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Dissecting the immune pathways stimulated following injection vaccination of rainbow trout (*Oncorhynchus mykiss*) against enteric redmouth disease (ERM)

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ABSTRACT

Enteric redmouth disease (ERM or yersiniosis) is one of the most important diseases of salmonids and leads to significant economic losses. It is caused by the Gram-negative bacterium *Yersinia ruckeri* but can be controlled by bacterin vaccination. The first commercial ERM vaccine was licenced in 1976 and is one of the most significant and successful health practices within the aquaculture industry. Although ERM vaccination provides complete protection, knowledge of the host immune response to the vaccine and the molecular mechanisms that underpin the protection elicited is limited. In this report, we analysed the expression in spleen and gills of a large set of genes encoding for cytokines, acute phase proteins (APPs) and antimicrobial peptides (AMPs) in response to ERM vaccination in rainbow trout, *Oncorhynchus mykiss*. Many immune genes in teleost fish are known to have multiple paralogues that can show differential responses to ERM vaccination, highlighting the necessity to determine whether all of the genes present react in a similar manner. ERM vaccination immediately activated a balanced inflammatory response with correlated expression of both pro- and anti-inflammatory cytokines (eg IL-1β1-2, TNF-α1-3, IL-6, IL-8 and IL-10A etc.) in the spleen. The increase of pro-inflammatory cytokines may explain the systemic upregulation of APPs (eg serum amyloid A protein and serum amyloid protein P) and AMPs (eg cathelicidins and hepcidin) seen in both spleen and gills. We also observed an upregulation of all the α-chains but only one β-chain (p40B2) of the IL-12 family cytokines, that suggests specific IL-12 and IL-23 isoforms with distinct functions might be produced in the spleen of vaccinated fish. Notably the expression of Th1 cytokines (IFN-γ1-2) and a Th17 cytokine (IL-17A/F1a) was also upregulated and correlated with enhanced expression of the IL-12 family α-chains, and the majority of pro- and anti-inflammatory cytokines, APPs and AMPs. These expression profiles may suggest that ERM vaccination activates host innate immunity and expression of specific IL-12 and IL-23 isoforms leading to a Th1 and Th17 biased immune response. A late induction of Th2 cytokines (IL-4/13B1-2) was also observed, that may have a homeostatic role and/or involvement in antibody production. This study has increased our understanding of the host immune response to ERM vaccination and the adaptive pathways involved. The early responses of a set of genes established in this study may provide essential information and function as biomarkers in future vaccine development in aquaculture.

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1. Introduction

Enteric redmouth disease (ERM, yersiniosis) is one of the most important diseases of salmonids and leads to significant economic losses [1,2]. The disease is caused by *Yersinia ruckeri*, a Gram-negative rod-shaped enterobacterium, first isolated from rainbow

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trout *Oncorhynchus mykiss* in the Hagerman Valley of Idaho, USA in the 1950s [3–5]. ERM infected rainbow trout show a general septicaemia with an inflammatory response in all tissues. The gills are the first route of entry for infection, with the spleen a major secondary lymphoid organ associated with the response [1,6].

Vaccination as a means of controlling ERM is one of the most significant and successful health practices within the aquaculture industry, and has helped to reduce the use of environmental unfriendly antibiotics to control this bacterial disease [7]. The first commercial fish vaccine for ERM was licensed in 1976, and was a bacterin prepared from formalin-killed whole cells of *Y. ruckeri.* A simple immersion is effective but intraperitoneal (ip) injection provides superior and long lasting protection against ERM [8]. However, ERM outbreaks have occurred recently due to the emergence of atypical biotypes of *Y. ruckeri* [2]. This issue can be resolved by including new isolates in the improved vaccine preparation, as shown with AquaVac® RELERA™ that contains both biotypes 1 and 2 of *Y. ruckeri* and provides better protection [8,9]. Moreover, distinct strains of *Y. ruckeri* are present in the environment and can evolve with the introduction of susceptible hosts and vaccination of salmonids in aquaculture [10,11]. As the production of global aquaculture continues to increase it is likely that vaccines against other bacterial disease will encounter similar issues and will need the development of improved formulations.

The development of fish vaccines has been largely empirical, based on whether a formulation is effective at increasing survival post-disease challenge [12]. This is unsatisfactory from both ethical and scientific perspectives. There is a clear need to establish methods to improve fish vaccine development, such as pre-screening of candidate vaccines in the early development phase and the quality control of vaccines. The immune system is a network of specialized cell types and tissues that communicates via cytokines and direct contact, to orchestrate specific types of responses to effect protection [13]. It is known in mammals that different vaccines induce distinct transcriptional signatures, representing the highly specialized defence mechanisms that can be elicited to cope with the different pathogens and insults a host may encounter. In this context, the early innate responses to vaccines are likely critical instructors for the development of adaptive immunity at later time points [14]. For the development of new vaccines, it is crucial to determine molecular signatures of vaccine-induced immune responses to gain a better understanding of the pathways involved and mechanisms that underpin protection, as well as to predict vaccine performance.

As a highly efficacious vaccine, the ERM vaccine provides a useful model for the investigation of fish immune responses to vaccines and bacterial diseases. Thus past studies have examined immune gene responses to ERM after vaccination [15,16] or after vaccination and challenge [9,17,18]. However, only a relatively small number of genes were investigated in these studies and often in the absence of information of the paralogues present [15–18]. With the recent release of salmonid genomes [19,20] and the identification of a large number of cytokine genes including the paralogues for IL-1β [21], TNF-α [22], IL-17A/F [23,24], IL-4/13 [25] and subunits of the IL-12 family [26–29] in salmonids, it is timely to revisit the early immune responses to ERM vaccination.

Hence, in this study ERM vaccination was used as a model to investigate the early cytokine responses in rainbow trout in two major and relevant immune organs, the spleen and gills. The expression of acute phase proteins (APPs) and anti-microbial peptides (AMPs) was also examined. We found that intraperitoneal injection vaccination induces an early balanced expression of pro- and anti-inflammatory cytokines and adaptive cytokines in the spleen, with a heightened expression of APPs and AMPs in both spleen and gills.

2. Materials and methods

2.1. Fish maintenance and rearing condition

Apparently healthy rainbow trout with no history of infection were purchased from the Mill of Elrich Trout Fishery (Aberdeenshire, Scotland, UK) and maintained in 1-m-diameter fibreglass tanks with recirculating freshwater at 14 + 1 °C at the Scottish fish immunology research centre, the University of Aberdeen, UK. At least 10 fish from each batch were screened for potential bacterial infection by taking head kidney swabs and growing on tryptic soy broth (Sigma, UK) agar plates. No bacterial growth was seen. Fish were fed (2% biomass) twice a day with a commercial diet (EWOS) and given two weeks for acclimation prior to vaccination.

2.2. Fish vaccination

The commercial vaccine AquaVac™ ERM (MSD Animal Health), a formalin-inactivated bacterin containing not less than 5×10^9 cells per ml of *Y. ruckeri* (Hagerman strain type 1), was used in this study. The vaccine trial was described previously [30]. Briefly, a group of 24 fish (mean ± SEM = 48.8 ± 1.5) were vaccinated by intraperitoneal injection (ip) of 0.1 ml of vaccine following manufacturer's instructions. The same number of fish were injected with phosphate buffer saline (PBS) as the control. The vaccinated and control groups were kept in separate 1-m-diameter fibreglass tanks in a single recirculating freshwater system at 14 + 1 °C. Fish handling and experimental protocols comply with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals, and were carried out under UK Home Office project licence PPL 60/4013, approved by the ethics committee at the University of Aberdeen.

2.3. Sampling, total RNA extraction and cDNA synthesis

Six fish from both the vaccinated and control groups were killed at 1, 3, 7 and 14 days post vaccination. Spleen and gills were taken from each fish and homogenised separately in TRI reagent (Sigma, UK). Total RNA extraction and cDNA synthesis was as described previously [17,31]. The synthesised cDNA samples were diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH8.0) and stored at −20 °C ready for real-time PCR analysis.

2.4. Gene expression analysis by real-time PCR

The expression of a set of genes for acute phase proteins (APPs), antimicrobial peptides (AMPs) and cytokines using real time PCR were performed as described previously [25,32,33]. Briefly, the PCR amplification was performed using a LightCycler® 480 Instrument II (Roche Applied Science) and 384 multiwell plates in a 10 μl reaction using SYBR® Green I (Invitrogen™, Carlsbad, USA) and IMMOLASE ™ DNA Polymerase (Bioline, UK), and expression levels calculated using the LightCycler® 480 software version 1.5. Elongation factor-1α (EF-1α), a house keeping gene, was used as an internal control. The sequences of primers used, and the DDBJ/ENA/GenBank accession number of the sequence the primers were designed against are listed for each gene in Table 1. At least one of each primer pair was designed to cross an intron and were tested to ensure that PCR products could only be amplified from cDNA samples and not from genomic DNA. The cp value (the crossing point at which the fluorescence crosses the threshold, means + SEM) of EF-1α was 11.31 + 0.25 (spleen samples) and 10.44 + 0.11 (gill samples). The expression of each gene was first normalized to that of EF-1α, and expressed as a fold change relative to the expression level of control fish at the same time points.

Table 1
Primers used for expression analysis by real-time PCR.

