesicles. Therefore OMVs consist of a similar protein composition as the outer membrane of the bacteria while additionally containing cytosolic and inner membrane proteins. (1) Interestingly, it was proven that OMVs are not an identical copy of the outer bacterial membrane (57). Some proteins of the outer membrane were found with high abundancy while others were not detected at all (58).

The fact that the proteins maintain their native conformation (59) and that OMVs can function as a natural adjuvant (4) gives OMVs great potential for the modulation of the immune response (3). E.g., it was shown that OMVs of different pathogens are able to interact with immune cells as well as epithelia cells via pattern recognition receptors (PRRs), for example Toll-like receptor (TLR) 4 or Nucleotide-binding oligomerization domain-containing protein 1 (NOD1), resulting in the inflammation and recruitment of immune cells (60).

For over 20 years, OMVs of *N. meningitidis* have been successfully used as parts of three different multicomponent vaccines, in combination with three purified outer membrane proteins, which illustrates the safety and potential of OMV based vaccines (1, 3-5). Furthermore, studies using model organisms such as *E. coli* have shown that OMVs are easily manipulated and that foreign proteins expressed in *E. coli* can be detected on the surface or in the lumen of derived OMVs (59, 61). Injection of OMVs, which contain surface-exposed foreign antigens, in mice results in an antibody response against the expressed foreign protein, which offers protection in a sepsis mouse model (61). Apart from the expression of foreign proteins on the surface or in the lumen of OMVs, by genetically engineering the bacterium of which the OMVs are derived from, also the exogenous addition of antigens to the surface of OMVs has been

accomplished (59). For this, bacteria were engineered to express OMVs containing tags to which foreign antigens can be added. In addition to the expression of single antigens also the possibility of expressing antigens of different pathogens has been discussed for OMVs, which would make them ideal candidates for multicomponent vaccines. (59)
All in all, these findings make OMVs an interesting antigen to be further explored as a vaccine candidate or possible vehicle for vaccine delivery in aquaculture.

2.4. Disease prevention in aquaculture

Due to the high economical losses associated with disease outbreaks in aquaculture (49, 52) different approaches of disease prevention have been established. Most commonly is the use of antibiotics (62) and vaccination (17) but also feeding with probiotics (63) and the use of frozen feed or the use of culture offspring instead of wild-caught foundation stock can lead to the reduction in disease occurrence (29). In this thesis disease prevention via antibiotic treatment and vaccination will be discussed.

2.4.1. Antibiotic treatment

Antibiotic treatment has been the traditional approach for disease treatment in aquaculture (62), especially when no protective vaccines are available. This is for example the case in the Chilean aquaculture for the fish pathogen *Piscirickettsia salmonis* (*P. salmonis*) where vaccination only results in moderate protection and treatment of *P. salmonis* infection is achieved using antibiotics (64). To illustrate this, the Norwegian aquaculture used 0.01 g of antibiotics to produce 1 ton of salmon, while the Chilean aquaculture used 526 g of antibiotics to produce 1 ton of salmon in 2016 (65).
Additionally, antibiotics are not only used to treat occurring diseases but are also applied as a prophylactic measure with the food (66), which has especially been reported for aquacultures in Vietnam and China (67). In 2018 approximately 67 different antibiotic compounds were used by the top 10 fish producing countries with as many as 15 different antibiotics being used per country. Due to the fact that antibiotics are most often applied via the feed, around 70 % of the used antibiotics diffuse into the environment, which can induce changes in the local ecosystem resulting in the development of antibiotic resistant microbes in the sediment, for example.(67)

Furthermore, the excessive use of antibiotics can result in the development of resistant bacteria (18), which has also been reported for *A. salmonicida* (68). Additionally, antibiotic resistant determinants can be transferred via horizontal gene transfer from aquatic pathogens to terrestrial pathogens, including human pathogens (69). Antibiotic residues could be detected in fish meat exceeding the maximum residue limits which has been reported to lead to allergic reactions as well as organ lesions in human consumers (67).

Norway is one of the top 10 fish producing countries and serves as a good example for sustainable aquaculture production (67). Early on in the development of the Norwegian aquaculture antibiotics were vastly used and remained the main source of disease prevention until the 1980s. Bacterial infections caused by *Vibrio spp.* and *Aeromonas spp.* nearly resulted in the complete collapse of the aquaculture industry in Norway and could only be prevented by the massive use of antibiotics (Figure 2) (18). Due to the invention and use of vaccines in the 1980s against for example vibriosis and furunculosis (70) the use of antibiotics could be drastically reduced (Figure 2). This shows the protective potential of these vaccines (70) and resulted in the development of protective vaccines against other diseases (17, 18). The increased use of vaccines resulted in a reduction of antibiotic use in the Norwegian aquaculture industry from approximately 48 t in the 1980s to only 0.5 t in 2014 (67).

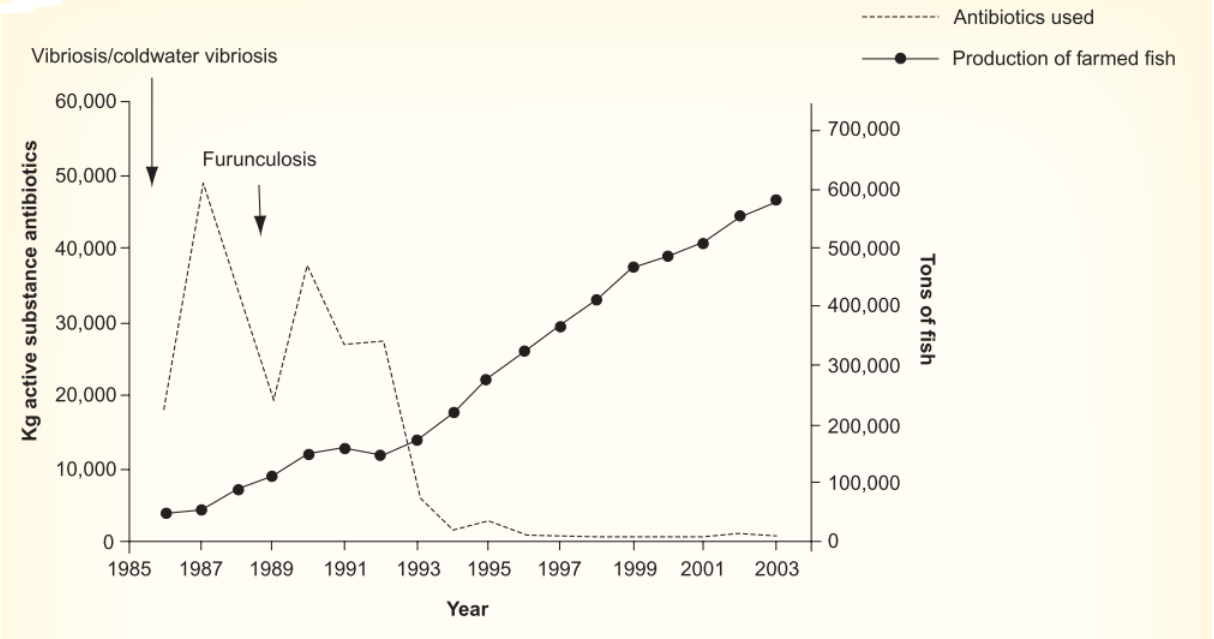

Figure 2: Use of antibiotics and produced fish in Norwegian aquacultures.
Arrows indicate the outbreaks of vibriosis induced by *Vibrio spp.* and furunculosis caused by *Aeromonas spp*. The dotted line indicates the amount of used antibiotics (kg) and the line with the big black dots indicates the amount of fish produced (t) (18).[2]

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2.4.2. Vaccines in aquaculture

By now, vaccines against 22 bacterial diseases and 6 viral diseases are available for over 17 fish species and vaccination regimes have been adapted for the different aquaculture conditions around the world (17, 25). For bacterial diseases the most common approach to vaccine development is the use of formalin inactivated bacteria in combination with adjuvants. Viral diseases are most commonly prevented by using either vector based or recombinant nonpathogenic microorganism based vaccines. (14)

In aquaculture, three main types of vaccines are used: injectable vaccines, immersion vaccines and oral vaccines (71).

2.4.2.1. Injectable vaccines

In general, the most commonly used vaccines are injectable vaccines (18). Injectable vaccines are either given intraperitoneally (i.p.) (Figure 3) or via intra muscular injection (i.m.) (14) and offer long-lasting protection (17). They are often the only available option. This is, e.g., the case for vaccines against furunculosis caused by *Aeromonas salmonicida (72)*, bacterial kidney disease due to *Renibacterium salmoninarum* infection (72), *Vibrio salmonicida* infection leading to cold-water vibriosis (72) or infectious hematopoietic necrosis caused by infectious hematopoietic necrosis virus (IHNV); the IHNV vaccine, however, is currently only licensed in Canada (17, 72). Injectable vaccines have the advantage that every fish is administered the correct dosage (18) resulting in high efficacies (17) and long-term protection (73). The disadvantages of injectable vaccines are that they are labor intensive and only fish above a certain size can be vaccinated (18). Furthermore, they result in high stress for the fish and are associated with adverse events such as granuloma formation and reduced growth rate resulting in production losses (74) or even mortality (21).

Figure 3: Delivery of intraperitoneal vaccines by hand.
Vaccination of *Atlantic salmon* done manually. Fish have to be anesthetized, vaccinated and placed back into their tanks within minutes after vaccination to ensure quick recovery and low stress levels (18).[3]

2.4.2.2. Immersion vaccines

Immersion vaccines are as widely used as injectable vaccines (14) and are either prepared as a dip vaccine where fish are placed in water containing a high antigen dose for a few minutes or as bath vaccines where fish are placed for longer periods in water containing highly diluted antigen (75). These vaccines do not need to be administered to individual fish and do not require individual handling of the fish (21). Therefore, they are less stressful for fish but usually require a booster (17) as they have been shown to be generally less protective when compared to injectable vaccines (21). The reason for this is not completely understood but the type of vaccine, inactivated or attenuated, the fish size, temperature and, most importantly, the vaccine dosage and time of immersion seem to influence the vaccine efficacy to a great extend (75). One way of increasing the vaccine efficacy has been the method of hyperosmotic infiltration, where fish are fist immersed for instance in a sodium chloride solution for a short period of time (71). This salinity shock results in a loss of body fluids of the fish so that when

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they are transferred to the vaccine bath a greater amount of water containing the vaccine antigen can enter the fish compared to fish which have not undergone hyperosmotic infiltration (76).

2.4.2.3. Oral vaccines

The first scientific description of an oral fish vaccine was reported already over 50 years ago when mixing food pellets with chemically inactivated *A. salmonicida* resulted in protection against experimental bacterial challenge (77). The first licensed fish vaccine was also an oral vaccine (1976) against enteric red mouth diseases caused by *Y. ruckeri* (71). Oral vaccines are either produced by coating food pellets with antigen or by mixing the antigen into the food pellets (71). Therefore, oral vaccination has been described as the ideal vaccination method for fish, as it results in the least amount of stress and is, at the same time, the least labor-intensive method (17, 18). Unfortunately, only few licensed vaccines are commercially available. Oral vaccines for rainbow trout against *Vibrio anguillarum* (*V. anguillarum*) and *Y. ruckeri* are available in Europe (17), whereas in Chile oral vaccines against *P. salmonis*, IPNV and infectious salmon anaemia virus (ISAV) are licensed for use in Atlantic salmon (17). The low number of available oral vaccines is due to the fact that high amounts of antigen are needed for vaccination, which, however, only results in low protection with short duration (18). Apart from that it is also hard to determine the vaccine dose each fish will take up, on the one hand this depends directly on the amount of vaccine pellets that are eaten by the fish and on the other hand on the amount of intact antigen which passes the gastric environment of the gut to be absorbed by the hindgut in order to induce an immune response. If the level of antigen is too low to induce a protective immune response with antibody production, it can result in oral tolerance and thus resulting in low protection of fish, even after subsequent use of injectable vaccines (21).

A variety of vaccines have been licensed for use in aquacultures, but for some diseases prophylactics do not exist or are still under development (78). This is also the case for VHSV, which is considered ''one of the most pathogenic viral diseases of finfish worldwide'' (42), but no commercial vaccines exist to prevent infection (79). Promising vaccine approaches have been described to prevent infection with VHSV ranging from the use of attenuated VHSV as an oral

vaccine (19) to the expression of VHSV G gene by probiotic bacteria which are mixed in with the feed (80).

Additionally, even injectable vaccines do not result in life long protection as has been noticed in Danish aquacultures, when, due to *A. salmonicida* infection, outbreaks of furunculosis re-occurred (11). The insufficient availability of vaccines, particularly oral vaccines, as well as the side effects associated with injectable vaccines illustrate the need for further advances in vaccine development for fish in aquacultures.

2.5. Immune system of fish

Fish have been described as the first phyla to have both an innate and adaptive immune system, therefore fish have been studied even before they became commercially attractive because of the fast growing aquaculture industry (81). Even though fish possess both types of the immune system, they also show major differences compared to mammals, for example in the presence of lymphoid organs (13) or the characteristics of certain immune cells (82, 83).

2.5.1. Immune organs in fish

One major difference in comparison to mammals is that fish do not have lymph nodes and a bone marrow. Instead, lymphoid organs are the head kidney, which is described as the bone marrow equivalent (13), the thymus, and the spleen as well as the mucosa associated lymphoid tissues (Figure 4) (13, 84).

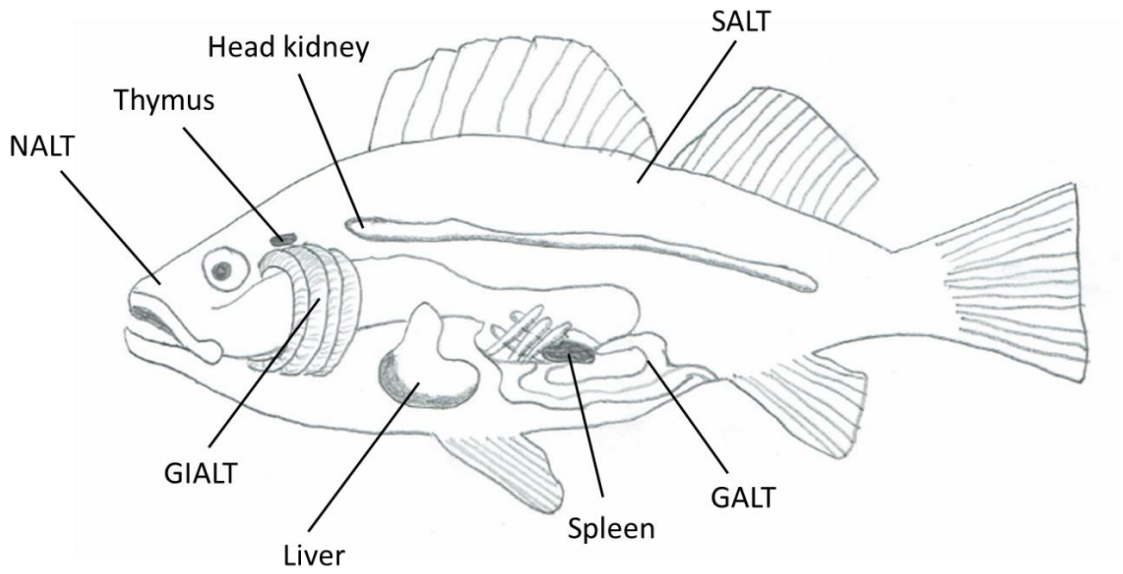

Figure 4: Schematic representation of lymphoid organs in fish.
Immune organs head kidney, thymus, liver and spleen are indicated as well as the mucosal immune organs skin-associated lymphoid tissue (SALT), gill- associated lymphoid tissue (GIALT), nasopharynx- associated lymphoid tissue (NALT) and gut-associated lymphoid tissue (GALT). Adapted from (13, 84).[4]

The mucus barrier in fish plays an important role in protecting the fish from infection as fish are constantly submerged and surrounded by possible pathogens, with for example 10^{10} viral particles per liter of aquatic habitat (13). So far, the skin-, gill-, nasopharynx- and gut-associated lymphoid tissue have been described in fish (75). They have an epithelial barrier which is covered in a mucus layer that prevents the attachment of pathogens to those tissues. (85). The mucosal surfaces in fish show many characteristics of the type I mucosal surfaces of, e.g., the intestines in mammals, since both surfaces contain macrophages, dendritic cells, T cells and B cells (86). Lymphoid tissues are in general less organized but still play an important role in antigen recognition, activation of the innate immune response and modulation of the adaptive immune system (75).

The head kidney is the main organ for hematopoiesis, phagocytosis, antigen processing as well as immune memory (13, 87). The head kidney consists of melanomacrophage centers, which are phagocytic cells that can retain foreign antigens for long periods of time (88). The melanomacrophage centers have been postulated to have similar functions as mammalian germinal centers and are supposed to be involved in the differentiation of memory B cells and plasma cells (88). The head kidney is the main organ for antibody secretion of proliferating B cells and plasma blasts while the posterior part consists of activated B cells and plasma blasts

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(89). Additionally, the head kidney can also release hormones such as corticosteroids, displaying a similar function to that of mammalian adrenal glands, while the posterior part of the kidney has renal function (13).

The thymus is located near the opercular cavity (Figure 4) and is responsible for thymocytes developing to functional T cells. The thymus, like the head kidney, also contains melanomacrophage centers. Apart from retaining antigens, these macrophages also phagocytose apoptotic cells in order to prevent the development of autoimmunity by recognizing self-antigens. (90) In contrast to mammals, the development and function of the thymus in fish is more dependent on hormonal and seasonal changes than on age (13).

The spleen is, similar to mammals, a secondary immune organ which consists of high amounts of mature B cells that can secret antibodies upon activation (91). Furthermore, the spleen is comprised of phagocytic cells which are able to take up antigen from the blood and retain them for long periods of time in the melanomacrophage centers (92) trapping antigens as is described for lymph nodes in mammals (93). Therefore, the spleen plays an important role in the antigen presentation and activation of the adaptive immune system in fish (12).

The immunological role of the liver in fish is greatly understudied but is believed to have similar functions as in mammals (30). This is mainly due to the fact that the liver receives blood from the gastro-intestinal tract as well as the systemic blood circulation, enabling the exchange of immunological information and detection of foreign particles in both blood streams (94). Furthermore, the presence of intrahepatic immune cells (94) as well as the increased expression of immune relevant gene expression after bacterial infection (30) characterize the liver as an important organ within the fish immune system.

In addition to the immune organs, fish possess many cells involved in immune response that are also known from mammals.

2.5.2. The innate immune system

The innate immune system of *Teleostei* "is based on cell phagocytosis and secretion of soluble antimicrobial molecules" (13) with the basic strategy of constantly expressing pattern recognition receptors (PRRs) to recognize PAMPs which results in the activation of an inflammatory response to eliminate pathogens (95). The innate immune system consists of the natural epithelial and mucus barrier (skin, gills, gut, and nose), the humoral part (complement system, pattern recognition receptors, and bactericidal enzymes) and the cellular response

(phagocytic leukocytes like dendritic cells and macrophages; non-specific cytotoxic cells such as neutrophils and natural killer cells) (81, 92). Some cells have additional functions in fish for instance the B cells that are able to phagocytose and kill foreign microbes (82). The same has been observed for fish thrombocytes, which, in contrast to those in mammals, also contain a nucleus (83, 96). For carp it was shown that thrombocytes are the dominating phagocytic cell type in the blood, and phagocytic thrombocytes could be detected in the spleen and in the head kidney suggesting a role in the antigen presentation and in the activation of the adaptive immune response (83).

Additionally, cells not described in mammals have been shown to be present in fish for example cells involved in cell mediated cytotoxicity (CMC) (85, 97, 98). Apart from the well-known neutrophils, which are also present in mammals, two additional cell types involved in CMC have been described in fish: nonspecific cytotoxic cells (NCCs) and NK-like cells, while NCCs are suggested to be the precursor of mammalian NK cells (98).

Whyte (2007) describes that if the pathogen can breach the natural barriers, it is met with soluble defense mechanisms for example anti-proteases, haemolysin and lysozyme. Additionally, innate immune cells such as macrophages, neutrophils, B lymphocytes and thrombocytes are able to recognize the foreign particles via PRRs (12).

PRRs include C-type lectin receptors, retinoic acid-inducible gene I (RIG-I)-like receptors, NOD-like receptors and TLRs (99). Upon recognition of PAMPs, uptake of the foreign particle into the early phagosome is initiated by the phagocytic cell and followed by fusing the early phagosome with the lysosome, forming the phagolysosome (100). The hydrolytic environment of the phagolysosome results in the degradation of the pathogen, providing for example proteins that can be presented via major histocompatibility complex (MHC) II or bacterial DNA which can be recognized by TLR9 (101).

Activation of phagocytosis also results in producing reactive oxygen species and reactive nitrogen intermediates which support inactivation of internalized pathogens and external pathogens (97, 102).

Apart from degrading foreign particles in the lysosome, the recognition of PAMPs by PRRs also results in activating signaling cascades leading to expression of cytokines (103). Even though the detailed pathway of pattern recognition in fish is not completely characterized (103), the TLRs have been studied to a great extent and the pathway used has been well described (Figure 5) (104). Brietzke et al. (2015) mentioned that there are over 20 TLRs described in fish

which upon activation dimerize and recruit the Myddosome which consists - among other factors - of myeloid differentiation primary response 88 (Myd88) and interleukin-1 receptor-associated kinase 1 (IRAK1) (Figure 5). Formation of the Myddosome results in activating the downstream factors and in activating NF-κB, which finally leads to expression of immune relevant genes (104). In fish cytokines, such as interleukins (ILs), also play a key role in the regulation of the innate and adaptive immune response (105). The cytokine network has been described in different reviews (105-109), and even though not for all cytokines discovered in mammals homologs could be found in fish (107), a good understanding of the possible interaction has been achieved. Upon infection of fish, tumor necrosis factor α (TNFα), IL-1β and IL-6 are the first cytokines to be released by, for example, macrophages, resulting in the recruitment of neutrophils via TNFα (109). Release of IL-6 leads to the proliferation of macrophages, the activation of the expression of antimicrobial peptides and regulating cytokine release (110). Also, the growth, proliferation and recruitment of lymphocytes, especially natural killer cells and B cells take place after release of IL-1β (111). Furthermore, additional pro-inflammatory factors are released, such as IL-8, leading to the recruitment of neutrophils, T cells and basophils as well as the activation of respiratory burst of neutrophils (112). Apart from that, the activation of inducible nitric oxide synthases (iNOS) leads to the induction of antimicrobial activity via production of nitric oxide (NO) (113) or mediation of inflammation (114). Besides those pro-inflammatory factors, also anti-inflammatory cytokines have been studied in fish, such as IL-10 and TGFβ. IL-10 inhibits, e.g., the secretion of TNFα and IL-8, while TGFβ regulates the release of NO (113). There are several more cytokines described in fish, such as IL-2, IL-4/13, IL-7, IL-12, IL-15, IL-17, IL-21 and interferon γ (IFNγ) which are more closely associated with the adaptive immune system but which are not in the focus of this thesis (105).

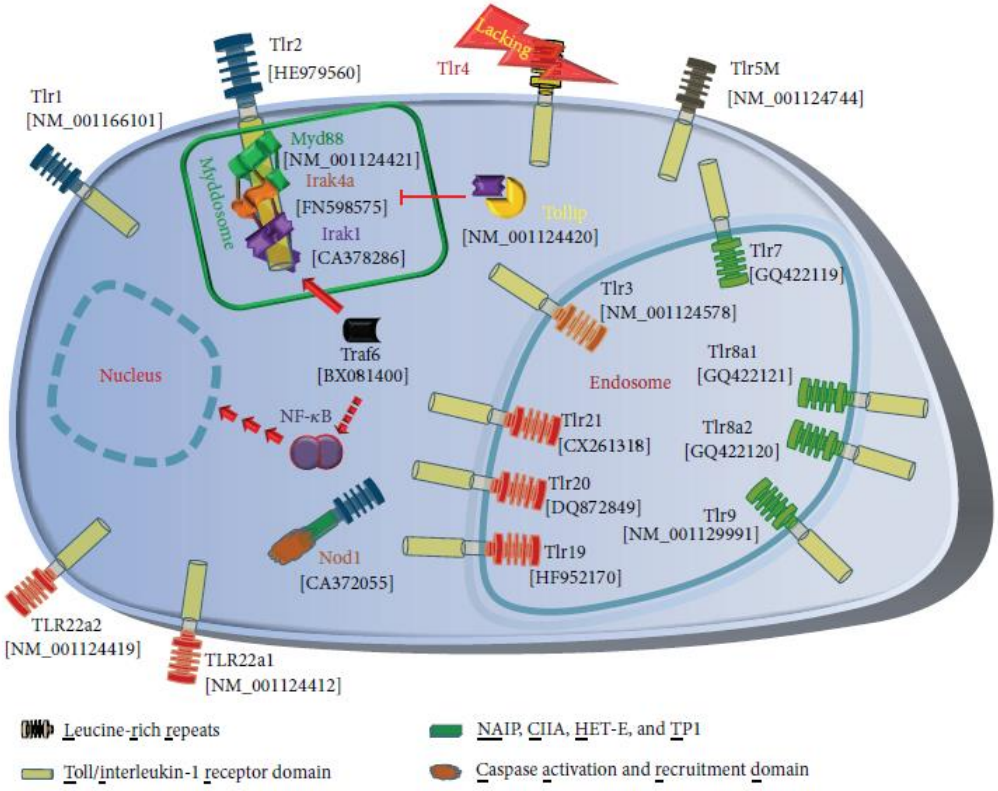

Figure 5: Schematic representation of pathogen recognition in trout.
"Toll-like receptors, Nod1, and downstream factors as known from trout are listed with their GenBank accession numbers. Different colors of the LRR regions factors indicate the membership to individual Tlr families (TLR1 (blue), Tlr3 (orange), Tlr5 (black), Tlr7 (green), and Tlr11 (red)). Notably, a Tlr4 ortholog is absent in salmonid fish (marked with a flash). The Myddosome consisting ofMyd88, Irak4a, and Irak1 (inside the green box) binds to the activated Tlr and recruits Traf6 and further downstream factors (indicated with a broken arrow), which in turn activate NF-κB. Tollip functionally inhibits Irak1 by preventing its recruitment into the Myddosome complex." (104)[5]

2.5.3. The adaptive immune system

While the innate immune system is believed to have been present for over 600 million years, the adaptive immune system is assumed to have arisen approximately 450 million years ago with the evolution of jawed vertebrates (115). The adaptive immune system is essential for protection against repetitive infections with the same pathogen, by mounting a faster and more efficient immune response than known from the innate immune response (95). This is for example achieved by the presence of MHC, recombination-activating gene (RAG)-1/2 and terminal deoxynucleotidyl transferase (TdT) which altogether lead to the generation of pathogen specific memory B and T cells (93, 115).

[5] Permission was granted by the publishers for the use of figures in dissertations.

As described earlier, the head kidney is the main organ for hematopoiesis which includes B lymphogenesis (91). Fish do not possess germinal centers were, for example in mammals, the production of plasma cells and memory B cells occurs (30) instead it is assumed that mature B cells develop to plasma B cells in the blood and spleen upon pathogen encounter, and migrate to the head kidney to become long-lived antibody secreting plasma cells (91, 115).

Antibodies produced by B cells can directly bind to foreign antigens (30), and inhibit entry of pathogens into cells, neutralize toxins released by the pathogens or activate the classical complement pathway (116). In order to mount a specific antibody response against pathogens, rainbow trout need 3 to 8 weeks (117, 118), depending on the temperature (117), instead of the approximately 10 days needed for example in mammals (119). Smith et al. (2019) explain that antibodies are present as a secreted form as well as a membrane-bound form. The membrane-bound form makes up the B cell receptor together with the signaling molecules Igα and Igβ (115).

Similar to mammals the diversity of B cell receptors, as well as T cell receptors, in fish is achieved by "site specific recombination events of variable region gene segments, termed V(D)J recombination" (120) as well as somatic hypermutation (121, 122). So far three immunoglobulin heavy (IgH) chain elements have been detected on the IgH loci in trout (121). The three Ig isotypes classified by the heavy chain of the immunoglobulin are: IgM, IgD and IgT. IgM and IgD share a V(D)J cassette resulting in the co-expression of IgM and IgD on most B cells in trout (123) while the IgT expression of B cells is completely independent of IgM and IgD as it has its own V(D)J gene cassette on the IgH locus preventing co-expression with other IgH chains (121).

In rainbow trout B cells expressing IgM^+/IgD^+ (IgM^+ B cells) have been shown to down-regulate IgD expression upon pathogen encounter (123), these B cells have been found in the blood (124) as well as in the lymphoid organs, liver, adipose tissue and mucosal surfaces (125). Recently B cells expressing IgM^-/IgD^+ (IgD^+ B cells) have been reported in the gills and the gut of rainbow trout and are either supposed to be a PRR via the Fc part of IgD (121) or to play a role in mucosal immunity (123, 126), but have not been studied to a great extend yet (123, 126). IgT has been described as an equivalent to mammalian IgA (123) and is assumed to play a role in mucosal immunity of gut and skin (127).

IgM^+ B cells make up 72–83 % of all B cells in the systemic lymphoid organs of rainbow trout and IgT^+ B cells make up 16–27 % of all B cells in these organs, whereas in the gut the

percentage of both IgT^+ and IgM^+ lymphocytes is around 50 % of all B cells with IgT^+ B cells usually being the dominant B cell subtype (30).

IgM concentration in serum is approximately 1000 times higher than that of IgT and therefore plays an important role in systemic immunity as well as "intestinal and cutaneous immune responses" (121). IgT is more closely associated with an immune response in gut and skin mucus (121).

Besides the three heavy chains, four immunoglobulin light (IgL) chains have been described to be expressed in the *Teleostei*, always in combination with the IgH chain, namely IgL1 (κG), IgL2 (κF), IgL3 (σ) and IgLλ (127), while only the expression of the first three has been described for trout B cells. Two IgH chains and two IgL chains connected via disulfide bonds make up one immunoglobulin (Ig) molecule (127).

Interestingly, there is no regular distribution of light chains expressed with the different heavy chains, and e.g., IgL1 (κG) is the main light chain expressed on IgM^+ B cells as well as IgT^+ B cells in the head kidney and blood of the rainbow trout. IgL3 (σ), however, was found to be expressed twice as much on IgT^+ B cells than IgM^+ B cells. Even though the detailed function of the different light chains in an immune response could not be determined so far, it was demonstrated that IgL3 (σ) is mainly expressed on B cells in the skin and the gut, which could indicate a role in mucosal immunity, as most of the B cells in the skin and gut are IgT^+ B cells. (127) Another difference compared to mammals is that in fish no class switching of B cells occurs, resulting in limited affinity maturation of the B cells after repeated challenges (122, 128).

Apart from the B cells also the T cells play an important role in adaptive immunity. T lymphocytes are involved in rejection of non-self, stimulation of phagocytosis and antibody production by B cells (13). All T cells express a T cell receptor for antigen recognition (129) as well as co-receptors such as CD3, CD4, CD8, CD28 and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) (130). As mentioned earlier the antigen specificity of the T cell receptor is achieved via V(D)J recombination (115) as well as somatic hypermutation (121, 122). Different to B cells, most T cells can only interact with antigens that were processed and are presented via MHC molecules on cell surfaces (30, 129). Leal et al. (2016) mentioned that T cells can be classified based on the expression of their T cell receptor chains as either αβ- or γδ-T cells. In mammals γδ-T cells make up only approximately 2 % of all T cells and are considered as innate-like

immune cells as they can recognize unprocessed antigen. The αβ-T cells can be characterized as either T cytotoxic cell expressing CD8 or T helper (Th) cells expressing CD4 (129). Mosley et al. (2013) described that, T cytotoxic cells can interact with antigens presented via MHCI and, as a result, they can kill, e.g., viral infected cells or cancerous cells. $CD4^+$ T cells on the other hand are responsible for the recognition of peptides presented via MHCII receptor and they can modify the immune response via release of cytokines and interaction with B cells (130). This is for example achieved by the release of IL-4, IL-5 and IL-13 by Th2 cells, which stimulate B cells to produce antibodies to control extracellular infections while Th1 cells release cytokines such as IFNγ and TNFα to target intracellular infections. (129)
Not only is the differentiation into different effector T cell subsets activated upon antigenic stimulation of T cells, but also the formation of memory T cells (30, 131).
In fish, genomic studies reveal the presence of most of the described T cell subsets, and though the functionality was not tested for each one, it is speculated that fish do possess all the different T cell subsets as described in mammals (129).

Compared to that of mammals, the adaptive immune system of fish shows some restriction e.g., in the needed time for the development of a pathogen specific immune response (85, 117), the absence of lymph nodes and germinal centers as well the lack of Ig class switching (85, 117, 132). Therefore the innate immune system is considered to be of high importance in combating initial infections in fish (85).

2.6. Rationale of experimental approach and aims of this thesis

To enable target-orientated vaccine development, in-depth knowledge of the immune system of fish is necessary. Over the last three decades it could be established that *Teleostei* have a sophisticated immune system comparable to that of vertebrates, with similar characteristics of an innate and adaptive immune system. Until now successful vaccination has been established by *in vivo* challenge trials as well as the antibody response to antigens used in these trials (11, 133). Furthermore, it is known that the innate immune response modulates and activates the adaptive immune response (105). Even though OMVs have been used in human vaccine, little is known about the immunostimulatory potential of OMVs derived from fish pathogenic bacteria such as *A. salmonicida*. Additionally, oral vaccination has been described

as the ideal vaccination route for fish, but no well-established model for testing oral vaccines in rainbow trout has been broadly used. Therefore, the two main questions of this work are:

1. Can outer membrane vesicles derived from the fish pathogenic bacterium *A. salmonicida* be used as a vaccine antigen?

2. Is it possible to establish an oral vaccination model of rainbow trout for the use of bacterial and viral antigens?

Many studies have looked at the immune response either after vaccination of fish or stimulation of fish derived cells to better understand the underlying processes of successful vaccination. As mentioned earlier, antibody production in temperate fish can take up to eight weeks (117, 118), but in some of the described challenge trials not all of the non-vaccinated fish succumbed to the challenge and showed mortality rates as low as 50 % (133). This again emphasizes the importance of the innate immune response in providing protection from bacterial and viral challenges and in modulating the adaptive immune system of fish.

After breaching the physical barrier, monocytes, macrophages and neutrophils are one of the first cells which will be encountered by the pathogens or vaccine antigens (12). Therefore studies analyzing successful vaccination strategies have also looked at the stimulation of innate immune cells to predict activation of the adaptive immune system and vaccination success (134).

Already in the early stages of fish vaccine development the importance of innate immune cells was shown, by Olivier et al. (1985), when coho salmon (*Oncorhynchus kisutch*) were i.p. injected with modified complete Freund's adjuvant (MFCA) and showed similar survival rates as fish vaccinated with MFCA and inactivated *A. salmonicida*. Due to the fact that the MFCA induced immune response was nonspecific, and as also challenge with different pathogens led to increased survival rates, it was suggested, that peritoneal macrophages play an important role against invading pathogens (135). This could be confirmed when rainbow trout were i.p. injected with inactivate *A. salmonicida* and then isolated macrophages showed enhanced killing of *A. salmonicida* when compared to macrophages isolated from PBS injected fish (136). Furthermore i.p. vaccination of rainbow trout with *A. salmonicida* resulted in an influx of myeloid cells, mainly monocytes, macrophages, and granulocytes, to the site of vaccination (15).

