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Communication

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

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Abstract: The teleost head kidney is a highly relevant immune organ, and myeloid cells play a major role in this organ's innate and adaptive immune responses. Because of their complexity, the early phases of the innate immune reaction of fish against bacteria are still poorly understood. In this study, naïve rainbow trout were stimulated with inactivated *A. salmonicida* and sampled at 12 h, 24 h and 7 d poststimulation. Cells from the head kidney were magnetically sorted with a monoclonal antibody mAB21 to obtain one (MAb21-positive) fraction enriched with myeloid cells and one (MAb21-negative) fraction enriched with lymphocytes and thrombocytes. The gene expression pattern of the resulting cell subpopulations was analysed using a panel of 43 immune-related genes. The results show an overall downregulation of the complement pathway and cytokine production at the considered time points. Some of the selected genes may be considered as parameters for diagnosing bacterial furunculosis of rainbow trout.

Keywords: *Aeromonas*; NF-κB; myeloid; inflammatory; innate immune response; rainbow trout

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1. Introduction

Aquaculture is the fastest-growing food production sector, accounting for about half of all food fish consumed globally [1]. Disease outbreaks have been a massive stumbling block to the growth of the aquaculture industry and have severely impacted the industry's economic expansion in many countries [2]. To prevent pathogenic outbreaks and ensure economic success, fish health management aims for achieving optimum conditions in aquaculture [1]. Although prophylactic methods are currently applied, bacterial, viral and parasitic diseases still occur [3]. Therefore, greater knowledge of the molecular host–pathogen interactions over the course of an infection is critical for developing molecular tools and effective vaccines to prevent and reduce losses because of disease outbreaks.

The fish innate immune system comprises mucosal barriers, several unspecific pathogen-resistance components (pattern recognition receptors, enzymes, complement components, etc.) and cells (monocytes/macrophages, dendritic cells, granulocytes) [4,5]. Upon intraperitoneal (i.p.) stimulation, the innate defence is immediately activated, and myeloid cells, including monocytes/macrophages and granulocytes, are attracted to the peritoneum. Subsequently, lymphoid cells, including B-, T- and NK cells, are activated, which are part of the adaptive immune system. The induction of adaptive immunity involves time-consuming processes, such as cell proliferation, cell differentiation and cell mobilisation [6].

Despite these defence mechanisms, bacteria such as *Aeromonas salmonicida* ssp. *salmonicida*, which is the causative agent of furunculosis, can still invade fish. This Gram-negative

bacterium causes haemorrhagic and necrotic lesions in the gills, gut and muscle that are highly lethal [7,8]. A closer examination of the virulence factors of *A. salmonicida* shows a type III secretion system (T3SS). This is a syringe needle–shaped protein complex responsible for the transfer of toxins from the bacterial cytoplasm to the cytoplasm of the host cell. The T3SS effector proteins AexT, AopH, Ati2, AopP, AopO, AopN and ExsE modulate the host's immune response by interfering with those inflammatory responses initiated by MAP kinases or NF-κB proteins [9–12] (Figure 1). The ability of macrophages to trigger inflammation and interact with other immune cells is significantly reduced when pathogens block the NF-κB signalling pathways [13].

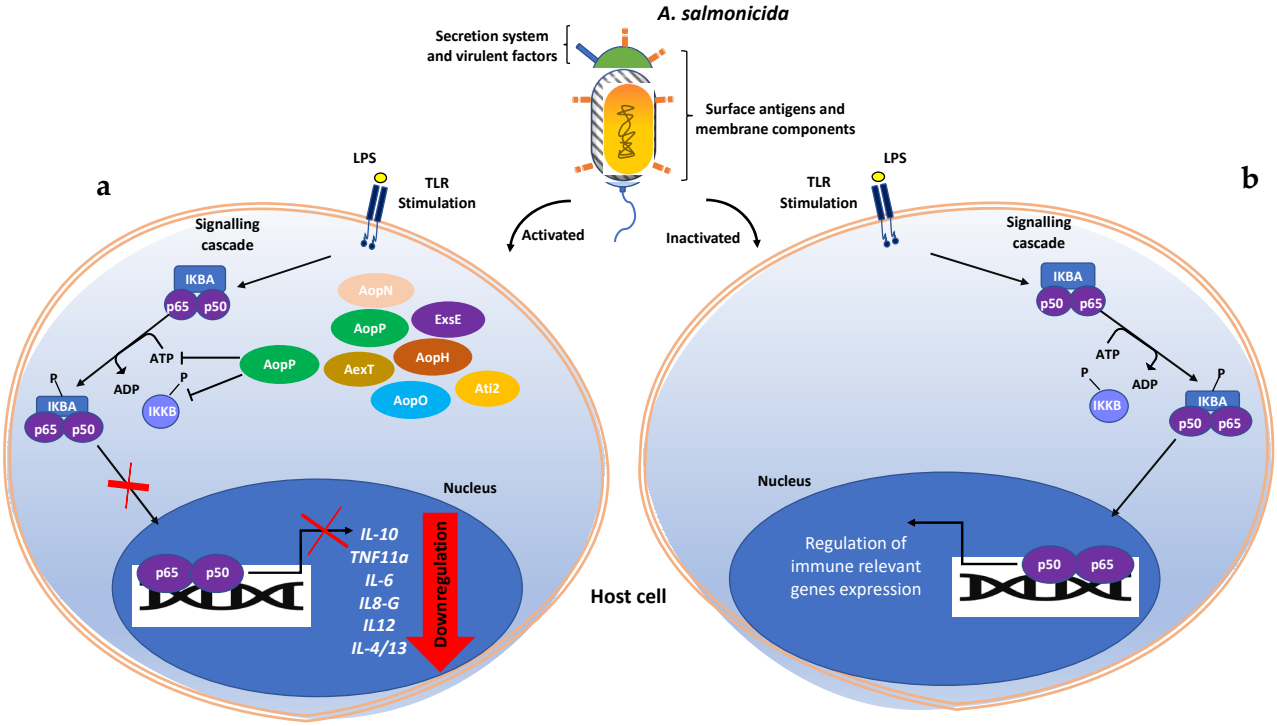

Figure 1. Graphic representation of (**a**) an infection with activated and (**b**) stimulation with inactivated *Aeromonas salmonicida* bacteria. In both scenarios, *A. salmonicida* activates the TLR-NF-κB pathway. (**a**) Previous investigations have documented the intracellular effects of AexT, Ati2 and AopP on inflammatory gene expression, whereas the impact of AopH, AopO and AopS has been predicted based on cytotoxic homologs from other bacterial species. (**b**) Stimulations with inactive bacteria do not contain an active T3SS system; the host cell response is directed against molecular antigens and induces immune-relevant gene expression. Abbreviations: LPS, lipopolysaccharide; TLR, toll-like receptor; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