Gene	Δcp[a](Spleen)	Δcp[a] (Gills)	Forward (5′ to 3′)	Reverse (5′ to 3′)	Acc. No.
House-keeping gene					
EF-1α	0[b]	0[b]	CAAGGATATCCGTCGTGGCA	ACAGCGAAACGACCAAGAGG	AF498320
Acute phase proteins and antimicrobial peptides					
SAA	8.16	8.34	GGTGAAGCTGCTCAAGGTGCTAAAG	GCCATTACTGATGACTGTTGCTGC	AM422447
SAP1	20.43	21.17	GCTGTTATGGTGACCTTCAAGATCTCTC	GCGTTTGTACAACAACAAATCATTGTC	X99385
SAP2	15.08	13.91	GGTTGTTATGCTGAACATCAAGATCTCTC	CCACCCTTTGATTGCATACACAGATT	EZ763346
CATH1	9.39	9.14	ACCAGCTCCAAGTCAAGACTTTGAA	TGTCCGAATCTTCTGCTGCAA	AY594646
CATH2	11.77	11.00	ACATGGAGGCAGAAGTTCAGAAGA	GAGCCAAACCCAGGACGAGA	AY542963
Hepcidin	11.73	16.19	GCTGTTCCTTTCTCCGAGGTGC	GTGACAGCAGTTGCAGCACCA	CA369786
LEAP2A	12.19	12.91	GGTTCCTGGTGTTTCTGGTGCT	AGTGGCCACCCCTGCAAAT	AY362186
β-defensin-1	17.99	19.57	CTGGTTTTCCTATTGCTTAATGTTGTGG	GAAATGAGAAACACAGCACAAGAATCC	AM282655
β-defensin-2	17.77	20.22	ATGGGGAGACTGGGTTTGGT	ACGCAAAGCACAGCATCTTAATCT	FM212656
β-defensin-3	17.96	24.42	GGCTCTTTTAGTCATTGCTTGTGGAATAC	CAGCATACATTCGGCCATGTACA	FM212657
β-defensin-4	20.58	18.13	TGGTGCTCCTCGCTTTCTTGG	TGGGCGACACAGCATACAAATC	FM212658
Cytokines					
IL-1β1	10.26	11.51	CCTGGAGCATCATGGCGTG	GCTGGAGAGTGCTGTGGAAGAACATATAG	AJ278242
IL-1β2	20.89	14.09	GAGCGCAGTGGAAGTGTTGG	AGACAGGTTCAAATGCACTTTATGGT	AJ245925
IL-1β3	11.18	11.77	CTG AAG GCC GTC ACA ATC CA	CTGGTCCTTACAGCGCTCCAA	AM181685
nIL-1Fm	10.04	9.41	CCCATTCCTCGTGACACCAG	CTGGACGACCTGGAGAGTGACT	AJ555869
IL-2	13.45	14.43	TGATGTAGAGGATAGTTGCATTGTTGC	GAAGTGTCCGTTGTGCTGTTCTC	AM422779
IL-4/13A	13.96	12.12	ACCACCACAAAGTGCAAGGAGTTCT	CACCTGGTCTTGGCTCTTCACAAC	FN820501
IL-4/13B1	17.21	13.28	GAGATTCATCTACTGCAGAGGATCATGA	GCAGTTGGAAGGGTGAAGCTTATTGTA	HG794522
IL-4/13B2	18.55	15.87	GAGACTCATCTATTGCGTATGATCATCG	TGCAGTTGGTTGGATGAAACTTATTGTA	HG794523
IL-6	14.45	18.56	GGGAGAAAATGATCAAGATGCTCGT	GCAGACATGCCTCCTTGTTGG	DQ866150
IL-8	9.58	8.72	AGAGACACTGAGATCATTGCCAC	CCCTCTTCATTTGTTGTTGGC	AJ310565
IL-10A	16.55	13.68	GGATTCTACACCACTTGAAGAGCCC	GTCGTTGTTGTTCTGTGTTCTGTTGT	AB118099
IL-10B	16.90	15.50	GGGATTCTAGACCACATCAAGAGTCC	GATGGGAGATTTAAAGTTGTGTGTTCC	FR691804
IL-11	10.86	14.38	CTCTCGCTGCTATTGGCCCA	TCTCGAATGCATGTTCCTTCAATAGAT	AJ535687
M17	12.14	10.58	GTGGACCTCTTAAAAACATACAAGCTCAG	GGATGGTGGCTGTAAGTCTGTCTG	FM866399
IL-12 p35A1	16.62	17.51	GGAACACCACATTCAGTGAGAGTGC	CGTCTGCAACTTGTGAGGAAGGAT	HE798148
IL-12 p35A2	20.73	19.77	GGAACACCACATTCAGTGAGAGTGA	CAACCTGTGAGGAAGACACCCA	HG917950
IL-12 p35B1	21.49	18.58	TGCCAAACGCCAAGCTTTATTTTG	GCTGTTGAGTGCTTTTGGTCTTTGG	HG917951
IL-12 p40B1	17.14	9.77	CCCTTCTACATCCGAGAAATAGTGAAAC	GTTGGTTTCACTTATAAACACCTTTTCCTT	HE798149
IL-12 p40B2	14.14	9.46	CCGTTCTACATACGAGAAATAGTGGAGA	TCAGAGTCACAGCTTTCCCTGG	HG917952
IL-12 p40C	12.14	12.13	TTAAAGACAACGGAAAGGAGGAGC	CCTCCCGTAACCACATTTTTCC	AJ548830
IL-23 p19	22.01	16.44	ACCTAAGAGCAGATTCAATGCCTTG	TCTTCCCAGCTCTTCACTTCCTG	KP410548
IL-27 p28A	19.88	18.47	GCAGCTGCTCAGGAGATATAAGGAGG	TCTCTCAGGTATGCTGGGTTTTGG	HG794528
IL-27 p28B	22.39	22.06	GCAGCTGCTCATGAGATATAAAGAGGA	GCTGCTCTCTGTTCCACCTTATCCAC	HG794529
EBI3	17.27	17.42	ACATCGCCACCTACAGTATGAAAGG	GGGTCCGGCTTCACAATGT	AJ620467
IL-15	7.95	7.69	TGGAATTGCTTCATAATATTGAGCTGCC	TGGTAGTTATCTGTGACCGACATGTCCTC	AJ628345
IL-17A/F1A	24.36	13.42	CAAACGTACACTTTTTGATGGTGCTG	GGGACTCATCATAGGTGGTGTTGGT	KJ921977
IL-17A/F1B	18.92	20.46	CTTCCAGTCTTTGACGGTGCTG	GGTTGTAAATAGGTCTGATGGAATGGAA	KJ921978
IL-17A/F2A	20.94	11.97	CACCCTGGACCTGGAAAAGCAC	GGCCACAGACAGGAAGGAGG	AJ580842
IL-17A/F2B	22.62	18.05	CCCTGGACCTGGAAACCCAT	GGCCACGGACAGGAAGGTGA	KJ921979
IL-17A/F3	18.89	11.41	CTGGTGCTGGGTCTGATCATGT	GGTCTCATCGTATGTGTCGCTGTATG	KJ921980
IL-17A/F4/N	25.20	18.46	AGAACTCAACATGCAACAGCTCCA	CGGTTCAAGTCAATTTTTTCCACGTA	KJ921981
IL-17C1	22.60	15.21	CTGGCGGTACAGCATCGATA	GAGTTATATCCATAATCTTCGTATTCGGC	FM955455
IL-17C2	16.84	16.70	CTGGCGGTACAGCATCGATA	CAGAGTTATATGCATGATGTTGGGC	FM955456
IL-17D	19.30	11.10	GAAGAAATCCTCGAGCAGATGTTTG	GGGTCGTGGGAGATCCTGTATG	AJ580843
IL-18	9.02	10.01	GAGCAATGCAAAGCAGATGATTG	CATGTTTTGAGCAGCCAATGTAGTC	AJ556990
IL-20	>25	19.37	CAAGAACCTGAGGCAATGTCACTG	TCTCCTATAGCCTTTACTGCTGCCG	FN386780
IL-21	16.45	15.00	AAAGTTATCAAAAACCTCAACAACCGAA	CCAGTCTACTGATGGCCTTTTGAAG	FM883702
IL-22	18.05	12.20	GAAGGAACACGGCTGTGCTATTAAAC	GATCTAGGCGTGCACACAGAAGTC	AM748538
IL-34	9.97	9.31	AGGCAGAAGACGTAACATGAAACACA	TCCACCCTCGCCCTCAGCTT	FN820429
IFNγ1	12.41	12.16	CAAACTGAAAGTCCACTATAAGATCTCCA	TCCTGAATTTTCCCCTTGACATATTT	AJ616215
IFNγ2	14.43	14.00	CAAACTGAAAGTCCACTATAAGATCTCCA	GGTCCAGCCTCTCCCTCAC	FM864345
TGF-β1A	8.13	9.90	CTCACATTTTACTGATGTCACTTCCTGT	GGACAACTGCTCCACCTTGTG	OMY7836
TGF-β1B	7.47	8.72	CATGTCCATCCCCCAGAACT	GGACAACTGTTCCACCTTGTGTT	FN822750
	13.09	13.29	TGTGTGGGGTCCTCTTAATAGCAGGTC	CCTCAATTTCATCCTGCATCGTTGA	AJ277604
TNF-α2	16.03	14.35	CTGTGTGGCGTTCTCTTAATAGCAGCTT	CATTCCGTCCTGCATCGTTGC	AJ401377
TNF-α3	14.94	16.51	GCTGCACTCTTCTTTACCAAGAAACAAG	CCACTGAGGACTTGTAATCACCATAGGT	HE798544
Master transcription factors					
T-bet	11.09	14.89	GGTAACATGCCAGGGAACAGGA	TGGTCTATTTTTAGCTGGGTGATGTCTG	FM863825
GATA3	11.12	5.29	CCAAAAACAAGGTCATGTTCAGAAGG	TGGTGAGAGGTCGGTTGATATTGTG	FM863826
RORγ[c]	9.04	8.61	ACAGACCTTCAAAGCTCTTGGTTGTG	GGGAAGCTTGGACACCATCTTTG	FM883712
Foxp3A	9.78	10.42	CCCAGAACCGAGGTGGAGTGT	TGACGGACAGCGTTCTTCCA	FM883710
Foxp3B	9.09	9.99	TCCTGCCCCAGTACTCATCCC	TGACGGACAGCGTTCTTCCA	FM883711

Notes
[a] Δcp is the average cp value of the target gene minus that of the house-keeping gene EF-1α in the control fish at day 1 post vaccination. A higher Δcp value indicates a lower expression level.
[b] The average cp of EF-1α is 11.31 + 0.25 (spleen) and 10.44 + 0.11 (gills).
[c] Primers amplify both RORγa and RORγb genes.