Myeloid cells recruited to the peritoneal cavity originated from the head kidney (137). Therefore, macrophages seem to be of great importance in the early immune response of fish.
This has led to the question:

> Can OMVs derived from *A. salmonicida* activate innate immune cells resulting in an inflammatory response?

In this context, different results have been obtained in previous studies. Stimulation of the monocyte/macrophage cell line RTS-11 (138) with LPS, which is generally used to mimic molecular patters of bacteria (139), results in increased expression of IL-8 mRNA (112). Furthermore LPS stimulation of HK derived leukocytes, which are the main source of macrophages in fish (110, 139, 140), leads to an increase in mRNA expression of pro inflammatory cytokines such as IL-1β, IL-6, TNFα as well as the anti-inflammatory cytokine IL-10 (139). Using recombinant bacterial proteins of *Y. ruckeri* an increased expression of IL-1β, IL-6, IL-8 and TNFα could be observed in head kidney derived leukocytes and RTS-11 cells within 24h after stimulation, when compared to the time matched controls (141).

Both gram-negative bacteria and derived OMVs are known to contain LPS (1, 40). Therefore the monocyte/macrophage cell line RTS-11 (138) in addition to peritoneal leukocytes, as the site of i.p. injection, and head kidney leukocytes, as the main source of macrophages in fish (110, 139, 140), were used to answer the question if OMVs derived from *A. salmonicida* activate innate immune cells so as to result in an inflammatory response.

OMVs have been part of human vaccines for decades (5) and their possible use as a vaccine delivery vehicle has been studied in model organisms such as *E. coli* (61). However, so far only little is known about the OMVs of fish pathogenic bacteria. OMV production was verified for fish pathogenic gram-negative bacteria such as *A. salmonicida*, *P. salmonis*, *Edwardsiella tarda* (*E. tarda*), *Francisella noatunensis* (*F. noatunensis*) and V. *anguillarum* (6-10). Furthermore, it was shown that injection of flounder *Paralichthys olivaceus* (*P. olivaceus*) with *E. tarda* derived OMVs resulted in a higher expression of mRNA of immune relevant genes such as IL1β, IL-6 and TNFα in head kidney cells when compared to fish vaccinated with formalin killed *E. tarda*, even though both groups showed similar survival rates after challenge. (9) I.p.

injection of OMVs derived from *V. anguillarum* into *P. olivaceus* resulted in increased mRNA levels of IL-1β, IL-6 and TNFα in cells of the head kidney and spleen (7).
This has led to the questions:

> Is the cellular immune response in rainbow trout after i.p. vaccination with OMVs derived from *A. salmonicida* comparable to the cellular immune response after vaccination with inactivated *A. salmonicida*?
>
> Does i.p. vaccination of rainbow trout with inactivated *A. salmonicida* or derived OMVs result in the same mRNA expression pattern in primary and secondary lymphoid organs?

So far, it was demonstrated that the bath challenge with virulent strains of *A. salmonicida* led to an increased expression of IL-1β, IL-8 and TNFα in cells derived from the intestine of rainbow trout while the expressions of those cytokines was reduced in cells derived from the head kidney. TGFβ expression was reduced in cells of both organs (142).
Apart from the description of the immune response on the gene level, also the cellular immune response has been investigated via monoclonal antibodies (mabs). Unfortunately, only few mabs are available for the rainbow trout so that only thrombocytes, monocytes/macrophages, T cells, $CD8\alpha^+$ T cells, $CD8\alpha^-$ T cells, IgM^+ B cells and subsets of the B cell light chains can be detected (15, 96, 143-146). Using those mabs it was demonstrated that after i.p. injection of a pathogenic strain of *A. salmonicida* the pre-dominating lymphoid cells in the peritoneal cavity were replaced by myeloid cells within 24h. At 72h the lymphoid cells were again the dominating cells with IgM^+ B cells making up over 50 % of the cell population. (15)

OMVs consist of a similar protein composition as the outer membrane of the bacteria while additionally containing cytosolic and inner membrane proteins (1). Furthermore, OMVs containing virulence factors and pathogen associated molecular patterns (PAMPs) (1, 2). Therefore, the peritoneal model of inflammation (15) was used to answer the question if the cellular immune response in rainbow trout after i.p. vaccination with OMVs derived from *A. salmonicida* is comparable to the cellular immune response after vaccination with inactivated *A. salmonicida*. During this trial also the question if i.p. vaccination of rainbow trout with

inactivated *A. salmonicida* or derived OMVs results in the same mRNA expression pattern in primary and secondary lymphoid organs was investigated.

Furthermore, i.p. vaccination of zebra fish with OMVs derived from *F. noatunensis* resulted in the protection from the challenge with the homologues bacteria strain (6). Similar results could be obtained when olive flounder were i.p. vaccinated with *E. tarda* derived OMVs and subsequently challenged.
This has led to the question:

> Does i.p. vaccination of rainbow trout with OMVs derived from *A. salmonicida* result in an *A. salmonicid*a specific antibody response?

The humoral response after i.p. injection of two commercial vaccines against furunculosis was tested in rainbow trout and it showed that protection directly correlated with the amount of specific antibodies present (133, 147).

Therefore, long-term *in vivo* trials were conducted to answer the question if i.p. vaccination of rainbow trout with OMVs derived from *A. salmonicida* result in an *A. salmonicid*a specific antibody response, possibly conferring protection.

Injectable vaccines are the most commonly used type of vaccine in aquacultures (18) even though the optimal vaccination method would be oral vaccination (17, 18). Despite the fact that successful oral vaccine approaches have been described (19, 80), only few oral vaccines are commercially available (17). It was shown that attenuated VHSV surrounded by polyethylene glycol (PEG) can be used as an oral vaccine and resulted in VHSV specific antibodies and protection after challenge (19).
In addition to the successful use of OMVs in human vaccines (5) they have also been described as easy to manipulate and being able to express antigens of different pathogens (59). This would make OMVs ideal vaccine candidates functioning as an antigen (60), adjuvant (4) and vaccine delivery vehicle (59).
This has led to the question:

Can OMVs be used as a bacterial and viral dual oral vaccine?

So far it was demonstrated that the injection of OMVs, which contain surface exposed foreign antigens, in mice results in an antibody response against the expressed protein, which offers protection in a sepsis mouse model (61). Additionally it was shown that the expression of VHSV G-Protein in an genetically engineered *A. salmonicida* strain and subsequent bath vaccination with this engineered bacterium resulted in protection when fish were challenged with VHSV (148).

To test the potentials of OMVs as multicomponent oral vaccine, an oral model for bacterial and viral antigens had to be established. For this purpose, the oral vaccine described for VHSV (19) was adapted in this thesis for the use with inactivated bacteria functioning as a surrogate antigen for OMVs.

3. Materials

3.1. Consumables

Table 1: Consumables used in this thesis.

Product	Manufacturer
1 ml TBC Syringe	Mediware
1.5 ml microcentrifuge tubes	Nerbe plus
2 ml cryogenic tubes	Greiner BIO-ONE
10 ml EDTA blood collection tube	Sarstedt
12 ml round bottom tubes	Greiner BIO-ONE
50 ml centrifuge tube	Sarstedt
75x12 mm sample tubes (FACS)	Sarstedt
96 well cell culture plates	Corning
96 clear well round bottom plates	Greiner BIO-ONE
96 well high binding plates (ELISA)	Greiner BIO-ONE
150 nm gaze	NeoLab
Needles 26G x 1/2 Luer-Lock	Medoject
Petri dishes	Sarstedt
Pipette tips	Sarstedt
T75 sealed cap cell culture flask	Corning

3.2. Equipment

Table 2: Equipment used in this thesis.

Equipment	Model	Manufacturer
Centrifuge	Centrifuge 5810R	Eppendorf
Centrifuge	Mikro 200R	Hettich Zentrifugen
Centrifuge	Rotina 35	Hettich Zentrifugen
ChemiDoc™ Touch Imaging System		Bio-Rad
Electrophoresis Systems		Bio-Rad
ELISA Reader	Infinite M200 PRO	Tecan

Equipment	Model	Manufacturer
Flow cytometer	FACSCanto II	Becton Dickinson
Homogenizer	Potter-Elvehjem	Harnstein
Incubator	MCO 19AIC	Sanyo
Inverse light microscope	AxioVert 200 M	Zeiss
Laminar flow cabinet	Safe 2020	Thermo Scientific
Magnetic hot plate stirrer	IKAMAG RCT	Bachofer
Microwave		Exquisit
Mortar and pestle		Jipo
PCR workstation	DNA/RNA UV-cleaner	LTF Labortechnik
pH-Meter	FiveEasy Plus	Mettler Toledo
Photometer	BioPhotometer	Eppendorf
Pipettes	Research/ Research Plus	Eppendorf
Power Mixer		Labinco
Real-Time Cycler	CFX96 Touch Real-Time PCR Detection System	Bio-Rad
RNAse free ddH_2O		Quiagen
Scalpel blade	No. 22	Mediware
Scissors		Chiru-Instrumente
Shakers		ThermoFisher Scientific
Spectrophotometer	NanoDrop Lite	Thermo Scientific
Tangential flow filtration	Äkta flux	GE Healthcare
Thermocycler	CFX-96 Real-Time System	Bio-Rad
Water bath		Thomas Scientific

3.3. Chemicals

Table 3: Chemicals used in this thesis.

Chemical	Concentration	Manufacturer
Aceton		Roth
Agarose		Sigma Aldrich
Aluminum oxide (Al_2O_3)		Roth

Chemical	Concentration	Manufacturer
β-mercaptoethanol		MP Biomedicals
BCA Assay		ThermoFisher Scientific
Benzocaine		Altmann Analytik
Boric acid		Sigma Aldrich
B-PER™ Complete Bacterial Protein Extraction Reagent		ThermoFisher Scientific
Buffer RLT		Qiagen
Chloroform		Roth
Collagenase D	0.30 U/mg	Sigma Aldrich
Dextrose		Sigma Aldrich
Disodium phosphate (Na_2HPO_4)		Roth
dNTP Mix	10 mM	Quiagen
Ethylenediaminetetraacetic acid (EDTA)		Serva
Ethanol		Roth
Ethidium Bromide	0.625 mg/mL	ThermoFisher Scientific
EXTRAzol		Blirt
Forelle Aroma Konzentrat		Jenzi
GeneRuler 50 bp DNA ladder		ThermoFisher Scientific
Glycerol		Roth
GlycoBlue™ Coprecipitant	15 mg/mL	Invitrogen
Ham's F12		Gibco
Iscove's Modified Dulbecco's Medium (IMDM)		Gibco
Isopropanol		Roth
Iron Oxide E172		Caelo
Luria Agar Base		Sigma Aldrich
Luria Broth (LB)		Sigma Aldrich
Magnesium chloride solution ($MgCl_2$)	25 mM	Merck
Magnesium hydroxide ($Mg(OH)_2$)		Roth
Methanol		Roth

Chemical	Concentration	Manufacturer
Monopotassium phosphate (KH_2PO_4)		Fluka Chemika
Penicillin-Streptomycin 100x	10.000 U/ml 10 mg/ml	Sigma Aldrich
Polyethylene glycol (PEG)	1000	Roth
Polyethylene glycol (PEG)	1500	Roth
Polyoxyethylene sorbitan monolaurate solution (Tween)	20	Merck
Potassium chloride (KCl)		Roth
Propidium iodide		ThermoFisher Scientific
Roti Histofix 10%		Roth
Skim milk powder (ELISA)		Hobbybäcker-Versand
Sodium bicarbonate ($NaHCO_3$)		Roth
Sodium chloride (NaCl)		Roth
Sulfuric acid (H_2SO_4)		Merck Millipore
Talcum		Caelo
TMB/E Solution (ELISA)		Merck Millipore
Triethylenediamine		Sigma Aldrich
Tris (hydroxymethyl)-aminomethan		Sigma Aldrich
Trypsin	1:250	Invitrogen
Tryptic soy agar		Merck Millipore
Tryptic soy broth		Merck Millipore

3.4. Media and buffers

Alsever's Trypsin-Versen-solution in 1L ddH_2O, pH 7.2 (Collection for Cell Lines and media in Veterinary Medicine, Insel Riems, Germany)

8.5 g NaCl
0.4 g KCl
1 g Dextrose
0.58 g $NaHCO_3$
1 g 1:250 Trypsin
0.2 g EDTA

Phosphate Buffered Saline (PBS), 10×, in 1 L a.dest, pH 7.2

80 g NaCl
2 g KCl
14.4 g Na_2HPO_4
2 g KH_2PO_4

Fluorescence maintenance buffer pH 8.6:

2.5 g Triethylenediamine
90 ml glycerol
10 ml 1x PBS
100 µl propidium iodide (2mg/ml)

PBST 1L, pH 7.2

500 µl Tween 20
Add to 1 l with 1 x PBS

TBE buffer 10x

108 g Tris (hydroxymethyl)-aminomethan
55 g boric acid
40 ml of 0.5 M EDTA, pH 8.0
Add to 1 l with distilled deionized water

2 % Agarose gel

100 ml 1x TBE
2 g agarose
5 µl Ethidiumbromid

ZB 5d,1L a.dest pH 7.2 (Collection for Cell Lines and media in Veterinary Medicine, Insel Riems, Germany)

4.76 g MEM (Earle's salts)
5.32 g MEM Eagle (Hanks' salts)
10 ml nonessential amino acids (100x),
120 mg Na-Pyruvat

ZB 5, pH 7.2 (Collection for Cell Lines and media in Veterinary Medicine, Insel Riems, Germany)

ZB5d with 10 % FCS

ZB 28d, pH 7.2 (Collection for Cell Lines and media in Veterinary Medicine, Insel Riems, Germany)

5.32 g Ham's F12
8.80 g IMDM
2.485 g NaHCO3

ZB 28, pH 7.2 (Collection for Cell Lines and media in Veterinary Medicine, Insel Riems, Germany)

ZB 28d with 10 % FCS

3.5. Antibodies

Table 4: Antibodies used in this thesis.

Antibody Name	Antigen	Reference /Manufacturer
mab 4C10	Trout IgM µ-chain	(149)
mab 42	Trout thrombocytes	(96)
mab 21	Trout pan-myeloid cells	(15)
mab D30	Trout pan-T cells	(150)
mab CD8-13-2	Trout-CD8α	(145)
mab 1.14	Trout-IgM	(146)
mab N2	Trout IgκG light chain (IgL1)	(144)
Bio282	Viral hemorrhagic septicemia virus N-protein	Bio-X Diagnostics
Goat anti-Rat IgG (H+L), Alexa Fluor 647	Rat IgG (H+L)	ThermoFisher Scientific
Goat IgG anti-Mouse IgG1 (Fc)-DyLight 405	Mouse IgG1	Dianova
Goat anti-Mouse IgG/IgM-POD	Mouse IgG/IgM	Novusbio
F(ab')2-Goat anti-Mouse IgG (H+L), Alexa Fluor 488	Mouse IgG (H+L)	ThermoFisher Scientific
Goat anti-Mouse IgG MicroBeads	Mouse IgG	Miltenyi Biotec

3.6. Kits

Table 5: Kits used in this thesis.

Kit Name	Manufacturer
Lightning-Link (R) R-PE Antibody Labeling Kit	Novus Biologicals
Lightning-Link (R) APC-Cy7 Antibody Labeling Kit	Novus Biologicals
Lightning-Link (R) PerCP Antibody Labeling Kit	Novus Biologicals
Lightning-Link (R) Fluorescein Antibody Labeling Kit	Novus Biologicals
Pierce™ Coomassie Plus (Bradford) Assay Kit	ThermoFisher Scientific
RT-qPCR, SensiFAST SYBR No-ROX One-Step Kit	Bioline
RNeasy Mini Kit	Qiagen
Phire Hot Start II DNA Polymerase	ThermoFisher Scientific
SuperScript™ III Reverse Transcriptase	ThermoFisher Scientific

3.7. Cells and microorganisms

Aeromonas salmonicida subspecies salmonicida strain JF2267[6]

This strain was isolated directly from an arctic char and contains a fully functional type 3 secretion system (151).

Aeromonas salmonicida subspecies salmonicida strain JF5505[1]

Re-isolated from dead fish inoculated with JF2267. Increased mortality compared to JF2267.

Outer membrane vesicels

OMVs were provided by Tobias Kroniger, University of Greifswald, Germany.

The *A. salmonicida* strain JF2267 was grown in LB medium in a 16 °C water bath under shaking at 170 rpm for ~40 hours. Bacteria were harvested by centrifugation (10000 g, 20 min, 4 °C). The supernatant was filtered twice using a 0.45 µm bottle-top filter membrane and was concentrated ~20-fold using tangential flow filtration with a nominal molecular weight cut-off of 100 kDa and ultracentrifuged afterwards (100000 g, 1 h, 4 °C). The pellet was washed in Tris EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.25) and the supernatant was discarded. Subsequently, the OMVs were purified by density gradient centrifugation with OptiPrep (60 %

[6] Kindly provided by Joachim Frey, University of Bern, Switzerland.

Iodixanol). The OMV containing pellet was resuspended in 2 ml 50 % Iodixanol, diluted with a solution of 0.85 % NaCl and 60 mM HEPES (pH 7.4). Then, 1 ml of 40 %, 30 % and 10 % Iodixanol diluted with 0.85 % NaCl and 10 mM HEPES (pH 7.4) were carefully layered from the highest to the lowest Iodixanol concentration above the 50 % solution layer. The samples were ultracentrifuged in a swing-out rotor (100000 g, 2 h ,4 °C). The third and fourth 1 ml fraction contained the OMVs and were transferred into new ultracentrifugation tubes, diluted 5- to 6-fold with TE buffer and ultracentrifuged again. Finally, the pellets of both fractions were pooled in TE buffer, transferred into a new reaction tube, and ultracentrifuged again. The protein content of the OMVs was determined by BCA assay according to manufacturer's instructions. OMVs were stored at -20 °C subsequently. (Personal communication with Tobias Kroniger, University of Greifswald, Germany)

Yersinia ruckeri strain 890823-2/1[7]

This strain was isolated from rainbow trout by Inger Dalsgard (Technical University of Denmark).

Viral hemorrhagic septicemia virus[8]

The attenuated VHSV strain ATT 150 belonging to the Genotype I (19) was used in this study. The virus was attenuated via 150 passages in EPC cells (152).

RTS-11[9]

RTS-11 is a cell line of spleen cells from rainbow trout which consists of small non-adherent monocytes and larger adherent macrophage like cells which are phagocytic (138). The cell line was cultured in ZB28 at 20°C and 5 % CO_2 in sealed cap T75 flasks. Cells were split when a confluent cell layer was reached.

[7] Kindly provided by Dr. Uwe Fischer, Friedrich-Loeffler-Institut, Insel Riems, Germany.
[8] Kindly provided by Dr. Sven Bergmann, Friedrich-Loeffler-Institut, Insel Riems, Germany.
[9] Kindly provided by Dr. Nils Bols, University of Waterloo, Canada.

EPC (Collection for Cell Lines and media in Veterinary Medicine, Insel Riems, Germany)
EPC cell line originated from ''epidermal herpes virus-induced hyperplastic lesions'' (153) from *Pimephales promelas* of the carp family (154). The cell line was cultured in ZB5 at 15°C and 5 % CO_2 in sealed cap T75 flasks. Cells were split when a confluent cell layer was reached.

4. Methods

In the following section the cultivation and storage of the microorganisms used for *in vitro* and *in vivo* experiments in this thesis are described.

4.1. Cultivation of *Aeromonas salmonicida*

4.1.1. Growth and storage of A. salmonicida

Bacteria were cultured in tryptic soy broth at 18°C shaking at 200 RPM for 24h. The next day the optical density (OD) was measured using a photometer and the bacterial suspension was diluted to an OD_{600} of 0.1 in tryptic soy broth and further cultivated until the suspension reached an OD_{600} of 0.6 - 0.8. Then, the suspension was centrifuged (3220 g, 5 min, 4°C) and re-suspended in tryptic soy broth containing 25 % glycerol. After dilution to the originally measured OD_{600} the bacterial suspension was transferred to 2 ml cryogenic tubes and stored at -80°C. Before storage, an aliquot was taken to determine the bacterial concentration. Briefly, a serial dilution of the aliquot was prepared and 20 µl of the dilutions 10^{5}, 10^{-6}, 10^{-7} and 10^{-8} were plated on tryptic soy agar plates in triplicate. The plates were incubated at 18°C for 48h and then the colonies were counted. The average of the three triplicates was calculated and multiplied by the dilution factor followed by multiplication with the factor 50 in order to determine CFU/ml (155).

4.1.2. Inactivation of *A. salmonicida*

Bacteria were grown as described in section 4.1.1. When the suspension had reached an OD_{600} between 0.6 and 0.8 an aliquot was taken for the determination of the bacterial concentration. The remaining culture was inactivated in 1.5 % formaldehyde for 1.5h at 4°C. Afterwards the bacterial suspension was centrifuged (3220 g, 5 min, 4°C). The supernatant was discarded and the pellet was washed twice with tryptic soy broth. The washing step consisted of the pellet being re-suspended in 10 ml of tryptic soy broth followed by centrifugation (3220 g, 5 min, 4°C). After the final washing step, bacteria were re-suspended to the measured OD_{600} in tryptic soy broth containing 25 % glycerol and stored at -80°C in 2 ml cryogenic tubes. Before storage, an aliquot was plated on tryptic soy agar plates and incubated for 48 h to confirm the complete inactivation of bacteria.

4.2. Cultivation of *Yersinia ruckeri*

Y.ruckeri strain 890823-2/1 was grown overnight in LB media at 20°C, shaking at 200 RPM. The next day the optical density was measured, and the bacteria were left to grow until OD_{600} 0.6 - 0.8 was reached. The suspension was centrifuged (3220 g, 5 min, 4°C), re-suspended in LB media containing 25 % glycerol and diluted to the originally measured OD_{600}. The bacterial suspension was transferred to 2 ml cryogenic tubes and stored at -80°C. Before storage, an aliquot was taken to measure the bacterial concentration as described in section 4.1.1. Plating of serial dilutions to determine the bacterial concentration was done on Luria base agar plates. Bacteria were inactivated as described in 4.1.2. but incubation with formaldehyde occurred for 24h instead of 1.5h.

4.3. Determination of the protein concentration of lysed bacteria

1 ml of bacterial suspension was centrifuged (5000 g, 10 min, 4°C). Supernatant was discarded and the bacterial pellet was re-suspended in 100 µl of B-PER™ lyses buffer and incubated for 15 min at room temperature. After incubation the suspension was centrifuged (20 min, 16000 g, 4°C). The supernatant was used for detection of protein concentration via a Pierce™ Coomassie Plus (Bradford) Assay Kit, as per the manufacturer's instructions.

4.4. Viral hemorrhagic septicemia virus (VHSV)

4.4.1. Propagation and storage of VHSV

VHSV was cultivated by infection of confluent EPC cells in a T75 sealed cap cell culture flask with 1 ml of VHSV with a titer of 10^7 tissue culture infection $dose_{50}$ ($TCID_{50}$). The infected cells were incubated for 10 days at 15°C. Next, the culture flask containing the infected cells was stored at -20°C overnight to disrupt any intact cells and ensure viral release into the supernatant. The next day the cell suspension was thawed and transferred to 50 ml centrifuge tube and, centrifuged (3220 g, 10 min, 4°C). The supernatant containing the virus particles was transferred to 2 ml cryogenic tubes and stored at -80°C. Stored supernatant could be used to infect a new flask of EPC cells.

4.4.2. Determination of tissue culture infection dose$_{50}$ of VHSV

To determine the $TCID_{50}$ of the cultured virus 2x 10^4 EPC cells were seeded in each well of a 96 well cell culture plate and incubated in 100 µl ZB5 overnight at 15°C. The next day the medium was aspirated and 90 µl of new ZB 5 medium was added. Afterwards 10 µl of a serial dilution from 10^1 to 10^8 of the cell supernatants containing the cultured virus were added to EPC cells in triplicate. The infected cells were incubated for 2 days at 15°C. Then, the medium was aspirated and the cells were fixed by adding 100 µl of ice-cold acetone-methanol (1:1; v:v) in each well and incubating at -20°C for 10 min. Cells were washed two times for 1 min with 1x PBS and one more time for 5 min with 1x PBS. Next, cells were incubated with the anti-viral hemorrhagic septicemia virus N-protein mab Bio282 at a 1:160 dilution in 1x PBS for 20 min at room temperature. The washing procedure described earlier was repeated and cells were incubated with goat anti-mouse IgG (H+L), Alexa Fluor 488, at a 1:1000 dilution in 1x PBS for 20 min at room temperature. The washing steps were repeated, and the cells were covered with 100 µl of fluorescence maintenance buffer and incubated overnight at 4°C.

The next day VHSV positive wells were detected by visualizing infected cells via an inverse light microscope and $TCID_{50}$ was calculated according to the Spearman-Kärber-Formula (156, 157):

$$\log_{10} TCID_{50}\text{-end point dilution} = (x_0 - d/2 + d \sum r/n)$$

With:

x_0 = $\log_{10}$ of the reciprocal of the lowest dilution, of which cells in cell culture wells were positive for VHSV.

d = $\log_{10}$ of dilution factor (1:10 = 1.0).

n = number of cell culture wells used for each viral dilution.

r = number of wells in which cells infected with VHSV could be detected, starting with x_0.

4.5. *In vitro* assays

In order to perform the *in vitro* assays, the cell line RTS-11 was used as well as leukocytes obtained from the peritoneal cavity and head kidney of rainbow trout. This section describes the extraction, harvesting, and stimulation of those cells.

4.5.1. Extraction of leukocytes from the head kidney

Leukocytes were extracted from different lymphatic organs of rainbow trout after fish were euthanized using an overdose of benzocaine. The head kidney was extracted using a scalpel and suspended in 5 ml 1x PBS containing 5 mM EDTA. The organ was disrupted using manual homogenization and the suspension was transferred into 12 ml round bottom tubes. The suspension was centrifuged (5 min, 625 g, 4 °C). The cells were re-suspended in 1 ml of 1x PBS containing 5 mM EDTA with the erythrocytes lysed by hypotonic lysis (158). Briefly, cells were incubated for 20 sec with 8 ml of ice-cold ddH_2O, while inverting the tube, immediately afterwards 1 ml of 10x PBS was added and the samples were filtered via gauze. Afterwards the samples were centrifuged (5 min, 625 g, 4°C), the supernatant was discarded, and the cells were re-suspended in 1 ml of ZB28, and lymphocyte-like cells were counted using a Neubauer haemocytometer to calculate cell number.

4.5.2. Extraction of leukocytes from the peritoneal cavity

The peritoneal cavity was opened carefully by cutting from above the pectoral fins towards the anus using a scalpel. 5 mL of 5 mM EDTA in ice cold 1x PBS was used to gently wash the peritoneal cavity, the washing volume was recovered and transferred to 12 ml round bottom tubes. The cells were further processed as described in 4.5.1.

4.5.3. Harvesting of RTS-11 cells

RTS-11 cells were harvested by first collecting the supernatant. The adherent cells were detached by adding 5 ml of ATV-D solution for 5 min, 10 ml of ZB28 was then added to recover all detached cells. Both the supernatant fraction as well as the detached cells were centrifuged at 110 g for 6 min. Both cell fractions were pooled in 1 ml of ZB28 for cell counting using a Neubauer chamber.

4.5.4. Stimulation of cells

All work done with cells was carried out in a laminar flow cabinet. 10^6 cells of either extracted leukocytes or RTS-11 cells were seeded in 24 well flat-bottomed plates in a total volume of 470 µl ZB28 and left overnight at 20°C to settle. The next day, cells were stimulated with 1x PBS, 5 µg *A. salmonicida* strain JF2267, 50 µg *A. salmonicida* strain JF2267 or 5 µg OMVs

derived from *A. salmonicida* strain JF2267 in 30 µl PBS. Before the bacteria were used for stimulation they were washed once with 1x PBS, centrifuged (5 min, 5000 g, 4°C), and suspended in the appropriate volume of PBS. After 2h, 4h, or 8h of incubation time the supernatant of the cells was transferred to 1.5 ml microcentrifuge tubes. Adherent cells were detached via 5 min incubation with 300 µl of ATV-D. Detached cells were transferred to the same microcentrifuge tube and centrifuged (5 min, 200 g, 4°C). The supernatant was discarded, and cells were re-suspended in 1 ml of EXTRAzol and stored at -80°C prior to RNA extraction.

4.6. *In vivo* experiments

The following section covers the animal trials, describing the method of vaccination, the extraction of immune organs as well as the preparation and isolation of leukocytes and serum for further analysis.

4.6.1. Animal trials

All animal trials (Table 6) were done in accordance with the current ethic rules and conducted under the internal number FLI 28/17, which is registered at the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern with the number 7221.3-2-042/17. Rainbow trout were kept in recirculating 300 l water tanks at 15°C and left for 1 week to acclimatize before the start of a trial. Trials 1, 2 and 4 were done in replicates.

Table 6: Conducted animal trials.

Trial name	Immunization groups	Time points sampled after vaccination	Tissues sampled	Fish weight (g)
1: Early immune response after vaccination with OMVs or bacteria **i.p. vaccination**	(1) 10 µg *A. salmonicida strain* JF2267 (2) 10^8 CFU *A. salmonicida* strain JF2267 (3) 10 µg OMVs derived from strain JF2267 (4) PBS	24h and 72h	Peritoneal cavity, head kidney, blood, and spleen	44 (± 14)

Trial name	Immunization groups	Time points sampled after vaccination	Tissues sampled	Fish weight (g)
2: Antibody response and long-term cellular response **i.p. vaccination**	(1) 10^8 CFU *A. salmonicida* strain JF2267 (2) 10 µg OMVs derived from strain JF2267	0d, 21d, 35d, 49d, 65d, 77d, 91d, 104d	Blood at all-time points; peritoneal cavity, head kidney, spleen, and thymus only at 65d	106 (± 49)
3: Comparison of two strains of *A. salmonicida* **oral vaccination**	(1) oral 10^7 CFU *A. salmonicida* strain JF2267/gram pellet (2) oral 10^7 CFU *A. salmonicida* strain JF5505/gram pellet (3) oral PBS	6h, 12h, 24h, 48h and 7d, 29d	gut, head kidney and spleen	41 (± 12)
4: Evaluation of the oral vaccination model via the use of viral and bacterial antigens **oral vaccination**	(1) 10^7 CFU *A. salmonicida* strain JF5505/gram pellet (2) 10^6 $TCID_{50}$/gram pellet VHSV (3) 10^7 CFU of *A. salmonicida* strain JF5505 per gram pellet and 10^6 $TCID_{50}$/gram pellet VHSV (4) Empty pellets	24h, 72h, 14d and 28d	gut, head kidney, spleen, and blood	187 (± 40)

4.6.1.1. Intraperitoneal vaccination

For i.p. vaccination, fish were anaesthetized via immersion in Benzocaine (30 mg/L) and immunized with 200 µl of 1x PBS containing the stimulants as indicated in table 6 using a 26G needle between pelvic and pectoral fins. Before being used for stimulation, bacteria were centrifuged (3220 g, 5 min, 4°C) and washed twice with 1 ml of 1x PBS, followed by centrifugation (3220 g, 5 min, 4°C). After the final centrifugation bacteria pellets were re-suspended in the appropriate amount of 1x PBS. The control fish received only 200 µl of 1x PBS.

4.6.1.2. Oral vaccination

For oral vaccination food pellets had to be manufactured. For this purpose, the appropriate amounts of polyethylene glycol 1000 and polyethylene glycol 1500 (Table 7) were measured into a 50 ml centrifuge tube and melted in a water bath. The polyethylene glycol mix was solidified at -70°C overnight. The next day the polyethylene glycol mix was placed in a mortar and incubated at 37°C to thaw and soften the mix. When the mix was soft enough the remaining ingredients (Table 7) were mixed in using a pestle. The antigens were added last and mixed in thoroughly using the pestle. Pellets were manufactured via cold extrusion using a manual

extruder and pellets were coated with talcum that was pre-stained black using iron oxide E172. Finally, pellets were coated with commercial fish oil to ensure good uptake. Fish were fed once with 1 g of the vaccine pellets per fish containing the appropriate antigens (Table 7). For trial 3 (Table 6) fish were starved for 36h before being orally vaccinated.

Table 7: Composition of oral vaccine pellets.

Reagent	Trial 3	Trial 4
Polyethylene glycol (PEG) 1000	27.5 g	27.5 g
Polyethylene glycol (PEG) 1500	7 g	7 g
Aluminum oxide (Al_2O_3)	3.5 g	3.5 g
Magnesium hydroxide ($Mg(OH)_2$)	3.5 g	3.5 g
Iron Oxide E172	0.01 g	0.01 g
Inactivated *A. salmonicida* strain JF5505 or JF2267	10^7 CFU/g	10^7 CFU/g
Attenuated VHSV	x	10^6 CFU/g
Inactivated *A. salmonicida* strain JF5505 and Attenuated VHSV	x	10^7 CFU/g and 10^6 CFU/g

4.6.1.3. Sampling and leucocyte preparation

Cells from all organs were used either for FACS analysis or stored at -80°C in 350 µl RLT Buffer (RNeasy Mini Kit) containing 1 % β-mercaptoethanol according to manufactures instructions for RNA extraction. Head kidney and cells from the peritoneal cavity were processed as described in 4.5.1. and 4.5.2., respectively.

4.6.1.3.1. Blood collection and serum preparation

For collecting blood fish were anaesthetized via immersion in Benzocaine (30 mg/L). Blood was taken from the caudal vein using a 26G needle. For trials 2 and 4 (Table 6) 200 µl blood was taken and transferred to 1.5 ml microcentrifuge tubes and left at room temperature until clotted in order to obtain serum. For all other trials 1 ml of blood was collected in 10 ml EDTA blood collection tubes and processed to obtain leukocytes via hypotonic lysis of erythrocytes (158) as described in 4.5.1. After the lysis the samples were centrifuged (5 min, 625 g, 4°C),

the supernatant was discarded, and the cells were re-suspended in 1x PBS containing 5 mM EDTA. Cells were counted using a Neubauer chamber.

After collection and clotting of blood as described above, samples were centrifuged (20 min, 8000 g, 4°C). The upper aqueous phase (sera) was transferred to new 1.5 ml microcentrifuge tubes and stored at -80°C until further analysis.

4.6.1.3.2. Leukocyte extraction

The leukocytes of spleen, thymus and gut were extracted as following. The spleen was extracted from the fish via scissors and surrounding fat tissue was removed. The organ was suspended in 5 ml 1x PBS containing 5 mM EDTA and homogenized to obtain a single cell suspension and further processed as described in 4.5.1. After the hypotonic lysis, cells were immediately counted using a Neubauer chamber.