Certain aspects of the early immune response to *A. salmonicida* of teleostean cells of myeloid origin remain unclear, for example, their expression profiles and their kinetics during the first 24 h poststimulation (p.s.) with the bacteria. To provide a better understanding of the early immune response to *A. salmonicida* in rainbow trout (*Oncorhynchus mykiss*), we investigated two leukocyte fractions from the head kidney at different time points after stimulation with *A. salmonicida*. To this end, we used a panel of 43 genes representing the most common markers for dendritic cells, macrophages, lymphocytes, thrombocytes, complement components and anti- and proinflammatory factors.

2. Materials and Methods

2.1. Ethics Statement

The experiment was approved by the State Office for Agriculture, Food Safety and Fisheries (approval number LALLF 7221.3-2-042/17), according to the German and Euro-

pean guidelines on animal welfare (Tierschutzgesetz, Tierschutz-Versuchstierverordnung, Directive 2010/63/EU).

2.2. A. Salmonicida for Stimulation Experiments

An aliquot of *A. salmonicida* ssp. *salmonicida* (A.s.s.)—the highly virulent strain JF 5505—from stock cryo-preserved batches was cultivated on tryptic soy broth media (TSB, Becton Dickinson, Heidelberg, Germany) at 15 °C for 24 h. Bacterial suspension was inactivated in 1.5% paraformaldehyde (PFA) for 1.5 h at 4 °C, and afterwards, a sample was plated out on TSB agar plates to prove successful inactivation. The inactivated A.s.s. bacteria were washed twice with TSB media by centrifugation at 4000× *g* for 10 min at 4 °C. The pellet was resuspended in TSB 25% glycerol at a concentration of 1.5×10^8 bacteria/mL. For intraperitoneal immunisation, the bacteria were washed once with phosphate-buffered saline (PBS) and set to a concentration of 1×10^7 bacteria/mL. Injections were prepared under aseptic conditions in sterile 1× PBS.

2.3. Fish

The Born strain of rainbow trout (*O. mykiss)* weighing 20 g to 100 g was purchased without gender selection from the commercial trout breeding farm Forellenzucht Uthoff GmbH, Neubrandenburg (Germany). The fish were kept in 300 L glass aquaria in a partially recirculating water system at a constant 12 °C and 12 h light:12 h dark period for both the summer and winter experiments. They were fed twice per day with commercial dry food pellets (Aminoforte, Kronen-Fisch, Wesel, Germany). A total of 24 trout were used for the experiments. All manipulations of the fish were done after anaesthesia with benzocaine. The fish received a single i.p. injection containing 100 µL of 1×10^7 bacteria/mL.

The highly virulent strain JF5505 of the inactivated *A. salmonicida* ssp. *salmonicida* was diluted in 200 µL PBS, and 5×10^5 CFU were i.p. injected using a 20 G needle. During this stimulation trial, the fish weighed between 30 g and 50 g. The control fish were injected with 200 µL of PBS. At 12 h, 24 h, and 7 d poststimulation, four fish per treatment group were euthanised using an overdose of benzocaine (100 mg/L, Sigma, Steinheim, Germany) (Figure 2) and sampled.

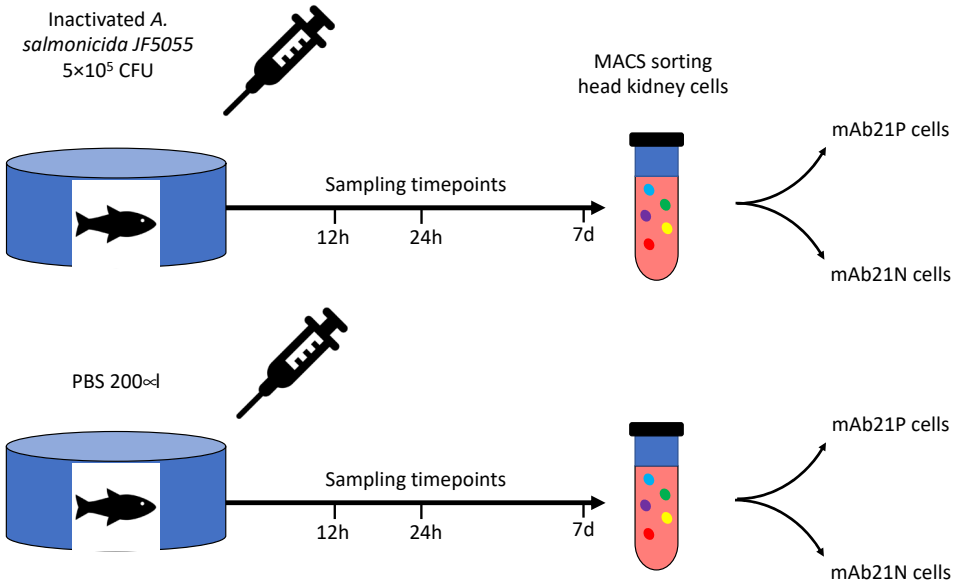

Figure 2. Overview of the exposure conditions, subsequent sampling at specific time points and magnetic separation with a monoclonal antibody (MAb21) against a myeloid lineage marker to separate a MAb21-positive (MAb21P) fraction of myeloid cells from a depleted MAb21-negative (MAb21N) cell fraction enriched in lymphocytes and thrombocytes.