2.5. Data and statistical analysis

For the statistical analysis, all of the gene expression data were first calculated as arbitrary units after normalization to the expression level of the house keeping gene EF-1α. Then the data were log2 transformed to improve the normality of real-time quantitative PCR measurements, as described previously [31]. One way-ANOVA and Bonferroni post hoc tests were used to analyse all of the expression data using IBM SPSS statistics 22 software (SPSS Inc., Chicago, IL, USA). Differences between vaccinated and control groups for each time point were considered statistically significant at $p < 0.05$. In addition, we also undertook a correlation analysis of vaccine modulated genes using the Spearman rank order correlation test to look for associations between expression patterns. The gene expression levels in spleen samples at day 1 and day 3 ($N = 12$) were used for this analysis, since the majority of gene expression changes seen were during this period.

3. Results

The expression of 63 genes, including the house keeping gene EF-1α, 11 APPs and AMPs, 46 cytokine genes and 5 master transcription factors, were analysed by real-time RT-PCR in this study. To give an indication of relative transcription level in the spleen and gills, ΔCp that is the Cp of the target gene minus that of EF-1α, were provided for the control fish at day 1 in Table 1.

3.1. ERM-vaccination activates an immediate expression of APP and AMP genes in both spleen and gills

APPs, eg serum amyloid A protein (SAA) and serum amyloid protein P (SAP), and AMPs, eg cathelicidins (CATH) and hepcidin, are evolutionarily conserved effector molecules of the innate immune system that have important roles in the resolution of infection and activation of the adaptive immune response [34,35] The expression of some APPs and AMPs has been shown to be induced by *Y. ruckeri* infection previously [36]. Thus their expression was examined first in response to ERM vaccination. Trout SAA was highly expressed in spleen and gills ($\Delta Cp = 8$, Table 1). Its expression was significantly induced in spleen by ERM vaccination at day 1, 3 and 7, and peaked at day 1 (89-fold) and in the gills at day 1 (18-fold, Fig. 1A). One of the SAP paralogues, SAP1 that was lowly expressed constitutively, was also induced in spleen at day 1 and 3 (Fig. 1B and C).

The constitutive expression of the AMPs, CATH1, CATH2 [35] and Hepcidin [34] was relatively high in spleen and gills ($\Delta Cp = 9$ to 16, Table 1). CATH1 expression was induced at day 1 and day 3 in spleen but was refractory in gills after ERM vaccination (Fig. 1D). CATH2 expression was highly induced and peaked at day 1 in both spleen (385 fold) and gills (32 fold) and the heightened expression lasted until day 7 in the spleen and day 3 in the gills after vaccination (Fig. 1E). Hepcidin expression was also induced at day 1 in both spleen (43 fold) and gills (59 fold, Fig. 1F) by ERM vaccination. The expression of other AMPs, including LEAP2A [37] and β-defensins [38] was refractory in both spleen and gills (Table 2 and S1).

3.2. ERM-vaccination induces an early correlated upregulation of pro- and anti-inflammatory cytokine expression in the spleen

A successful vaccine is expected to activate the innate immune system to express pro- and anti-inflammatory cytokines. Several such cytokines have been investigated previously [15,16]. However, many other cytokines discovered recently, especially the paralogues, have not been examined after ERM vaccination. Therefore, a large number of cytokine genes including all the known paralogues have been investigated in this study. Three active IL-1β paralogues are present in salmonids [21]. IL-1β1 and IL-1β3 have relatively high constitutive expression in spleen and gills, compared to IL-1β2 (Table 1). The expression of both IL-1β1 and IL-1β2 was induced at day 1 and 3, and peaked at day 1 in the spleen (Fig. 2A-B), but was not modulated in the gills (Table S1), of vaccinated fish. IL-1β3 expression was refractory (Table 2 and S1). In terms of fold change, IL-1β2 expression was more inducible (peaked at 49 fold) than IL-1β1 (peaked at 17 fold). At least three TNF-α paralogues are present in rainbow trout [22]. All the three genes (TNF-α1-3) were up-regulated in spleen at day 1, with TNF-α2 remaining elevated at day 3 in the spleen, but no changes were seen in the gills after vaccination (Fig. 2C-E). The other pro-inflammatory cytokines investigated included IL-8 [39], and three IL-6 family members (IL-6, IL-11 and M17) [40,41]. The expression of all these genes was induced and peaked at day 1 in the spleen (Fig. 2F-I) after vaccination but in gills only IL-6 expression was induced to a small extent (3 fold) at day 1 (Table S1).

Several genes with regulatory roles, including two paralogues of IL-10 [42] TGF-β1 [43] and FoxP3 [44], and the novel fish IL-1 family member nIL-1Fm [45] were also investigated. IL-10A expression was up-regulated from days 1 and 3 and peaked at day 1 (301 fold) (Fig. 2J) in the spleen but was not modulated in the gills of vaccinated fish, however IL-10B expression was refractory in both tissues (Table 2 and S2). The expression of nIL-1Fm was also induced at day 1 in the spleen by vaccination (Fig. 2K). The expression of both TGF-β1 paralogues was refractory in both spleen and gills after vaccination (Table 2 and S1). However, the expression of the two trout master transcription factors Fox3A and Fox3B, important for mammalian TGF-β1 expression in mammals, was decreased at day 1 in the gills and spleen respectively (Fig. 2L, Table 2 and S1).

Correlation analysis of vaccination-modulated gene expression in the spleen at day 1 and 3 revealed that the expression of the major pro-inflammatory cytokines (IL-1β1, IL-1β2, TNF-α paralogues, IL-6, IL-8, IL-11 and M17) and anti-inflammatory cytokines (IL-10A and nIL-1Fm) was highly correlated (Table 3). Their expression was also correlated with that of APPs (SAA) and AMPs (CATH1, CATH2 and hepcidin) (Supplementary Table S2).

3.3. ERM-vaccination activates an early Th1 and Th17-type response but a later Th2-type response in the spleen

Activation of specific Th responses is important for vaccine-mediated immunity [46]. The expression of the key Th-response specifying cytokines has only focused on IFN-γ1 after ERM vaccination to date [15,16]. With the recent success of identification of the complete repertoire of these cytokines, including three IL-4/13 paralogues and six IL-17A/F paralogues [24,25] in rainbow trout, we examined, for the first time, the repertoire of Th response related cytokines after ERM vaccination. The Th1 specifying cytokines, IFN-γ1 and IFN-γ2 were induced at day 1 (8 fold) in the spleen of vaccinated fish (Fig. 3A-B). Two IFN-γ inducing cytokines, IL-18 [47] and IL-21 [31] were also significantly induced to some extent at day 1 in the spleen, but not in the gills, of the vaccinated fish (Table 2 and S1). Of the six potential Th17 cytokines in rainbow trout, the expression of IL-17A/F1A, 2A and 3 was upregulated at day 1 in spleen, with IL-17A/F2A remaining elevated at day 3, but no changes were apparent in the gills of vaccinated fish (Fig. 3E-G). However, IL-17A/F1B, 2B and IL-17N (or IL-17A/F4) were refractory (Table 2 and S1). In terms of inducibility, IL-17A/F1A (105 fold) and 17A/F3 (19 fold) were more responsive than IL-17A/F2A (7 fold). Three potential Th2 cytokines, IL-4/13A, IL-4/13B1 and IL-4/13B2 are present in salmonids [25]. A small but significant induction of