To remove the thymus, the gills were cut off using scissors and the thymus was removed by inserting the scissors beneath the thymus and cutting around the organ. The organ was mechanically disrupted using a glass homogenizer after being re-suspended in 5 ml 1x PBS containing 5 mM EDTA. The homogenate was filtered via gauze and centrifuged (5 min, 625 g, 4°C). Cells were re-suspended in 1 ml of 1x PBS containing 5 mM EDTA and leukocytes were counted using a Neubauer chamber.

Leukocytes of the gut were obtained by extracting the whole organ and gently removing the remaining food. After washing in 1x PBS for 15 min on ice with gentle agitation the gut was cut longitudinally and incubated in 5 ml of 1 mM EDTA containing 1 mM β-mercaptoethanol in 1x PBS for 30 min at 4°C with soft agitation. After washing the gut pieces three times with 1x PBS they were treated with 0.15 mg/mL Collagenase D in ZB28d for 30 min at room temperature under soft agitation. Medium and gut pieces were placed in a glass homogenizer and mechanically disrupted. The homogenate was filtered through gauze into two 12 ml black cap round bottom tubes. The cells were further processed as described in 4.5.1., omitting the hypotonic lysis.

4.7. Analysis of antibody kinetic and cellular response

To analyze the humoral and cellular response after vaccination antibody titers were detected via enzyme-linked immunosorbent assay (ELISA) and the cellular response was measured via

mabs using flow cytometry to analyze the cellular distribution of certain cell types after vaccination.

4.7.1. Enzyme-linked immunosorbent assay for detection of specific antibody development specific to bacteria

The ELISA protocol used here was adapted from Veenstra et al. (159) and Köllner et al. (160). 96 well high binding plates were coated with 1 µg per well of formaldehyde inactivated *Aeromonas salmonicida* strain JF2267, *Aeromonas salmonicida* strain JF5505 or *Y. ruckeri* strain 890823-2/1 in 50 µl of 1x PBS and incubated at 4°C overnight. The antigen solution was discarded the next day and plates were blocked with 200 µl PBST containing 5 % skim milk for 1h at room temperature. Afterwards, serial dilutions of fish sera diluted in PBST containing 1 % skim milk was added to all wells, except the control wells, and incubated for 1h at room temperature. Next, 50 µl of anti-trout-IgM µ-chain mab 4C10 supernatant was added to all wells at a 1:100 dilution in PBST containing 1 % skim milk and incubated for 1h. Binding of the monoclonal antibody 4C10 was detected by adding 50 µl of goat anti-mouse IgG/IgM-POD per well at a 1:5000 dilution in PBST containing 1 % skim milk and incubated for 1h. After each 1-hour incubation step plates were washed 3 times with 200 µl of PBST. 50 µl of TMB/E solution per well was added to all wells as a substrate for the peroxidase and incubated for 30 min at room temperature in the dark. The reaction was stopped by adding 50 µl of 4N H_2SO_4 to all wells. Plates were read at 450 nm using the Infinite M200 PRO.

4.7.1.1. Enzyme-linked immunosorbent assay for detection of specific antibody development specific to VHSV

To detect antibodies specific to VHSV 2x 10^4 EPC cells were seeded in each well of a 96 well cell culture plate and incubated in 100 µl ZB 5 overnight at 15°C. The next day the medium was aspirated and 100 µl of new ZB 5 medium containing 10^5 $TCID_{50}$ of VHSV were added to all cells except the control well which received 100 µl of ZB 5 medium. Cells were incubated for 2 days at 15°C, the medium was aspirated and the cells were fixed by adding 100 µl of ice-cold acetone-methanol (1:1; v:v) in each well and incubating at -20°C for 10 min. Afterwards the plates were washed 3 times with 200 µl of PBS per well and subsequently blocked with

200 µl PBST containing 5 % skim milk for 1h at room temperature. The next steps were conducted as described for the detection of antibodies specific to bacteria (Section 4.7.1.).

4.7.2. Flow cytometry

For flow cytometry analysis the prepared leukocytes were labelled with different lineage-specific mabs (Table 8). Briefly, after cell counting using a Neubauer chamber 200000 cells were seeded in 96 well clear round bottom plates and, centrifuged (110 g, 3 min, 4°C). Cells were re-suspended in 50 µl of 1x PBS containing 5 mM EDTA and the appropriate monoclonal antibodies which were not conjugated to a fluorochrome (Table 8). Cells were incubated for 30 min at 4°C and washed with 200 µl 1x PBS containing 5 mM EDTA and centrifuged (110 g, 3 min, 4°C). The supernatant was discarded and 50 µl of 1x PBS containing 5 mM EDTA and the appropriate monoclonal antibodies, recognizing the specific isotypes of the first antibodies, conjugated to fluorochromes. After 30 min incubation the cells were washed with 200 µl of 1x PBS containing 5 mM EDTA and centrifuged (110 g, 3 min, 4°C). In the last incubation step directly labelled antibodies were added to the cells for 30 min at 4°C in a volume of 50 µl of 1x PBS containing 5 mM EDTA. Then, cells were washed with 200 µl of 1x PBS containing 5 mM EDTA and centrifuged at 110 g for 3 min and 4°C. After the last incubation and washing step, cells were suspended in 200 µl of 1x PBS containing 5 mM EDTA and transferred to 75x12 mm sample tubes and measured using a FACSCanto II with BD FACSDiva™ software version 8.0.

Table 8: Antibodies and fluorochromes used in the different animal trials.

Trial	Antibodies	Fluorochromes
1 and 2	• Anti-trout thrombocytes mab 42 • Anti-trout pan myeloid cells mab 21 • Anti-trout IgM mab 1.14 • Anti-trout IgK light chain mab N2 • Anti-trout pan T cell mab D30 • Anti-trout CD8α mab CD8-13-2	• APC-Cy7/ RPE-Cy7 (directly labelled) • RPE (directly labelled) • PerCp (directly labelled) • FITC (directly labelled) • DyLight405 • Alexa 647
3	• Anti-trout thrombocytes mab 42 • Anti-trout pan myeloid cells mAb 21	• RPE-Cy7 (directly labelled) • RPE (directly labelled)
4	• Anti-trout thrombocytes mab 42 • Anti-trout pan myeloid cells mab 21	• RPE (directly labelled) • FITC (directly labelled)

4.7.2.1. Gating strategy

To analyze the cellular distribution in the lymphoid organs the gating strategies displayed in figures 6 and 7 were used. Auto-fluorescent cells were excluded from the analysis by looking at two colors that were not used in the staining of the cells (Figure 6 A). Cells within the range of a fluorescence of 10^3 were used for further analysis and further characterized by size by looking at forward scatter (FSC) and sideward scatter (SSC). Due to size and granularity small debris and large cell aggregation were excluded from further analysis (Figure 6 B). Next the height and the area of the FSC of cells was used to exclude cell doublets from further analysis (Figure 6 C). After using this strategy to obtain single cell leukocytes, large granulated cells were defined as **myeloid cells** (FSC_{high}/SSC_{high} - cells) containing mainly myeloid cells but also progenitor cells and plasma cells (161). Smaller less granulated cells were defined as **lymphocytes** (FSC_{low}/SSC_{low} - cells) containing lymphocytes and thrombocytes (Figure 6 D) (144).

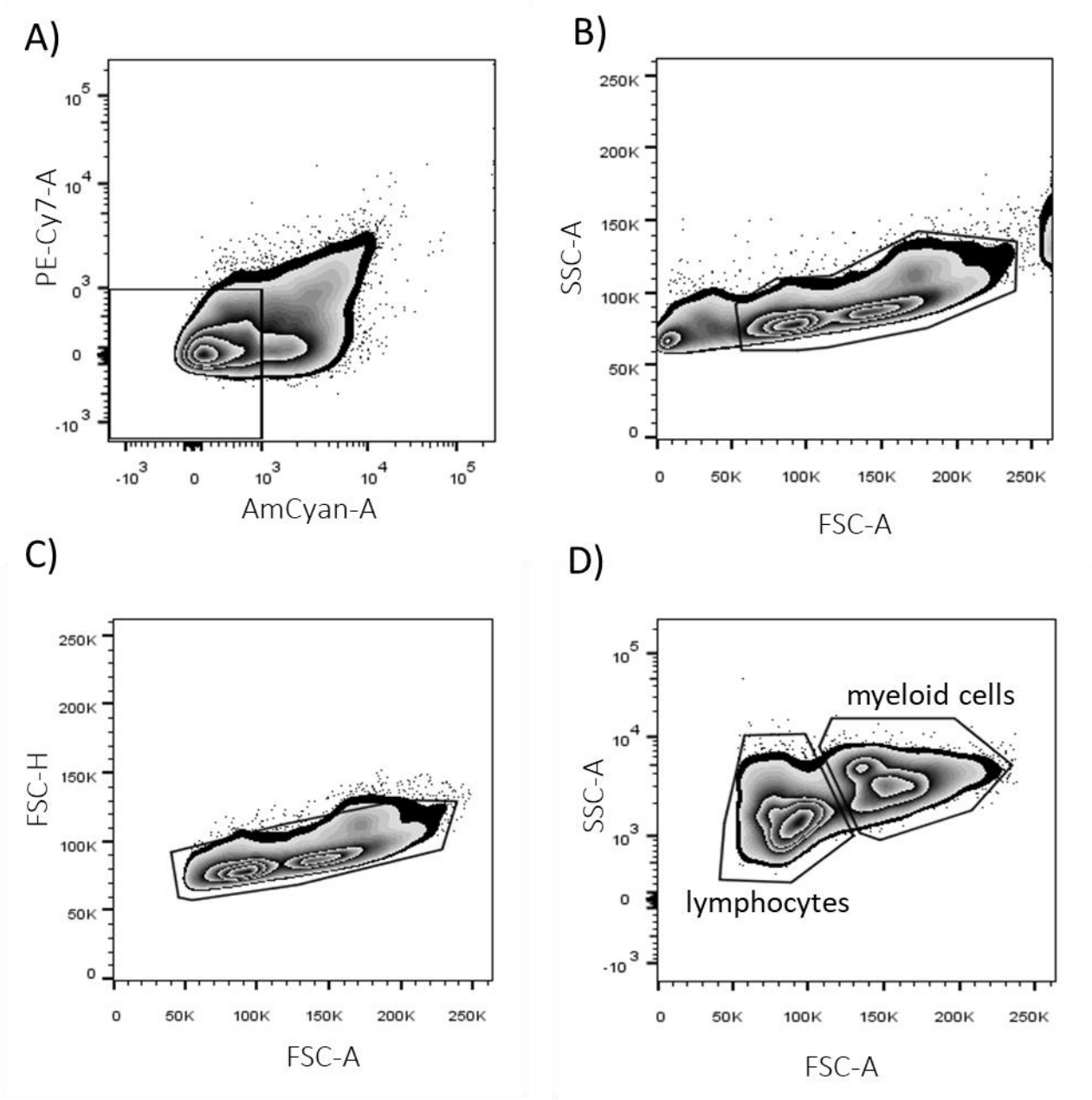

Figure 6: Gating strategy used to determine single cell events in different organs after vaccination of rainbow trout.

Gating strategy is shown here with the example of leukocytes isolated from the head kidney. A) Two colors not used for staining of the antibodies were used to eliminate auto fluorescent cells. B) Gating of leukocytes according to SSC-A and FSC-A, excluding debris and cell clumps. C) Exclusion of doublets via FSC-H and FSC-A. D) Contour plot showing the leukocyte population used for further analysis, with lymphocytes and myeloid cells being labeled.

To further characterize those morphologically distinguished cells, monoclonal antibodies (mabs) were used. The denoted FSC_{high} and SSC_{high} cells contain mainly myeloid (granulocytes and monocytes (162)) and **monocytes/macrophages** could be characterized with the anti-trout pan myeloid cells mab 21 from the FSC_{high} and SSC_{high} cells alone (Figure 7 A). Due to

their small size and low amount of granulation, **thrombocytes** are found in the FSC_{low} and SSC_{low} gate (96) and could be clearly identified by using the anti-trout thrombocytes mab 42 (Figure 7 B).

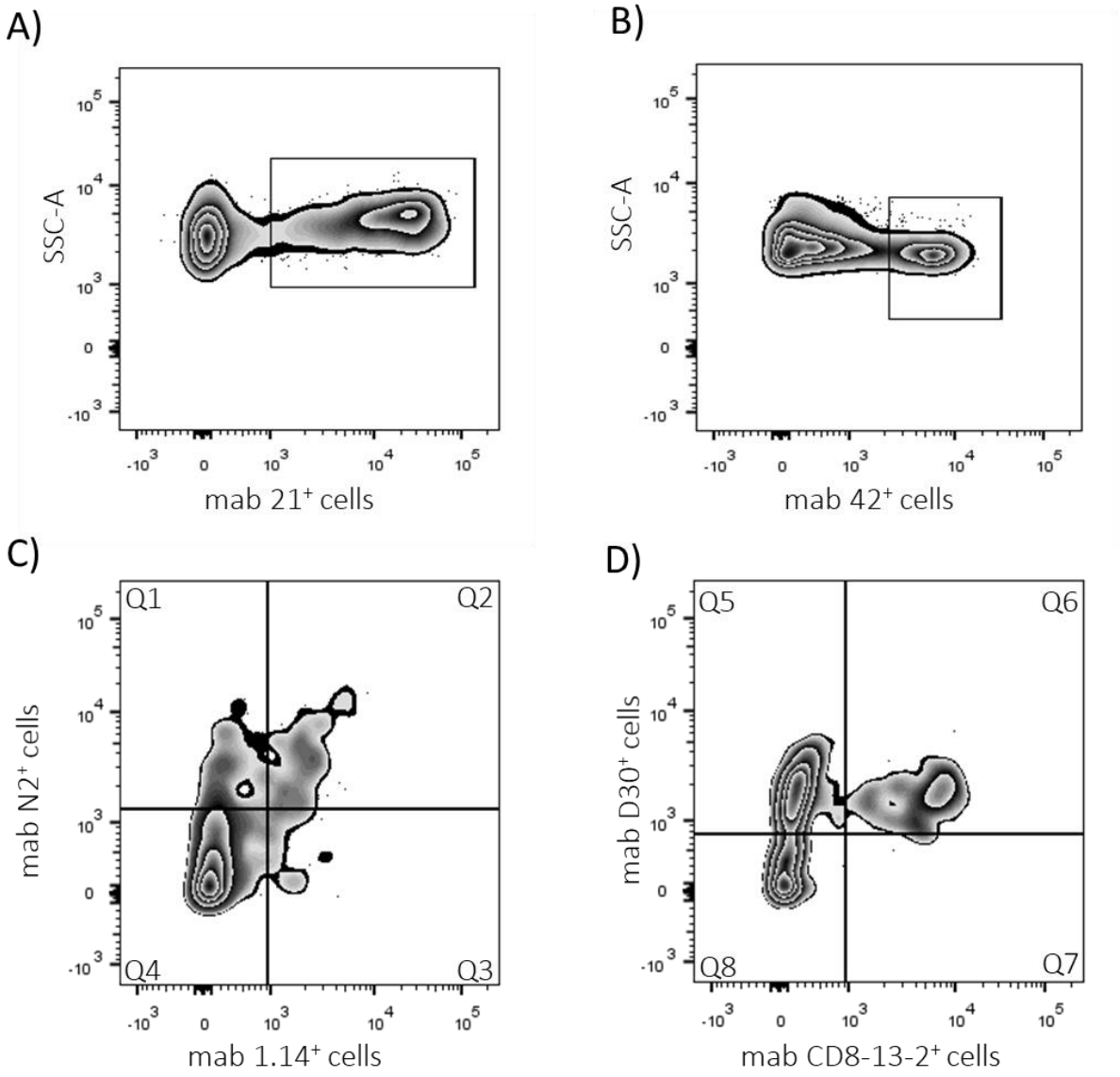

Figure 7: Gating strategy used to analyze the cellular distribution in percentage in different organs after stimulation of rainbow trout.

A) anti-trout pan myeloid cells mab 21 positive stained cells were only determined from the FSC_{high}/SSC_{high} - cell population. B-D) Cell populations determined from the FSC_{low}/SSC_{low} - cell population; anti trout thrombocytes mab 42 stained cells (B), anti-trout IgM mab 1.14 (C: Q2 and Q3) anti-trout IgK light chain mabN2 (C: Q1 and Q2), anti-trout pan T cell mab D30 positive cells (D: Q5 and Q6) and anti-trout CD8α mab CD8-13- positive cells (D: Q6).

In addition to thrombocytes, B and T cells were also analyzed based on the characterized FSC_{low} and SSC_{low} cells (Figures 7 C and D). The B cells were differentiated as **IgM^+ B cells** characterized by anti-trout IgM mab 1.14^+ single positive B cells and anti-trout IgM mab 1.14^+ and

anti-trout IgK light chain mab $N2^+$ double positive B cells (Figure 7 C: Q2 and Q3); or as **IgT^+ B cells** characterized by anti-trout IgM mab 1.14^- but anti-trout IgK light chain mab $N2^+$ single positive B cells (Figure 7 C: Q1).

For the T cells it was possible to distinguish between **$CD8^-$ T cells**, detected only by the anti-trout pan T cell mab D30 (Figure 7 D: Q5, containing $CD4^+$ T cells) and **$CD8^+$ T cells,** characterized by anti-trout pan T cell mab D30 and anti-trout CD8α mab CD8-13-2 double positive cells (Figure 7 D: Q6). For the flow cytometry analysis at least 10.000 events of single leukocyte cells were recorded (Figure 6 C) and the percentage of positive cells for recorded antibodies was used to analyze changes between treatment groups.

4.8. Analyzes of mRNA pattern after stimulation

To investigate changes in the mRNA expression pattern after *in vitro* and *in vivo* stimulation the RNA was extracted from the cell line RTS-11 as well as leukocytes derived from the peritoneal cavity and head kidney.

4.8.1. RNA extraction

For RNA extraction of cell lines and leukocytes the EXTRAzol kit was used. The manufactures instructions were modified as described below. Samples were thawed and 200 µl ice-cold chloroform was immediately added, microcentrifuge tubes were capped tightly and shaken for 20 sec. After incubation at 4°C for 3 min, the samples were centrifuged (15 min, 13550 g, 4°C). The upper aqueous phase was transferred to a new 1.5 ml microcentrifuge tube and 500 µl of ice-cold isopropanol containing 3.4 µl of GlycoBlue were added. After 10 min incubation at 4°C, the samples were centrifuged (10 min, 13550 g, 4°C). Supernatant was discarded and RNA was washed with 1 ml of ice-cold 75 % ethanol. The solution was briefly vortexed at maximum speed using a power mixer. The samples were centrifuged (5 min, 9000 g, 4°C), the supernatant was removed, and the blue pellet was dissolved in 30 µl of RNAse free ddH_2O. After determining the RNA concentration of each sample using a spectrophotometer, the RNA was stored at -80°C. For RNA extraction of cells stored in RLT Buffer from animal trials, the RNeasy Mini Kit was used according to manufactures instructions.

4.8.2. Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

The extracted RNA was diluted to 25 ng/µl and the SensiFAST SYBR No-ROX One-Step Kit was used for all qPCR amplifications. Working in a PCR workstation a master mix for the number of samples needed was prepared as described in table 9. The diluted RNA samples (25 ng/µl) were added outside of the PCR workstation directly into the prepared reaction mix. The qPCR protocol described in table 10 was used for all amplifications using a real time cycler and the results were visualized with the software Bio-Rad CFX Maestro. The copy numbers for each primer pair were calculated based on the linear regression of the standard curve, obtained when calculating the primer efficiency (104). The fold change of the individual genes was calculated as following: The relative gene expression cycle threshold (Ct) values obtained from RT-qPCR were normalized using the Ct values obtained for the housekeeping gene Elongation Factor (EF)-1α, the Ct values of the PBS control group was subtracted and the computed results log_2 transformed.

Table 9: Reaction mix per sample for RT-qPCR.

Reagent	Volume
SensiFAST SYBR No-ROX One-Step Mix	5 µl
10 µM Forward Primer	0.4 µl
10 µM Reverse Primer	0.4 µl
Reverse transcriptase	0.1 µl
RiboSafe RNase Inhibitor	0.2 µl
DEPC-H_2O	1.9 µl
RNA template	2 µl
	10 µl final volume

Table 10: Protocol for RT-qPCR.

PCR Program	Temperature	Time	Cycles
Reverse transcription	45°C	10 min	1
Initial denaturation	95°C	2 min	1
Denaturation	95°C	5 sec	35

Annealing	62.5 - 65 °C*	10 sec	
Elongation and signal acquisition	72°C	5 sec	
Product dissociation	95°C	5 sec	1
Signal acquisition melt curve	65°C	5 sec	1
	95°C	30 sec	1
*Depending on the annealing temperature of the primer pair.			

4.9. Primer validation for RTq-PCR

The primers listed in table 7 were used for amplification of the selected genes. To ensure that the chosen primer pairs worked as described they had to be verified in the used setting. For this purpose, the annealing temperature, the size of the amplicon as well as the primer efficiency of each primer pair were tested.

Table 11: Primers used for RT-qPCR.

Name	Sense (3´-5´)	Antisense (3´-5´)	Size (bp)	Reference
Myd88	GATCAAGAATTACGAGGATTGCC	CCTCAATGAGAACTCGGAGATC	155	(104)
IRAK1	ATGGACAGTATCTCCGATGTGG	TGCAGCTTGTCCAGTACGTTCA	173	(104)
TNFα	GATACCCACCATACATTGAAGCA	ATTTGGTTCCCCTGTAGCTCGA	162	(104)
TGFβ	ATCAGGGATGAACAAGCTGAGG	TTCGCACACAGCAACTCTCCG	152	(104)
IL-1β	AAGTCTTTAAGCAACTGACTAAGC	TGCACTTTCAGAGGTGTTCTTTAT	182	(104)
iNOS	CGAATGGAGCTATCGTCAGACC	CGGGAACGTTGTGGTCATAATACC	234	(163)
EF1α	CAAGGATATCCGTCGTGGCA	ACAGCGAAACGACCAAGAGG	327	(164)
IL-6	CTGTCTGCCAGTGAGAGGAA	CCACTCTGTCCCCGTAGAC	57	(165)
IL-8	AGAGACACTGAGATCATTGCCAC	CCCTCTTCATTTGTTGTTGGC	162	(141)
IL-10A	GGATTCTACACCACTTGAAGAGCCC	GTCGTTGTTGTTCTGTGTTCTGTTGT	167	(141)
OligodT	-	TTTTTTTTTTTTTT	-	Eurogentec

4.9.1. Establishing the annealing temperature and verifying the size of the amplicon

The annealing temperature of each primer was obtained by performing a gradient PCR (Table 12).

Table 12: Protocol for gradient PCR.

PCR Program	Temperature	Time	Cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	5 sec	35
Annealing	65 - 58°C	5 sec	
Elongation	72°C	10 sec	
Final elongation	72°C	1 min	1

cDNA was synthesized from the isolated RNA using superscript 2 reverse transcriptase (Table 13) and primer specific DNA was amplified using Phire hot start II DNA-polymerase (Table 14).

Table 13: Protocol for cDNA synthesis.

Reagent	Volume	Incubation time and temperature
DEPC-H_20	6 µl	10 min 65°C
OligodT 10 µM	1 µl	
DNTP 10 nm	1 µl	
RNA template	5 µl	
		2 min on ice
5x reverse transcriptase buffer	4 µl	60 min 45°C 15 min 70°C
0.1 M DTT	1 µl	
Superscript 2 reverse transcriptase	1 µl	
25 mM $MgCl_2$	2 µl	

In this step the correct amplicon size was verified by applying 10 µl of the PCR reaction mix to a 2 % agarose gel and running it for 1h at 110 volts in an electrophoresis system. The gel was visualized using a ChemiDoc.

Table 14: Reaction mix per sample for gradient PCR.

Reagent	Volume
5x Buffer	4 µl
10 µM Forward Primer	0.4 µl
10 µM Reverse Primer	0.4 µl
DNTP 10 nm	0.1 µl
Phire polymerase	0.2 µl
DEPC-H_2O	12.9 µl
cDNA template	2 µl
	20 µl final volume

4.9.2. Determining the efficiency of the primer pairs for use in RT-qPCR

Efficiency was determined using RNA extracted from unstimulated RTS-11 cells as described in section 4.8.1. and was calculated based on serial dilutions of RNA ranging from 25 ng/µl to 0.0025 ng/µl. For each primer pair the standard curve was generated in Excel (2019) by linear regression analysis using the Ct- values obtained from RT-qPCR for each amplification reaction versus the $\log_{10}$ RNA copy number (166). The primer efficiency was than calculated using the free software provided by ThermoFisher Scientific (167).

4.10. Statistical analysis

Statistical analysis was performed using the software GraphPad Prism 5.01. In the *in vitro* experiments the copy numbers obtained after computing the RT-qPCR Ct values as described in section 4.8.2. were assessed using either a two-way analysis of variance (ANOVA) (Bonferroni post-test) or one-way ANOVA (Dunnett's multiple comparisons test), depending on the number of groups, which were compared to the PBS control group.

For the *in vivo* experiments different statistical analysis were used depending on the circumstances, as described below.

In animal trial 1 (Table 6) significant changes after i.p. injection with 10 µg OMVs, 10 µg of inactivated *A. salmonicida* strain JF2267 or 10^8 CFU of inactivated *A. salmonicida* strain JF2267 in comparison to the PBS injected group were determined using a one-way ANOVA (Dunnett's multiple comparisons test). While the copy numbers obtained after computing the RT-qPCR Ct values for the head kidney and spleen after stimulation with 10 µg OMVs or 10 µg of inactivated *A. salmonicida* strain JF2267 as described in section 4.8.2. were assessed using a two-way analysis of variance (ANOVA) (Bonferroni post-test) in comparison to the PBS control group.

In animal trial 2 (Table 6) significant differences in the long-term cellular response after i.p. injection of either 10 µg OMVs or 10^8 CFU of inactivated *A. salmonicida* strain JF2267 as well as the antibody titers were detected using an unpaired t-test.

In animal trials 3 and 4 (Table 6) significant differences between the experimental groups and the PBS control group were computed using a two-way analysis of variance (ANOVA) (Bonferroni post-test). For all statistical tests a confidence interval of 95 % was used and significance was determined when $p < 0.05$.

5. Results

To answer whether OMVs derived from the fish pathogenic bacterium *A. salmonicida* can be used as a vaccine antigen with potential use as an oral vaccine, different *in vitro* and *in vivo* experiments were conducted.

First, the immune stimulatory potential of OMVs was evaluated *in vitro* by analyzing the mRNA expression of immunoregulatory cytokines. For this purpose, the immortalized monocyte/macrophage cell line RTS-11 as well as leukocytes derived from the peritoneal cavity (representing cells at the site of vaccination), and head kidney leukocytes (as the primary lymphoid organ in fish and main source of macrophages) (110, 139, 140) were used. The *in vitro* experiments were followed by i.p. injection of rainbow trout with OMVs, using the established model of peritoneal inflammation (15), to investigate the early cellular response, mRNA expression of immunoregulatory cytokines in primary and secondary lymphoid organs, and the antibody response in the serum. The second part of the *in vivo* results deals with the adaption of the oral model for vaccination with VHSV (19), to be used for immunization with inactivated bacteria and a combination of inactivated bacterial and viral antigens together.

To perform these experiments and obtain conclusive results, the methods were established and validated first. Therefore, the results section will start with the cultivation of VHSV, and the validation of the primers used for RT-qPCR.

5.1 Establishing and validating methods used

5.1.1. Titration of VHSV

To determine the appropriate amount of VHSV, the titer of the VHSV stocks had to be determined as described in section 4.4. The nuclei of the cells were stained red since the fluorescence maintenance buffer contained propidium iodide. The viral hemorrhagic septicemia N-protein could be detected in the cell cytoplasm via the specific mab Bio282 and subsequent staining with Alexa Fluor 488, using an inverse light microscope (Figure 8 E-F).

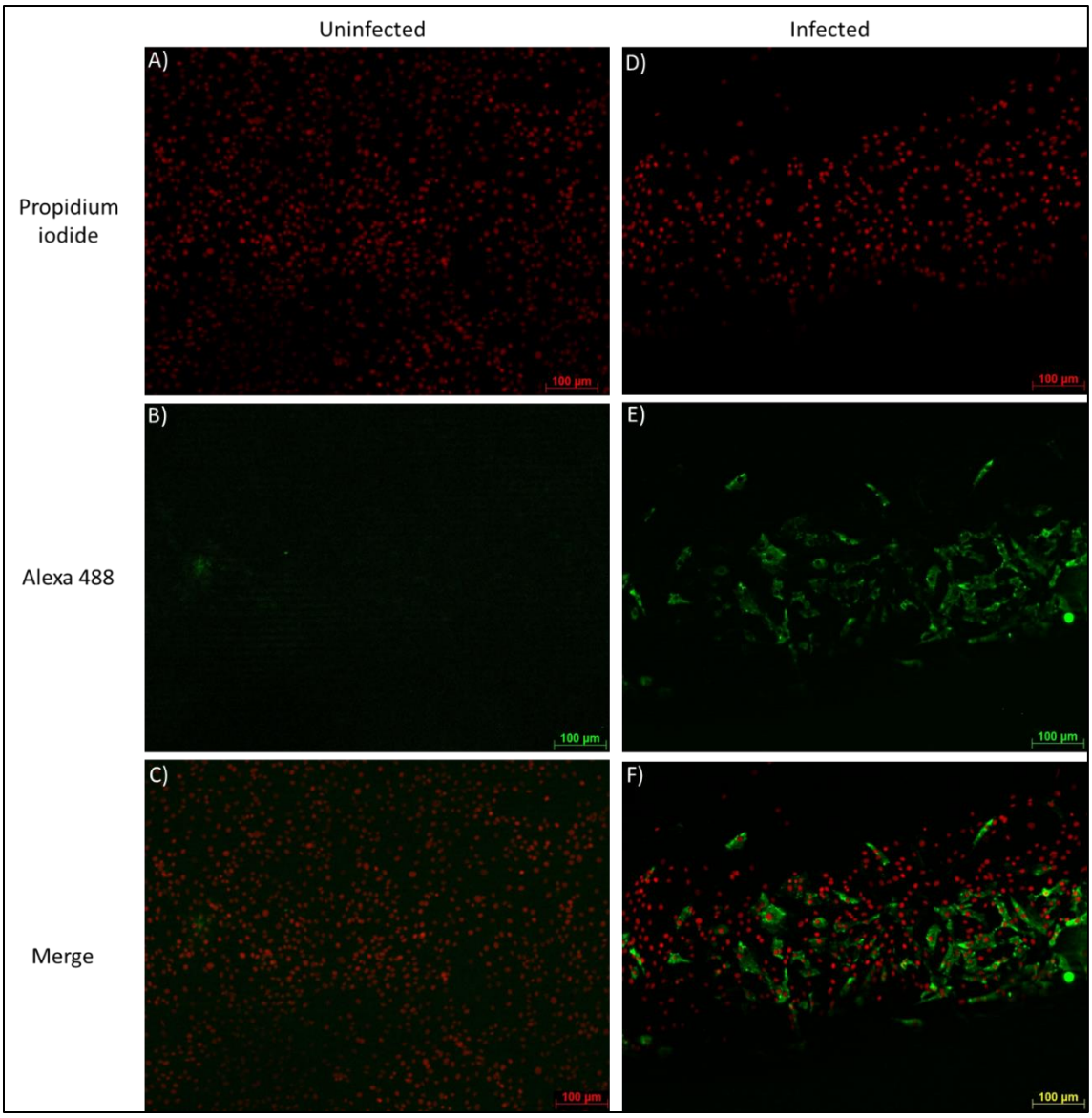

Figure 8: Uninfected EPC cells and EPC cells infected with VHSV.

EPC cells were used to determine the $TCID_{50}$ of VHSV stocks. A serial dilution of the VHSV stocks was done and 10 µl of each dilution was used to infect one well of EPC cells. After 2 days of incubation cells were fixed using acetone-methanol. An indirect ELISA using the VHSV N-protein specific mab Bio 282 and goat anti-mouse IgG (Alexa Fluor 488) (green) was used to detect infected wells and nuclei were counterstained with propidium iodide (red). A-C: uninfected EPC cells, D-F: EPC cells infected with VHSV.

All wells in which cells infected with the virus could be detected were counted as positive (Table 15) and used for the calculation of the $TCID_{50}$ according to the Spearman-Kärber-Formula (156, 157):

$$\log_{10} TCID_{50}\text{-endpoint dilution} = (x_0 - d/2 + d \sum r/n)$$

With:

x_0 = the $\log_{10}$ of the reciprocal of the lowest dilution, of which cells in cell culture wells were positive for VHSV.

d = $\log_{10}$ of dilution factor (1:10 = 1.0).

n = number of cell culture wells used for each viral dilution.

r = number of wells in which cells infected with VHSV could be detected, starting with x_0.

Table 15: Results of viral serial dilution as an example for $TCID_{50}$ calculation.

Viral dilution	Number of cell culture wells	Number of cell culture wells in which VHSV was detected
10^{-5}	4	4
10^{-6}	4	2
10^{-7}	4	0

After determining the number of cell culture wells that contained cells infected with VHSV the Spearman-Kärber-Formula (156, 157) was used to calculate the $TCID_{50}$:

With:

$x_0 = 5$

d = $\log_{10}(10) = 1.0$

n = 4

r = 6

$$\log_{10} TCID_{50}\text{-end point dilution} = (5 - 1/2 + 1 \sum 6/4)$$

$$= 10^{6.0}\ TCID_{50}/100\ \mu l$$

$$= \underline{10^{7.0}\ TCID_{50}/1\ ml}$$

5.1.2. Primer validation for use in RT-qPCR.

The gene expression pattern after stimulation with either *A. salmonicida* derived OMV or inactivated *A. salmonicida* was first analyzed *in vitro* using the cell line RTS-11 as well as isolated leukocytes from different organs of rainbow trout. Therefore, the efficacy of the qPCR and the selected primers had to be validated as described in section 4.9. This was done for each primer pair and is shown with primers used to amplify the housekeeping gene EF1α. A gradient PCR was performed for the primers specific to EF1α to determine the optimal annealing temperature for the EF1α molecule (Figure 9).