2.4. Sorting of Head Kidney Cells

Pooled suspensions of viable leukocytes from the head kidney of four fish per treatment were prepared, as described previously [13]. Afterwards, 1×10^6 cells were incubated with the monoclonal antibody 21 (MAb21, previously validated as specifically recognising a lineage marker on all cells from myeloid lineage) for 30 min at 4 °C. Then, the cells were washed with 700 µL of MACS Buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) and sedimented by centrifugation for 5 min at 300× *g* at 4 °C. The cell pellet was resuspended in 200 µL of antimouse IgG magnetic beads (Miltenyi Biotec, Germany) following the manufacturer's recommendations.

After a final washing step with 700 µL of MACS buffer (as described above), the cells were resuspended in 500 µL of MACS buffer (Miltenyi Biotec, Germany). For magnetic separation in the autoMACS Proseparator (Miltenyi Biotec, Germany), the Possel_S programme was used. The resulting enriched cell fraction (MAb21P, myeloid cells) and depleted cell fraction (MAb21N, lymphocyte and thrombocytes enriched) were centrifuged, and the pellets were resuspended in 350 µL of lysis buffer RLT (Qiagen, Hilden, Germany) for further RNA extraction and gene expression analysis.

2.5. Primer Design and Biomark qPCR Measurements

The qPCR oligonucleotide primers (Table 1) were designed using pyrosequencing assay design software (v.1.0.6; Biotage, Uppsala, Sweden). All the analysed genes were selected based on previous publications on teleosts. Each primer pair was tested prior to RT-qPCR measurements in a standard PCR reaction using the HotStar-Taq (Qiagen, Germany) following a standard protocol. The resulting PCR products were visualised on agarose gels to assess product size and quality. Subsequently, primer pairs were tested in a quantitative PCR analysis using the LightCycler-96 system (Roche, Mannheim, Germany).

Table 1. Primers used in the study, amplicon length and function of each analysed gene. The left column reports the specific cell population.

Cell Population	Gene Symbol	Primer 5′–3′	Length (bp)	Accession No.	Function	Ref.
Dendritic cells	*TAP1*	CACTCCTGGCAGGGGCTACTT	176	XM_021559784	Antigen presentation	[14]
		CCTTATTTCATACGCTTTGGAGC				
	TAP2	CATCTGTGAGACGTTTATCCCTT	99	XM_024386707	Antigen presentation	[15]
		TCATGTACGCCATTGGAGGCAT				
	CD83	GTCTGCATTCTAGCTGCCTACT	128	XM_021593617	Immune cell interactions	[16]
		ACGTAAGCCTGGGGTCCAGTA				
	CD209	ATCTCTCAGGTACCGGAAGAGT	127	HG428763	Intercellular adhesion, antigen uptake	[17]
		GACTGTCTGGAGAGAGGAGCA				
Macrophages	*LYG*	GCAGGTTGACAAGCGCTACCA	118	BT073825	Hydrolyzation of the bacterial cell wall	[18]
		AAAGGGGGAATTTCAGCCTACAA				
	DAA (MHCII)	CAGTGATTCAGATGGAGTGAATAT	131	FR688130	Cell surface proteins with a key role in adaptive immunity	[19]
		AGATTTCCTTCCCTGGATATTATG				
	CD80/86	GCGTCGGCTGCTTCGAAGGT	152	NM_001160477	Co-stimulation of T-cells	[20]
		AGACTCCCAAACCACCTGTATG				
	CD68	GACACTGGAAAGACAGGAGTATT	115	XM_021578316	Scavenger receptor and antigen processor	[21]
		TTCAAGGAGGGCTTCATCACCT				
	SPIC	CACCTGGTCCTGCATCAGAAG	127	NM_001124513	Immune gene expression	[22]
		CTGGGACTATCACGCCACTCA				