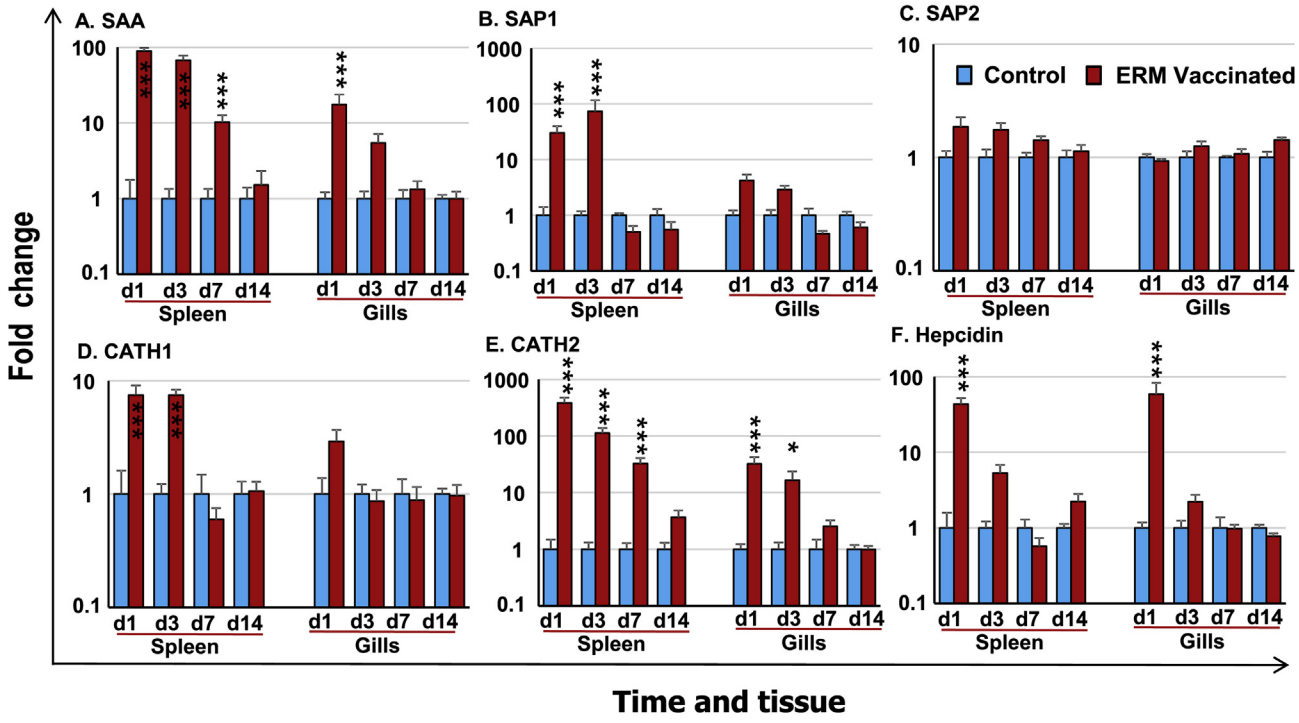

Fig. 1. Modulation of the expression of APP and AMP genes in the spleen and gills by ERM vaccination. Two groups of rainbow trout were vaccinated by ip injection with AquaVac ERM (red bars) or PBS as control (blue bars). The fish were killed at days 1, 3, 7 and 14, and spleen and gills collected for gene expression analysis by real-time RT-PCR (as described in the Materials and Methods). Modulated expression was expressed as a fold change calculated as the mean expression levels in vaccinated fish normalized to that of time-matched controls in the same tissue. The means + SEM of six fish are shown. The relative significance of a Bonferroni post hoc test after a significant one way-ANOVA between the vaccinated and control groups at the same time point is shown above/within the bars as: *$p < 0.05$, and ***$p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Fold change of transcript expression of studied genes in spleen after ERM vaccination not shown in Figs. 1–4. Asterisks indicate significant differences between vaccinated fish and controls as**$p < 0.01$ and***$p < 0.001$ (One-way-ANOVA with Bonferroni correction).

Gene	D1	D3	D7	D14
LEAP2A	0.58	0.53	0.81	0.22
β-defensin-1	0.14	0.27	0.27	0.21
β-defensin-2	2.57	0.35	0.17	1.07
β-defensin-3	1.25	0.43	0.46	0.41
β-defensin-4	1.68	0.75	0.73	0.48
IL-1β3	1.11	1.22	0.95	0.92
IL-10B	1.33	0.67	0.59	0.62
IL-12 p40B1	1.21	0.84	0.66	1.41
IL-12 p40C	1.08	0.77	1.34	1.56
IL-27 p28B	11.06***	2.16	0.44	0.99
EBI3	0.75	0.48	1.45	1.38
IL-15	1.65	0.99	0.72	0.86
IL-17A/F1b	5.90	4.44	3.09	0.58
IL-17A/F2b	1.41	3.00	0.38	1.78
IL-17A/F4/N	15.95	0.97	3.15	4.52
IL-17C1	2.93	2.00	1.21	0.68
IL-17C2	1.48	2.00	0.82	1.52
IL-17D	0.50	0.29**	0.48	0.32
IL-18	1.72***	1.10	0.70	0.82
IL-20	1.66	0.24	0.04	0.37
IL-21	2.51**	0.61	1.18	1.49
IL-22	1.97	2.24	0.84	1.02
IL-34	1.59	0.52	0.69	1.04
TGF-β1A	1.42	1.07	0.83	0.81
TGF-β1B	2.43	0.74	0.58	0.48
Foxp3A	0.43***	0.74	1.26	0.76

the expression of IL-4/13B1 (4 fold) and IL-4/13B2 (3 fold) was found at day 3 only in the spleen of vaccinated fish (Fig. 3J-K). IL-4/13A expression was refractory (Fig. 3I), as was the expression of the master transcription factors (T-bet, GATA3 and RORγ) [48,49] and the T cell cytokine IL-2 [50] (Fig. 3C, D, H, and L) in the spleen. Of these genes only RORγ at day 14 was modulated in the gills but again to a very small degree (Table S2).

The expression of Th1 cytokines (IFN-γ1 and 2) and the Th17 cytokines (IL-17A/F1A and IL-17/A/F3) was highly correlated ($R = 0.72–0.90$, $p < 0.001$) but there was a lack of correlation with the other Th17 cytokine IL-17A/F2A, and the Th2 cytokine IL-4/13B1, the predominantly expressed IL-4/13B isoform (Table 4). The expression of IFN-γ1, IFN-γ2 and IL-17A/F1A was also correlated to IL-21, the majority of pro-inflammatory cytokines (IL-1β1-2, TNF-α1-3, IL-6, IL-8, IL-11 and M17), anti-inflammatory cytokines (IL-10A and nIL-1Fm), APPs and AMPs (Table S2).

3.4. ERM-vaccination induces specific isoforms of the IL-12 family members in the spleen

IL-12 family cytokines play key roles in immunity bridging the innate and adaptive immune systems. Each cytokine consists of an α-chain (p19, p28 and p35), and a β-chain (p40 and EBI3) in mammals. The orthologues of mammalian p35 and p40 are increased in teleosts fish, and salmonids in particular, due to the whole genome duplication events in these lineages [25]. Rainbow trout has three isoforms of p35 and p40, and two p28 isoforms, that potentially make additional IL-12 family cytokines [51]. The constitutive expression of α-chains (p35A1, p35A2, p35B1, p19, p28A and p28B) was lower ($\Delta Cp = 16–22$, Table 1) than that of the β-chains (P40A, P40B2, p40C and EBI3, $\Delta Cp = 9–17$, Table 1). The expression of all the α-chains was significantly increased in spleen and peaked at day 1 (13.6-fold for p35A1, 37.5-fold for p35A2, 58.7-