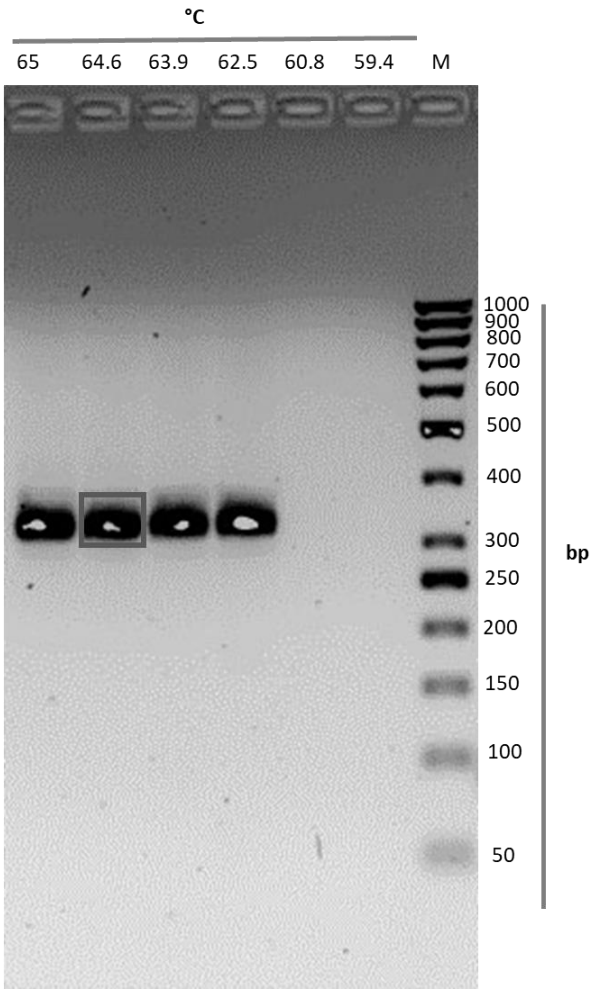

Figure 9: Amplicons of a gradient PCR performed with the primer pair for amplification of EF1α.
Agarose gel electrophoresis was used to determine the size of the amplified cDNA with primers specific for EF1α with a length of 327 bp (Table 11) 10 µl of PCR reaction were applied to each lane, M: molecular marker (10 µl), relevant sizes are indicated. Optimal amplicon is marked with ☐, and optimal temperature can be taken from the gradient displayed above the gel picture.

After the optimal temperature and the correct size of the amplicon were determined, the primer efficiency had to be calculated as described in section 4.9.2. An RT-qPCR with the primers specific for EF1α on a serial dilution of RNA ranging from 25 ng/µl to 0.0025 ng/µl was

conducted in duplicate, and the obtained Ct-values (Table 16) were used to generate a standard curve (Figure 10). Using the slope of the equation for the regression curve, the primer efficiency was calculated (Table 17). During the RT-qPCR on the serial dilution of RNA, a melt curve was generated to analyze the specificity of the primer pair, ensuring that only one DNA product is amplified (168) (Figure 11). As described for the example of EF1α, the primer efficiency was calculated for each primer pair (Table 17).

Table 16: Results of RT-qPCR for generation of a standard curve for EF1α.

RNA (ng/µl)	Log_{10} (RNA)	Average Ct-value
25	1.39794	20.62
2.5	0.39794	24.36
0.25	- 0.602006	27.66
0.025	- 1.60206	30.77
0.0025	- 2.60206	33.32

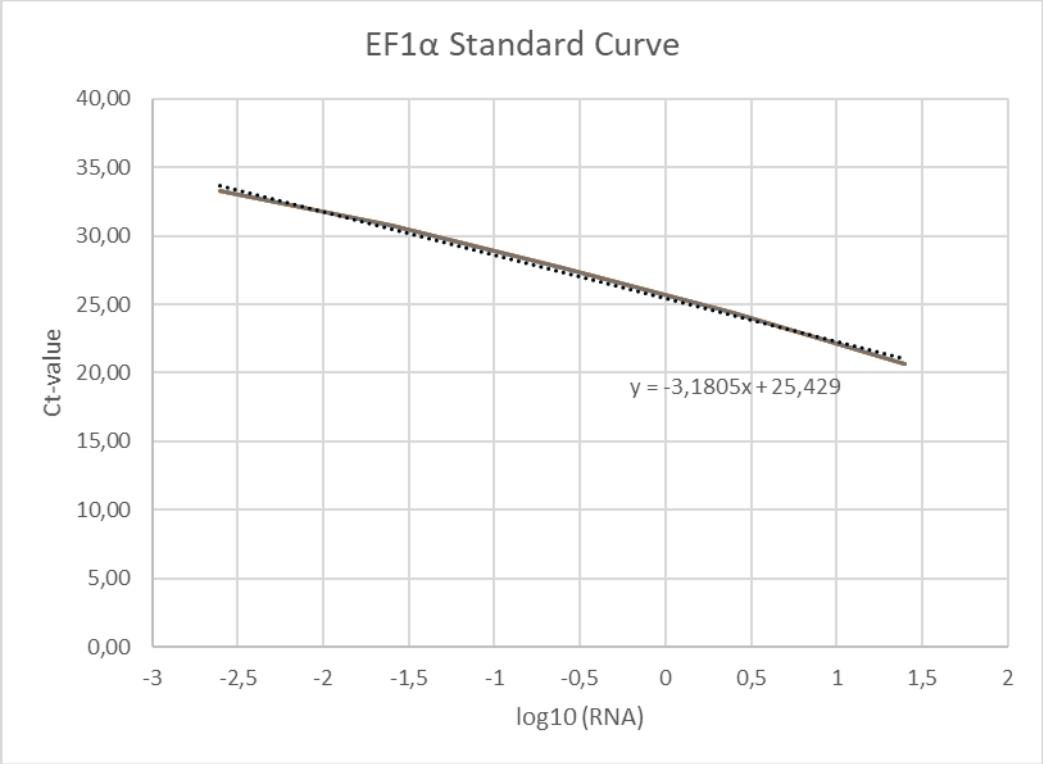

Figure 10: Standard Curve of EF1α.
Ct-values obtained after RT-qPCR with a serial dilution of RNA extracted from unstimulated RTS-11 cells were plotted against the log_{10} of the used RNA dilutions resulting in a standard curve (grey line). The regression line (black dotted line) was calculated in Excel, and the appropriate equation is shown below the curve. The slope of the equation has been used to determine the primer efficiency using the free software provided by ThermoFisher Scientific (167).

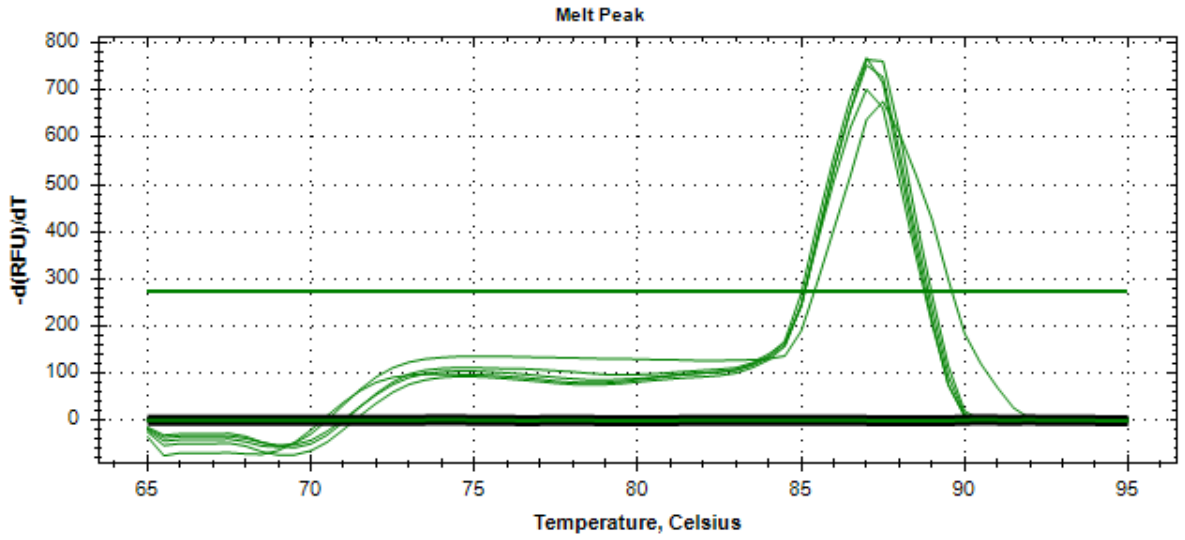

Figure 11: Melt Curve of RT-qPCR of EF1α amplification.
As the final step of the RT-qPCR, a melt curve was performed to ensure the amplification of one DNA molecule. The melt curve is automatically computed by the software (Bio-Rad CFX Maestro) used by the real-time cycler (CFX96 Touch), and the melting temperature for the EF1α amplicon is 87.5 °C. Therefore, the melt curve is depicted as the "rate of change in relative fluorescence units (RFU) with time (dt) versus temperature in Celsius (C°)" (169).

The calculated efficiency of the primer pairs ranges from 91.94 % to 116.96 % (Table 17). An efficiency of at least 90 % is expected for robust DNA amplification (170), so all primer pairs could be used in this study.

Table 17: Primer efficiency and optimal temperature of primers used in this study.

Name	Tm (°C)	Efficiency (%)
Myd88	65	91.94
IRAK1	64.6	94.30
TNFα	64.6	106.55
TGFβ	62.5	105.03
IL-1β	62.5	106.52
EF1α	64.6	106.26
IL-6	64.6	113.07
IL-8	62.5	104.41
IL-10A	62.5	101.32
iNOS	62.5	116.96

5.2. *In vitro* analyzes of the immune stimulatory potential of OMVs derived from *A. salmonicida*

5.2.1. Analyzes of the immune stimulatory potential of OMVs

The first step in target-orientated vaccine development had to be to investigate if OMVs can induce a similar stimulation response as inactivated bacteria. For this purpose, the monocyte/macrophage cell line RTS-11 and leukocytes from the head kidney and the peritoneal cavity were stimulated with OMVs and inactivated bacteria. Based on granularity and size, the cell composition of the different leukocytes used for the *in vitro* experiments was obtained as described in section 4.7.2.1. The monocyte/macrophage cell line RTS-11 shows a homogeneous cell population (Figure 12 A). In contrast, leukocytes from the head kidney and peritoneal cavity clearly show lymphocytes and myeloid cells, with myeloid cells being the dominant cell type in the head kidney and lymphocytes dominating the peritoneal cavity (Figure 12 B and C).

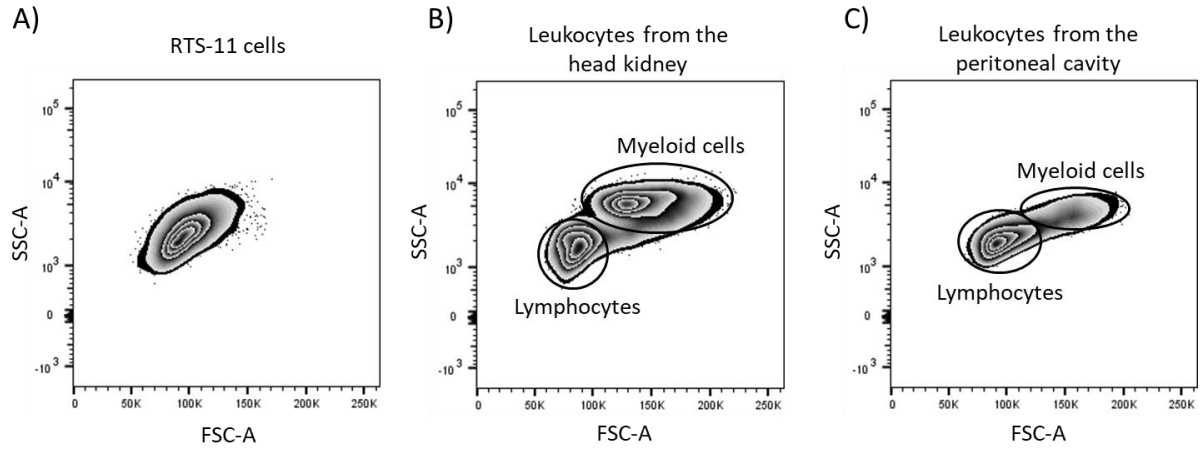

Figure 12: Leukocyte composition of the different cells used for *in vitro* experiments based on their granularity (SSC-A) and size (FSC-A).
The monocyte-macrophage cell line RTS-11 (A), leukocytes from the head kidney (B), and leukocytes from the peritoneal cavity (C) of naïve rainbow trout were isolated and analyzed for their granularity (SSC-A) and size (FSC-A) using FACSCanto II.

5.2.1.1. Stimulation with bacterial antigen results in significant changes in the mRNA expression of the cell line RTS-11

As described earlier, the cell line RTS-11 has been successfully used as a simplified *in vitro* model to study the impact of bacterial and viral stimuli on monocyte/macrophage-like cells of rainbow trout (110, 112, 171-174). Here the potential of OVMs to induce a change in the

mRNA expression of early activation markers of inflammation was analyzed, in the cell line RTS-11, to understand further the role of macrophages as the first line of defense against pathogens.

Because OMVs consist of similar protein composition, as found in the bacteria they are derived from, it was expected that stimulation with OMVs would lead to similar results as stimulation with bacteria. Therefore, RTS-11 cells were treated as described in section 4.5.3. and RNA was isolated using the EXTRAzol kit (Section 4.8.1.). The extracted RNA was used to amplify genes of interest using the primer pairs listed in table 11 and the RT-qPCR conditions described in table 10. As this was the first experiment conducted, all the available primer pairs were used to identify the possible genes involved in the immune response to bacteria and OMVs.

A heat map of the gene expression pattern of regulatory, pro-, and anti-inflammatory molecules of RTS-11 cells was generated (Figure 13).

	Regulatory		**Pro-inflammatory**					**Anti-inflammatory**	
2h	Myd88	IRAK1	IL-1β	IL-6	IL-8	TNFα	iNOS	IL-10	TGFβ
OMV	2,6	1,8	9,2	11,2	74,9	2,8	8,7	1,5	1,0
AS 5 µg	2,8	1,9	4,8	3,0	9,4	1,8	1,6	1,7	1,6
AS 50 µg	2,7	2,6	**22,2**	13,5	100,3	6,3	1,2	13,7	1,0
4h	Myd88	IRAK1	IL-1β	IL-6	IL-8	TNFα	iNOS	IL-10	TGFβ
OMV	1,7	2,7	8,5	2,8	71,8	3,1	1,0	12,6	1,0
AS 5 µg	2,4	2,6	12,2	16,6	**89,4**	6,8	2,4	346,7	1,7
AS 50 µg	4,1	**6,6**	**29,7**	**201,8**	**219,0**	**29,1**	-1,3	**1216,1**	1,3
8h	Myd88	IRAK1	IL-1β	IL-6	IL-8	TNFα	iNOS	IL-10	TGFβ
OMV	1,6	2,6	10,2	2,7	99,0	4,7	**-1,4**	3,7	-1,4
AS 5 µg	2,2	3,0	7,8	76,1	**132,0**	8,6	**-1,6**	90,2	-1,9
AS 50 µg	2,5	4,4	**33,9**	**374,4**	**229,8**	**36,1**	**1,3**	**551,7**	-1,5

Figure 13: Response of immune-relevant genes after stimulation of RTS-11 cells using different vaccine antigens.

RTS-11 cells were stimulated with 5 µg *A. salmonicida* strain JF2267 (AS 5 µg), 50 µg *A. salmonicida* strain JF2267 (AS 50 µg) or 5 µg OMVs derived from *A. salmonicida* strain JF2267 (OMV). The fold change of different genes compared to the PBS control group was measured by RT-qPCR. In addition, all genes were normalized to the housekeeping gene EF1α. ▬ indicates increased expression and ▬ indicates decreased expression of the gene compared to the PBS control. Statistically significant differences in expression ($p < 0.05$) are highlighted in bold, calculated by using a two-way ANOVA, based on copy numbers (N = 6).

The stimulations with OMVs and the stimulation with inactivated bacteria induced a similar gene response pattern. This can, for example, be seen 2h after stimulation in the increased

expression of IL-1β, IL-6 and IL-8. The expression of regulatory genes is not drastically changed after stimulation with the different antigens. In contrast, IL-10 gene expression is significantly increased only after stimulation with 50 µg of inactivated *A. salmonicida* (Figure 13). Thus, even though a substantial increase in mRNA expression can be seen for pro-inflammatory genes after stimulation with the three different antigens, this change is not significant for all of them. This could be explained by the large variation of the obtained copy numbers, used for the statistical calculations. To visualize this, the copy numbers of IL-8 have been shown for the individual experiments 4h after stimulation for each of the used antigens (Figure 14). Stimulation with all the used antigens increased copy numbers of IL-8, but this change was not significantly different from the PBS control group, due to the extensive range.

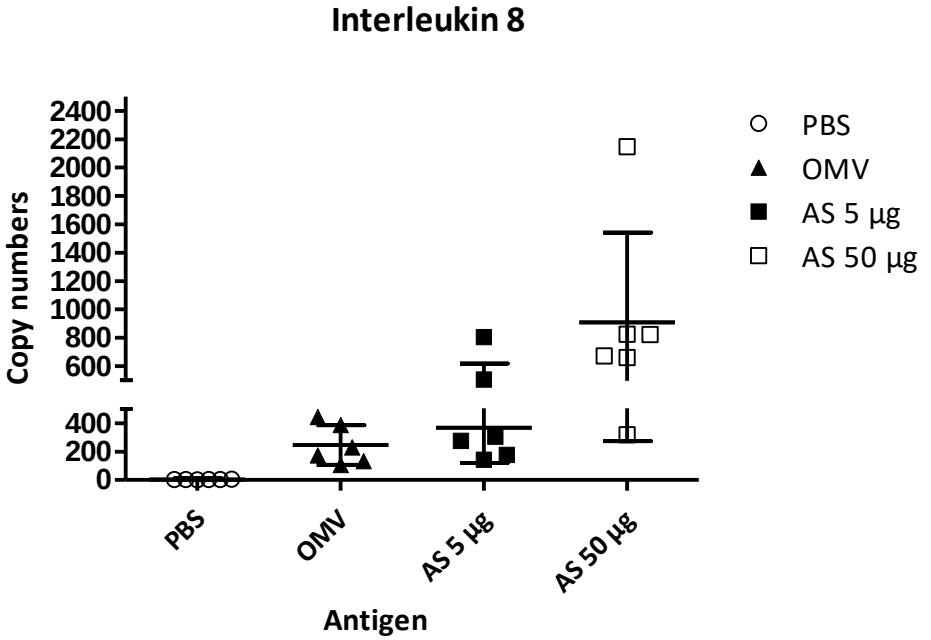

Figure 14: Copy numbers of IL-8 gene 4h after stimulation of RTS-11 cells with different antigens.
RTS-11 cells were stimulated with 5 µg *A. salmonicida* strain JF2267 (AS 5 µg), 50 µg *A. salmonicida* strain JF2267 (AS 50 µg) or 5 µg OMVs derived from *A. salmonicida* strain JF2267 (OMV). Copy numbers of IL-8 were calculated using the Ct-values obtained after RT-qPCR and the equation of the linear regression line of the standard curve. The computed copy numbers were normalized to the housekeeping gene EF1α.

5.2.1.2. Stimulation with *A. salmonicida* and derived OMVs significantly alters mRNA expression of leukocytes from the head kidney

Stimulation of the immortalized monocyte/macrophage cell line RTS-11 with inactivated bacteria and derived OMVs resulted in a similar change in mRNA expression of early activation markers of inflammation (Figure 13). The head kidney is the main lymphoid organ in fish (13,

87), and the leukocyte population is dominated by myeloid cells (Figure 12). Leukocytes of the head kidney have been used as a source for fish macrophages in different studies (110, 139, 140) and have been suggested to be recruited to the site of vaccination (137). Therefore, leukocytes from the head kidney have been used here to analyze if the findings obtained for the cell line RTS-11 could be replicated *ex vivo*.

After obtaining leukocytes from the head kidney as described in sections 4.5.1., the cells were stimulated and processed as described in sections 4.5.4. Primer pairs listed in table 11 and the qPCR conditions listed in table 10 were used to amplify genes of interest using the extracted RNA. Only the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNFα as well as the anti-inflammatory cytokine IL-10, were investigated as they showed the most substantial changes in RTS-11 cells (Figure 13).

Again, it could be shown that stimulation of leukocytes from the head kidney with OMVs leads to similar results obtained after stimulation with inactivated bacteria (Figure 15).

	Pro-inflammatory				**Anti-inflammatory**
2h	IL-1β	IL-6	IL-8	TNFα	IL-10
OMV	17,4	1,9	14,5	3,3	1,8
AS 5 µg	2,4	1,5	3,1	1,3	1,9
AS 50 µg	**42,4**	3,8	12,0	5,7	4,0
4h	IL-1β	IL-6	IL-8	TNFα	IL-10
OMV	**18,7**	**11,0**	**13,6**	10,7	7,9
AS 5 µg	6,2	1,9	4,3	1,9	4,8
AS 50 µg	**68,4**	**13,6**	**21,1**	4,5	106,0
8h	IL-1β	IL-6	IL-8	TNFα	IL-10
OMV	8,3	1,7	4,4	5,6	2,5
AS 5 µg	5,6	3,8	4,3	6,5	3,2
AS 50 µg	**19,6**	**13,9**	7,8	**31,6**	**66,5**

Figure 15: Response of immune-relevant genes in the head kidney cells after stimulation with different vaccine antigens.

Head kidney cells were stimulated with 5 µg *A. salmonicida* strain JF2267 (AS 5 µg), 50 µg *A. salmonicida* strain JF2267 (AS 50 µg) or 5 µg OMVs derived from *A. salmonicida* strain JF2267 (OMV). The fold change of different genes compared to the PBS control group was measured by RT-qPCR. In addition, all genes were normalized to the housekeeping gene EF1α. ▬ indicates increased expression and ▬ indicates decreased expression of the gene compared to the PBS control. Statistically significant differences in expression ($p < 0.05$) are highlighted in bold, calculated by using a two-way ANOVA, based on copy numbers (N = 5).

For leukocytes from the head kidney, stimulation with OMVs and 50 µg of inactivated bacteria resulted in significant changes of pro-inflammatory genes at 4h and again, only stimulation of

the leukocytes with 50 µg of inactivated bacteria results in substantial changes of IL-10 expression (Figure 15). Unlike the results obtained after stimulation of RTS-11 cells (Figure 13), stimulation with 5 µg of *A. salmonicida* strain JF2267 only showed a low increase in the expression of the investigated genes (Figure 15).

5.2.1.3. Only stimulation with inactivated bacteria leads to significant changes in the mRNA profile of leukocytes from the peritoneal cavity

Injection into the peritoneal cavity is one possible mode of vaccine delivery and has been described to result in a protective immune response after vaccination with bacterial antigens (175). Leukocytes of the peritoneal cavity mainly consist of lymphoid cells (Figure 12), but recruitment of myeloid cells, particularly macrophages, has been reported after i.p. injection of bacterial antigens (15). Here, cells derived from this organ were analyzed for their ability to be stimulated by OMVs to understand how the recruitment of myeloid cells to the peritoneal cavity might be activated. Significant changes in gene regulation were mainly observed at 4h after stimulation of RTS-11 cells and leukocytes from the head kidney (Figures 13 and 15). Therefore, only this time point was investigated for primary cells derived from the peritoneal cavity.

After obtaining leukocytes from the peritoneal cavity as described in sections 4.5.2., the cells were stimulated and processed as described in sections 4.5.4. and 4.8.1. Primer pairs listed in table 11 and the qPCR conditions listed in table 10 were used to amplify genes of interest using the extracted RNA.

Similar to the previous experiments with RTS-11 cells (Figure 13) and leukocytes from the head kidney (Figure 15), stimulation with 50 µg of inactivated bacteria results in increased expression of pro-inflammatory cytokines and anti-inflammatory cytokines at 4h. However, the expression of the investigated genes is only slightly increased 4h after stimulation with OMVs (Figure 16).

	Pro-inflammatory				Anti-inflammatory
4h	IL-1β	IL-6	IL-8	TNFα	IL-10
OMV	4,2	2,9	1,9	5,0	2,2
AS 50 µg	**11,2**	**44,4**	**4,5**	**30,8**	**89,2**

Figure 16: Response of immune relevant genes after stimulation of cells derived from the peritoneal cavity.

Cells obtained from the peritoneal cavity were stimulated with PBS, 50 µg *A. salmonicida* strain JF2267 (AS 50 µg), or 5 µg OMVs derived from *A. salmonicida* strain JF2267 (OMV) and the fold change of selected immune-relevant genes compared to the PBS control group was measured by RT-qPCR. All genes were normalized to the housekeeping gene EF1α. ▬ indicates increased expression and ▬ indicates decreased expression of the gene compared to the PBS control. Statistically significant differences in expression ($p < 0.05$) are highlighted in bold, calculated by using Dunnett's multiple comparisons test, based on copy numbers (N = 3).

5.3. *In vivo* results

To obtain an idea of the immune stimulatory potential of OMVs derived from *A. salmonicida*, rainbow trout cells known to be involved in the initial immune response after i.p. vaccination were investigated. The *in vitro* experiments were used as a model for the initial reaction after vaccination, looking at macrophages (responsible for bacterial clearance and antigen presentation), and leukocytes of the peritoneal cavity (as the first cells to encounter the antigen after i.p. vaccination). The *in vitro* experiments showed that stimulation of leukocytes from the peritoneal cavity with OMVs resulted only in a low change in the expression pattern of the investigated genes (Figure 16). However, stimulation of leukocytes from the head kidney and the monocyte/macrophage cell line RTS-11 resulted in a change of the expression of early inflammatory markers indicating the immunogenic potential of OMVs derived from *A. salmonicida* (Figures 13 and 15). Knowing that *in vitro* experiments can only reflect some factors involved in an immune response and the fact that bacterial antigens have been shown to induce drastic changes of the leukocyte composition in the peritoneal cavity, the cellular distribution of the leukocyte composition in the peritoneal cavity of rainbow trout after i.p. vaccination with OMVs was analyzed *in vivo*.

5.3.1. I.p. vaccination with OMVs

As mentioned earlier, previous research using the peritoneal cavity as a model for stimulation has shown that the peritoneal cavity of naïve fish is dominated by leukocytes of the lymphocyte lineage, lymphocytes (FSC_{low}/SSC_{low}). After stimulation or infection, a drastic influx of myeloid cells (FSC_{high}/SSC_{high}) could be observed in the first 12h to 24h followed by domination of

the lymphocytes, again at 72h after stimulation (15). The same model was used to investigate the cellular response after vaccination with OMVs locally (peritoneal cavity) and in the lymphoid organs (head kidney and spleen) as well as the blood. Furthermore, it is known that the percentage of cells, especially myeloid cells, changes throughout the day in fish (176). Additionally, it has been reported that injection of PBS alone can result in changes in the cellular distribution of leukocytes in the peritoneal cavity (15, 143). Therefore, time-matched PBS vaccinated fish are used as controls for each time point in all the *in vivo* trials where PBS has been used as a solvent.

5.3.1.1. I.p. vaccination with OMVs and inactivated bacteria results in a similar local response of myeloid cells.

In animal trial 1 (Table 6), fish were injected with 10 µg of OMVs derived from *A. salmonicida* strain JF2267 and compared to fish injected with either 10 µg or 10^8 CFU of *A. salmonicida* strain JF2267. This animal trial was conducted to see if injection with OMVs induced the same cellular response observed for injection with inactivated bacteria.

The change of the leukocyte composition has been investigated via FACS using lineage-specific mabs as described in section 4.7.2.

To better visualize the changes in the leukocyte population observed after vaccination, the leukocyte population of naïve fish was compared to the leukocyte population of fish 24h after i.p. vaccination with the bacterial antigen (Figure 17).

The described influx of myeloid cells 24h after i.p. vaccination with bacterial antigen (15) was also observed in the conducted animal trials of this thesis (Figure 17 B). As an example of the characterization of cells stained with a single mab, the myeloid cells positive for the anti-trout pan myeloid cells mab 21 are depicted (Figure 17 C and D). In addition, the distinction between IgM^+ B cells and IgT^+ B cells via the use of the mabs 1.14 and N2 is shown as an example for the characterization of cells with two mabs´s (Figure 17 E and F). The percentages obtained for the individual cell population are shown as bar graphs in the following sections of this thesis.

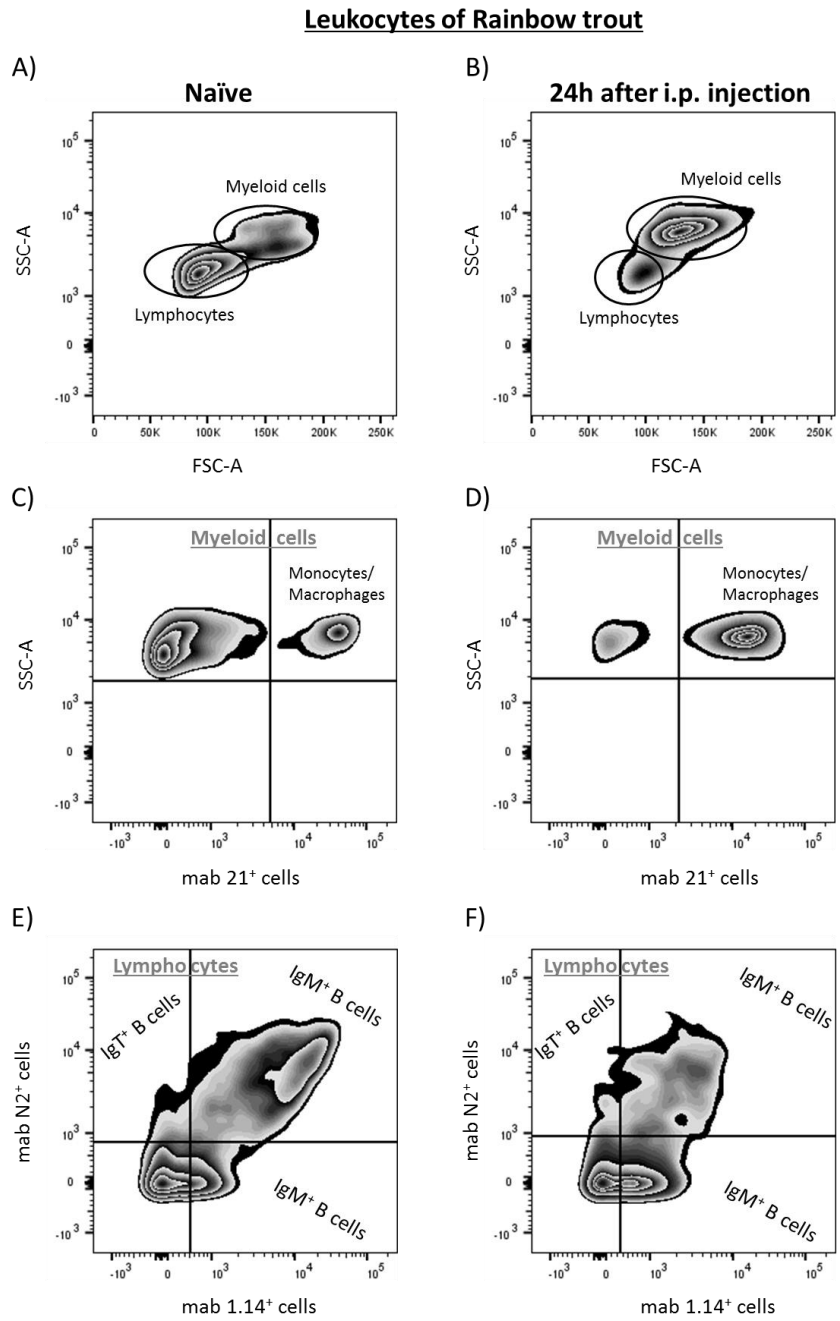

Figure 17: Changes observed in the leukocyte composition 24h after i.p. vaccination with bacterial antigen.

Flow cytometry analyzes of extracted leukocytes from the peritoneal cavity (A-D), and the blood (E and F) was done using FACSCanto II, comparing naïve fish (A, C and E) to fish injected with bacterial antigen, 10^8 CFU of inactivated *A. salmonicida* (B and D) or 10 µg of inactivated *A. salmonicida* (E). The change in the leukocyte composition based on the SSC and FSC (A and B), monocytes/macrophages (anti-trout pan myeloid cells mab 21^+ cells) (C and D), IgM^+ B cells (characterized by anti-trout IgM mab 1.14^+ single positive B cells and anti-trout IgM mab 1.14^+ and anti-trout IgK light chain mab $N2^+$ double-positive B cells) and IgT^+ B cells (mab $N2^+$) (E-F) are shown as examples for cells positive for a single mab and cells characterized by two mabs.

First the percentages of the leukocytes in the peritoneal cavity after antigenic stimulation were analyzed (Figure 18). 24h after stimulation, a significant decrease in the percentage of

lymphocytes for the group stimulated with 10^8 CFU of *A. salmonicida* could be observed. This was accompanied by a substantial increase of myeloid cells in the peritoneal cavity at this time point (Figure 18 A and C).

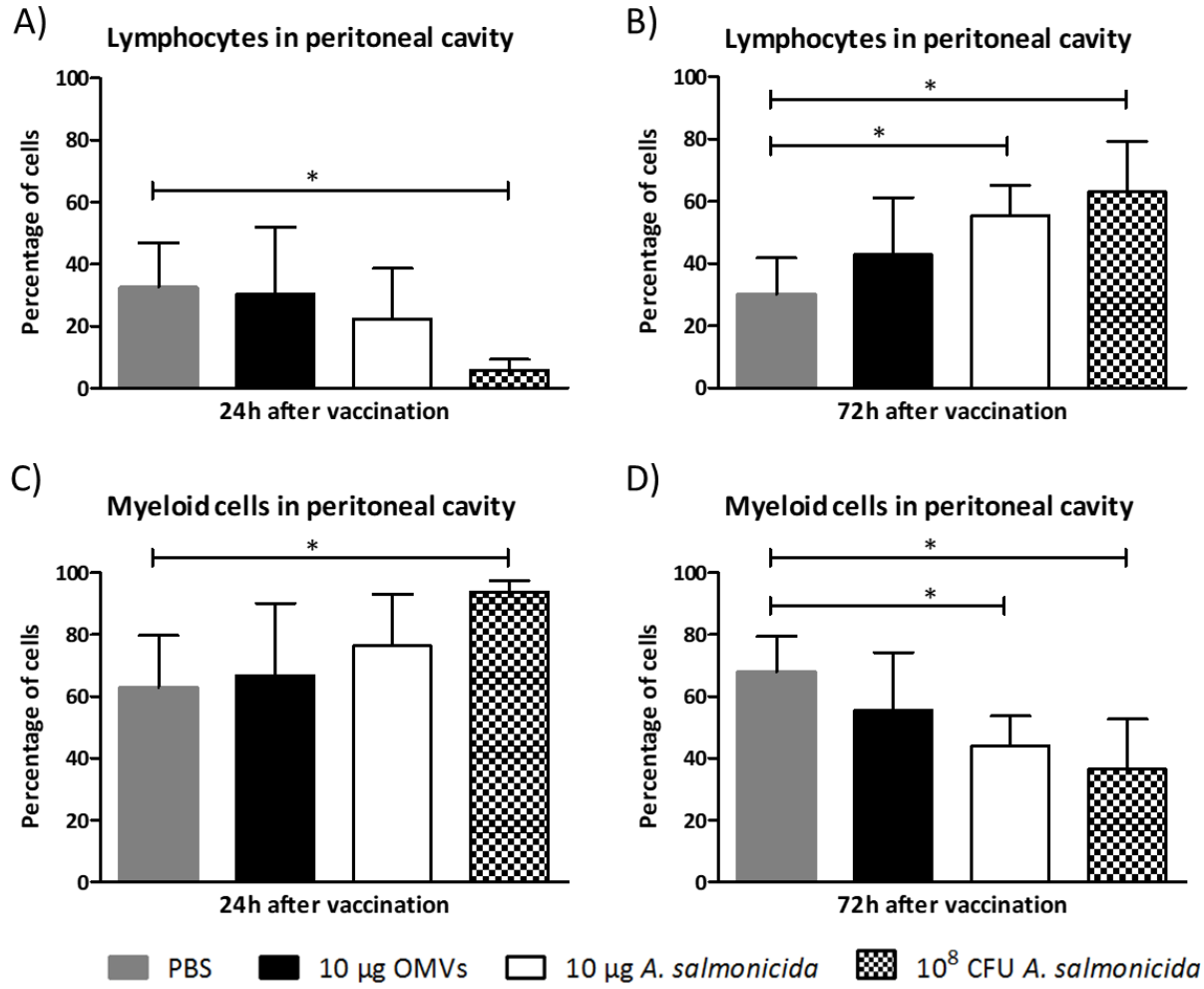

Figure 18: Lymphocytes and myeloid cells in the peritoneal cavity 24h and 72h after vaccination.
Fish were injected with either PBS (N=9), 10 µg OMVs (N=8), 10 µg of inactivated *A. salmonicida* (N=9) or 10^8 CFU of inactivated *A. salmonicida* (N=6). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of lymphocytes 24h (A) and 72h (B) after vaccination as well as of myeloid cells 24h (C) and 72h (D) after vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using Dunnett's multiple comparisons test.