Table 1. *Cont.*

Cell Population	Gene Symbol	Primer 5′–3′	Length (bp)	Accession No.	Function	Ref.
B- and T-cells	*CD79B*	TGAACCTCTCAGTGGCTTTAAAC	111	XM_021565350	Signalling through B-cell receptors	[23]
		TCTGTGTGGTGTCGGACCGAA				
	PAX5	AAGTATCCGTCCCGGGGTGAT	144	NM_001124682	Development of lymphoid progenitors	[24]
		GACTATTGGCTGAGAGAGTGTG				
	TARP	GAGAAGTGGAGTGGGACCAGA	105	XM_021619909	Antigen presentation	[25]
		AGGTGAGTGACGGGGGACAC				
Pan-T-cells and Thrombocytes	*GAPDH*	TCAACGGATTTGGCCGTATTGG	134	NM_001124246	Influence cytokine production	[26]
		GTTCAAGTATGACTCCACCCAC				
	CS	CACCTTCAACGAGGTTTACCCC	131	XM_021610150	Metabolic regulator	[27]
		GAGATGTGCTTTTGGATCTTGTC				
	CD18	AGTGAGTTGAGGGTTACATAGGA	148	XM_021579244	Leukocyte migration and adhesion	[28]
		GTATGTTCCAAAACAGTGATCAAC				
	CD41	CAGTTTAGCAACAAAACCATCAGT	161	XM_021624569	Leukocyte migration and adhesion	[29]
		TGACACAAAAGGTGATGAGGTTTA				
	CD36	GACTGTTACAAAGGAATCGGTCAT	112	XM_021577070	Ligand binding	[30]
		ACAGTTCTGGCTCTTTGACGTG				
Natural Killer cells	*GZMB*	TCCTTTCCTCTGCTGGAGCCT	94	XM_021598076	Destruction of infected and/or transformed cells	[31]
		TATATGGTCTCTCTGCAACACAG				
	PRF1	GCGGGTATTACAGCTATCGAGTA	161	XM_021558434	Destruction of infected and/or transformed cells	[32]
		ATTACATTACCAAGGTGAGCCTG				
	KLRD1 (*CD94*)	TGGGGCAACGATCGGCTCAAA	114	XM_021559366	"Missing-self" discrimination	[33]
		GAATCCTGCCACAGCAGTGGA				
Complement system	*CFD*	GACAAGTCATGAGCCCCAAG	151	NM_001246346	Serine protease activity of the alternative complement pathway	[34]
		GTGCCGAAAGTGGGTATTGT				
	CFH	GCTGGACCAAGACACTTGGC	166	NM_001124410	Complement regulation	[34]
		CCTCTACCGGGGGTTGGTG				
	CFI	ACCCAGTGTTTGCAAGAGAACC	167	XM_021593383	Inactivation of C3b/C4b factors	[34]
		CAGTTGGCGATCAGAGAGACG				
	C3-1	AGCCTCTGACCAGGGAGATATT	164	L24433	Opsonisation, elimination of pathogens	[34]
		GACGATGTCAGGGAGTTTGAAC				
	C1r/s	AACCAGAGGGGACTCTGTCCA	182	NM_001124380	Initiation of classical complement pathway	[35]
		TGGACAGAGTCCCCTCTGGTT				
Immune regulation	*NFKBIA*	AACCCTGGAGGAAAACAGTGAC	153	NM_001124368	Inhibition of NF-κB pathways	[36]
		GAACAATCAGAGACAGACGGCG				
	SERPINB1	TACCAGTTCGTTGAGACGTTCC	116	NM_001124515	Reduction of tissue damage; cell differentiation; immune activation	[37]
		ATCAAGAACCTATTGGCGGAGG				
	SERPING1	AAGGAATGACGAACGGCAAACG	169	NM_001124379	Activation of the C1 complex	[37]
		TCAGCTGTCTCACAGTAGTACAT				
	CD59	GATTGAGTGGGCAAAGTATTGTAT	167	XM_021606996	Inhibition of the membrane-attack complex	[38]
		CATACCCTGTTACATAACATTGCT				
	IL10	TGCCCAGTGCAGACGTGTACC	137	NM_001245099	Anti-inflammatory function	[39]
		TACACCACTTGAAGAGCCCCG				
	SOD2	TCCCTGACCTGACCTACGAC	201	XR_00247449	Oxidative stress	[40]
		GAGGTTTAATGGAGGAGGCC				
	A2M	GGGAGGAAGGATGAGATGAGTA	184	XM_021582312	Inhibition of cytokine-induced inflammation	[41]
		CTAACAGTGGAGCTTCAGGACC				

Table 1. *Cont.*

Cell Population	Gene Symbol	Primer 5′–3′	Length (bp)	Accession No.	Function	Ref.
Inflammation	*RIPK2*	TGTTGGCGAAAGGGAGAGGAAT GTACATGAGCAATGGCTCTCTG	105	KJ184523	Modulation of innate and adaptive immune responses	[42]
	NKIRAS2a	TGCATGTCTGCCTGTCTCTTTTT TGAGCCCGCAATATGATTGGCA	201	XM_021557705	Regulation of NF-κB signalling	[43]
	RANKL	GAGAGCATCGACTGGGAAAATGT TGTTCTGGGTACTCTGACACCA	125	XM_021620403	Regulation of interactions between T-cells and dendritic cells	[44]
	IL2RG (*CD132*)	ACCCCCAATGTAAACTGCCTGA TTTCAGCAGCAGGTTCATCAAAG	112	NM_001124356	Cytokine signalling involved in the stimulation of phagocytosis	[45]
	IL6	GTGTTAGTTAAGGGGAATCCAGT CCTTGCGGAACCAACAGTTTGT	128	NM_001124657	Proinflammatory cytokine and anti-inflammatory myokine	[46]
	CXCL8	ATATAACACTTGTTACCAGCGAGA ATTACTGAGGAGATGAGTCTGAG	106	HG917307	Chemoattraction	[47]
	IL12	ACATTCAGTGAGAGTGCGTGTC ACAAGGGGATCCTTCCTCACAA	118	HE798148	Differentiation of naïve T-cells	[48]
	IL4/13	CTGTCAGAGGAACTTCTGGAAAC GTGAAAAATGACGCGTTTGGTGA	131	NM_001246341	Regulation of inflammatory processes	[49]
	IL2RB (*CD122*)	AGAGGACAGTGGCGGTAATGAT CTCACAACCTCCAAGGACTGTT	94	XM_021622445	Cytokine signalling involved in T-cell-mediated immune responses	[50]
	IL1B	GAGAGTGCTGTGGAAGAACATAT ATGAATGAGGCTATGGAGCTGC	157	NM_001124347	Inflammation	[51]

The cell pellets were resuspended in 350 µL RLT buffer (Qiagen, Germany) and stored at −70 °C. RNA was isolated from these samples in separate tubes using TRIzol (Invitrogen, Karlsruhe, Germany) and subsequently purified with the ISOLATE II RNA Micro Kit ((Bioline/Meridian Bioscience, Luckenwalde, Germany). The concentrations of the individual RNA aliquots were adjusted at 2 ng/5 µL or 5 ng/5 µL. After cDNA synthesis using the reverse transcription master mix (Fluidigm, South San Francisco, CA, USA), the cDNA aliquots were individually preamplified in 13 (5 ng RNA input) or 15 cycles (2 ng RNA input) using the PreAmp master mix (Fluidigm, CA, USA) and subsequently treated with exonuclease I (New England BioLabs, Ipswich, MA, USA). Multiplex RT-qPCR was conducted using the Biomark HD system and EvaGreen fluorescence dyes (Bio-Rad, Hercules, CA, USA), as previously described [15]. In brief, the 48.48 Fluidigm gene expression biochips were first primed in the MX integrated fluidic circuit (IFC) controller (Fluidigm, South San Francisco, CA, USA) before being loaded with the preamplified cDNA samples and eventually analysed using the Biomark HD instrument (Fluidigm, CA, USA). The raw RT-qPCR results were retrieved with instrument-specific analysis software (v. 3.0.2; Fluidigm, CA, USA). The geometric means of the copy numbers of the reference genes *EEF1A1* [16] and *RPS5* [17] were used to normalise the expression data.