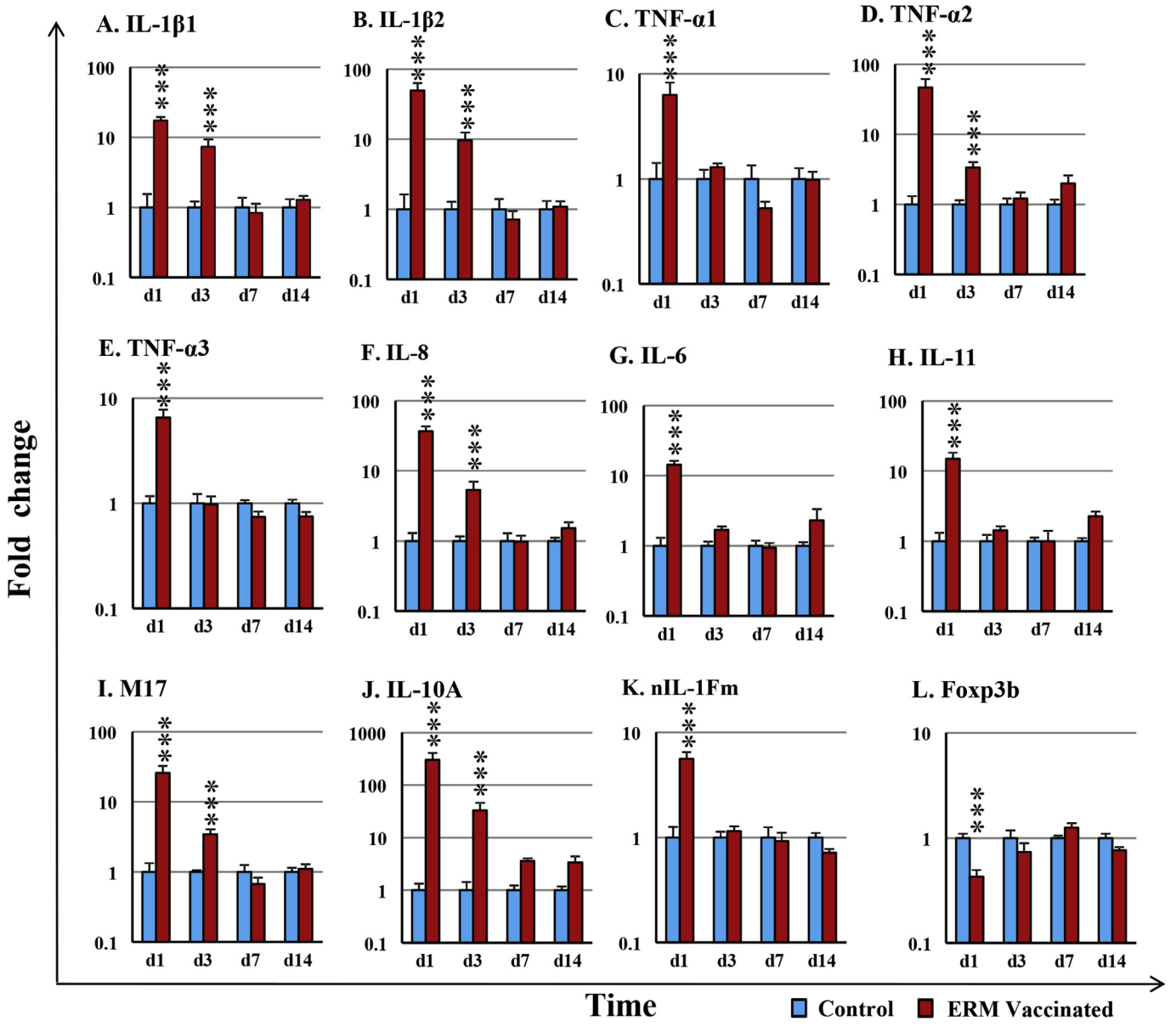

Fig. 2. Modulation of the expression of pro- and anti-inflammatory cytokines and Foxp3b in spleen by ERM vaccination. Two groups of rainbow trout were vaccinated by ip injection with AquaVac ERM (red bars) or PBS as control (blue bars). The fish were killed at day 1, 3, 7 and 14, and spleen and gills collected for gene expression analysis by real-time PCR (as described in the Materials and Methods). Modulated expression was expressed as a fold change calculated as the mean expression levels in vaccinated fish normalized to that of time-matched controls in the same tissue. The means + SEM of six fish are shown. The relative significance of a Bonferroni post hoc test after a significant one way-ANOVA between the vaccinated and control groups at the same time point is shown above/within the bars as: $^{*}p < 0.05$, and $^{***}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fold for p35B1, 1502.0-fold for p19, 52.6-fold for p28A and 11.1-fold for p28B), with upregulated levels remaining at day 3 except for p35A1 and p28B, in the spleen (Fig. 4A-E). No modulation of these genes occurred in the gills and expression of the β-chains was refractory in both spleen and gills, with the exception of p40B2 that was up-regulated 3.4-fold at day 1 in the spleen (Fig. 4F, Table 2 and S1). These expression profiles suggest that specific isoforms of IL-12 and IL-23 could be increased in the spleen after ERM vaccination.

The upregulated expression of the α-chains in the spleen was significantly correlated and also correlated to that of Th1 (IFN-γ1/2) and Th17 (IL-17A/F1A) markers (Table 4). The increased expression of most of the IL-12 family α-chains was also correlated to the pro- and anti-inflammatory cytokines (IL-1β1-2, TNF-α1-3, IL-6, IL-8, IL-11, M17, IL-21, nIL-1Fm and IL-10A), AMPs (CATH1, CATH2 and hepcidin) and APPs (SAA and SAP1, Table S2).

3.5. Other cytokine genes

Other cytokine genes examined included IL-15 [52], two IL-17C paralogues [53], IL-20 [54], IL-22 [55] and IL-34 [56] and were refractory in both spleen and gills in response to ERM vaccination (Table 2 and S1). IL-17D, which was lowly expressed in the spleen, was inhibited at day 3 in the spleen of vaccinated fish (Table 2).

4. Discussion

Through the analysis of the expression of a large number of immune genes after ERM injection vaccination in rainbow trout, we

Table 3
The Spearman's rho correlation coefficient (R) and the 2-tailed significance (p) between gene expression levels of the pro and anti-inflammatory cytokines in the spleen of vaccinated fish at day 1 and day 3 post-injection. R in bold suggests a significant Spearman rank ordered correlation.

		IL-1β1	IL-1β2	TNF-α1	TNF-α2	TNF-α3	IL-8	IL-6	IL-11	M17	IL-18	IL-21	nIL-1Fm	IL-10A
IL-1β1	R	1.000	**0.925**	**0.846**	**0.907**	**0.949**	**0.954**	**0.818**	**0.939**	**0.837**	0.415	**0.724**	**0.905**	**0.691**
	p		0.000	0.001	0.000	0.000	0.000	0.001	0.000	0.001	0.180	0.008	0.000	0.013
IL-1β2	R	**0.925**	1.000	**0.774**	**0.811**	**0.888**	**0.872**	**0.799**	**0.806**	**0.768**	0.439	0.544	**0.774**	**0.600**
	p	0.000		0.003	0.001	0.000	0.000	0.002	0.002	0.004	0.154	0.068	0.003	0.039
TNF-α1	R	**0.846**	**0.774**	1.000	**0.870**	**0.809**	**0.847**	**0.882**	**0.821**	**0.747**	**0.668**	**0.789**	**0.902**	0.499
	p	0.001	0.003		0.000	0.001	0.001	0.000	0.001	0.005	0.018	0.002	0.000	0.099
TNF-α2	R	**0.907**	**0.811**	**0.870**	1.000	**0.895**	**0.912**	**0.891**	**0.947**	**0.863**	**0.627**	**0.704**	**0.924**	**0.613**
	p	0.000	0.001	0.000		0.000	0.000	0.000	0.000	0.000	0.029	0.011	0.000	0.034
TNF-α3	R	**0.949**	**0.888**	**0.809**	**0.895**	1.000	**0.907**	**0.855**	**0.904**	**0.853**	0.519	**0.723**	**0.865**	**0.656**
	p	0.000	0.000	0.001	0.000		0.000	0.000	0.000	0.000	0.084	0.008	0.000	0.020
IL-8	R	**0.954**	**0.872**	**0.847**	**0.912**	**0.907**	1.000	**0.823**	**0.94**	**0.896**	0.508	**0.780**	**0.949**	**0.779**
	p	0.000	0.000	0.001	0.000	0.000		0.001	0.000	0.000	0.092	0.003	0.000	0.003
IL-6	R	**0.818**	**0.799**	**0.882**	**0.891**	**0.855**	**0.823**	1.000	**0.828**	**0.787**	**0.724**	**0.747**	**0.830**	**0.599**
	p	0.001	0.002	0.000	0.000	0.000	0.001		0.001	0.002	0.008	0.005	0.001	0.039
IL-11	R	**0.939**	**0.806**	**0.821**	**0.947**	**0.904**	**0.940**	**0.828**	1.000	**0.903**	0.485	**0.743**	**0.961**	**0.745**
	p	0.000	0.002	0.001	0.000	0.000	0.000	0.001		0.000	0.110	0.006	0.000	0.005
M17	R	**0.837**	**0.768**	**0.747**	**0.863**	**0.853**	**0.896**	**0.787**	**0.903**	1.000	0.465	**0.669**	**0.907**	**0.861**
	p	0.001	0.004	0.005	0.000	0.000	0.000	0.002	0.000		0.128	0.017	0.000	0.000
IL-18	R	0.415	0.439	**0.668**	**0.627**	0.519	0.508	**0.724**	0.485	0.465	1.000	0.428	**0.589**	0.165
	p	0.180	0.154	0.018	0.029	0.084	0.092	0.008	0.110	0.128		0.165	0.044	0.607
IL-21	R	**0.724**	0.544	**0.789**	**0.704**	**0.723**	**0.780**	**0.747**	**0.743**	**0.669**	0.428	1.000	**0.782**	**0.613**
	p	0.008	0.068	0.002	0.011	0.008	0.003	0.005	0.006	0.017	0.165		0.003	0.034
nIL-1Fm	R	**0.905**	**0.774**	**0.902**	**0.924**	**0.865**	**0.949**	**0.830**	**0.961**	**0.907**	**0.589**	**0.782**	1.000	**0.731**
	p	0.000	0.003	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.044	0.003		0.007
IL-10A	R	**0.691**	**0.600**	0.499	**0.613**	**0.656**	**0.779**	**0.599**	**0.745**	**0.861**	0.165	**0.613**	**0.731**	1.000
	p	0.013	0.039	0.099	0.034	0.020	0.003	0.039	0.005	0.000	0.607	0.034	0.007	

observed a systematic activation of anti-microbial defences in both spleen and gills, but a specific activation of inflammatory cytokines and specific IL-12 family members that leads to a Th1/Th17 biased immune activation in the spleen.

4.1. Activation of pro- and anti-inflammatory cytokines in the spleen after ERM vaccination

In this study, we comparatively examined immune gene expression in spleen and gills after ERM vaccination. The choice of spleen over head kidney was because transcriptome changes mainly happen transiently in spleen after bacterin vaccination in fish as shown by microarray analysis [57]. The gill tissue was chosen because it is a major lymphoid tissue in salmonids [58] and an important immune organ relevant to bacterial infections such as ERM [1,2,6]. Vaccination with a bacterin mimics a bacterial infection thereby activating the innate immune system and initiating an adaptive immune response without the development of severe disease. Thus, a number of genes known to be activated by *Y. ruckeri* infection (Table 5) were found to be activated by ERM vaccination in this study. However, key differences of the host responses between ERM vaccination and *Y. ruckeri* infection exist. First, only a subset of genes are commonly activated by both ERM vaccination and infection. For example, the major pro-inflammatory cytokines IL-1β1-2, TNF-α1-3, IL-6, IL-8, IL-17A/F (1A, 2A and 3) and IFN-γ1, and anti-inflammatory cytokine IL-10A were activated by both vaccination and infection. In contrast, IL-2, IL-17C1-2 and TGF-β1A were up-regulated only by infection and were refractory to vaccination (Table 5). Secondly, the kinetics of gene activation was different. For example, the activation of gene expression by ERM vaccination was rapid and peaked at day 1 for most of the genes, whilst during infection the activation of inflammatory genes such as IL-1β, TNFα, and SAA occurs later [36]. Thirdly, the activation of pro- and anti-inflammatory cytokine expression happened mainly in the spleen by vaccination but also in the gills by infection (Table 5). Although direct comparisons of transcript levels between different experiments could be complicated by multiple factors, such as the age and life history of the fish, and the pathogen strains used, these comparisons do suggest that different pathways have been activated by vaccination and pathogenic challenge.