At 72h after vaccination, the percentage of lymphocytes is significantly increased, leading to a decreased percentage of myeloid cells. This was also significant for the group stimulated with 10 µg of *A. salmonicida*. The cellular distribution of fish stimulated with OMVs follows the same trend observed for fish stimulated with inactivated bacteria (Figure 18). The change in the percentage of lymphocytes and myeloid cells at 24h and 72h, respectively, is also significant compared to time. To make this change clear, the data shown in figure 18 is presented differently in figure 19.

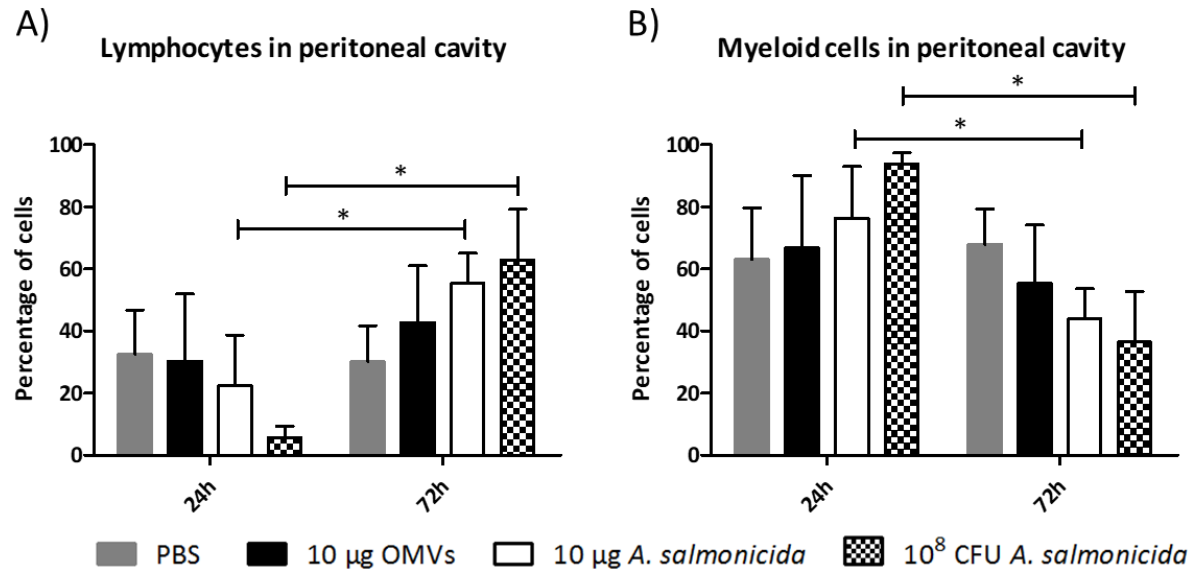

Figure 19: Lymphocytes and myeloid cells in the peritoneal cavity 24h and 72h after vaccination.
Fish were injected with either PBS (N=9), 10 µg OMVs (N=8), 10 µg of inactivated *A. salmonicida* (N=9) or 10^8 CFU of inactivated *A. salmonicida* (N=6). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of lymphocytes 24h and 72h (A) after vaccination and myeloid cells 24h and 72h (B) after vaccination was compared to each other over time. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using Dunnett's multiple comparisons test.

When analyzing the reaction of specific cell types, this trend becomes even more apparent. For example, the anti-trout pan myeloid cells mab 21 has been used to analyze monocytes/macrophages of the myeloid cells. At 24h, only stimulation with 10^8 CFU of inactivated *A. salmonicida* resulted in a significant increase of the monocytes/macrophages (Figure 20). However, 72h after vaccination, a substantial increase of the monocytes/macrophages can be seen after injection of 10 µg of OMVs and 10 µg of inactivated *A. salmonicida*, compared to the PBS group (Figure 20).

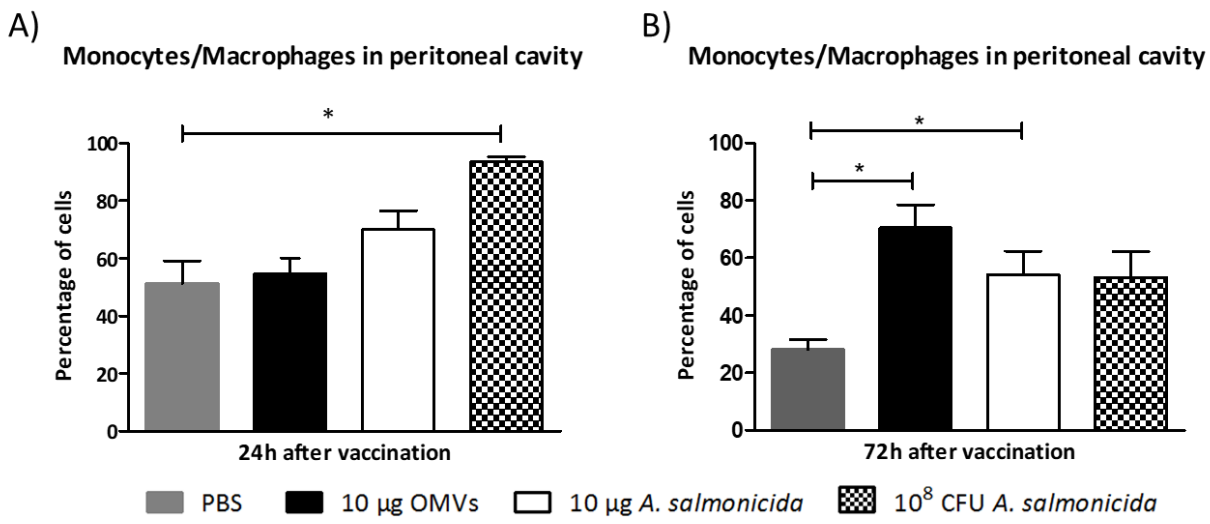

Figure 20: Monocytes/macrophages in the peritoneal cavity 24h and 72h after vaccination.
Fish were injected with either PBS (N=9), 10 µg OMVs (N=8), 10 µg of inactivated *A. salmonicida* (N=9) or 10^8 CFU of inactivated *A. salmonicida* (N=6). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of myeloid cells (anti-trout pan myeloid cells mab 21^+ cells) 24h (A) and 72h (B) after vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using Dunnett's multiple comparisons test.

In addition to the monocytes/macrophages, IgM^+ B cells also showed significant changes after injection with both concentrations of inactivated bacteria (Figure 21). Vaccination with either concentration of inactivated bacteria has led to a considerable increase in the percentage of IgM^+ B cells 72 h after vaccination (Figure 21 A and B), while the percentage of IgT^+ B cells did not change in any of the observed groups (Figure 21 C and D). All other cells analyzed with lineage-specific mabs did not show significant changes in their distribution in the peritoneal cavity (data not shown).

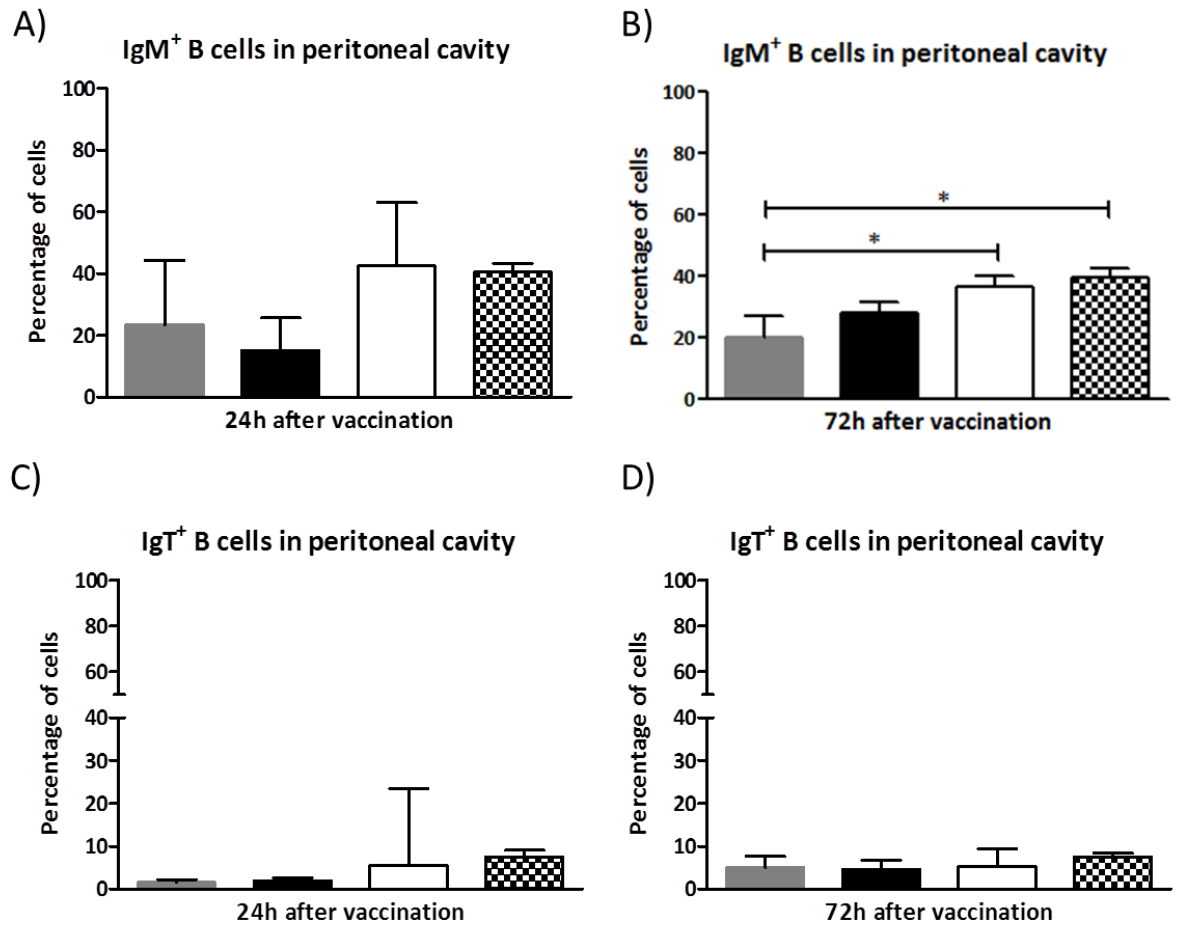

Figure 21: IgM$^+$ B cells and IgT$^+$ B cells in the peritoneal cavity 24h and 72h after vaccination.
Fish were injected with either PBS (N=9), 10 µg OMVs (N=8), 10 µg of inactivated *A. salmonicida* (N=9) or 10^8 CFU of inactivated *A. salmonicida* (N=6). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage IgM$^+$ B cells (characterized by anti-trout IgM mab 1.14$^+$ single positive B cells and anti-trout IgM mab 1.14$^+$ and anti-trout IgK light chain mab N2$^+$ double-positive B cells) 24h (A) and 72h (B) and IgT$^+$ B cells (mab N2+ single positive cells) 24h (C) and 72h (D) after vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using Dunnett's multiple comparisons test.

5.3.1.2. Analysis of the cellular composition in lymphoid organs and blood shows a different response after i.p. vaccination with OMVs and inactivated bacteria

Previous studies have shown a significant increase of B cells in the head kidney of Atlantic salmon after i.p. vaccination with a multivalent commercial vaccine against bacterial and viral diseases (177). It has also been shown that i.p. injected foreign particles, such as *A. salmonicida* antigens are transported to the head kidney and spleen (178-180). Additionally, it was proven that, i.p. injection of microparticles resulted in a migration of cells from the site of injection to lymphoid organs such as the head kidney and spleen (181). Therefore, the cellular

composition characterized by the lineage-specific mabs was investigated in the head kidney, spleen, and blood to analyze if i.p. vaccination with OMVs also changes the leukocyte composition in other organs.

No overall response could be observed in the different organs, and it seems that they follow a different pattern depending on the antigen used for i.p. vaccination. For example, in the blood, only 72h after immunization with 10 µg of OMVs, a significant decrease in the percentage of lymphoid cells accompanied by a substantial increase in the percentage of myeloid cells could be observed (Figure 22 A and B).

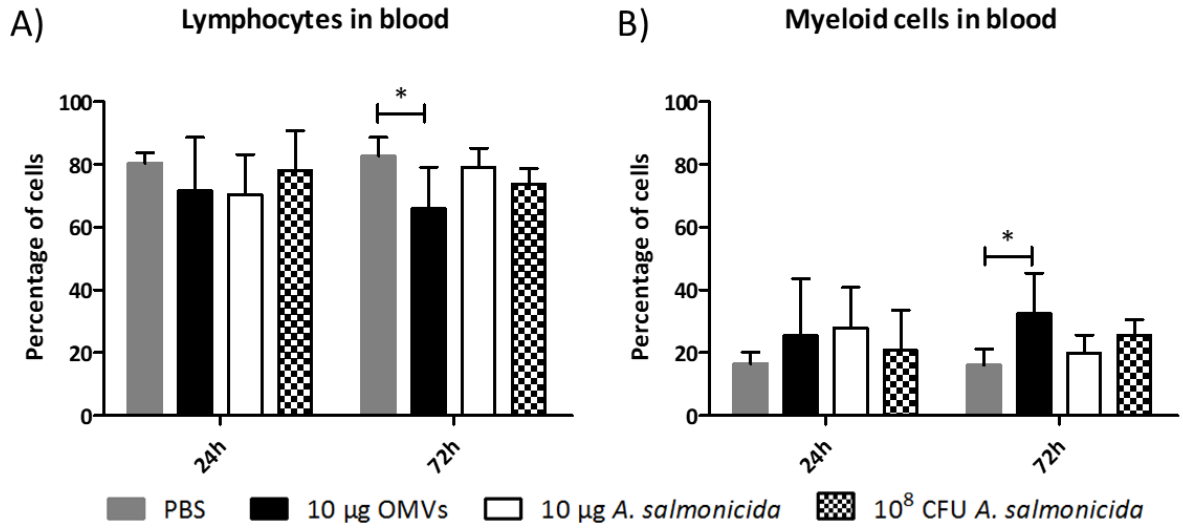

Figure 22: Lymphocytes and myeloid cells in the blood 24h and 72h after vaccination.
Fish were injected with either PBS (N=9), 10 µg OMVs (N=8), 10 µg of inactivated *A. salmonicida* (N=9) or 10^8 CFU of inactivated *A. salmonicida* (N=6). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of lymphocytes (A) and myeloid cells (B) 24h and 72h after vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using Dunnett's multiple comparisons test.

While in the head kidney an increase of lymphoid cells could be observed at 24h and 72h after injection of OMVs, only injection of 10 µg of inactivated *A. salmonicida* led to a significant decrease in the percentage in lymphoid cells accompanied by a significant increase in the percentage of myeloid cells at 24h and 72h (Figure 23 A and B). In the spleen, no changes in the percentage of lymphocytes or myeloid cells could be detected in any of the groups (data not shown).

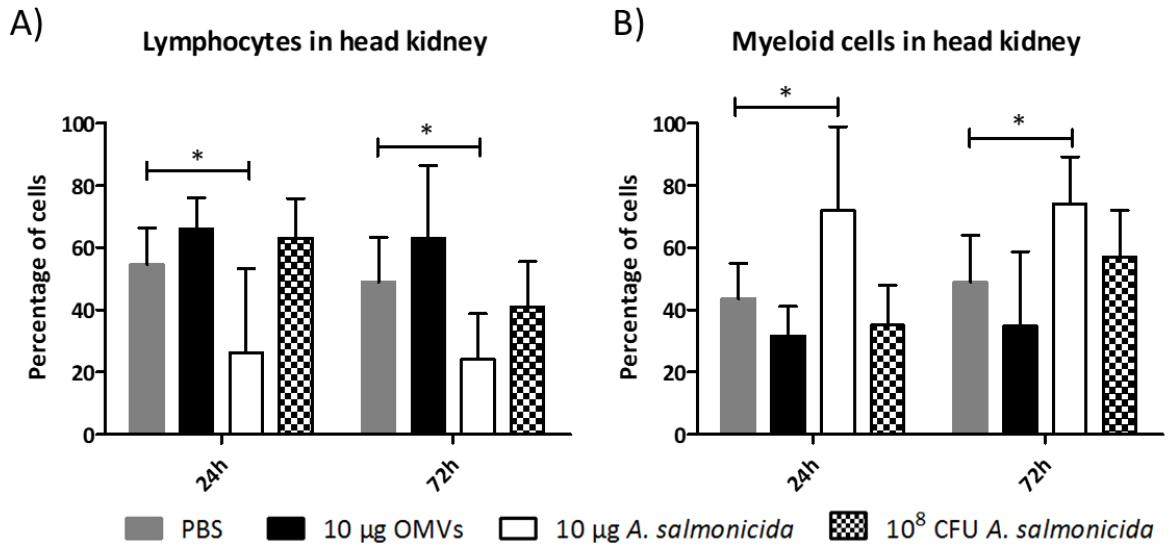

Figure 23: Lymphocytes and myeloid cells in the head kidney 24h and 72h after vaccination.
Fish were injected with either PBS (N=9), 10 µg OMVs (N=8), 10 µg of inactivated *A. salmonicida* (N=9) or 10^8 CFU of inactivated *A. salmonicida* (N=6). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of lymphocytes (A) and myeloid cells (B) 24h and 72h after vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using Dunnett's multiple comparisons test.

Looking at the leukocyte populations recognized by lineage-specific mabs in the different organs, the percentage of monocytes/macrophages was significantly altered in the spleen after injection of 10 µg of OMVs, resulting in a significant increase in the percentage of this cell population (Figure 24).

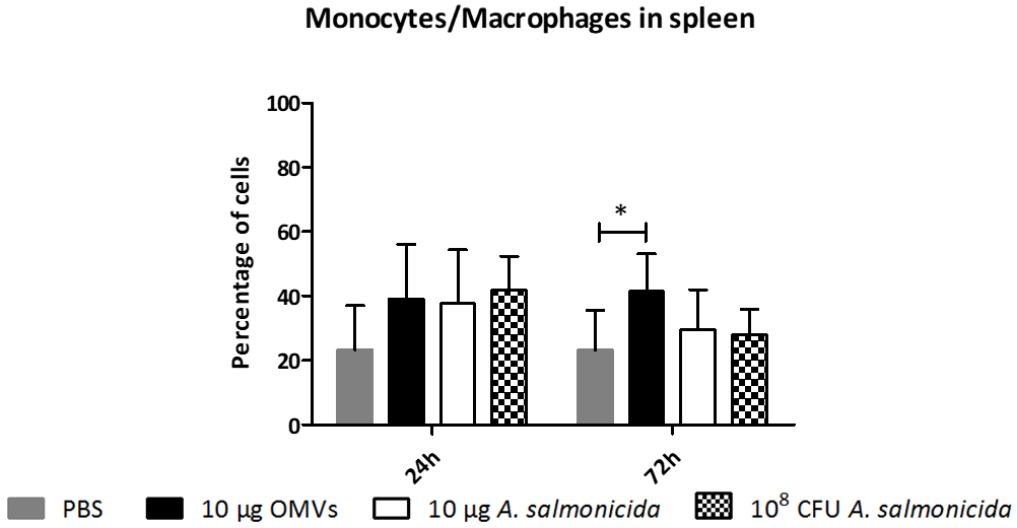

Figure 24: Monocytes/macrophages in the spleen 24h and 72h after vaccination.
Fish were injected with either PBS (N=9), 10 µg OMVs (N=8), 10 µg of inactivated *A. salmonicida* (N=9) or 10^8 CFU of inactivated *A. salmonicida* (N=6). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of monocytes/macrophages (mab 21^+ cells) 24h and 72h after vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using Dunnett's multiple comparisons test.

In the blood, a significant decrease in the percentage of monocytes/macrophages could be observed at 24h followed by an increase at 72h, compared to the PBS group (Figure 25). However, no significant changes of monocytes/macrophages could be detected in the head kidney in either of the investigated groups (data not shown).

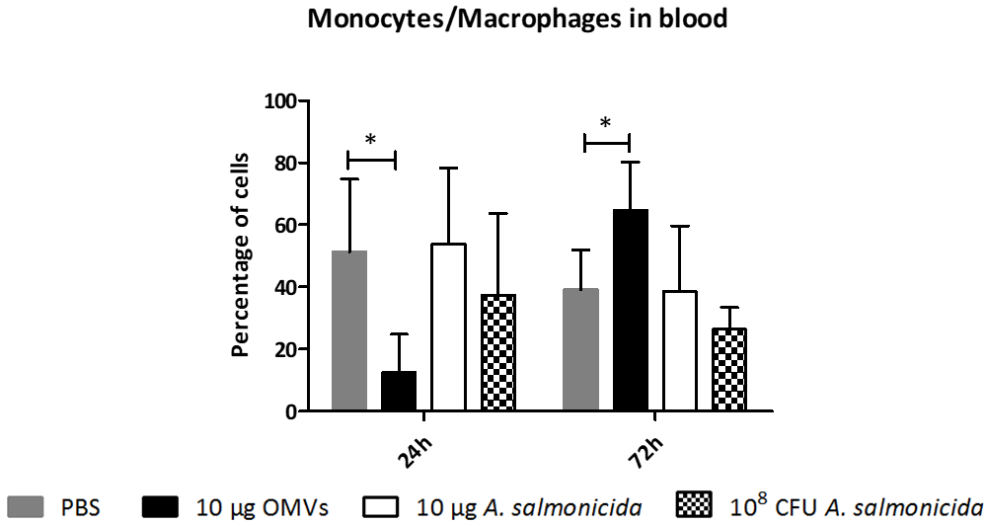

Figure 25: Monocytes/macrophages in the blood 24h and 72h after vaccination.
Fish were injected with either PBS (N=9), 10 µg OMVs (N=8), 10 µg of inactivated *A. salmonicida* (N=9) or 10^8 CFU of inactivated *A. salmonicida* (N=6). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of monocytes/macrophages (mab 21^+ cells) 24h and 72h after vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using Dunnett's multiple comparisons test.

In addition to the monocytes/macrophages, B cells were also analyzed via lineage-specific mabs as shown in figure 17 E and F.

When looking at IgM^+ B cells in the blood, only injection with 10 µg of OMVs resulted in a significant decrease in the percentage of these cells in the blood at 24h (Figure 26 A). While the percentage of IgT^+ B cells in the blood was only significantly increased 24h after injection with 10^8 CFU of inactivated *A. salmonicida* (Figure 26 B).

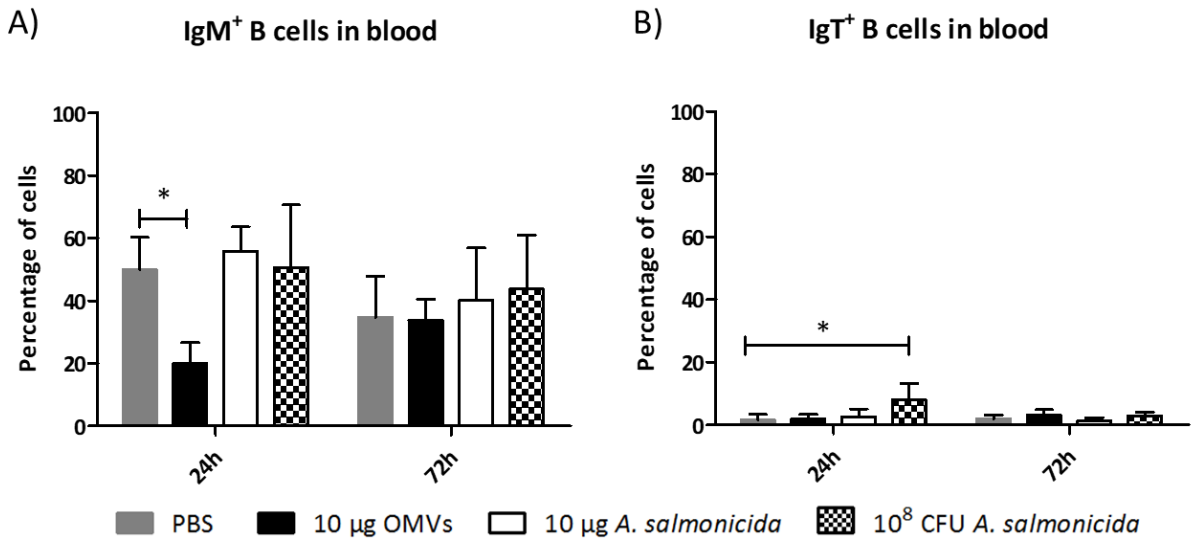

Figure 26: IgM⁺ B cells and IgT⁺ B cells in the blood 24h and 72h after vaccination.
Fish were injected with either PBS (N=9), 10 µg OMVs (N=8), 10 µg of inactivated *A. salmonicida* (N=9) or 10^8 CFU of inactivated *A. salmonicida* (N=6). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage IgM⁺ B cells (characterized by anti-trout IgM mab 1.14⁺ single positive B cells and anti-trout IgM mab 1.14⁺ and anti-trout IgK light chain mab N2⁺ double-positive B cells) (A) and IgT⁺ B cells (mab N2⁺ single positive cells) (B) 24h and 72h after vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using Dunnett's multiple comparisons test.

In the spleen, only injection of 10^8 CFU of inactivated *A. salmonicida* led to a significant increase in the percentage of the IgM⁺ B cells 24h after vaccination (Figure 27 A), with no substantial changes in the percentage of IgT⁺ B cells observed after immunization with either antigen (Figure 27 B).

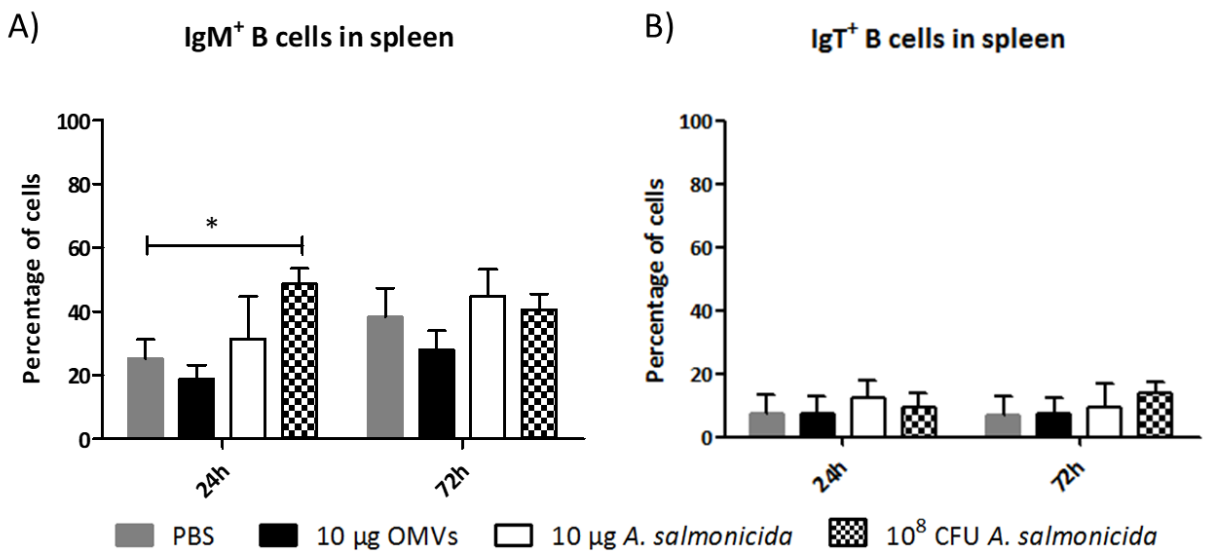

Figure 27: IgM⁺ B cells and IgT⁺ B cells in the spleen 24h and 72h after vaccination.
Fish were injected with either PBS (N=9), 10 µg OMVs (N=8), 10 µg of inactivated *A. salmonicida* (N=9) or 10^8 CFU of inactivated *A. salmonicida* (N=6). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage IgM⁺ B cells (characterized by anti-trout IgM mab 1.14⁺ single positive B cells and anti-trout IgM mab 1.14⁺ and anti-trout IgK light chain mab N2⁺ double-positive B cells) (A) and IgT⁺ B cells (mab N2⁺ single positive cells) (B) 24h and 72h after vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using Dunnett's multiple comparisons test.

All other cells analyzed with lineage-specific mabs did not show significant changes in their distribution in the blood or lymphoid organs (data not shown).

The first animal trial has shown that vaccination with OMVs derived from *A. salmonicida* results in a similar local immune response compared to injection with inactivated *A. salmonicida* even though dose-dependent and temporal differences in the response could be observed.

5.3.1.3. Vaccination with OMVs results in distinct cytokine response in the head kidney and spleen

Previous research has shown that i.p. injection of flounder with OMVs derived from pathogenic fish bacteria *E. tarda* or *V. anguillarum* resulted in significant changes in the mRNA expression pattern of immune relevant genes such as IL-1β, IL-6 and TNFα in the spleen and head kidney (7, 9).

To see whether injection of rainbow trout with OMVs derived from *A. salmonicida* would also change the mRNA expression of immune relevant genes, the RNA of leukocytes of the head

kidney and spleen of fish used in animal trial 1 was extracted as described in section 4.8.1. and section 4.8.2.

The cytokine response 24h and 72h after vaccination with 10 µg of OMVs derived from *A. salmonicida* or 10 µg of inactivated *A. salmonicida* was analyzed in the head kidney and spleen (Figures 28 and 29).

In the head kidney, drastic changes in the mRNA expression of the investigated genes could only be seen at 24h post vaccination with both antigens (Figure 28). Vaccination with OMVs resulted in solid increases in the expression of all analyzed pro-inflammatory genes and IL-10. At the same time, vaccination with 10 µg of inactivated *A. salmonicida* resulted in significant increases in the expression of the analyzed anti-inflammatory genes and a substantial increase in IL1β expression.

	Regulatory	**Pro-inflammatory**			**Anti-inflammatory**	
24h	Myd88	IL-1β	IL-6	IL-8	IL-10	TGFβ
10 µg OMV	-1,5	**13,0**	61,8	**11,9**	**40,1**	1,0
10 µg AS	1,2	21,2	3,7	1,8	**20,0**	**2,8**
72h	Myd88	IL-1β	IL-6	IL-8	IL-10	TGFβ
10 µg OMV	-1,5	-1,6	-1,3	-2,2	3,2	1,2
10 µg AS	-2,1	2,0	-2,4	-1,6	1,1	-1,1

Figure 28: Response of immune relevant genes in the head kidney 24h and 72h after vaccination with OMVs.

Rainbow trout were vaccinated with either 10 µg of *A. salmonicida* strain JF2267 (AS) or 10 µg of derived OMVs. 24h and 72h after vaccination, head kidney leukocytes were isolated, and RNA was extracted. The fold change of different genes compared to the average of the PBS control group was measured by RT-qPCR. All genes were normalized to the housekeeping gene EF1α. ▬ indicates increased expression and ▬ indicates decreased expression of the gene compared to the PBS control. Significant differences in expression ($p < 0.05$) are highlighted in bold, calculated by using a two-way ANOVA based on copy numbers (OMVs: N = 8; *A. salmonicida:* N = 9).

In the spleen, the changes in mRNA expression profile lasted until 72h after vaccination for IL-1β, IL-6 and IL-10 (Figure 29). The same trends can be observed for both vaccination groups with increased mRNA expression of nearly all analyzed cytokines except for TGFβ in both groups. However, only vaccination with 10 µg of *A. salmonicida* strain JF2267 resulted in a significant change of mRNA expression of IL-8 (24h) and IL-10 (72h) compared to the PBS control group.

	Regulatory	Pro-inflammatory			Anti-inflammatory	
24h	Myd88	IL-1β	IL-6	IL-8	IL-10	TGFβ
10 µg OMV	39,8	57,4	4,7	15,4	58,7	1,9
10 µg AS	14,5	34,4	80,2	**18,1**	37,0	2,0
72h	Myd88	IL-1β	IL-6	IL-8	IL-10	TGFβ
10 µg OMV	1,9	18,5	8,0	2,6	10,3	1,2
10 µg AS	-2,5	70,9	1,0	2,1	**14,4**	1,3

Figure 29: Response of immune relevant genes in the spleen 24h and 72h after vaccination with OMVs.
Rainbow trout were vaccinated with either 10 µg of *A. salmonicida* strain JF2267 (AS) or 10 µg of derived OMVs. 24h and 72h after vaccination, spleen leukocytes were isolated, and RNA was extracted. The fold change of different genes compared to the average of the PBS control group was measured by RT-qPCR. All genes were normalized to the housekeeping gene EF1α. ▬ indicates increased expression and ▬ indicates decreased expression of the gene compared to the PBS control. Significant differences in expression ($p < 0.05$) are highlighted in bold, calculated by using a two-way ANOVA based on copy numbers (OMVs: N = 8; *A. salmonicida*: N = 9).

Considering these findings from the first animal trial, it is apparent that OMVs derived from *A. salmonicida* are recognized as an antigen, resulting in a similar cellular response and activation of immune-relevant genes in lymphoid organs as observed after injection of inactivated *A. salmonicida*.

5.3.2. I.p. vaccination with OMVs result in an *A. salmonicida* specific antibody response

After observing similar results for stimulation with inactivated *A. salmonicida* and OMVs derived from this bacterium, animal trial 2 (Table 6) was conducted to analyze whether vaccination with OMVs results in an *A. salmonicida* specific antibody response and if the induced antibody titers are comparable to fish injected with inactivated *A. salmonicida*. Blood was taken over a 3 month period and serum was obtained as described in section 4.6.1.3.1. The antibody titer specific to *A. salmonicida* was detected as described in section 4.7.1.