2.6. Data Analysis

Heatmaps and data visualisation were performed using MacOS GraphPad 9 or Windows, GraphPad Software, San Diego, CA, USA (https://www.graphpad.com, accessed on 1 October 2021).

3. Results

3.1. Basal Expression Profiles of Selected Immune Genes in Two Fractions Enriched in Myeloid Cells or Lymphocytes and Thrombocytes

For the three sampling time points of 12 h, 24 h and 7 d after stimulation with inactivated *A. salmonicida*, head kidney cells were separated into a MAb21P fraction enriched in myeloid cells and a depleted MAb21N cell fraction comprising mainly lymphocytes and

thrombocytes (Figure 3). The purity after magnetic sorting was higher than 95%. Both fractions were used to profile a panel of 43 immune genes using multiplex qPCR.

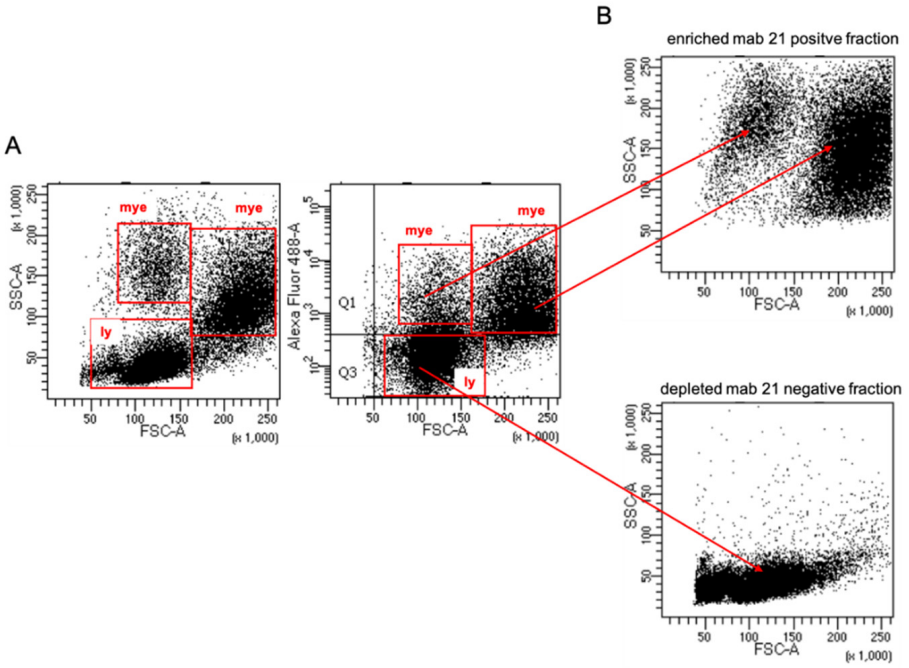

Figure 3. (**A**) Magnetic-activated sorting of head kidney cells from rainbow trout using the myeloid lineage marker specific monoclonal antibody MAb21 resulted in (**B**) an enriched myeloid cell fraction (MAb21-positive myeloid cells) and a depleted lymphoid cell fraction (MAb21-negative lymphocytes and thrombocytes).

The basal expression of the selected lineage marker genes validated the successful separation of the myeloid MAb21-positive cell fraction (expressing *CD209*, *LYG* and *SPI-1*) from the MAb21-negative fraction containing thrombocytes (*CD36*) and B-lymphocytes (*CD79b*, *PAX5*) and T-lymphocytes (*TCR*) (Figure 4).

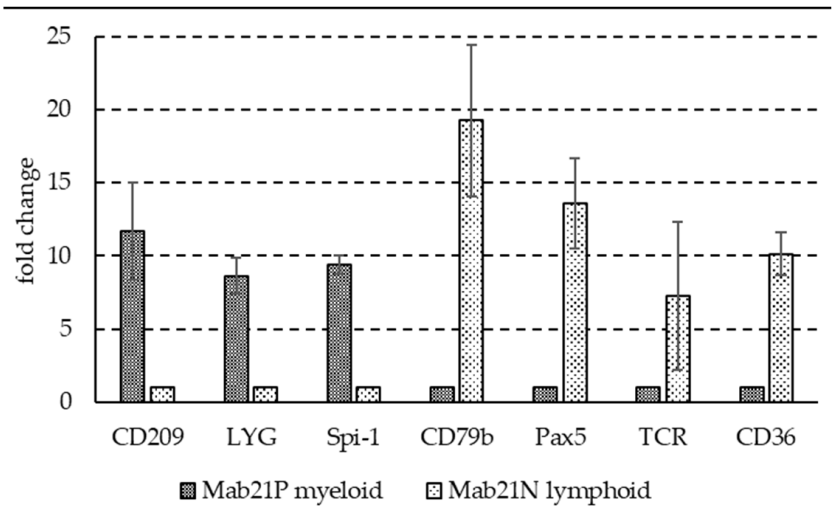

Figure 4. Basal expression of the selected lineage marker genes in MAb21-positive myeloid cells and MAb21-negative lymphoid cells.

A comparison of the kinetics of these genes after i.p. injection of PBS indicated that the average basal expression did not change significantly (data not shown).

3.2. Immune Gene Expression Profiling in Two Fractions Enriched in Myeloid Cells or Lymphocytes and Thrombocytes after Stimulation with A. salmonicida

The average expression ratios of the *A. salmonicida*–treated group compared with the PBS-treated control groups are presented in heatmaps as log2 fold change values (Figures 5 and 6).