The differences between ERM vaccination and pathogenic *Y. ruckeri* infection observed could be attributed to (1) dose effects, (2) factors released during infection [2,3] but absent in the vaccine preparation, (3) different stress responses due to the damage caused by pathogenic infection and vaccination, and (4) the evasion mechanisms of the pathogen. The ERM vaccination used a high dose, ie equivalent to 5×10^8 cfu/fish, whilst infection studies typically use lower doses, eg 5×10^5 cfu/fish [17,45]. It is well known that the host immune response (eg cytokine transcript levels) is dose (pathogen load) dependent [36]. Thus, after ERM vaccination we observed an immediate activation of a set of APPs, AMPs, and pro- and anti-inflammatory cytokines in the spleen. However, after low (er) dose infection, the bacteria multiply and spread in different target tissues, with associated tissue damage likely responsible for the relatively late kinetics of gene expression and the broad activation of immune genes in multiple tissues (eg in both the spleen and gills). Some of the differences, however, might be due to immune evasion of the pathogen. For example, IL-4/13B1-2 were shown to be induced *in vitro* in HK cells by bacterin exposure and *in vivo* in the spleen after vaccination. In contrast, their expression was inhibited in gills by *Y. ruckeri* infection [25].

Despite the differences, the expression of the major pro-inflammatory cytokines (IL-1β1, IL-1β2, and TNF-α paralogues, IL-6, IL-8, IL-11 and M17) and anti-inflammatory cytokines (IL-10A and nIL-1Fm) was highly upregulated in the spleen (but not in the gills) immediately after ERM vaccination (Figs. 2–3). These changes are highly correlated (Table 3), suggesting an effective activation of the innate immune system was established with a balanced inflammatory response.

4.2. Activation of IL-12 family cytokines may lead to a Th1/Th17 biased adaptive immune response

Among a wide range of cytokines, the IL-12 family (IL-12, IL-23,

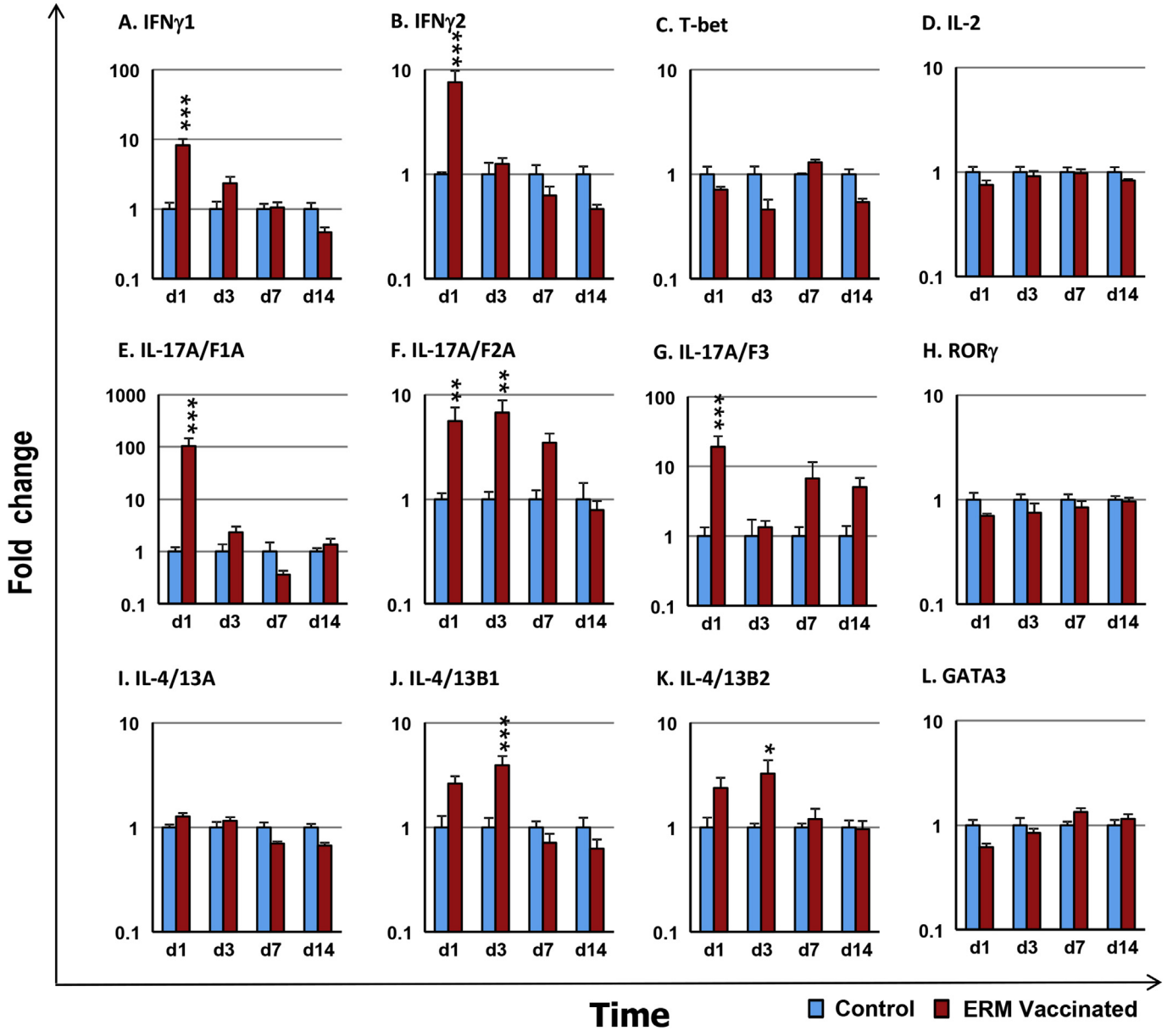

Fig. 3. Modulation of the expression of genes associated with T helper cells in spleen by ERM vaccination. Two groups of rainbow trout were vaccinated by ip injection with AquaVac ERM (red bars) or PBS as control (blue bars). The fish were killed at day 1, 3, 7 and 14, and spleen and gills collected for gene expression analysis by real-time PCR (as described in the Materials and Methods). Modulated expression was expressed as a fold change calculated as the mean expression levels in vaccinated fish normalized to that of time-matched controls in the same tissue. The means + SEM of six fish are shown. The relative significance of a Bonferroni post hoc test after a significant one way-ANOVA between the vaccinated and control groups at the same time point is shown above/within the bars as: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

IL-27 and IL-35) has unique structural, functional, and immunological characteristics that have made this family as important immunological playmakers. Each IL-12 family member is composed of an α-chain (p19, p28 and p35) and a β-chain (p40 and Ebi3). Whilst IL-12 (p35/p40), IL-23 (p19/p40) and IL-27 (p28/EBI3) are secreted by activated antigen presenting cells (APC) during antigen presentation to naïve T cells, IL-35 (p35/EBI3) is a product of regulatory T and B cells in mammals [26,60,61]. IL-12 and IL-23 are proinflammatory or prostimulatory cytokines, whereas IL-27 and IL-35 are inhibitory cytokines. With our recent success in cloning and characterizing all of the subunits of the IL-12 family in salmonids [26–29], this is the first study to examine their involvement in a fish vaccination model. Although the constitutive expression of α-chains (p35A1, p35A2, p35B1, p19, p28A and p28B) was low, their expression was significantly increased in the spleen of vaccinated fish. The expression of the β-chain p40B2 was also up-regulated, but not p40B1, p40C or EBI3. This expression profiles suggest that specific isoforms of IL-12 and IL-23 containing p40B2 could be produced in the spleen after ERM vaccination.

$CD4^+$ T cells, also known as T-helper (Th) cells, play an important role in orchestrating adaptive immune responses to pathogens and vaccines. When naive $CD4^+$ T cells recognize a foreign antigen-derived peptide presented in the context of major histocompatibility complex (MHC) class II on APCs, they undergo massive proliferation and differentiation into distinct Th cell subsets such as Th1, Th2, Th17 and induced T-regulatory (iTreg) cells in mammals

Table 4
The Spearman's rho correlation coefficient (R) and the 2-tailed significance (p) between gene expression levels of the cytokines involved in Th cell development in the spleen of vaccinated fish at day 1 and day 3 post-injection. R in bold suggests a significant Spearman rank ordered correlation.