The individual development of the *A. salmonicida* specific antibody titer was followed over time, and most vaccinated fish responded to the vaccination by producing *A. salmonicida* specific antibodies, except for one fish in each vaccine group which showed only a slight development of *A. salmonicida* specific antibodies (Figure 30 A and B). At 49d post-immunization, a peak in the antibody titer was seen for all fish of both groups. At 65d post-vaccination, a decline in the antibody titer could be detected, and 3 fish from each group were sacrificed

(Figure 30 A) to analyze the long-term cellular response after vaccination (Section 5.3.2.1.). The antibody titer of the remaining fish immunized with OMVs further declined towards the end of the trial. However, this trend is not so distinct in the group vaccinated with 10^8 CFU of inactivated *A. salmonicida*. No significant differences between groups could be detected for the average antibody titer 65 days after vaccination (Figure 31).

Overall, this data indicates that vaccination with 10 µg of OMVs derived from *A. salmonicida* results in the development of *A. salmonicida* specific antibodies. Furthermore, the antibody titers can reach similar magnitudes as observed from fish vaccinated with 10^8 CFU of inactivated *A. salmonicida*. Again, this demonstrates the immune stimulatory potential of OMVs.

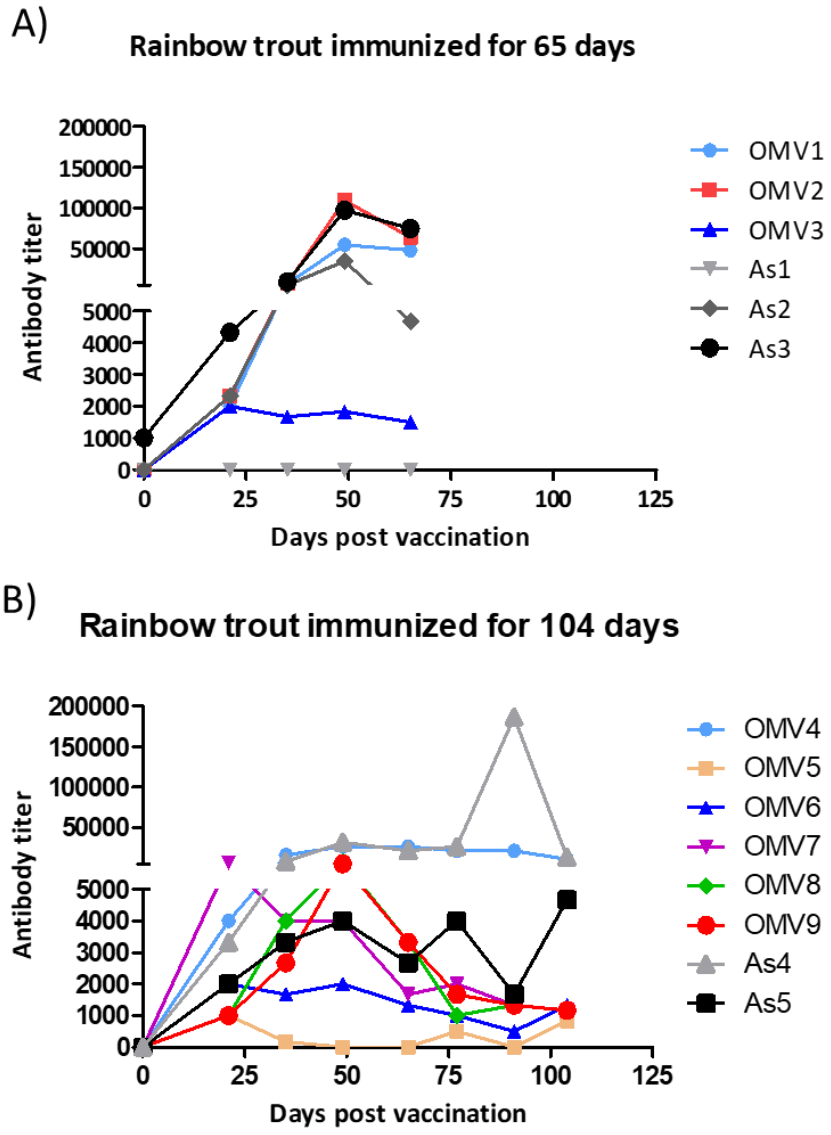

Figure 30: Antibody titers of rainbow trout immunized with either *A. salmonicida* or derived OMVs. Fish were immunized for 65 days with either 10 µg OMVs (N=3), or 10^8 CFU of inactivated *A. salmonicida* (As) (N=3) (A) or 104 days (B) with either 10 µg OMVs (N=6), or 10^8 CFU of inactivated *A. salmonicida* (As) (N=2). Blood was taken at indicated times from the caudal vein, and antibodies were detected in serum via ELISA plates coated with 1 µg of *A. salmonicida*.

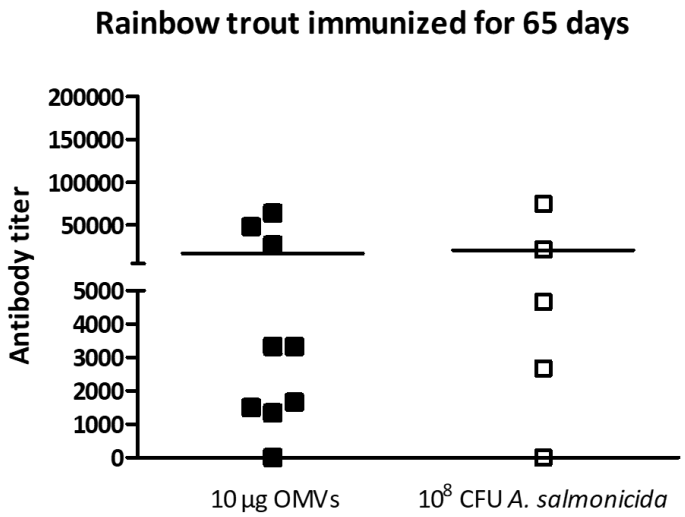

Figure 31: Average antibody titer of rainbow trout 65d after immunization with either *A. salmonicida* or derived OMVs.

Fish were immunized for 65 days with either 10 µg OMVs (N=9), or 10^8 CFU of inactivated *A. salmonicida* (N=5). Blood was taken 65 days after vaccination from the caudal vein, and antibodies were detected in serum via ELISA plates coated with 1 µg of *A. salmonicida*. Data were analyzed with an unpaired t-Test, which did not detect significant differences between both groups. Black bars indicate the arithmetic mean.

5.3.2.1. Long-term cellular response after i.p. vaccination with OMVs

To investigate if a different long-term cellular response could be the reason that 10 µg of OMVs induce a similar humoral immune response as vaccination with inactivated bacteria, the cellular immune response at 65d after vaccination was analyzed.

In general, the cellular response after i.p. vaccination with 10 µg of OMVs or 10^8 CFU of inactivated *A. salmonicida* did not show significant differences in the leukocyte composition of the peritoneal cavity or thymus (data not shown).

In the spleen, a significantly higher percentage of IgM^+ B cells was detected in the group vaccinated with 10^8 CFU of inactivated *A. salmonicida* when compared to the group vaccinated with 10 µg of OMVs (Figure 32).

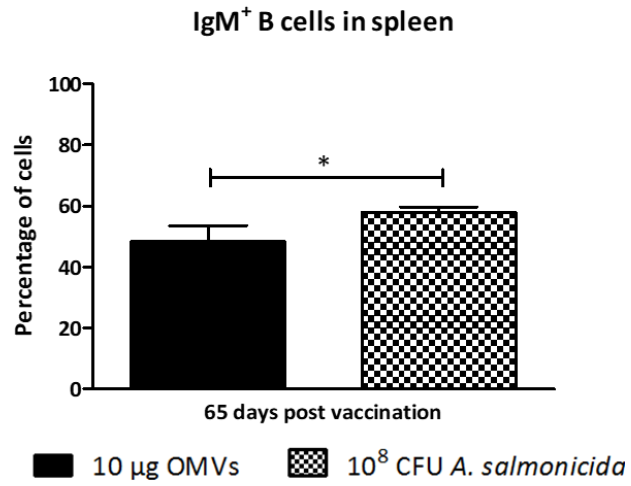

Figure 32 IgM⁺ B cells in the spleen 65d after vaccination.
Fish were injected with either 10 µg OMVs (N=3) or 10^8 CFU of inactivated *A. salmonicida* (N=3). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of IgM⁺ B cells (characterized by anti-trout IgM mab 1.14 single positive and anti-trout IgM mab 1.14 and anti-trout IgK light chain mab N2 double-positive cells) after vaccination was compared among the two groups. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using an unpaired t-test.

When looking at $CD8^-$ T cells, vaccination with 10 µg of OMVs resulted in a significantly higher percentage of these cells in the head kidney (Figure 33 A) and blood (Figure 33 B) compared to the group vaccinated with 10^8 CFU of inactivated *A. salmonicida*.

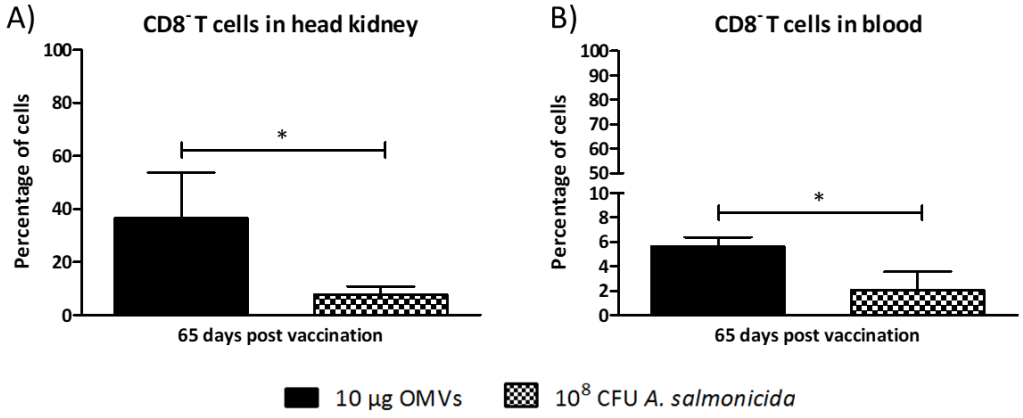

Figure 33: $CD8^-$ T cells in the head kidney and blood 65d after vaccination.
Fish were injected with either 10 µg OMVs (N=3) or 10^8 CFU of inactivated *A. salmonicida* (N=3). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. $CD8^-$ T cells (anti-trout pan T cell mab D30 positive cells) in the head kidney (A) and blood (B) 65d after vaccination was compared among the two groups. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using an unpaired t-test.

The increased percentage of $CD8^-$ T cells after vaccination with 10 µg of OMVs compared to the percentage found after vaccination with 10^8 CFU of inactivated *A. salmonicida* could

indicate a higher percentage of $CD4^+$ T cells after vaccination with OMVs, which could result in an increased stimulation of B cells to produce antibodies.

5.3.3. Oral vaccination with *A. salmonicida*

After establishing that OMVs can be used as a vaccine antigen for i.p. vaccination, the question of whether this would also be possible in an oral immunization model had to be investigated. For this purpose, the oral immunization model with VHSV (19) was adapted for use with inactivated bacteria. To evaluate the successful adaption of the oral vaccination model the cellular response to two different strains of *A. salmonicida* was compared after oral. vaccination.

5.3.3.1. Local response after oral vaccination.

In animal trial 3 (Table 6), fish were vaccinated with two different strains of *A. salmonicida* via the oral route using handmade vaccine pellets (Table 7). As mentioned in section 3.7. the strain JF2267 was directly isolated from arctic char and therefore is characterized as a ''wild type'' strain, which has been used for most of the experiments in this thesis. The strain JF5505 is a re-isolate of the strain JF2267, which induced increased mortality after an infection and is titled ''high virulent re-isolate''.
To directly compare both strains, the local cellular response was analyzed in the gut after oral immunization. In addition, the systemic cellular response was analyzed using leukocytes from the lymphatic organs, head kidney and spleen.

When looking at the leukocyte distribution in the gut after oral vaccination (Figure 34), opposite to i.p. vaccination (Figure 18), no significant changes occur within the first 24h. Immunization with vaccine pellets containing 10^7 CFU of strain JF2267 resulted in a substantial decrease in the percentage of myeloid cells in the gut at 48h compared to the PBS control group (Figure 34 A). Interestingly, the exact opposite can be seen when the vaccine pellets contained strain JF5505 with the percentage of myeloid cells significantly increasing 48h after vaccination (Figure 34 A). 7d after oral vaccination, a significant increase in the percentage of lymphocytes could be seen for fish vaccinated orally with strain JF5505 (Figure 34 B).

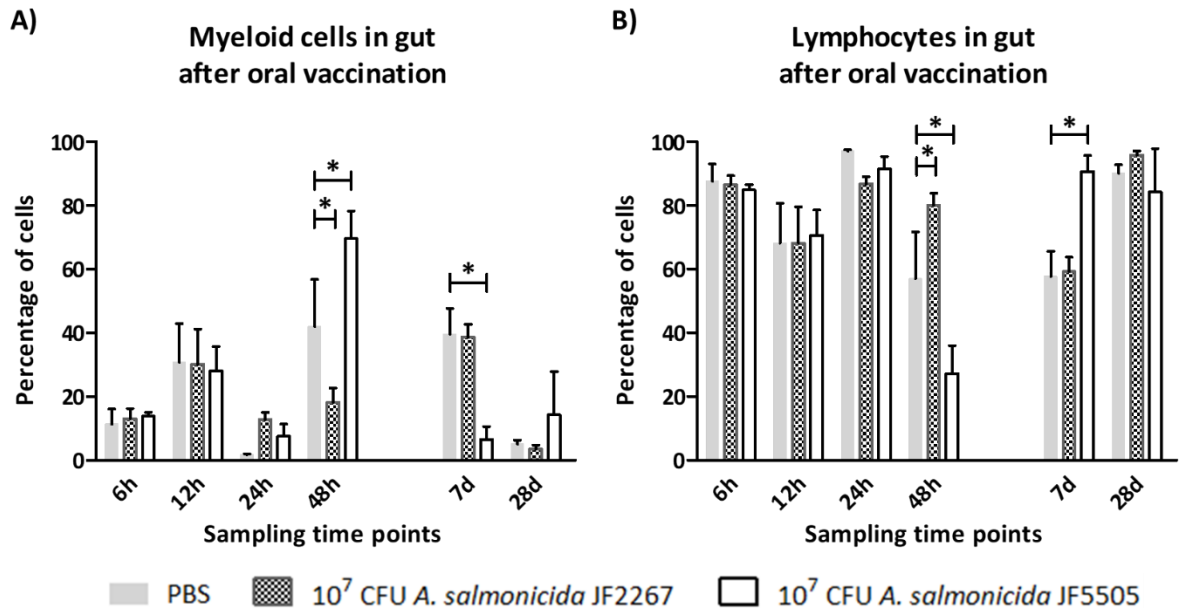

Figure 34: Kinetics of myeloid cells and lymphocytes in the gut after oral vaccination.
Fish were either orally vaccinated with pellets containing either sterile PBS (N=4), 10^7 CFU of *A. salmonicida* strain JF2267 (N=4) or 10^7 CFU of *A. salmonicida* strain JF5505 (N=4). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of lymphocytes in the peritoneal cavity (A) and gut (B) after vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using a two-way ANOVA (Bonferroni post-test).

Of the leukocyte populations analyzed with lineage-specific mabs only the percentage of thrombocytes was significantly changed after oral vaccination with both strains of *A. salmonicida* resulting in a significant drop in the percentage of thrombocytes in the gut at 48h and a significant increase in the percentage of thrombocytes at 7d (Figure 35).

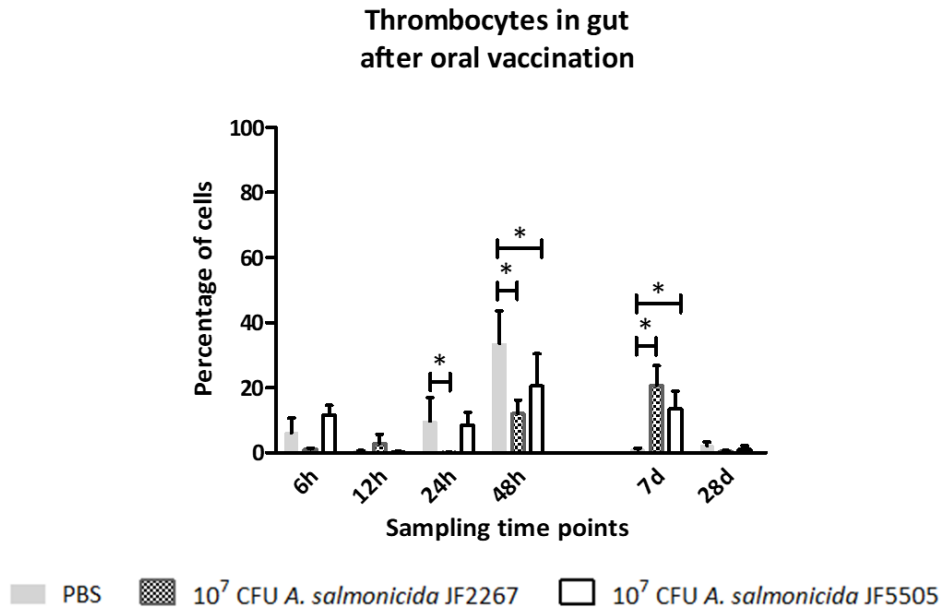

Figure 35 Thrombocytes in the gut after oral vaccination.
Fish were orally vaccinated with pellets containing either sterile PBS (N=4), 10^7 CFU of *A. salmonicida* strain JF2267 (N=4) or 10^7 CFU of *A. salmonicida* strain JF5505 (N=4). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of thrombocytes (mab 42^+ cells) in the gut after vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using Dunnett's multiple comparisons test.

5.3.3.2. Analysis of the cellular composition in lymphoid organs after oral vaccination with different *A. salmonicida* strains.

To analyze the immune response in lymphoid organs after oral vaccination, the leukocyte populations of the head kidney and spleen were characterized. No distinct pattern could be observed when comparing the cellular response after oral immunization in both organs. For example, a significant decrease in the percentage of myeloid cells could be seen after oral vaccination with strain JF5505 in the head kidney at 6h (Figure 36 A), while vaccination with either strain of *A. salmonicida* resulted in no significant changes within the first 48h in the spleen (Figure 37).

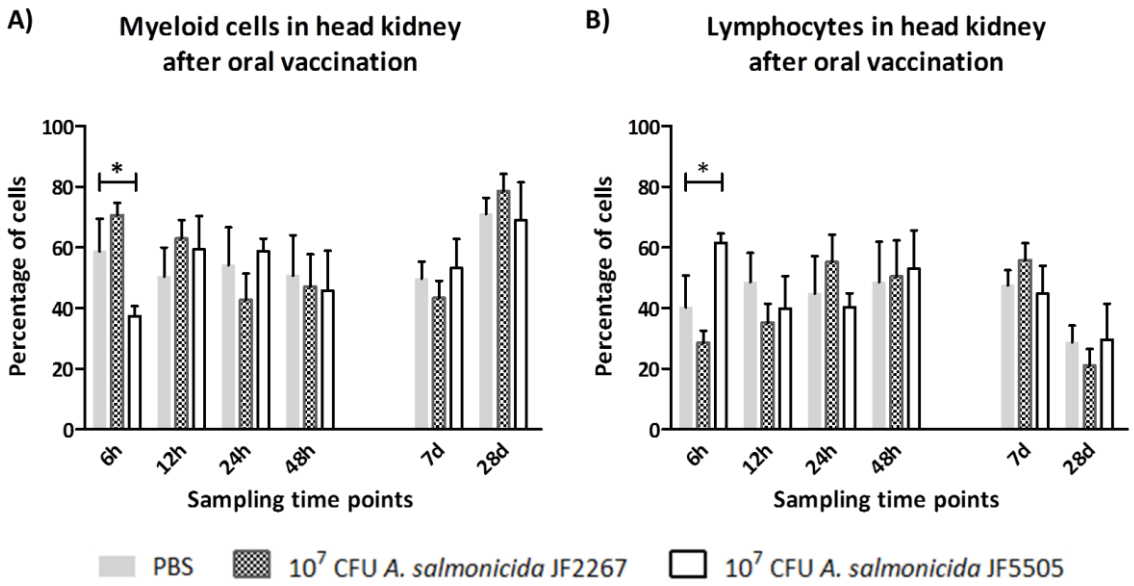

Figure 36: Kinetics of leukocytes in the head kidney after oral vaccination.
Fish were orally vaccinated with pellets containing either sterile PBS (N=4), 10^7 CFU of *A. salmonicida* strain JF2267 (N=4) or 10^7 CFU of *A. salmonicida* strain JF5505 (N=4). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of myeloid cells (A) lymphocytes (B) in the head was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using a two-way ANOVA (Bonferroni post-test).

In the spleen, a significant decrease in the percentage of myeloid cells could be seen at day 7 after vaccination with either strain of *A. salmonicida* when compared to the PBS group (Figure 37 A). Also, vaccination with strain JF2267 led to a significant decrease in the percentage of lymphocytes 28d after vaccination (Figure 37 B).

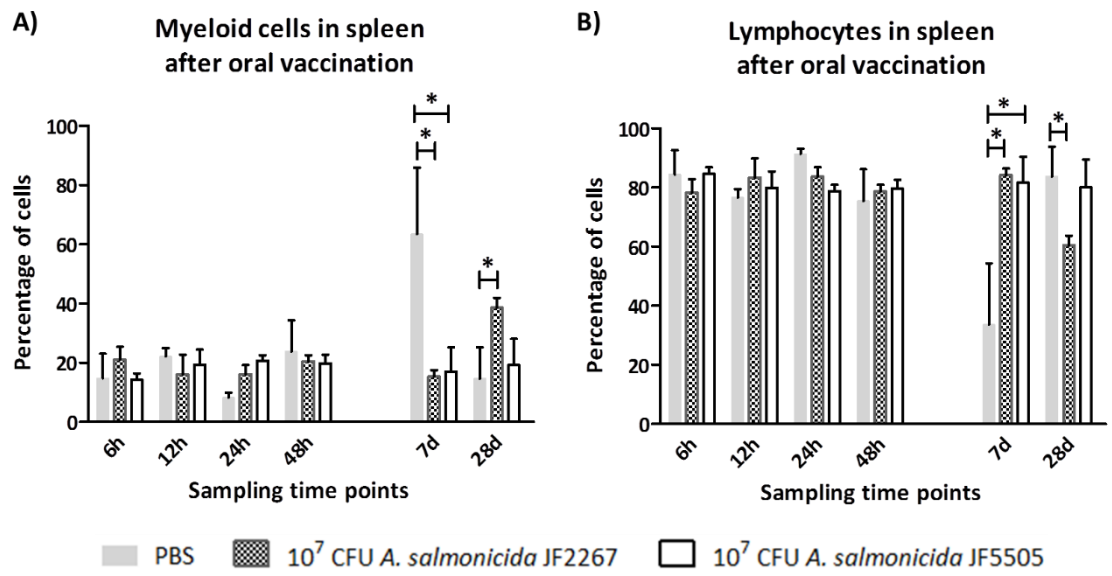

Figure 37: Kinetics of lymphocytes in the spleen after i.p and oral vaccination.
Fish were orally vaccinated with pellets containing either sterile PBS (N=4), 10^7 CFU of *A. salmonicida* strain JF2267 (N=4) or 10^7 CFU of *A. salmonicida* strain JF5505 (N=4). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of myeloid cells (A) lymphocytes (B) in the head was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using a two-way ANOVA (Bonferroni post-test).

Even though no overall pattern could be observed for the lymphocytes and myeloid cells in the head kidney and only to some extent in the spleen after vaccination with either strain of *A. salmonicida*, the analysis of the leukocyte population using lineage-specific mabs showed similarities in the distribution of monocytes/macrophages and thrombocytes after vaccination with either strain of *A. salmonicida* (Figures 38 - 40).

In the head kidney, oral vaccination with strain JF2267 led to a significant decrease in the percentage of monocytes/macrophages 24h after immunization and a considerable increase in the percentage of these cells 7d after vaccination (Figure 38). Furthermore, the significant increase of mab 21 positive cells could also be observed 7d after oral vaccination with strain JF5505 (Figure 38). In contrast, oral vaccination with either strain led to a significant decrease in the percentage of thrombocytes at 7d in the head kidney (Figure 39).

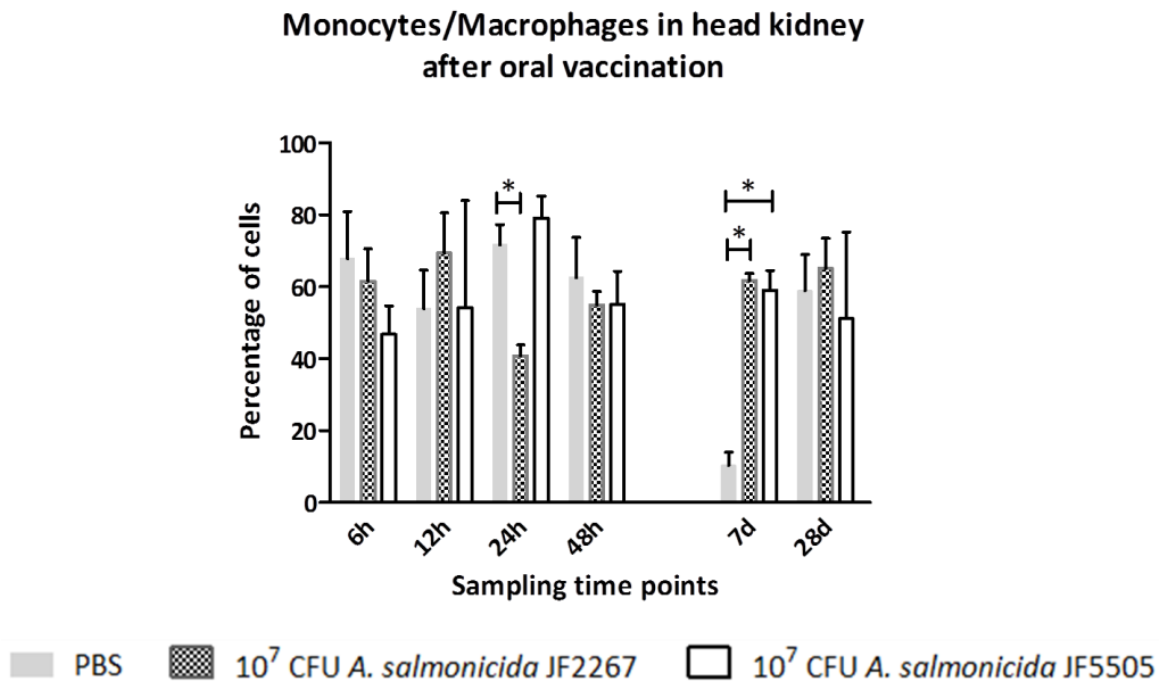

Figure 38: Monocytes/macrophages in the head kidney after oral vaccination.
Fish were orally vaccinated with pellets containing either sterile PBS (N=4), 10^7 CFU of *A. salmonicida* strain JF2267 (N=4) or 10^7 CFU of *A. salmonicida* strain JF5505 (N=4). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of monocytes/macrophages (mab 21^+ cells) in the head kidney after oral vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using two-way ANOVA.

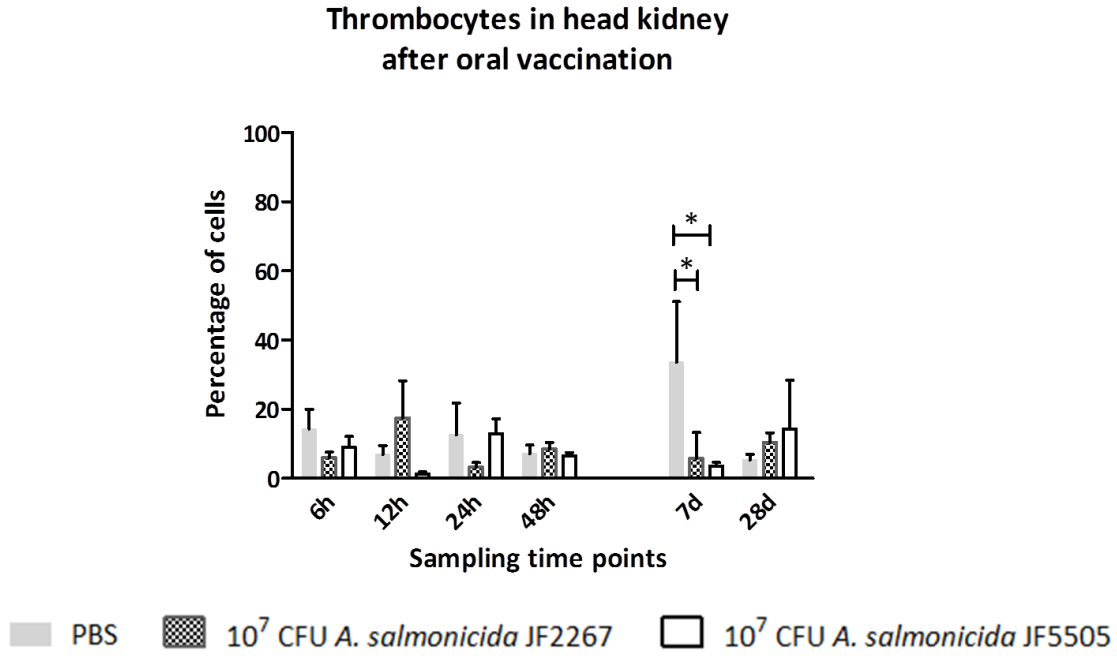

Figure 39: Thrombocytes in the head kidney after oral vaccination.
Fish were orally vaccinated with pellets containing either sterile PBS (N=4), 10^7 CFU of *A. salmonicida* strain JF2267 (N=4) or 10^7 CFU of *A. salmonicida* strain JF5505 (N=4). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of thrombocytes (mab 42^+ cells) in the head kidney after oral vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using two-way ANOVA.

The percentage of thrombocytes in the spleen is significantly increased 6h after oral vaccination with strain JF2267, in comparison, vaccination with strain JF5505 led to a significant increase in the percentage of thrombocytes 24h and 48h after vaccination (Figure 40).

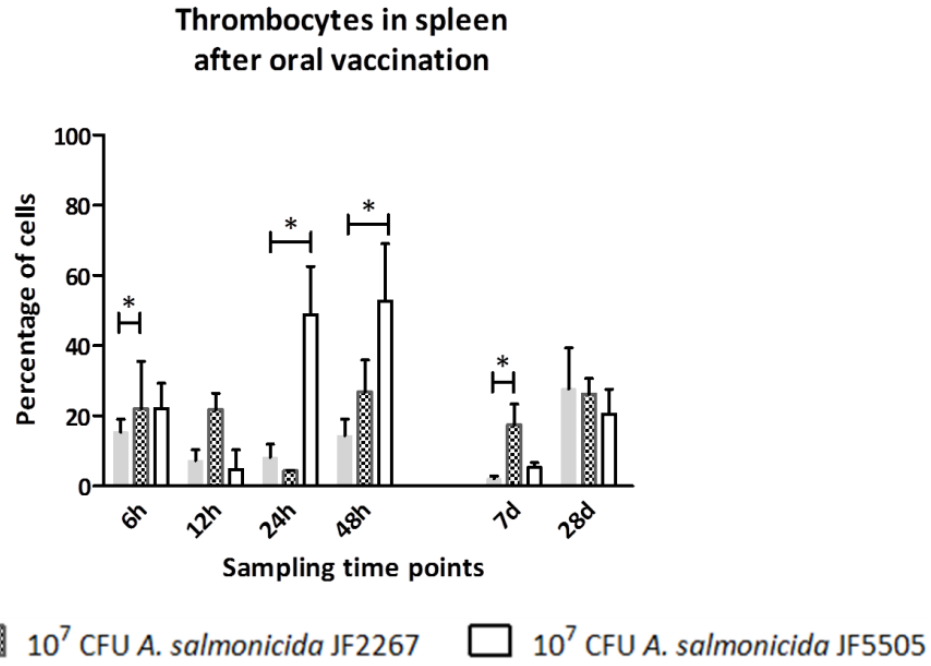

Figure 40: Thrombocytes in the spleen after oral vaccination.
Fish were orally vaccinated with pellets containing either sterile PBS (N=4), 10^7 CFU of *A. salmonicida* strain JF2267 (N=4) or 10^7 CFU of *A. salmonicida* strain JF5505 (N=4). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of thrombocytes (mab 42^+ cells) in the spleen after oral vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using two-way ANOVA.

The results of this animal trial show that the local cellular response differs between the used strains of *A. salmonicida*, especially during the early time points after vaccination. However, a more distinct response to *A. salmonicida* strain JF5505 could be seen when looking at the monocytes/macrophages and thrombocytes, especially in the gut and spleen (Figures 35 and 40) after oral vaccination. This was accompanied by a superior immune reaction to the *A. salmonicida* strain JF5505 in the spleen, which plays an essential role in antigen presentation and activation of the adaptive immune system in fish (12). Therefore, the use of *A. salmonicida* stain JF5505 strain was further investigated in a second oral vaccine trial to test the robustness and reproducibility of the oral vaccination model.

5.3.4. Second oral vaccination trial leads to different cellular response

After adapting the established rainbow trout model for oral vaccination with VHSV (19) to be used for bacterial antigens (Section 5.3.3.), animal trial 4 (Table 6) was conducted to analyze the cellular response of fish orally vaccinated with *A. salmonicida* strain JF5505, VHSV or a mix of both pathogens. Next to morphometric analyzes of the cellular response, the cellular reaction of cells characterized with lineage-specific mabs 21 and 42 was analyzed after 24h and 72h in the gut, head kidney and spleen, and at 14d and 28d in the head kidney and spleen.

The local response in the gut showed that oral vaccination with bacteria and virus increased the percentage of lymphocytes 24h after immunization, accompanied by a decrease of the myeloid cells at that time point. However, this cellular response was only significant for fish vaccinated with 10^6 $TCID_{50}$ of VHSV (Figure 41).