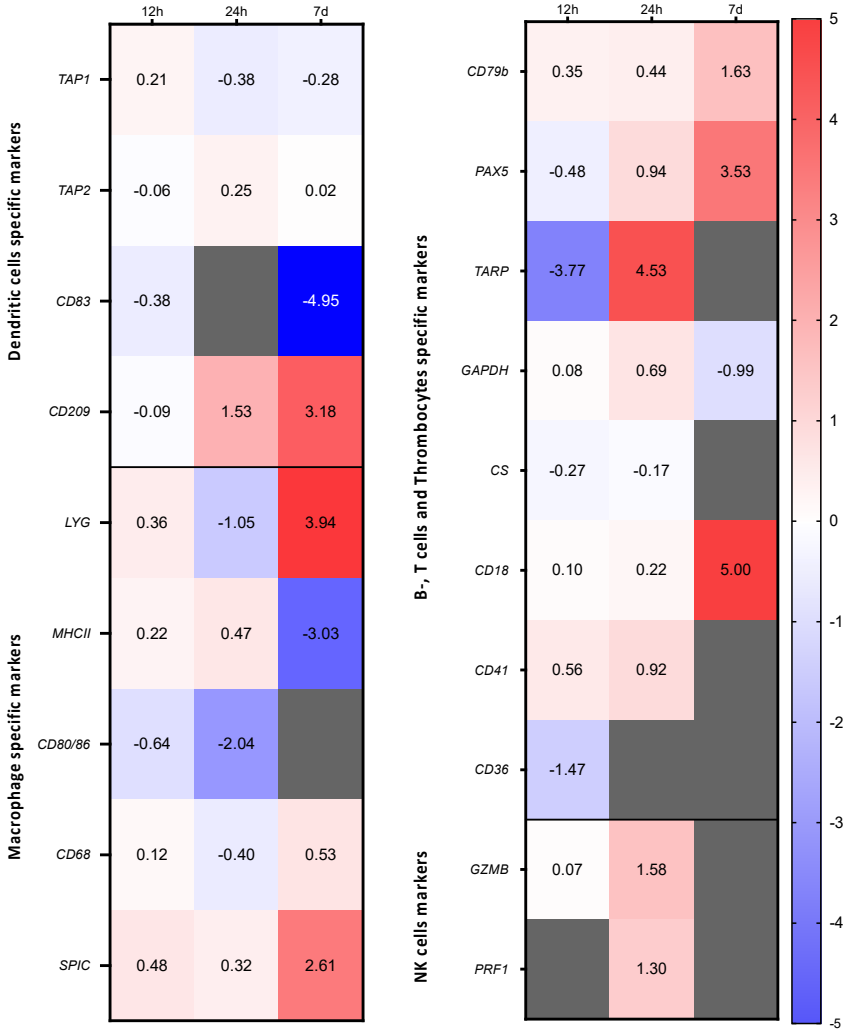

Figure 5. Expression modulation of immune genes specific to myeloid cells (left panel) and to lymphocytes and thrombocytes (right panel) after peritoneal injection of *A. salmonicida* into rainbow trout. The heat map is representative of four samples and illustrates the average expression ratios as log2 FC values of the *A. salmonicida*–stimulated group relative to the control group (n = 4) for the immune genes listed at the left margin of each panel. *EEF1A1b* and *RPS5* were used as reference genes to normalise the data. Grey fields indicate that the calculation of FC values failed because of undetectable expression in the treated or control groups. Increased and decreased transcript levels in the samples of the stimulated group compared with the controls are indicated by red and blue fields, respectively, according to the legend on the right.

Generally, the expression patterns in both the MAb21P and MAb21N fractions did not change strongly after intraperitoneal stimulation.

Compared with the PBS control groups, *CD209* was upregulated in the MAb21P fraction at 24 h p.s. (log2 FC = 1.5) and 7 d p.s. (log2 FC = 3.2), while *CD80/86* was downregulated at 24 h p.s. (log2 FC = −2). After 7 d p.s., the expression levels of *LYG*, *DC-SIGN* and *SPIC* (log2 FC > 2.6) increased. *CD83* underwent a strong downregulation at

7 d p.s. (log2 FC = −5), accompanied by a downregulation of *DAA/MHCII* at 7 d p.s. (log2 FC = −3) (Figure 5).