		IFNγ1	IFNγ2	IL-17A/F1A	IL-17A/F2A	IL-17A/F3	IL-4/13B1	IL-4/13B2	P35A1	P35A2	P35B1	P19	P28A	P28B	P40B2
IFNγ1	R	1.000	**0.830**	**0.904**	0.564	**0.753**	0.011	**0.652**	**0.605**	**0.840**	**0.775**	**0.789**	**0.881**	**0.793**	0.385
	p		0.001	0.000	0.056	0.005	0.974	0.022	0.037	0.001	0.003	0.002	0.000	0.002	0.217
IFNγ2	R	**0.830**	1.000	**0.741**	0.394	**0.720**	−0.014	0.425	**0.845**	**0.839**	**0.900**	**0.646**	**0.715**	**0.620**	0.456
	p	0.001		0.006	0.205	0.008	0.966	0.169	0.001	0.001	0.000	0.023	0.009	0.032	0.136
IL-17A/F1A	R	**0.904**	**0.741**	1.000	0.528	**0.755**	−0.210	0.386	**0.694**	**0.86**	**0.609**	**0.877**	**0.904**	**0.830**	0.186
	p	0.000	0.006		0.078	0.005	0.513	0.215	0.012	0.000	0.035	0.000	0.000	0.001	0.563
IL-17A/F2A	R	0.564	0.394	0.528	1.000	0.500	0.127	0.534	0.397	**0.682**	0.473	0.530	0.490	0.215	−0.223
	p	0.056	0.205	0.078		0.098	0.695	0.074	0.201	0.015	0.121	0.076	0.106	0.502	0.487
IL-17A/F3	R	**0.753**	**0.720****	**0.755**	0.500	1.000	0.168	0.498	0.528	**0.670**	0.529	0.523	**0.578**	0.420	0.102
	p	0.005	0.008	0.005	0.098		0.602	0.099	0.078	0.017	0.077	0.081	0.049	0.174	0.753
IL-4/13B1	R	0.011	−0.014	−0.210	0.127	0.168	1.000	0.491	−0.412	−0.021	0.018	−0.453	−0.298	−0.347	0.207
	p	0.974	0.966	0.513	0.695	0.602		0.105	0.183	0.948	0.957	0.140	0.347	0.269	0.519
IL-4/13B2	R	**0.652**	0.425	0.386	0.534	0.498	0.491	1.000	0.002	0.465	0.475	0.151	0.290	0.246	0.401
	p	0.022	0.169	0.215	0.074	0.099	0.105		0.996	0.128	0.119	0.639	0.361	0.441	0.196
P35A1	R	**0.605**	**0.845**	**0.694**	0.397	0.528	−0.412	0.002	1.000	**0.795**	**0.762**	**0.746**	**0.691**	0.550	0.102
	p	0.037	0.001	0.012	0.201	0.078	0.183	0.996		0.002	0.004	0.005	0.013	0.064	0.751
P35A2	R	**0.840**	**0.839**	**0.860**	**0.682**	**0.670**	−0.021	0.465	**0.795**	1.000	**0.830**	**0.759**	**0.779**	**0.608**	0.155
	p	0.001	0.001	0.000	0.015	0.017	0.948	0.128	0.002		0.001	0.004	0.003	0.036	0.631
P35B1	R	**0.775**	**0.900**	**0.609**	0.473	0.529	0.018	0.475	**0.762**	**0.830**	1.000	**0.624**	**0.646**	0.460	0.290
	p	0.003	0.000	0.035	0.121	0.077	0.957	0.119	0.004	0.001		0.030	0.023	0.133	0.361
P19	R	**0.789**	**0.646**	**0.877**	0.530	0.523	−0.453	0.151	**0.746**	**0.759**	**0.624**	1.000	**0.942**	**0.780**	0.030
	p	0.002	0.023	0.000	0.076	0.081	0.140	0.639	0.005	0.004	0.030		0.000	0.003	0.926
P28A	R	**0.881**	**0.715**	**0.904**	0.490	**0.578**	−0.298	0.290	**0.691**	**0.779**	**0.646**	**0.942**	1.000	**0.896**	0.262
	p	0.000	0.009	0.000	0.106	0.049	0.347	0.361	0.013	0.003	0.023	0.000		0.000	0.411
P28B	R	**0.793**	**0.620**	**0.830**	0.215	0.420	−0.347	0.246	0.550	**0.608**	0.460	**0.780**	**0.896**	1.000	0.513
	p	0.002	0.032	0.001	0.502	0.174	0.269	0.441	0.064	0.036	0.133	0.003	0.000		0.088
p40B2	R	0.385	0.456	0.186	−0.223	0.102	0.207	0.401	0.102	0.155	0.290	0.030	0.262	0.513	1.000
	p	0.217	0.136	0.563	0.487	0.753	0.519	0.196	0.751	0.631	0.361	0.926	0.411	0.088	

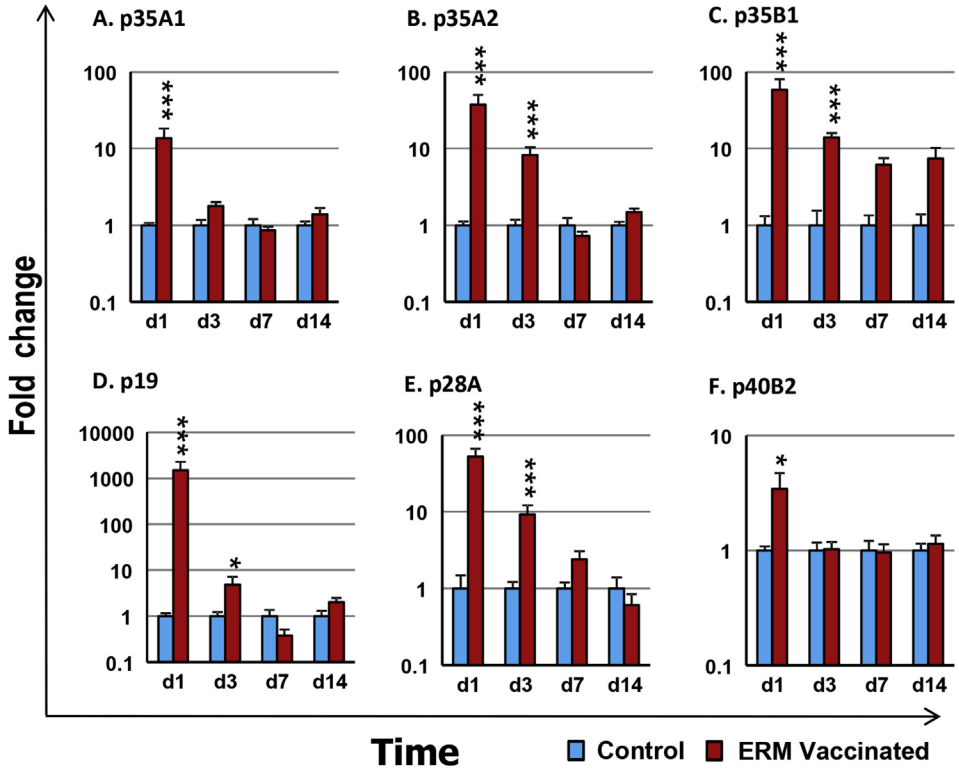

Fig. 4. Modulation of the expression of subunits of IL-12 family in spleen by ERM vaccination. Two groups of rainbow trout were vaccinated by ip injection with AquaVac ERM (red bars) or PBS as control (blue bars). The fish were killed at day 1, 3, 7 and 14, and spleen and gills collected for gene expression analysis by real-time PCR (as described in the Materials and Methods). Modulated expression was expressed as a fold change calculated as the mean expression levels in vaccinated fish normalized to that of time-matched controls in the same tissue. The means + SEM of six fish are shown. The relative significance of a Bonferroni post hoc test after a significant one way-ANOVA between the vaccinated and control groups at the same time point is shown above/within the bars as: *p < 0.05, and ***p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Comparison of gene expression modulated by ERM vaccination and *Y. ruckeri* infection in the spleen and gills of rainbow trout. "↑" = up-regulation, "↓" = down regulation and "-" = no change.

Gene	ERM vaccination		*Y. ruckeri* infection	
	Spleen	Gills	Spleen	Gills
SAA	↑	↑	↑ [9,36]	ND
SAP1	↑	–	ND	ND
SAP2	–	–	ND	ND
CATH1	↑	–	ND	ND
CATH2	↑	↑	↑ [9]	ND
Hepcidin	↑	↑	ND	ND
LEAP2A	–	–	ND	ND
β-defensin-1	–	–	ND	- [38]
β-defensin-2	–	–	ND	- [38]
β-defensin-3	–	–	ND	↑ [38]
β-defensin-4	–	–	ND	- [38]
IL-1β1	↑	–	↑ [9,17,36,45,62]	↑ [17]
IL-1β2	↑	–	↑ [45]	ND
IL-1β3	–	–	ND	ND
nIL-1Fm	↑	–	↑ [45]	ND
IL-2	–	–	↑ [17,50]	↑ [17,50]
IL-4/13A	–	–	- [9]	↓ [25]
IL-4/13B1	↑	–	ND	↓ [25]
IL-4/13B2	↑	–	ND	↓ [25]
IL-6	↑	↑	↑ [9,17,59]	↑ [17]
IL-8	↑	–	↑ [59]	ND
IL-10A	↑	–	↑ [9,17,59]	↑ [17]
IL-10B	–	–	- [42]	↑ [17]
IL-11	↑	–	↑ [17,59]	↑ [17]
M17	↑	–	↑ [45]	ND
IL-12 p35A1	↑	–	↑ [26]	ND
IL-12 p35A2	↑	–	ND	ND
IL-12 p35B1	↑	–	ND	ND
IL-12 p40B1	–	–	↑ [26]	ND
IL-12 p40B2	↑	–	ND	ND
IL-12 p40C	–	–	↑ [26]	ND
IL-23 p19	↑	–	↑ [28]	ND
IL-27 p28A	↑	–	ND	ND
IL-27 p28B	↑	–	ND	ND
EBI3	–	–	ND	ND
IL-15	–	–	ND	ND
IL-17A/F1A	↑	–	↑ [24]	- [24]
IL-17A/F1B	–	–	- [24]	- [24]
IL-17A/F2A	↑	–	↑ [23,24]	- [24]
IL-17A/F2B	–	–	- [24]	- [24]
IL-17A/F3	↑	–	↑ [24]	- [24]
IL-17A/F4/N	–	–	↑ [24]	- [24]
IL-17C1	–	–	↑ [53]	ND
IL-17C2	–	–	↑ [53]	ND
IL-17D	↓	–	ND	ND
IL-18	↑	–	ND	ND
IL-20	–	–	↑ [54]	ND
IL-21	↑	–	↑ [31]	ND
IL-22	–	–	↑ [17,55]	↑ [17]
IL-34	–	–	ND	ND
IFNγ1	↑	–	↑ [9,17,36,59]	↑ [17]
IFNγ2	↑	–	ND	ND
TGF-β1A	–	–	↑ [17,36]	↑ [17]
TGF-β1B	–	–	ND	ND
TNF-α1	↑	–	↑ [9,36]	↑ [17]
TNF-α2	↑	–	↑ [17,22]	↑ [17]
TNF-α3	↑	–	↑ [22]	ND
T-bet	–	–	↓ [17,47], ↑ [9]	↑ [17]
GATA3	–	–	↓ [9,17,48]	↑ [17]
RORγ	–	–	↑ [49]	↑ [49]
Foxp3A	–	–	ND	ND
Foxp3B	↓	–	ND	ND