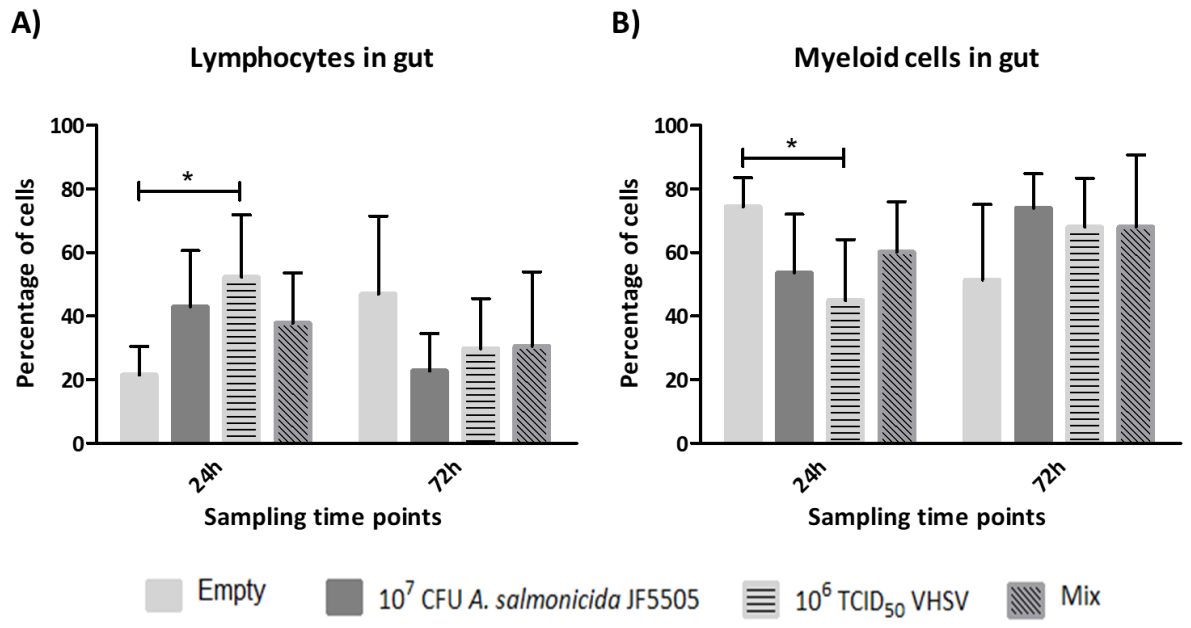

Figure 41: Lymphocytes and myeloid cells in the gut.
Fish were fed with food pellets containing either sterile PBS (N=4), 10^7 CFU of *A. salmonicida* strain JF5505 (N=8), 10^6 $TCID_{50}$ of VHSV (N=8) or a mix of both antigens (N=8). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of lymphocytes (A) and myeloid cells (B) after vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using two-way ANOVA.

When looking at the leukocytes characterized via lineage-specific mabs it could be shown that only vaccination with bacteria led to a significant increase of monocytes/macrophages (mab 21^+ cells) in the gut 24h after immunization (Figure 42 A). On the other hand, the

percentage of thrombocytes (mab 42^{+} cells) was also only significantly increased 72h after vaccination with inactivated bacteria (Figure 42 B).

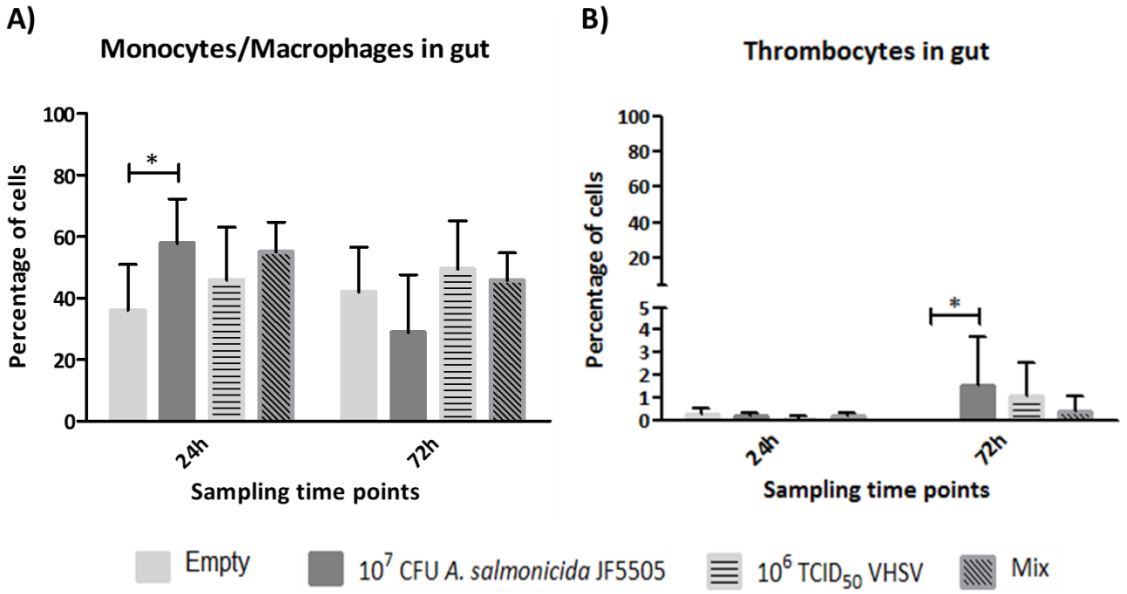

Figure 42: Monocytes/macrophages and thrombocytes in the gut.
Fish were fed with food pellets containing either sterile PBS (N=4), 10^7 CFU of *A. salmonicida* strain JF5505 (N=8) 10^6 $TCID_{50}$ of VHSV (N=8) or a mix of both antigens (N=8). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of monocytes/macrophages (mab 21^{+}) (A) as well thrombocytes (mab 42^{+}) (B) after vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using two-way ANOVA.

In addition to the local response, the systemic immune response was also investigated in this trial by looking at the cellular distribution in the head kidney and spleen. No significant difference could be observed in the spleen for any of the experimental groups (data not shown). But in the head kidney, all 3 vaccinated groups showed a significant increase in the percentage of myeloid cells 14d after vaccination accompanied by a considerable decrease in the percentage of the lymphocytes (Figure 43 A and B).

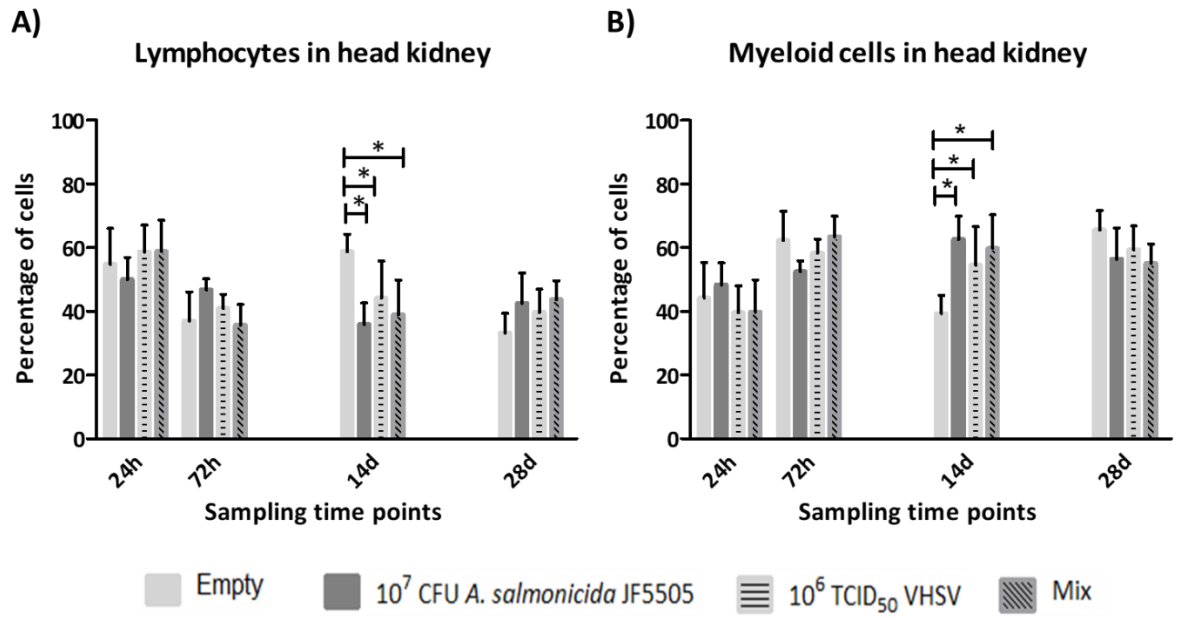

Figure 43: Lymphocytes and myeloid cells in the head kidney.
Fish were fed with food pellets containing either sterile PBS (N=4), 10^7 CFU of *A. salmonicida* strain JF5505 (N=8), 10^6 $TCID_{50}$ of VHSV (N=8) or a mix of both antigens (N=8). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of lymphocytes (A) and myeloid cells (B) after vaccination was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using two-way ANOVA.

In the head kidney and spleen, a significant increase in the percentage of thrombocytes could be observed 72h post-vaccination (Figure 44). However, in the head kidney, this was due to vaccination with VHSV (Figure 44 A), and in the spleen this change only occurred in the group fed with inactivated bacteria (Figure 44 B).

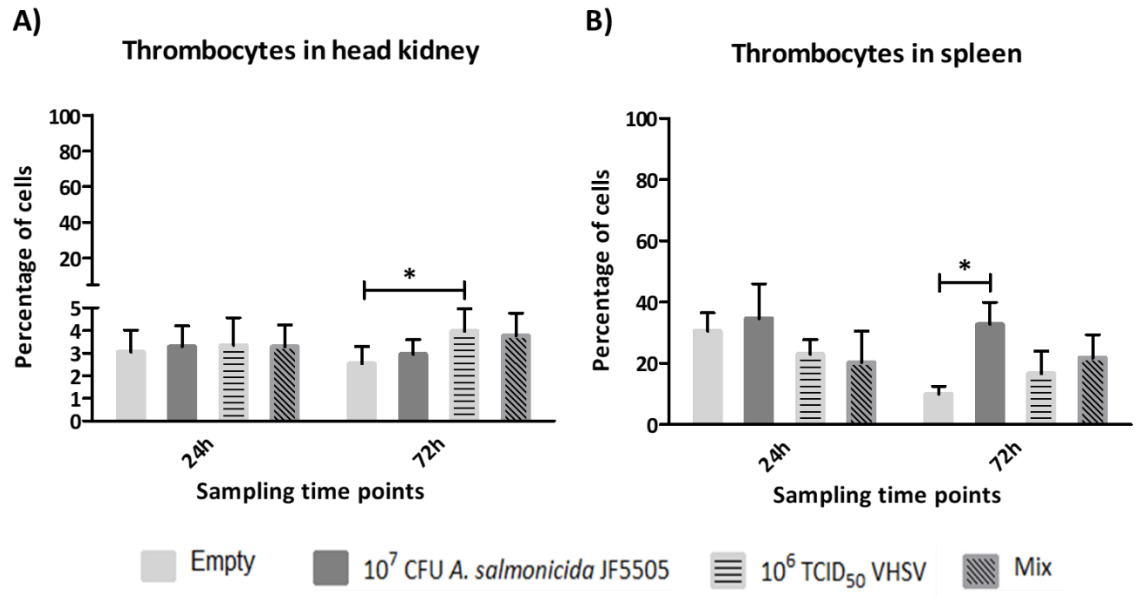

Figure 44: Thrombocytes in the head kidney and spleen after oral vaccination.
Fish were fed with food pellets containing either sterile PBS (N=4), 10^7 CFU of *A. salmonicida* strain JF5505 (N=8), 10^6 $TCID_{50}$ of VHSV (N=8), or a mix of both antigens (N=8). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of thrombocytes (mab 42^+) in the head kidney (A) and spleen (B) was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using two-way ANOVA.

For the monocytes/macrophages, a significant increase of cells was only observed in the spleen 3d after vaccination with inactivated bacteria (Figure 45).

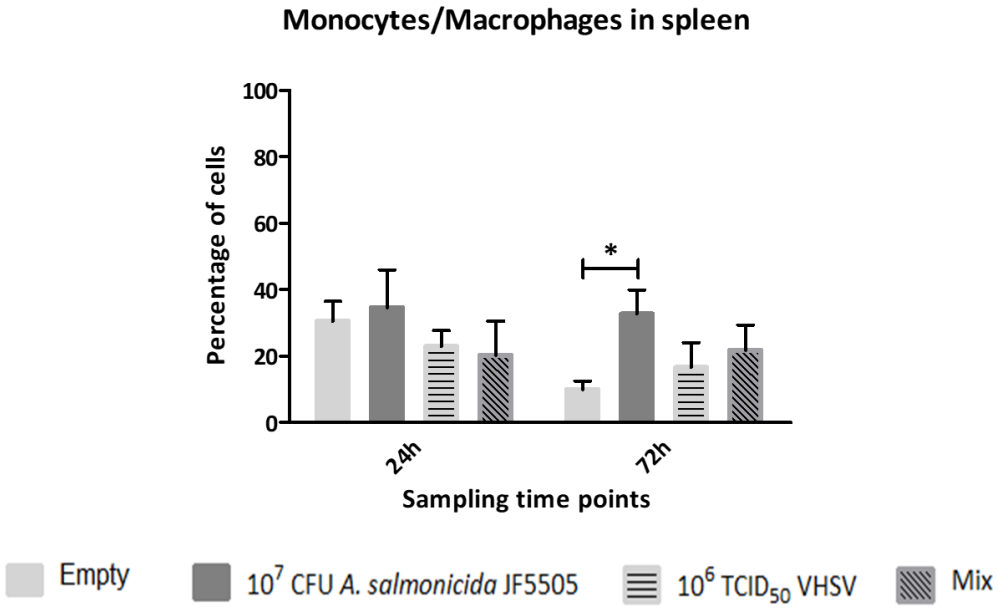

Figure 45: Monocytes/Macrophages in the spleen after oral vaccination.
Fish were fed with food pellets containing either sterile PBS (N=4), 10^7 CFU of *A. salmonicida* strain JF5505 (N=8), 10^6 $TCID_{50}$ of VHSV (N=8) or a mix of both antigens (N=8). Flow cytometry analyzes of the extracted leukocytes were done using FACSCanto II. The percentage of monocytes/macrophages (mab 21^+) in the spleen was compared to the PBS control fish. Significant differences in expression ($p < 0.05$) are indicated with asterisk (*), calculated by using two-way ANOVA.

Taken together, the results of animal trial 4 indicate the difficulties of designing a successful oral vaccine, which is reliable in the activation of the immune response and could be used with different vaccine antigens, as nearly no response was seen, for example, after vaccination with the mixed vaccine pallets. Furthermore, no antibody titer specific to *A. salmonicida* strain JF5505 or VHSV could be detected in the sera 28d post-vaccination obtained from trial 4 (data not shown).

6. Discussion

Aquaculture is the fastest-growing livestock-producing sector worldwide (22) and faces many bacterial or viral-induced infections (14). Even though several vaccines exist for different fish species raised in aquaculture (17, 25), they can result in adverse events (74) or even mortality (21) and do not always lead to long-lasting protection from pathogens (11). To look at a new approach for the vaccination of fish, the possibility of using OMVs has been investigated in this study as they can function as a natural adjuvant (4) and a potent activator of the immune system (3). Furthermore, they have been safely used in human vaccines for over 20 years (1, 3-5), and OMVs of different fish pathogenic bacteria have also been investigated for their immune stimulatory potential (6-10), leading to the two main questions of this thesis with varying subsequent questions:

1. Can outer membrane vesicles derived from the fish pathogenic bacterium *A. salmonicida* be used as a vaccine antigen?
    - Can OMVs derived from *A. salmonicida* activate innate immune cells and thus cause an inflammatory response?
    - Is the cellular immune response in rainbow trout after i.p. vaccination with OMVs derived from *A. salmonicida* comparable to the cellular immune response after immunization with inactivated *A. salmonicida*?
    - Does i.p. vaccination of rainbow trout with inactivated *A. salmonicida* or derived OMVs result in the same mRNA expression pattern in primary and secondary lymphoid organs?
    - Does i.p. vaccination of rainbow trout with OMVs derived from *A. salmonicida* result in an *A. salmonicida* specific antibody response?
2. Is it possible to establish an oral vaccination model of rainbow trout for the use of bacterial and viral antigens?
    - Can OMVs be used as an oral combination vaccine against bacterial and viral pathogens?

These questions have been established based on the rationale described in the introduction (Section 2.6.). Furthermore, different *in vitro* and *in vivo* experiments have been conducted to

answer those questions (Section 5.) and the results will be discussed in the following part of the thesis, starting with examining the used methods.

6.1. Establishment and validation of selected methods

6.1.1. Establishing the viral titer detection

To determine the tissue culture infection dose of viruses different methods can be used. For example, quantitative PCR is the most sensitive method; it visualizes replicating and inactivated viral genome copies (182), which can result in overestimating the $TCID_{50}$. Apart from using PCR-based methods in order to detect the viral load, the cytopathic effect (CPE) can be used, which looks at morphological changes of the infected cells, which often result in cell lysis (183). Cell lysis can take between 3 to 6 days depending on the cell line infected with VHSV (184) and, may result in counting cells that have not altered due to CPE but due to other reasons (183). Therefore, the $TCID_{50}$ of VHSV was determined by using a fluorescence-based assay that enabled the staining of a viral protein and of the nucleus of the cells (Section 4.4.2.), thus producing overlays that ensured to detect only cells infected with the virus (Figure 8). The fluorescence-based method of viral detection is the most distinct method for differentiating infected from non-infected cells (183) and, what is more, it is also time-efficient, allowing for the determination of the $TCID_{50}$ 3 days after the infection of the cells.

6.1.2. Optimization of RNA isolation and validation of RT-qPCR

Initial isolation of RNA at room temperature from the obtained cells following the manufactures` instructions did not result in sufficient RNA. Therefore, the protocol was adapted by performing the individual steps at 4°C and using ice-cold reagents, which had been successfully described for RNA isolation of rainbow trout tissue and cells using different reagents (185). The amounts of RNA obtained after adapting the protocol could be used for the experiments and the validation of the primers. The primers used for the experiments were taken from the relevant literature (104, 141, 163-165), but were still tested for their efficiency and accuracy as described in section 4.9. All primer pairs reached an efficiency of above 90 % (Table 17), which is necessary for robust DNA amplification (170). Other sources recommend an efficiency of around 100 %, ranging from 95 % to 105 % efficiency (186). The primer pairs used in this thesis reached an efficiency from 91.94 % to 116.69 %. It is assumed that an

efficiency of 100 % results in the highest sensitivity of the primer pair (186), but the visualization of the amplicons in an agarose gel, pictured for EF1α (Figure 9), showed that all used primers amplified the product of the correct size.

Furthermore, the optimal annealing temperature for each primer pair was detected using a temperature gradient PCR, pictured for EF1α (Figure 9). The range of the gradient was based on the fact, that the optimal annealing temperatures are between 57°C and 63°C (187), and that higher temperatures prohibit unspecific primer binding, while too high temperatures result in low product yield (188). Additionally, during the RT-qPCR protocol, the melting curve of the amplified product was detected (Figure 11), which verified that only one peak was present, indicating high specificity of the primer pair (170). The copy number of the genes of interest was calculated using the individual efficiencies for each primer pair, considering the differences in amplification efficiency when changes in the mRNA expression profile were analyzed. The key cytokines, which are one of the first to be released, such as IL-1β, IL-6 and IL-8 and IL-10 (109), were analyzed for all the experiments, while certain genes were added depending on the experiment as well as the available amount of RNA.

6.1.3. Rationale for used cell lines

The *in vitro* experiments were conducted on the immortalized cell line RTS-11, and on leukocytes from the head kidney and peritoneal cavity. As mentioned previously (Section 2.6.), early in the study of vaccine efficacy in fish, the importance of macrophages, especially after peritoneal vaccination, was observed (15, 135, 136). Therefore, the monocyte/macrophage cell line RTS-11 was used to get a first idea of the immune response of fish-derived macrophages to OMVs. The cell line RTS-11 consists of large, granular cells that can be found in suspension or are adherent to the cell culture surface (138). These granular cells have been characterized as macrophages as they are phagocytic and stain positive for non-specific esterase (138), an enzyme commonly used to distinguish macrophages from phagocytic granulocytes (189). Furthermore, smaller non-adherent cells are present in the culture, as well as the large granular cells, and these smaller cells are characterized to be at an ''earlier stage of macrophage development'' (138). Additionally, it was demonstrated that the stimulation of RTS-11 cells with LPS, generally used to mimic molecular patterns of bacteria (139), a PAPMP also found in OMVs (1, 2), results in an increased expression of inflammatory cytokines, for example, IL-8

(112). To sum up, all this indicates that the stimulation of RTS-11 cells with OMVs could change the mRNA expression pattern.

Not only the cell line RTS-11, but also leukocytes derived from the head kidney have been investigated after the stimulation with OMVs. The reason for using leukocytes of the primary lymphatic organ of fish was the fact that the head kidney is the main source of macrophages in fish (110, 139, 140). Similar to RTS-11 cells, it could also be shown for leukocytes from the head kidney that they respond to stimulation with LPS with an increase in mRNA expression of pro-inflammatory cytokines such as IL-1β, IL-6, TNFα as well as the anti-inflammatory cytokine IL10 (139). Furthermore, it was shown that macrophages from this organ are recruited to the peritoneal cavity after vaccination (137). Additionally, leukocytes of the peritoneal cavity have been used to observe changes in the mRNA expression pattern after the stimulation of those leukocytes with OMVs, as the peritoneal cavity is the main site of vaccine administration in fish.

6.1.4. Rationale for choosing the analyzed genes

The focus was set on the early cytokine response during this study, up to 72h after stimulation. It is known that after breaching the physical barrier, monocytes, macrophages and neutrophils are one of the first cells which will be encountered by the pathogens or vaccine antigens (12). And it was shown that upon the infection of fish TNFα, IL-1β and IL-6 are the first cytokines to be released by, for example, macrophages (109), which made them interesting cytokines to be studied in the context of an OMV induced immune response. In rainbow trout, it was shown that macrophages express TNFα after the stimulation with LPS (190). Another reason for looking at the expression of TNFα is the fact that *A. salmonicida* can also replicate intracellular (40, 55, 56). From mice, it is known that TNFα expression is essential to survive an infection with intracellular bacteria (191). For rainbow trout, it was proven that TNFα expression activates the recruitment of neutrophils (109) and leads to increased phagocytic activity, respiratory burst activity and nitric oxide (NO) production of macrophages (192).

IL-1β is also known to be expressed by macrophages of rainbow trout (193, 194), and its expression leads to growth, proliferation and recruitment of lymphocytes, especially natural killer cells and B cells (111), as well as to the stimulation of its expression (195). Furthermore, it was shown that the antibody titer against *Aeromonas hydrophyla* could be significantly increased when the IL-1β protein was part of the vaccination protocol (195).

As described by Costa et al. (2011) IL-6 results in the proliferation of macrophages, induces antimicrobial peptides and regulates cytokine release. The production of IL-6 can be observed in many cell types, but at the site of inflammation, monocytes and macrophages are the primary source of IL-6 production. In RTS-11 cells and rainbow trout macrophages, IL-6 expression could be induced via, e.g., LPS and recombinant IL-1β (110).

IL-8 is also produced by different cell types, including macrophages (112), resulting in the recruitment of neutrophils, T cells, and basophils and the activation of respiratory burst of neutrophils (112). Furthermore, as mentioned earlier (Section 2.5.2.), IL-8 expression could be induced in RTS-11 cells, stimulated with LPS (112). Additionally, it was shown that recombinant IL-8 functions as an attractant for leukocytes from the head kidney *in vitro*, and i.p. injection in rainbow trout resulted in an increased number of leukocytes, mainly neutrophils, in the peritoneal cavity (196).

As mentioned above, TNFα can activate an increased production of NO by macrophages (192); additionally, iNOS expression could be induced in rainbow trout macrophages via LPS and attenuated *A. salmonicida* (140). Due to this fact the expression of iNOS was also investigated. Next to the antimicrobial activity of iNOS via the production of NO (113), it also plays an essential role in vasodilation (140).

Apart from the beneficial function of pro-inflammatory cytokines during infection, adverse effects can occur upon overproduction which can, e.g., lead to sepsis-like symptoms, which has been reported for IL-1β and TNFα (191, 197). That is why a balanced immune response is important, e.g., via regulation of pro-inflammatory cytokines by release of anti-inflammatory cytokines such as IL-10 and TGFβ.

TGFβ has, in general, been associated with the "suppressing potentially damaging effect of the host inflammatory response while promoting tissue repair"(198). The processes that TGFβ suppresses includes B and T cell proliferation, suppression of macrophage activity (198), and regulation of the release of NO (113).

IL-10 is in addition to TGFβ, the most important anti-inflammatory cytokine, which is produced by nearly all leukocytes. Still, the cells that produce the most IL-10 *in vivo* are monocytes, macrophages, and T-helper cells (199). Macrophages, for example, secrete IL-10 after activating TLRs via PAMPs, for instance, LPS (199). This could also be shown for leukocytes from the head

kidney of rainbow trout stimulated with LPS (139). On the other hand, IL-10 inhibits, for example, the secretion of TNFα and IL-8 (113).

In *in vitro* and *in vivo* experiments, LPS is generally used to mimic molecular patterns of bacteria (139). This is also the case in the context of fish experiments, even though no ortholog to TLR4, the receptor that recognizes LPS in mammals, has been described in fish (104). It is postulated that LPS recognition in fish occurs via other PRRs such as beta-2 integrins (200). Activation of beta-2 integrins via PAMPs results in the activation of NF-κB via MAP kinases (200) and in the expression of immune-relevant genes (104). Since the recognition of LPS in fish is not characterized (200) and it is known that activation of TLRs results in the recruitment of Myd88 and IRAK1 in order to form the Myddosome (104), these factors have been analyzed as well to determine the possible involvement of TLRs in recognition of OMVs.

6.2. Stimulation with OMVs and inactivated bacteria induced a similar gene response pattern.

Due to the fact that the monocyte/macrophage cell line RTS-11 had been successfully used to investigate the effect of bacterial and viral stimuli on, e.g., the mRNA profile (110, 112, 171-173). This cell line has also been used to analyze if the stimulation with OMVs lead to a change in the expression of key cytokines (Section 5.2.1.1.). Previous studies could show that stimulation of RTS-11 cells with LPS led to an increased expression of IL-1β, IL-6, IL-8 and IL-10 (112, 201). Gram-negative bacteria such as *A. salmonicida* and their derived OMVs contain LPS as an essential part of their outer membrane (10).Therefore, a similar response was expected for the stimulation of RTS11 cells with inactivated *A. salmonicida* and OMVs. Stimulation with OMVs and inactivated bacteria induced a similar gene response pattern in RTS-11 cells, increasing the expression of pro-inflammatory genes IL-1β, IL-6 and IL-8.

At the same time, stimulation with inactivated bacteria also led to an increased expression of IL-10 (Figure 13). These results show that inactivated bacteria and OMVs are recognized by RTS-11 cells as a stimulus which activates the expression of immune-relevant genes, which has been indicated by the use of LPS as a substitute for bacterial molecular patterns (139). For human macrophages, it was demonstrated that the stimulation with OMVs results in activation of NF-κB, resulting in an increased expression of TNFα, IL8 and IL1β (202). Even though

the receptor for the recognition of LPS has not been characterized in fish (104), the successful use of LPS as a PAMP inducing cytokine production (112, 201) indicates that recognition of LPS by fish macrophages, and therefore also detection of OMVs is possible.

Even though the stimulation with OMVs resulted in an increased expression of pro-inflammatory cytokines during each repetition of the experiment (Figure 14), but this change was not significant compared to the PBS control (Figure 13). However, this is due to the extensive range of the obtained copy numbers; a clear induction of the cytokine expression can be seen after the stimulation of RTS-11 cells with OMVs (Figures 13 and 14). Recombinant IL-8 was shown to function as a chemoattractant for neutrophils derived from the head kidney of rainbow trout (196). Neutrophils were attracted to recombinant IL-8 in a range from 0.1 to 10 ng/ml (196). This indicates that a biological activity of the analyzed cytokines might still be possible even though an extensive range of mRNA expression of the studied cytokines was detected.

After obtaining the indication that OMVs can induce a pro-inflammatory cytokine response in a rainbow trout-derived cell line, *ex vivo* experiments with leukocytes of the head kidney, which have been described as the primary source of macrophages (110, 139, 140), were conducted. The stimulation of leukocytes derived from the head kidney with OMVs resulted in a similar picture as detected in the stimulation with inactivated *A. salmonicida*. Again, an increased expression of pro-inflammatory cytokines IL-1β, IL-6, IL-8 could be observed, especially 4h after the stimulation with both antigens, and the change in pro-inflammatory cytokines was significantly higher when compared to the PBS control group (Figure 15). This confirmed that OMVs could be recognized by rainbow trout leukocytes, possible monocytes/macrophages, and that they can induce a pro-inflammatory cytokine response. Monocytes/macrophages were analyzed for their potential to respond to the stimulation with OMVs as they are one of the first cells which pathogens will encounter upon infecting the host (12).

Still, i.p injection is one of the primary vaccine delivery methods; therefore, leukocytes derived from the peritoneal cavity were stimulated with OMVs. Only a slight increase in the expression of pro-inflammatory genes could be observed after the stimulation with OMVs (Figure 16).

In contrast, the stimulation with inactivated *A. salmonicida* resulted in an increased expression of all investigated genes (Figure 16). While monocytes/macrophages can recognize OMVs (202) and a similar response in cytokine expression could be observed after the stimulation with 5 µg of OMVs and 50 µg of inactivated *A. salmonicida* (Figures 13 and 15), this seems not

to be the case for leukocytes of the peritoneal cavity, which consist mainly of B cells (15). For B cells derived from humans, it was demonstrated that the stimulation with OMVs derived from gram-negative bacteria results in an increased expression of IL-6. Still, it could also be shown that the stimulation with OMVs does not activate the B cells to respond in a manner that would help with pathogen clearance (203). On the contrary, stimulation with OMVs has led to B cells responding in a way that facilitates bacterial colonization (203). To determine if this is also the case for B cells derived from rainbow trout, further experiments are necessary, e.g., the stimulation of magnetic isolated IgM^+ B cells with OMVs.

Nevertheless, it is known that OMVs generally reflect the outer membrane composition of bacteria (1) but are not an identical copy of the bacterial membrane with cytosolic and inner membrane proteins incorporated and with some proteins of the outer membrane being present while others are absent (1, 57). During the *in vitro* experiments, 5 µg of OMVs were used and were able to initiate a signaling cascade similar to that of 50 µg of inactivated bacteria. OMVs of other pathogens like *E. coli*, *Tannerella forsythia*, *Bordetella pertussis* and the fish pathogen *E. tarda* contain a high number of key virulence factors (9, 204-206). So far, for *A. salmonicida*, only the fact that OMVs are produced has been published (10), but the assumption that these OMVs also contain a high number of key virulence factors could explain why lower amounts of OMVs lead to similar results as high amounts of inactivated bacteria.

Here, the immune stimulatory potential of OMVs, which has been described for different fish pathogens (6-9), was shown for OMVs derived from *A. salmonicida*.

6.3. Immune stimulatory potential of OMVs *in vivo*

The *in vitro* results showed that the OMVs purified from the *A. salmonicida* strain JF2267 activate a signaling cascade, leading to significant changes in the mRNA expression of the investigated key cytokines present in the early immune response. Therefore, the potential of OMVs for cellular recruitment, changes in mRNA expression patterns in different primary and secondary lymphoid organs, and antibody development were investigated in further animal trials.

6.3.1. Intraperitoneal vaccination with OMVs triggers recruitment of myeloid cells to the site of injection

Korytář et al. (2013) showed that after i.p. injection of a pathogenic strain of *A. salmonicida,* the pre-dominating lymphoid cells in the peritoneal cavity are replaced by myeloid cells within 24h. 72h after vaccination, the lymphoid cells are again the dominating cells, with IgM^+ B cells making up over 50 % of these cells (15). The model of peritoneal inflammation (15) was also used to investigate the cellular response after i.p. injection of OMVs. In animal trial 1 (Table 6), injection of 10 µg of OMVs derived from *A. salmonicida* strain JF2267 was compared to the PBS control group. Additionally, 2 more groups of fish were injected with 10 µg or 10^8 CFU of inactivated *A. salmonicida* strain JF2267 as a positive control.

The cellular response in the peritoneal cavity showed that the model used for the experiment followed the pattern described by Korytár et al. (2013) injection of either concentration of inactivated bacteria into the peritoneal cavity resulted in significant changes of lymphocytes and myeloid cells (Figure 18). As described previously (15), the myeloid cells were the dominant cell type 24h after i.p. injection. In comparison, the lymphocytes were the predominant cell type at 72h after injection in the peritoneal cavity (Figure 19). Injection of 10^8 CFU of inactivated *A. salmonicida* strain JF2267 resulted in significant changes of the leukocyte population at 24h post-vaccination. In contrast, injection of 10 µg of inactivated *A. salmonicida* strain JF2267 resulted in substantial changes of the lymphocytes and myeloid cells at 72h, compared to the PBS control group, indicating a dose-dependent response (Figure 18). This was not surprising as a dose-dependent response is commonly found and therefore considered as a "central concept in many biological disciplines" (207). One example is the determination of the minimum infectious dose of a pathogen, with the assumption that the organisms do not cause disease or adverse events under a certain threshold (208). Another example is testing the bactericidal effect of different disinfections, which was first described by Robert Koch (207).
Even though no significant changes after the vaccination with OMVs were observed for the leukocyte populations in the peritoneal cavity, a substantial increase in the percentage of monocytes/macrophages 72h after vaccination could be seen in the peritoneal cavity after vaccination with 10 µg of OMVs and 10 µg of inactivated *A. salmonicida* strain JF2267 (Figure 20). This is different from the observation in the *in vitro* experiments (Figures 13 and 15), where the low concentration of OMVs (5 µg) used for stimulation led to similar results

observed for the high dosage (50 µg) of inactivated bacteria, showing that *in vitro* experiments can be reasonable indications for possible outcomes of *in vivo* experiments but are not directly transferrable. In previous studies, the increase of monocytes/macrophages in the peritoneal cavity detected with the mab 21 as well as phagocytic cells was seen 24h after vaccination (15, 181), which was the case for injection with 10^8 CFU of inactivated *A. salmonicida*, again showing a dose-dependent response to the vaccination with inactivated bacteria (Figure 20 A). Furthermore, it could be demonstrated that i.p. injection of turbot with particles of 0.5 µm resulted in the first peak of phagocytic cells already 6h after injection followed by a second peak at 24h (181). This could indicate that the model used would have to be expanded by looking at earlier times to analyze the complete cellular response to i.p. vaccination with OMVs. The early recruitment of macrophages to the peritoneal cavity is necessary for the efficient clearance of the bacterial antigen and has been reported in different fish species (15, 209-211). This conservation of the innate immune response shows its importance during evolution (15). Furthermore, the importance of innate immune cells, especially macrophages, was demonstrated, when rainbow trout were i.p. injected with inactivated *A. salmonicida*, and isolated macrophages showed an enhanced killing of *A. salmonicida* compared to macrophages isolated from PBS injected fish (136).

After the influx of macrophages has occurred, the percentage of lymphocytes is increased in the peritoneal cavity (15). Of this cell population, the B cells are of particular interest due to their ability to also phagocytose foreign microbes (82).
Looking at the IgM^+ B cells in the peritoneal cavity, only the injection of inactivated bacteria resulted in a significant change of these cells at 72h after vaccination (Figure 21). This response to bacterial antigen has been shown previously using *A. salmonicida*, *E. coli*, or LPS derived from *E. coli* (15, 143). The IgM^+ B cells dominating the peritoneal cavity also become the primary phagocytic cell type, taking over the role of the macrophages (143). Interestingly, the injection of 1 µg of LPS resulted in a significant increase of IgM^+ B cells on the following day, suggesting a slower reaction to the whole microorganism than to PAMPs (143). With OMVs being considerably smaller than whole microorganisms (1, 2), one can deduce that the investigated cellular response would need to be expanded by earlier time points to observe the full scope of the response to i.p. injection of OMVs.