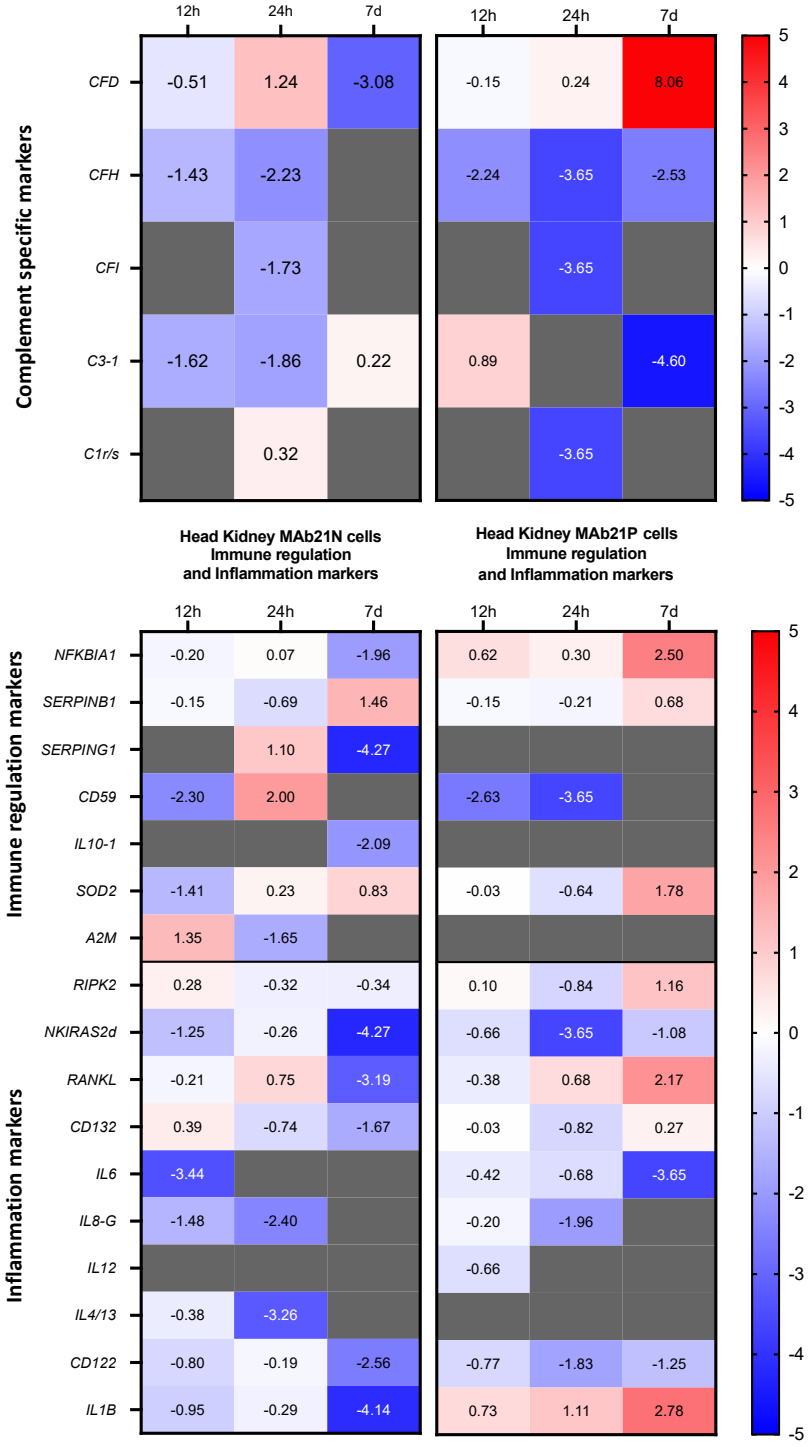

Figure 6. Expression modulation of genes specific to inflammation and immune regulation after p.i. injection of inactivated *A. salmonicida* into rainbow trout. The heat map is representative of four samples and illustrates the average expression ratios as log2 FC values of the stimulated group relative to the control groups (n = 4) for the given immune genes. *EEF1A1b* and *RPS5* were used as reference genes to normalise the data. Grey fields indicate that the calculation of FC values failed because of undetectable expression in the treated or control groups. Increased and decreased transcript levels in the samples of stimulated rainbow trout compared with controls are indicated by red and blue fields, respectively, according to the legend on the right.

In the PBS control groups, we did not detect *CFI*, *IL10* or *IL12* at 12 h p.s.; *CFH*, *C3-1*, *C1r/s*, *CD59*, *SERPING1*, *CFI*, *A2M* or *IL12* at 24 h p.s.; and *IL10* at 7 d p.s. *IL4/13* was not detectable in the controls across all time points.

In the stimulated groups, we did not detect *C1r/s*, *SERPING1* or *IL4/13*, at 12 h p.s.; *CFH*, *C1r/s*, *CD59*, *CFH*, *C3-1*, *C1r/s*, *CD59*, *SERPING1*, *CFI* or *IL4/13* at 24 h p.s.; and *CD80/86*, *C1r/s*, *CD59*, *CFI* or *CXCL8* at 7 d p.s. *A2M* and *IL12* were not detectable in stimulated fish across all time points.

3.3. Expression Profiles of Characteristic Markers in the Cell Fraction Enriched with Lymphocytes and Thrombocytes

The transcript level of *TARP* was strongly reduced in the MAb21N fraction 24 h p.s. (log2 FC = −3.8), followed by a marked increase 7 d p.s. (log2 FC = 4.5). *PAX5* was mildly upregulated in the MAb21N fraction at 24 h p.s. (log2 FC = 0.9), but its expression further increased at 7 d p.s. (log2 FC = 3.5), together with *CD18* (log2 FC = 5). *CD79B* was modestly upregulated across all time points (log2 FC between 0.4 and 1.6). The expression levels of *PRF1* increased at 24 h p.s. (log2 FC = 1.3) (Figure 5).

In the PBS control groups, we did not detect *PRF1*, *IL10* or *IL12* at 12 h p.s.; *CD36*, *CD94*, *IL10*, *CD59*, *A2M* or *IL12* at 24 h p.s.; and *CD41*, *CD36*, *GZMB*, *PRF1*, *CD94*, *CD59*, *SERPING1*, *A2M*, *IL6*, *CXCL8*, *IL12* or *IL14/13* at 7 d p.s.

In the groups stimulated for 12 h, we did not detect *CD94* or *SERPING1*; both *IL10* and *IL6* were not detectable at 24 h p.s.; and *TARP*, *CD36*, *GZMB*, *CD59*, *SERPING1*, *IL6* and *IL12* were all not detected at 7 d p.s.

3.4. Expression Patterns of Complement- and Cytokine-encoding Genes in the Two Cell Fractions from the Head Kidney

At 12 h p.s., the levels of *CD59* (log2 FC = −2.3) and *SOD2* (log2 FC = −1.4) were reduced in the MAb21N fraction. The *SERPING1* level was upregulated at 24 h p.s. (log2 FC = 1.1) and strongly downregulated at 7 d p.s. (log2 FC = −4.3). *A2M* was initially upregulated at 12 h p.s. in the MAb21N (log2 FC = 1.4), followed by a downregulation at 24 h p.s. (log2 FC = −1.7).

The genes encoding cytokines were generally downregulated in both fractions, except for *IL1B*. This gene was upregulated with a log2 FC of 1.1 (24 h) and 2.8 (7 d) in the MAb21P fraction but simultaneously strongly downregulated (log2 FC of −4.1 at 7 d) in the MAb21N fraction.

In both fractions, *CXCL8* and *IL4/13* were downregulated after 24 h p.s. (log2 FC < −1.5). *IL10* and *IL12* were downregulated only at one time point during stimulation in the MAb21N (7 d p.s.) and MAb21P fractions (12 h p.s.), respectively.