[46]. Whilst IL-12 and IL-23 are critical for Th1 and Th17 cell development, respectively, IL-35 has immunosuppressive effects that are mediated through regulatory T and B cells. IL-27 displays both pro- and anti-inflammatory activities. It promotes the differentiation of Th1 and IL-10-producing Tr1-like regulatory cells, but inhibits Th2 and Th17 [62]. Whilst the extent to which the mammalian Th cell paradigm is conserved is still unclear in fish [46], it provides a framework to investigate the immune responses to vaccination in fish [63,64]. The expression of Th1 cytokines (IFN-γ1 and 2), Th17 cytokines (IL-17A/F1A, 2A and 3), and Th2 cytokines (IL-4/13B1 and 2) was increased in the spleen after ERM vaccination. It is notable that the increased expression of IFN-γ1 and 2, and IL-17A/F1A was earlier and higher than Th2 cytokines (Fig. 4). Furthermore, their upregulated expression was significantly correlated to that of the α-chains of the IL-12 family, and pro-/anti-inflammatory cytokines in the spleen of the vaccinated fish (Table 4). It is known that rainbow trout IL-12 isoforms can induce IFN-γ expression [26]. The correlated expression of IL-12 and IL-23 with Th1 and Th17 cytokines and pro-*anti*-inflammatory cytokines may suggest that ERM vaccination activates a balanced inflammatory response with the expression of IL-12 and IL-23 leading to a Th1 and Th17 biased immune response in the spleen. The ability of IL-23 to induce Th17 cytokine expression remains to be proven since no bioactivity analysis of this cytokine has been performed in any fish species.

In mammals the differentiation of Th cell subsets and expression of lineage specifying cytokines depend on the induction of lineage-specific master transcription factors, including T-bet for Th1, GATA3 for Th2, and RORγt for Th17 [45,65]. Although the expression of Th1, Th2 and Th17 cytokines was found increased in the spleen of ERM vaccinated fish, the expression of T-bet, GATA3 and RORγ was refractory. However, the lack of transcriptional factor expression change at a tissue level doesn't exclude their role in the regulation of cytokine gene expression in a specific cell type. Indeed, the master transcription factors can be expressed in other cell types in addition to Th cells, and their expression can coexist in the same cell and is dynamic and quantitative [65]. Thus at the mixed tissue level, changes of gene expression in a specific cell type(s) may be diluted by the presence of other abundant cell types or by the changes in other cell types. The possibility to isolate relevant fish leucocyte populations, such as $CD4^+$ or $CD8^+$ cells, and study their responses has become possible in fish recently using antibodies [66–68] or transgenic fish [69]. For example in trout infected with *Y. ruckeri* 4 days earlier, upregulation of Th1 (IL-2, IFN-γ) and Th17 cytokines (IL-17A/F1a, IL-21, IL-22) is apparent in splenic CD4-1+/CD4-2+ cells and CD4-1-/CD4-2+ cells [70]. Such studies will undoubtedly be directed towards elucidating the responses in vaccinated fish in the near future.

4.3. Systematic activation of anti-microbial defences by ERM vaccination

APPs are an integral part of the acute phase response. They are secreted by the liver in response to a variety of injuries and can also be expressed in extrahepatic tissues. APPs favour the systemic regulation of defence, coagulation, proteolysis, and tissue repair [62]. AMPs are integral components of innate immunity, and one of the first lines of host defence against bacterial infection. The up-regulation of APPs and AMPs in both spleen and gills suggests a systemic activation of innate immune system by ERM vaccination, that provide non-specific protection after vaccination.

It is known that many proinflammatory cytokines can induce the expression of APPs and AMPs in mammals and in fish. In rainbow trout, IL-6 induces hepcidin and CATH2 but not CATH1 in the macrophage cell line RTS-11 [32]. In contrast TNF-α3 induces hepcidin and CATH1 but not CATH2 in HK macrophages [22], whilst IL-1β can induce the expression of both CATH1 and CATH2 (unpublished results). Adaptive cytokines such as IL-4/13 can also induce APPs (eg SAP1), and AMPs (eg hepcidin, and CATH1 but not CATH2) in HK cells [25]. The expression of a major APP (eg SAA) and

AMPs (eg hepcidin and CATH2) was positively correlated with the expression of major pro-inflammatory cytokines (Table S2), suggesting that the activation and release of pro-inflammatory cytokines may lead to the induction of APPs and AMPs following vaccination. In agreement with this notion, IL-6, that specific induces CATH2 and hepcidin, was the only up-regulated proinflammatory cytokine in gills after ERM vaccination, and may account for the high levels of expression of these two AMPs (Fig. 1).

4.4. Differential expression of paralogous genes

Many immune genes in teleost fish are known to have multiple paralogues, especially in salmonids that have undergone an additional whole genome duplication event. For example, there are three genes for IL-1β and TNF-α in salmonids [21,22] and three each of the p35 and p40 genes, that potentially could make 9 heterodimeric IL-12 isoforms with different functions [26,27]. Thus, it is necessary to determine whether all of the genes present react in a similar manner. In this study it is clear that major differences occur between different paralogues in response to vaccination. The biggest differences were seen when one of the paralogues was responsive and the other not, as with IL-1β1 and 2 vs IL-1β3, IL-4/13A vs IL-4/13B1 and 2, IL-10A vs IL-10B, IL-12 p40B2 vs p40B1 and p40C, IL-17A/F1A vs IL-17A/F1B, IL-17A/F2A vs IL-17A/F2B, and SAP1 vs SAP2. Differential responses of paralogues have been seen in responses to PAMPs [22,33], infection [24,26] and cytokine stimulation [25,26]. These differences likely reflect differences in the promoters, with some of the paralogues becoming more or less responsive to particular signalling pathways, perhaps in particular cell types, or genes that are being pseudogenised. The differential responses of IL-12 p40 paralogues is of particular interest. Two isoforms of rainbow trout rIL-12 have been made as recombinant proteins that differ in the p40 chain (ie p40B or p40C). These proteins can induce IFN-γ expression in HK cells but only the isoform containing p40C was able to also induce IL-10 [26]. The induction of all α-chains but only one β-chain (ie p40B2) suggests that specific IL-12 and IL-23 isoforms are produced after ERM vaccination that may have different functions (as seen with IL-12 isoforms) critical for a Th1/Th17 biased response.

4.5. Implications for vaccine development in fish

Using an efficacious bacterial model vaccine, this study has revealed that host innate immunity is activated by ERM vaccination as evidenced by the correlated upregulation of pro- and anti-inflammatory cytokines in the spleen and the systemic increase of APPs and AMPs. Specific IL-12 members are induced that may drive the Th1/Th17 biased immune responses observed. As an efficacious vaccine must activate innate immunity and initiate specific adaptive pathways, the early responses of the set of genes studied here may provide essential information and function as biomarkers in future vaccine development for fish, potentially allowing a screening method for vaccine candidates and formulations before more expensive mortality testing.

4.6. Conclusions

In summary, ERM vaccination immediately activates a balanced inflammatory response with correlated expression of both pro- and anti-inflammatory cytokines in the spleen. The increase of pro-inflammatory cytokines may lead to the systemic upregulation of APPs and AMPs in both spleen and gills. We also observed an upregulation of all the IL-12 cytokine family α-chains, but only one β-chain (p40B2) which suggests specific IL-12 and IL-23 isoforms with distinct functions might be produced in the spleen of vaccinated fish. Notably the expression of Th1 cytokines (IFN-γ1-2) and a Th17 cytokine (IL-17A/F1A) were up-regulated and correlated to that of the IL-12 family α-chains, the majority of pro- and anti-inflammatory cytokines, APPs and AMPs. These expression profiles may suggest that ERM vaccination activates host innate immunity and expression of specific IL-12 and IL-23 isoforms leading to a Th1 and Th17 biased immune response. This study has increased our understanding of the host immune response to ERM vaccination and the adaptive pathways involved. The early responses seen may provide useful biomarkers for future vaccine development in aquaculture.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.07.056.

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