6.3.2. The systemic cellular response differs after i.p. vaccination with OMVs and inactivated *A. salmonicida*

Even though the local cellular response in the peritoneal cavity after the injection of bacterial or viral stimuli has been analyzed previously, it still not known, if the influx of monocytes/macrophages and later lymphocytes is due to the proliferation of these cells or to the recruitment from other organs and if the decrease in myeloid cells is due to an increase in lymphocytes or if the myeloid cells migrate to lymphoid organs (15, 143). So far, it was shown that after stimulation with *E. coli* and VHSV, some of the local IgM^+ B cells differentiate towards plasmablasts/plasma cells (143). Previous studies also showed that B cells in the head kidney of Atlantic salmon significantly increase after i.p. vaccination with a multivalent commercial vaccine against bacterial and viral diseases (177). Additionally, in the head kidney and spleen foreign particles could be detected after i.p. injection (178, 179). This was also the case after injection of *A. salmonicida* antigens (180). Furthermore, i.p. injection of microparticles resulted in a migration of cells from the injection site to lymphoid organs such as the head kidney and spleen (181). To analyze the migration of cells in rainbow trout after i.p. vaccination with OMVs derived from *A. salmonicida* strain JF2267 and inactivated bacteria the cellular response in blood, head kidney and spleen were investigated.

24h and 72h after vaccination with 10 µg of inactivated bacteria, a significant increase of myeloid cells was observed in the head kidney (Figure 23). This could correlate with the reported detection of *A. salmonicida* antigens in the head kidney (180), suggesting that bacterial antigens are processed, and this again is what the head kidney as the primary lymphoid organ in fish is mainly responsible for (13). While previous studies could show that the source of the myeloid cells recruited to the peritoneal cavity is the head kidney (137), a decrease in the percentage of myeloid cells in the head kidney was not observed in this study. It might have happened at an earlier time, which was not investigated here. The spleen is a secondary lymphoid organ in fish and has been described to have similar functions as lymph nodes in mammals; e.g. presentation of foreign antigens to cells of the adaptive immune system (93). Additionally, phagocytic cells have been reported to migrate here after the uptake of foreign particles (181). The significant increase of monocytes/macrophages in the spleen 72h after the vaccination with 10 µg of OMVs (Figure 24) could also suggest that macrophages have taken up OMVs in the peritoneal cavity and migrated to the spleen for antigen presentation.

The monocytes/macrophages of the blood also showed significant changes after i.p. vaccination of OVMs (Figure 25), which is not surprising as the blood is the primary way of migration between lymphoid tissues (212) and could indicate migration of macrophages from the peritoneal cavity to the spleen, which has been previously reported for i.p. injection of nanoparticles (181). The percentage of IgM^+ B cells in the blood was also significantly decreased 24h after vaccination with OMVs (Figure 26 A). This cell type is also known to phagocytose bacteria (82), and a recruitment to the site of injection was reported previously after i.p. injection of bacterial antigens (15, 143). However, only a slight increase in the percentage of IgM^+ B cells after vaccination with OMVs could be observed in the peritoneal cavity at 72h (Figure 21 B). It has been reported previously that the injection of PBS alone can result in changes in the cellular distribution of leukocytes in the peritoneal cavity, mainly B cells, (15, 143). In order to analyze if the difference in the percentage of IgM^+ B cells seen in the peritoneal cavity is significant, a larger sample size could be used as one possible factor to increase the power of the statistical test (213).

The percentage of IgM^+ B cells also significantly changed in the spleen after the injection of 10^8 CFU of inactivated *A. salmonicida* (Figure 27); in previous studies, "macrophages-like cells" (180) have been described as the phagocytic cells to take up and present bacterial particles in the spleen and head kidney (180). But this was before the phagocytic capability of fish B cells was known (82), and the results obtained here suggest that B cells take up antigen and present it in the spleen.

Apart from the IgM^+ B cells, IgT^+ B cells are also known to phagocytose bacteria (82). However, this population was only increased in the blood 24h after the injection of 10^8 CFU of inactivated *A. salmonicida* (Figure 26 B). In which way the IgT^+ B cells are involved in the uptake of foreign particles after i.p. vaccination needs to be analyzed further since no changes of this cell type have been observed in the peritoneal cavity in this study (Figure 21 C and D), and only the phagocytic capacity of B cells isolated from systemic lymphoid organs was proven so far (214).

Interestingly, even though a dose-dependent response could be seen after vaccination with inactivated bacteria, both concentrations significantly altered the leukocyte population in the peritoneal cavity (Figure 18). Therefore, it was expected to see a similar cellular response in the systemic organs blood, head kidney and spleen. The different responses observed after i.p. vaccination with different bacterial concentrations or OMVs have not been further

investigated in this study. Still, it should be analyzed further to determine, e.g., which specific cells play a role in the initiation of the adaptive immune response.

These results show that OMVs derived from *A. salmonicida* have an immunostimulatory potential *in vitro* and *in vivo*; even though differences in the cellular response after the injection of bacteria and OMVs have been observed, especially on a systemic level, also similarities could be seen. For example, 72h after the injection of 10 µg of inactivated *A. salmonicida* or 10 µg of OMVs, a significant increase in monocytes/macrophages (mab 21^+ cells) in the peritoneal cavity can be seen when compared to the PBS group. Activation of these cells has been described as essential for a successful immune response after vaccination (135, 136).

6.3.3. mRNA expression of early inflammatory cytokines in lymphoid organs is altered after vaccination with inactivated bacteria and derived OMVs

The response on mRNA level has been investigated to get the first insight into the pathways activated after vaccination with OMVs next to the cellular response. As mentioned previously, the head kidney and spleen are the lymphoid organs responsible for antigen processing and activation of the adaptive immune system (13, 93), which is essential for a successful vaccination (30). Therefore, the cytokine response of the early inflammatory genes described above has been analyzed in the head kidney and spleen after vaccination with OMVs and 10 µg of inactivated *A. salmonicida*.

How important the early release of, e.g., IL-1β, IL-6, IL-8, IL-10 and TGFβ is in order to activate cells responsible for bacterial clearance but also for preventing the damaging effects of pro-inflammatory cytokines has been described in sections 2.5. and 6.1.4.

After the vaccination with 10 µg of OMVs, an increase of IL-1β, IL-6, IL-8 and IL-10 could be seen 24h after immunization in the spleen and head kidney, and for IL-1β and IL-10 at 72h in the spleen (Figures 28 and 29). Vaccination with 10 µg of inactivated bacteria also resulted in a similar cytokine response in the spleen (Figure 29). Still, only an increased expression of IL-1β, IL-10 and TGFβ was observed in the head kidney (Figure 28).

IL-1β and IL-6 are the first cytokines to be released by, e.g., macrophages after an infection (109), and previous research by Gyeong-Eun et al. (2009) and Park et al. (2011) could show that i.p. vaccination of olive flounder with OMVs derived from other gram-negative bacteria, *E. tarda* and *V. anguillarum*, resulted in an increased expression of IL-1β and IL-6 in the head

kidney and spleen. It was suggested that due to their composition, OMVs function as a LPS reservoir and therefore activate the expression of pro-inflammatory cytokines after vaccination (7, 9). Furthermore, OMVs contain many virulence factors, which could potentially be recognized by PRR activating a signaling cascade, which results in the expression of pro-inflammatory cytokines (9); for example, expression of the fish-specific TLR22 (215) was significantly increased after vaccination with OMVs (9). TLR22 was also upregulated in the head kidney after the infection of rainbow trout with *A. salmonicida,* which could be another potential way of recognizing *A. salmonicida* in rainbow trout, resulting in the expression of pro-inflammatory cytokines (104). The observed upregulation of Myd88 after the vaccination with either inactivated *A. salmonicida* or *A. salmonicida* derived OMVs in the spleen (Figure 29) could suggest an activation of the Myddosome, which binds to activated TLR´s (104).

IL-10 expression was significantly increased after both vaccination with OMVs and inactivated bacteria and is known to inhibit, e.g., the activation of IL-8 (113), which could previously be shown for IL-10 and TGFβ in the spleen of rainbow trout vaccinated and challenged with *Y. ruckeri*. The explanation for the activation of IL-10 and TGFβ was, that an unbalanced immune response, with only increased production of pro-inflammatory cytokine, could contribute to disease development (216).

The differences observed after the vaccination with 10 µg of inactivated *A. salmonicida* and 10 µg of OMVs, especially concerning the ability to induce an increased expression of pro-inflammatory genes in the head kidney, could indicate that 10 µg of inactivated bacteria used here do not have the same immunostimulatory potential as 10 µg of OMVs. A dose dependent change in gene expression was also described in other fish species. In orange-spotted grouper, it was demonstrated that a change in mRNA expression of pro-inflammatory genes was only observed in fish receiving a high vaccine dose (217). Additionally, it could be demonstrated that the infection of the Japanese flounder with a low dosage of intracellular pathogens led to an upregulation of the expression of, e.g., IL-1β in the spleen. In contrast, this change in expression in the head kidney is only observed after the infection with a 10 times higher dosage of intracellular bacteria (218). After infection of the Asian Seabass with *Streptococcus iniae*, an altered mRNA profile in the spleen but not in the head kidney within 7 days after the injection was shown (219). These studies indicate that a dose-dependent response is observed after the vaccination with inactivated bacteria as well as OMVs and a time-dependent reaction in the different organs.

Significant changes in the mRNA profile of inflammatory genes expressed early on in an immune response could be detected as soon as 6h (7) and as late as 7d (219) after vaccination, which could mean that significant changes in the mRNA expression in the lymphoid organs could also have occurred during earlier or later times after vaccination. To analyze this further, earlier time points should also be investigated for vaccination of rainbow trout with OMVs derived from *A. salmonicida*.

In addition, these results also show the immunostimulatory potential of OMVs derived from *A. salmonicida*, which are not only able to induce an upregulation of key inflammatory cytokines in vitro (Section 5.2.) and a recruitment of macrophages (Section 5.3.1.1.), which are essential for a successful immune response after vaccination (135, 136), but also induce the expression of IL-1β, IL-8 and IL-10 in vivo (Section 5.3.1.3.). IL-1β is strongly associated with the recruitment and proliferation of, e.g., natural killer cells and B cells (111). IL-8, for instance, is responsible for the recruitment and the activation of neutrophils as well as T cells (112), which on the one hand again shows the importance of activation of the innate immune system for the clearance of bacteria via neutrophils and, on the other hand, the importance of the adaptive immune cells in bacterial clearance such as cytotoxic T cells or further activation of B cells via $CD4^+$ T cells (105).

6.3.4. OMVs derived from *A. salmonicida* result in pathogen-specific antibody response after i.p. vaccination

After providing evidence that OMVs can induce a cellular immune response as well as changes in the mRNA expression of key regulatory cytokines, the questions remained whether this activation would also result in *A. salmonicida* specific antibodies. Previous studies could show that i.p. vaccination of zebrafish with OMVs derived from *F. noatunensis* resulted in the protection from a challenge with the homologues bacteria strain (6). Additionally, vaccination of olive flounders with OMVs derived from *E. tarda* also protected the olive flounders from subsequent challenges (9). So far only experiments on mice showed that antigen-specific antibodies are raised against antigens, which are exposed on the surface of OMVs (4) as well as heterologous proteins expressed in their lumen (220). The results obtained in this study show that the vaccination of rainbow trout with OMVs derived from *A. salmonicida* results in an *A. salmonicida* specific antibody response (Figure 30). The antibody titer is similar in both groups, despite the different amounts of antigen used for vaccination (Figure 31), which could

be explained by the fact that OMVs can function as a natural adjuvant (4). From mice, it is known that OMVs are recognized by TLRs, taken up by antigen-presenting cells, and are presented to $CD4^+$ T cells, which activate B cells to produce antigen-specific antibodies (60, 221). Furthermore, it has been postulated that IL-17 and IFNγ producing $CD4^+$ T cells are required to combat *E. coli* induced sepsis after vaccination with OVMs in mice (60), indicating that a Th1 response is induced after immunization with OMVs (222). A Th1 response is mainly associated with the clearance of intracellular bacteria and viruses (222) and the induction of antibody production of the type IgG2a or IgM (223). A Th2 response, determined by cytokines such as IL-4 and IL-5, is mainly associated with antibody production (IgE) (223) and with the clearance of extracellular pathogens (222). To analyze if the vaccination with OMVs causes a Th1 response in rainbow trout further research is necessary, e.g., the analyzes of the expression of IL-17 and IFNγ compared to IL-4 and IL-5.

This study also shows that not all vaccinated fish developed specific antibodies against *A. salmonicida*: one fish vaccinated with 10^8 CFU of inactivated *A. salmonicida* showed no response at all and one fish vaccinated with 10 µg of OMVs showed only a very inadequate response (Figure 30). The phenomenon of non-responders has been previously described in fish (224) and is not exclusive to fish vaccination, as not every vaccine induces protective antibodies in each vaccine recipient (225, 226). Therefore, herd immunity, which was first described in 1923 (225), can also be applied here. Herd immunity describes the phenomenon that when approximately 75 %, depending on the infectivity of the disease, of the high-risk population are vaccinated, a disease outbreak could be prevented, even though non-vaccinated or individuals not responding to the vaccine might get sick (225). In the case of challenge experiments, it was demonstrated that non-responding fish were 83 times more likely to succumb to the challenge than vaccinated fish (224).

The gold standard for vaccine efficacy testing in the fish industry is the survival after vaccination and challenge (227); the use of ELISA to evaluate vaccine efficacy against *A. salmonicida* infection has been suggested due to the strong correlation of induced antibody titer and protection (228).

Keeping this in mind, the same pattern and similar antibody titer obtained after vaccination with 10 µg OMVs and 10^8 CFU of inactivated bacteria (Figures 30 and 31) indicate that vaccinations with OMVs derived from *A. salmonicida* could protect from an infection with this

fish pathogen. Furthermore, even though the correlation between specific antibody response and protection against the particular pathogen has been debated in teleost (229), several studies in different fish species could show that a specific antibody response confers protection (230-232), which indicates that vaccination with OMVs derived from *A. salmonicida* can protect from subsequent challenges, which has also been shown for vaccination and challenge with OMVs of other fish pathogenic bacteria (6, 9).

To sum up, the results of these first two animal trials prove that OMVs derived from the pathogenic bacterium *A. salmonicida* alone have immunostimulatory potential. Vaccination with OMVs derived from *A. salmonicida* results in a local and systemic cellular response followed by the activation of signaling cascades, which leads to the production of cytokines and the development of strain-specific antibodies. Furthermore, those antibodies are strongly associated with protection from infection with at least homologous strains, as shown in other fish-associated bacteria.

6.3.5. No pronounced correlation between cellular response and antibody response could be observed in the long-term after i.p. vaccination with *A. salmonicida* or derived OMVs

To further investigate why a low amount of OMVs results in a similar humoral immune response comparable to that of 10^8 CFU of inactivated bacteria, 3 fish of each group were randomly selected and sacrificed 65d after vaccination, which was after the peak of specific antibodies against *A. salmonicida* had been detected in most fish (Figure 30). As mentioned earlier the humoral immune response after vaccination with OMVs has been well investigated (4, 220). For the long-term cellular immune response few aspects were investigated so far, e.g., the detection of memory B and T cells (233). Unfortunately, a long-term study directly comparing the cellular response after vaccination with bacteria or derived OMVs has not been published to my knowledge. Still, short-term studies that compare the activation of macrophages and dendritic cells after stimulation with both *Salmonella typhimurium* (*S. typhimurium*) and *S. typhimurium* derived OMVs could show a similar response in MHCII and CD86 upregulation, and increased expression of NO, TNFα and IL-12 in both groups (221).

In this animal trial, only a few differences were observed on a cellular level after the vaccination with 10^8 CFU of inactivated bacteria or OMVs.

The spleen is, similar to mammals, a secondary immune organ that consists of high amounts of mature B cells that can secret antibodies upon activation (91). Rainbow trout immunized with 10^8 CFU of inactivated showed a significant increase in the percentage of IgM^+ B cells in the spleen compared to fish vaccinated with 10 µg of OMVs (Figure 32). Interestingly, in the previous trial, the group immunized with 10^8 CFU of inactivated *A. salmonicida* was the only one in which the percentage of IgM^+ B cells significantly increased in the spleen 24h after the vaccination (Figure 27 A). Whether the percentage of IgM^+ B cells remained substantially higher than the percentage of IgM^+ B cells in the group vaccinated with OMVs, and what implications this might have on protection, would need to be investigated in further animal trials looking at more time points after vaccination. In humans, it could be shown that long-lived memory B cells persist in the spleen after vaccination with the vaccinia virus (234), while in trout, it was described that weeks after vaccination, antigen-specific antibodies are mainly produced by plasma cells in the head kidney (87).

Previous studies could show that vaccination with OMVs resulted in an enhanced activation of antigen-presenting cells followed by an enhanced activation of T cells (235), resulting in the production of IL-17 and IFNγ producing $CD4^+$ T cells (60, 221).

Due to the limited antibody repertoire available to characterize immune cells in fish, it was only possible to distinguish between $CD8^+$ and $CD8^-$ T cells (Section 4.7.2.1.). The $CD8^-$ T cells can either be $CD4^+$ αβ- or γδ T cells whereby γδ T cells make up only a tiny percentage of T cells (129). Therefore, it could be assumed that the majority of $CD8^-$ T cells are $CD4^+$ T cells (16). The percentage of $CD8^-$ T cell, possible $CD4^+$ T cells, was significantly increased in the head kidney and blood 65d after vaccination with OMVs, compared to the group vaccinated with 10^8 CFU of inactivated bacteria (Figure 33).

As mentioned earlier, vaccination with OMVs has been associated with induction of Th1 response after vaccination with OMVs (222), resulting in IL-17 and IFNγ producing $CD4^+$ T cells (60).

The fact that vaccination with OMVs led to a significant increase in the percentage of $CD4^+$ T cells compared to vaccination with inactivated bacteria could indicate that vaccination with OMVs strongly activates $CD4^+$ T cells. The activation of $CD4^+$ T cells could result in an increased stimulation of B cells to produce antibodies, which might explain the solid immune

response observed after vaccination with OMVs, similar to vaccination with a high dose of inactivated bacteria. Supporting this theory is the fact that OMVs function as a reliable adjuvant inducing a T cell response after vaccination and are discussed to be a more effective adjuvant as, e.g., the aluminum adjuvants which mainly trigger B cell immunity (235).

Taken together, the results of the first two animal trials indicate that OMVs derived from *A. salmonicida* achieve their highly immunogenic potential via activation of myeloid cells in the peritoneal cavity, which possibly migrate to the lymphatic organs and activate $CD4^+$ T cells resulting in the activation of B cells and in the production of *A. salmonicida* specific antibodies.

6.4. Challenges of oral vaccination

As described earlier (Section 2.4.2.3.) oral vaccination is considered the ideal vaccination method (17, 18), but only a few licensed vaccines are commercially available (17). One possible reason for this is that high amounts of antigen are needed for vaccination, which only results in low protection with a short duration (18). One of the main challenges of oral vaccines is the delivery of intact antigen to the hindgut, through the gastric environment of the gut (21). To prevent the digestion of the antigen by the gastric acid in the anterior part of the gut, different methods such as encapsulation in alginate microspheres (236), acid-stable coating with, e.g., copolymer (237), or surrounding the antigen with polyethylene glycol (PEG) (19) have been described.

In animal trials 3 and 4 (Table 6), PEG was used to protect the bacterial and viral antigen from the gastric environment in the gut. The main objective of animal trial 3 was to determine whether the established oral model for vaccination of *O. mykiss* with viral antigen (19) could be adapted for a vaccination with *A. salmonicida*. Instead of the attenuated virus, two different strains of inactivated *A. salmonicida* were used. As previously described, the strain JF2267 is characterized as a virulent "wild type" and the strain JF5505 as a "high-virulent re-isolate" because inoculation with 50 CFU leads to 80 % mortality in fish (Frey et al. unpublished), while for *Aeromonas salmonicida* "wild type" strains injection of $5*10^4$ CFU is used in challenge models (238). The oral vaccine pellets (Table 7) were manufactured by hand, as described previously (19), so that 1 g of food pellets contained 10^7 CFU of *A. salmonicida*, assuming that the fish would take up 1 g of food pellets. A dosage of $5*10^5$ CFU is sufficient for activating the cellular immune response after i.p. vaccination with inactivated *A. salmonicida* (15). The

higher dosage was chosen for the oral vaccine as it is known that besides the degradation of the antigen in the gut (21), which was prevented by the use of PEG (19), another great challenge of oral vaccines is the exact uptake of a certain vaccine dose (21). Therefore, the rationale of using 10^7 CFU in the oral pellets was that even if not all fish eat 1 g of vaccine, a high enough amount of antigen will be absorbed by the hindgut and presented to immune cells, this amount of antigen should be comparable to the amount of antigen delivered via, e.g., the i.p. vaccination route (75, 86, 239).

The data obtained from animal trial 3 indicated that vaccination with inactivated *A. salmonicida*, especially the "high-virulent re-isolate" strain JF5505, results in a cellular response comparable to i.p. vaccination (Figures 34 - 40). For example, the percentage of myeloid cells in the gut was significantly increased 48h after oral vaccination with strain JF5505 (Figure 34 A), which is known from the i.p. model to be essential for the bacterial clearance (15, 209-211) and for the subsequent antigen presentation (178, 179). 7d after oral vaccination with strain JF5505, the percentage of lymphocytes was significantly increased compared to the PBS control group. This domination of the lymphocytes has also been described after i.p. vaccination (15). It was shown that B cells become the main phagocytic cell type, taking over the role of the macrophages (143). The mucosal surfaces of fish are made up of loose formations of macrophages, dendritic cells, T cells and B cells (86, 240). For the gut, it has been reported that antigen uptake takes place via enterocytes (241), M-like cells, as well as directly via dendritic cells and macrophages either by "extending pseudopods through the tight junctions of the epithelium" (239) or by directly migrating into the intestinal lumen (239). IgT^+ B cells have been described as the dominant B cell subtype in the gut (30, 121), but so far, phagocytic capacity could only be shown of B cells isolated from systemic lymphoid organs (214). Whether the increase of lymphocytes observed after the oral vaccination with inactivated *A. salmonicida* is also associated with a rise in potentially IgT^+ B cells and if they also become the main phagocytic cell types needs to be investigated further. The local response after oral vaccination appears later than after i.p. injection (Figures 18 - 21). So far, it could only be shown for fish that the route of vaccination determines the type and magnitude of the mucosal immune response (242) and that mucosal vaccination results in lower antibody production than i.p. vaccination (243). Similar effects have been observed in mice after oral and i.p. vaccination and the reduced antibody production, after the oral vaccination, was explained by a delayed antigen availability to the immune system after oral vaccination (244).

After oral vaccination with inactivated *A. salmonicida*, an increase in the percentage of thrombocytes is seen in the gut on day 7 (Figure 35). So far, the involvement of thrombocytes in the immune response of the gut has not been discussed to my knowledge, even though their phagocytic potential has been described previously (83). It was shown that thrombocytes in the spleen are involved in initiating the immune response after exposure to toxic substances (245).

Interestingly, 24h and 48h after oral vaccination with strain JF5505, the percentage of thrombocytes was also significantly increased in the spleen (Figure 40). At the same time, thrombocytes were significantly reduced in the head kidney on day 7 post-vaccination (Figure 39). This could indicate the involvement of thrombocytes in oral vaccination. However, which role thrombocytes play in antigen clearance or presentation and activation of the adaptive immune response in the gut needs to be analyzed further. Apart from the thrombocytes, the percentage of all lymphocytes is increased 7d after oral vaccination in the spleen (Figure 37 B). Why differences are observed in the cellular response with the two different strains of *A. salmonicida* cannot be explained by the conducted study. Since the strain JF5505 originated from the strain JF2267, it was expected that they would be recognized by the same PRR and that both *A. salmonicida* strains would activate a similar cellular immune response. So far, it was demonstrated that the "high virulent re-isolate" strain JF5505 contains about 300 more genes than the "wild type" strains JF2267 (Jaros et al. unpublished). Whether this could explain a different cell response, e.g., via different protein expression patterns of the bacteria, needs to be investigated further.

Having observed a response after oral vaccination that was similar to that seen after i.p. vaccination, especially with the influx of myeloid cells followed by a domination of the lymphocytes at the vaccination site, the reproducibility of the model was tested by investigating a dual oral vaccine (Table 6). Oral vaccine pellets were manufactured by hand as described previously (19) and the pellet composition for the groups of inactivate bacteria, attenuated VHSV and a mix of both can be seen in table 7.

No consistency between the different groups could be observed after vaccination in the analyzed organs, except for a significant increase in the percentage of myeloid cells 14d after vaccination in the head kidney (Figure 43 B). For all the other organs, the response differs between the individual groups with no apparent explanation. The attenuated virus has been

described to be able to replicate and persist in the gut of rainbow trout for at least 21 days and increase the expression of, e.g., CD4 mRNA (19), which might explain the significant increase in the percentage of lymphocytes in the gut 24h after oral vaccination (Figure 41), as $CD4^+$ T cells can be found in the gate of FSC_{low}/SSC_{low} - cells (144). The same amount of virus was used in the “mixed pellet” groups, but no further similarities could be observed between both groups (Figures 41, 42, 43, 44 and 45). Furthermore, the use of the mixed pellets did not result in a cellular response similar to either the bacteria or viral pellets alone. For a viral mucosal vaccine it was shown that simultaneous delivery of the pre-mixed antigens resulted in a weaker immune response when compared to the subsequent delivery of the vaccines, which could indicate interference of the antigens (246) and may explain the lack in the cellular response seen in this trial. The significant increase in the percentage of thrombocytes of the spleen seen 24h after oral vaccination in animal trial 3 (Figure 40) is also not seen in the second animal trial (Figure 44). Additionally, no VHSV or *A. salmonicida* specific antibodies could be detected in the sera obtained 28d after oral vaccination (data not shown).

The high variability of the immune response after oral immunization, which has been described as the main challenge of successful oral vaccines (20), can also be seen in the animal trials of this thesis. One of the main challenges related to oral vaccines has been that all fish take up an equal dose of the vaccine antigen (21). This could explain the different results obtained after oral vaccination with inactivated bacteria in the animal trials. Additionally, the low efficacy of oral vaccines is hindered by the "highly tolerogenic gut environment" (247), which prevents mounting an immune response against all foreign particles taken up with the food. This phenomenon is also known as oral tolerance, which is most often is induced by repetitive feeding of low dosages of antigen (241). Therefore, one round of feeding was chosen in the conducted trials in this thesis. However, it could also be shown that feeding fish for 3 days per week with vaccine pellets results in a more robust immune response than feeding 5 days per month (241). The low pH in the gut provides an additional barrier to successful vaccine delivery (21), and even though the use of PEG packaged antigen has been successfully used for delivery of attenuated VHSV (19) and bacterial antigens (71), only few licensed oral vaccines exist. Many other forms of oral vaccine delivery, such as the expression of bacterial proteins in microalgae or alginate microparticles and the linking of antigen to nanoparticles (71) have been described, but have not been reproducible or transferable to different fish

species (243). Even though many of these approaches are promising, inconsistent responses to oral vaccines have been reported (18), as well as low protection and short duration of oral vaccines (18). Therefore, oral vaccines are, up until today, mainly used as a boost or prime vaccines (247).

7. Conclusion and Outlook

The main questions of this thesis were to determine if outer membrane vesicles derived from the fish pathogenic bacterium *A. salmonicida* could be used as a vaccine antigen, and if an oral model of vaccination of rainbow trout could be established for bacterial and viral antigens for testing the use of new vaccine candidates, such as OMVs, for oral immunization.

These questions were answered by analyzing the mRNA expression pattern of early inflammatory markers. It was demonstrated that remarkable similarities can be found after the stimulation with inactivated *A. salmonicida* and derived OMVs, especially in the activation pattern of IL-1β, IL-6, and IL-10 in RTS-11 cells and leukocytes of the head kidney. Differences in the activation of those and other early inflammatory markers after stimulation with inactivated bacteria or OMVs could be seen for leukocytes of the peritoneal cavity. Additionally, after i.p. vaccination of rainbow trout, significant changes in the mRNA expression of IL-8 and IL-10 in the head kidney could only be observed in fish vaccinated with inactivated bacteria while in the spleen vaccination with OMVs led to more significant changes on the mRNA level. Furthermore, the influx of myeloid cells into the peritoneal cavity after i.p. vaccination with OMVs and inactivated bacteria could be seen, and *A. salmonicida* specific antibodies were detected in trout serum after the immunization with *A. salmonicida* derived OMVs. These results strongly indicate the potential of OMVs derived from *A. salmonicida* as a possible vaccine candidate, which confirms what has been reported for OMVs derived from other fish pathogens (6, 7, 9). Even though a strong correlation between antibody titer and survival could be observed after vaccination with *A. salmonicida* (228), challenge trials are still considered the gold standard for testing vaccine efficacy (227), which is why future vaccine trials using OMVs should also test the survival of fish after challenge. Next to this, the differences after vaccination, seen in the systemic cellular immune response and stimulation with inactivated *A. salmonicida* or derived OMVs should be investigated further. While it is known that OMVs do not exactly resemble the outer membrane of bacteria (1, 57), it has not been described, which proteins might be missing from the OMVs derived from *A. salmonicida* that could explain the differences in the immune response. The fact that the OMVs can be very heterogenic in their size, attributed to different protein compositions, results in different ways of uptake into host cells (2). It could be shown that OMVs in the range of 20 to 100 nm derived from the gram-negative bacterium *Helicobacter pylori* contained fewer and less diverse bacterial proteins and were taken up via caveolin-mediated endocytosis (2, 248). Large OMVs of 90 to 450 nm were

taken up via macropinocytosis and endocytosis. Those large OMVs were made up of approximately 100 more different proteins than smaller OMVs, while nearly all the proteins were exclusive to the larger sized OMVs, and only around 20 were detected in both sizes of OMVs (2). Therefore, the protein composition of different size OMVs derived from *A. salmonicida* should be investigated further. For this, a proteomic analysis of the used OMVs as well as analyzes of the size of OMVs, which have been shown to result in different protein composition (2), should be studied further and might also explain the high scattering of the results in the *in vitro* experiments. OMVs have been used in combination with purified bacterial proteins in the licensed vaccine for humans for over 20 years (5), and even though their immunogenic potential has been described (1, 2, 6-9, 59, 61), it could also be shown that vaccination with OMVs alone did not result in the same protection from a challenge when compared to a combination of OMVs and bacterial components, indicating the need of additional bacterial components to confer a protective immune response (249). More recently, it could also be shown that OVMs derived from the pathogenic bacterium *Pseudomonas aeruginosa* contain DNA which was delivered to host cells (250), this could have tremendous implications for the use of OMVs in future vaccines, considering, e.g., the risk of horizontal gene transfer (69). Therefore, the possibility of DNA delivery to host cells via OMVs would need to be investigated for all OMVs that should be incorporated into vaccines.

The establishment of an oral vaccination model in trout for bacterial antigens proved to be challenging. The comparison of oral and i.p. vaccine administration has led to the conclusion that on a local level (peritoneal cavity and gut), a similar cellular immune response is activated after oral and i.p. vaccination, especially when looking at the myeloid cells and lymphocytes. The observed delay in the local immune response might be explained by the difference in the cellular composition of the peritoneal cavity and the gut. The peritoneal cavity is dominated by lymphoid cells (15), while the gut consists of a diffuse arrangement of macrophages, dendritic cells, T cells and B cells (86). However, the model could not be reproduced, and therefore testing of OMVs as an oral vaccine was not conducted, showing the difficulties of designing a successful oral vaccine for fish. Interestingly, the problem of developing oral vaccines is not exclusive for fish, and, when looking at the human setting, only few oral vaccines exist, e.g., oral vaccines against polio (247) or cholera (251).

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9. Publication

Sarais F., Rebl H., Verleih M., Ostermann S., Krasnov A., Köllner B., Goldammer T. & Rebl A.

Characterization of the teleostean κB-Ras family: The two members NKIRAS1 and NKIRAS2 from rainbow trout influence the activity of NF-κB in opposite ways.

Fish & Shellfish Immunology, Volume 106, 2020, doi.org/10.1016/j.fsi.2020.08.052

Sarais F., Montero R., Ostermann S., Rebl A., Köllner B. & Goldammer T.

The Early Immune Response of Lymphoid and Myeloid Head-Kidney Cells of Rainbow Trout (Oncorhynchus mykiss) Stimulated with Aeromonas salmonicida.

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10. Scientific presentations

2019

8th FLI Junior Scientist Symposium
Jena, Germany; (25. - 27. September)

Poster Presentation: "Bacterial outer membrane vesicles of A. salmonicida induce a proinflammatory immune response in vitro and in vivo. " Ostermann S., Kroniger T. & Köllner B.

19th International Conference on Disease of Fish and Shellfish
Porto, Portugal; (09. - 13. September)

Poster Presentation: "Bacterial outer membrane vesicles of A. salmonicida induce a proinflammatory immune response in vitro and in vivo. " Ostermann S., Kroniger T. & Köllner B.

2018

7th FLI Junior Scientist Symposium
Greifswald, Germany; (24. - 26. September)

Talk: "*Comparing two models for characterization of the innate immune response of Oncorhynchus mykiss after stimulation with bacterial antigens."* Ostermann S., Montero R. T., & Köllner B.

6th European Veterinary Immunology Workshop
Utrecht, Netherlands; (05. - 07. September)

Poster Presentation: "Bacterial outer membrane vesicle - target and carrier for oral vaccination of fish against fish pathogenic bacteria and viruses." Ostermann S., Montero R. T., & Köllner B.

14th Congress of International Society for Developmental and Comparative Immunology
Santa Fe, New Mexico, USA; (17. - 21. June)

Talk: *"Comparing two models for characterization of the innate immune response of Oncorhynchus mykiss after stimulation with bacterial antigens."* Ostermann S., Montero R. T., & Köllner B.

Fish Immunology Workshop
Wageningen, Netherlands; (29. April - 03. May)

Poster Presentation: "Comparing two models for characterization of the innate immune response of Oncorhynchus mykiss after stimulation with bacterial antigens." Ostermann S., Montero R. T., & Köllner B.

2017 9th Autumn School – Current Concepts in Immunology (DGfI)
Merseburg, Germany; (08. - 10. October)

Poster Presentation: "Bacterial outer membrane vesicle - target and carrier for oral vaccination of fish against fish pathogenic bacteria and viruses." Ostermann S., Montero R. T., & Köllner B.

2016 Snyder Trainee Research Day
University of Calgary, Canada (16th of December)

Poster Presentation: "Transferrin binding protein B of Mannheimia haemolytica as a vaccine candidate against bovine respiratory disease."

ImmuNet Research Day
Edmonton, Canada; (9th of June)

Poster Presentation: "Intra- and interspecies cross reactivity of Mannheimia haemolytica transferrin binding protein B."

Banff Conference on Infectious Diseases
Banff, Canada; (01. - 04. June)

Poster Presentation: "Intra- and interspecies cross reactivity of Mannheimia haemolytica transferrin binding protein B."

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