RIP2K, *SOD2* and *RANKL* were upregulated (log2 FC ≥ 1.2) in the MAb21P fraction 7 d p.s. *NFKBIA* levels were downregulated (log2 FC = −2) in the MAb21N fraction but upregulated (log2 FC = 2.5) in the MAb21P fraction at 7 d p.s. *SERPINB1* levels were mildly downregulated in both cell fractions (log2 FC < −0.7), followed by a mild upregulation at 7 d p.s. in the MAb21N (log2 FC = 1.5) and MAb21P fractions (log2 FC = 0.7).

The expression of the complement genes in both fractions was generally downregulated, except for *CDF*, which showed a strong upregulation (log2 FC > 8) at 7 d p.s. in the MAb21P fraction (Figure 6).

4. Discussion

In morphology and function, the head kidney of fish corresponds to the bone marrow of mammals and is one of the most important haematopoietic organs in bony fish. Macrophages and other cells of myeloid origin differentiate in the head kidney. These cells have a core function in innate immunity against a diverse and broad array of pathogens [52] because they are involved in phagocytosis, radical production and cytokine secretion, much like their mammalian counterparts [4].

The aim of the current study was to profile the expression of the specific genes involved in the early immune response of rainbow trout after intraperitoneal stimulation with

inactivated *A. salmonicida* ssp. *salmonicida* in two fractions of the head kidney: myeloid cells (MAb21P) and lymphocytes/thrombocytes (MAb21N).

Our results show an overall downregulation of the genes characteristic for cells of myeloid origin, except for *LYG*, *SPIC* and *CD209*, which appeared progressively higher expressed at the later time points analysed. *CD209* is an important mediator of dendritic cell/T-cell clustering and T-cell activation [53]; *SPIC* regulates haematopoietic cell differentiation, proliferation and apoptosis [22]; and *LYG* is involved in the response against bacterial infections [54]. The upregulation of the genes encoding *CD209*, *SPIC* and *LYG* indicates an effective activation of the early immune response, which is supported by the increased expression of the inflammation marker *IL1B*. The downregulated levels of *CD83*, *CD80/86* and *DAA* in the cells of myeloid origin may result from restricted NF-κB activation [55] and might indicate the reduced potential of leukocytes to produce cytokines in the head kidney [56–59]. It is more likely that the observed results indicate that the cells of myeloid origin migrate to the peritoneum to promote the first phase of inflammation at the site of stimulation, thereby leaving only a remaining fraction of unstimulated cells in the head kidney [60].

In the fraction enriched in lymphocytes and thrombocytes, the initially down- and subsequently upregulated level of transcripts coding for T-cell receptor gamma (*TARP*) could be explained by a process known as 'state-dependent inactivation'. Following its full downregulation, the T-cell receptor complex adapts to conditions of continuous stimulation [61]. *PAX5* and *CD79B* were progressively upregulated after stimulation. A similar regulation has been previously detected in the Chinese sucker (*Myxocyprinus asiaticus*) after being stimulated with inactivated *Aeromonas hydrophila*, suggesting increased levels of signal transduction and B-cell activation [23]. In Nile tilapia challenged with inactivated *A. hydrophila* [62], integrin β (*CD18*) was regulated during the early stimulation phase, indicating the recruitment and activation of leukocytes.

The downregulation of *CD36* may reflect a specific signalling pathway that activates thrombocytes [63]. Unlike their human counterparts, fish thrombocytes are nucleated cells that can phagocyte and regulate the immune response [64]. Thrombocytes from rainbow trout express genes that encode the proteins involved in activation and aggregation, antigen presentation and immune modulation [65]. *CD36* can be downregulated by toll-like receptor (TLR) ligands through TLR-induced cytokines [66], as observed in previous studies [29,67,68].

The early phase of stimulation revealed modulated levels of *PRF1* and *GZMB* in the MAb21N fraction, suggesting the regulation of apoptotic mechanisms by cytotoxic T-lymphocytes and NK cells [31,69].

Complement evasion strategies by pathogenic organisms have previously been reported for different Gram-negative bacteria [35,70]. The expression of complement components in myeloid cells was strongly inhibited in the first 12 h p.s. The strong downregulation of *CFH* and *CFI* at each time point analysed might indicate that the inhibition of the complement system by *A. salmonicida* is not unique to the early stage of an infection. It has been reported that several bacteria, including *A. salmonicida*, express and modify surface antigens such as outer membrane proteins, capsules and lipopolysaccharide (LPS) to inhibit the complement system [71,72]. Additionally, *A. salmonicida* may promote the immunosuppressive state of fish [73]. Although we used a highly virulent strain of *A. salmonicida*, the cells were inactivated. We cannot rule out the possibility that the membrane antigens of *A. salmonicida* interfered with the proinflammatory response, as reflected by the downregulation of *IL6* and a moderately increased *IL1B* level at the early time points. These proinflammatory cytokines promote the transcription of complement-related genes [74], which were consequently also at low levels. In the myeloid-depleted cell fraction, we did not observe any relevant regulation patterns in the first two time points analysed.

The expression of diverse proinflammatory factors was downregulated in both fractions during the earlier stimulation. The expression of *A2M* coding for A2-macrotubulin was obviously completely inhibited in the myeloid cell fraction across all phases of stim-

ulation. The acquisition of metal ions by pathogens is vital for their survival. Because A2-macrotubulin binds to iron, zinc and copper, its downregulation may be a further hallmark of the suppressed early immune response by *A. salmonicida* [41]. All of the above genes maintained essentially the same expression pattern 7 d after stimulation.

5. Conclusions

In conclusion, gene expression profiling in two head kidney cell fractions determined the transcriptional response to inactivated *A. salmonicida* at different time points. Our results have shown that despite the increased expression levels of myeloid cell markers, the expression of proinflammatory and anti-inflammatory cytokines was lower than in the control groups, which may reflect the migration of activated myeloid cells into the peritoneum. The current study provides valuable information about the immune components that should be targeted when developing and evaluating innovative vaccine formulations to provide effective protection against bacterial pathogens such as *A. salmonicida*. This will improve the knowledge of the early phase of the immune response to understand the regulation of immune-related genes upon inactivated bacteria stimulations.

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