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A time-course study of gene expression and antibody repertoire at early time post vaccination of Atlantic salmon

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ABSTRACT

The majority of studies of vaccine responses in Atlantic salmon have focused on several weeks after vaccination, and employed a limited number of marker genes. In this study, novel techniques were used to examine a broad panel of expressed genes and antibody repertoire of Atlantic salmon following vaccination. Salmon parr were vaccinated with a multivalent oil-based vaccine, and blood plasma and head kidney were sampled at several time-points between 0–35 days post vaccination. Saline-injected fish were used as control at all time-points. Microarray analyses showed increased expression of immune genes from the first day to the end of study in the head kidney of vaccinated fish. Genes up-regulated in the late phase included several leukocyte markers and components of the oxidative burst complex. A suite of genes that can take part in B cells differentiation were up-regulated from day 14, at which time secretory IgM transcripts also peaked. This coincided with marked increased plasma titres of non-vaccine specific antibodies binding to a hapten-carrier antigen DNP-KLH, while antibodies to bacterial components of the vaccine, *Moritella viscosa* and *Aeromonas salmonicida,* first showed significantly elevated antibody levels at day 21, and at a markedly lower magnitude than the non-vaccine specific titres. Sequencing of the variable region of IgM heavy chain (CDR3) revealed higher cumulative frequencies of unique clonotypes in vaccinated salmon starting from day 14 when specific antibodies were first detected. Reduced sequence variance of CDR3 suggested expansion of recently emerged clonotypes. Overall, the results presented here follow a broad panel of gene expression, immunoglobulin sequencing and plasma antibody titres in the first few weeks after vaccination of Atlantic salmon, pointing to a potentially important contribution of non-vaccine specific antibody responses early in the vaccine response.

1. Introduction

Vaccines play an essential role in commercial aquaculture (Sommerset et al., 2005; Biering et al., 2005; Hastein et al., 2005; Brudeseth et al., 2013). The formulation and improvement of vaccines require both empirical approaches and profound understanding of the immune processes that occur after vaccination. Processes taking place in the lymphatic organs of vaccinated fish, and local innate responses at the injection site have been extensively explored using microscopy and expression of marker genes with emphasis on the early events (Munang'andu et al., 2014; Secombes, 2008; Wang and Secombes, 2013; Ye et al., 2011, 2013; Yamaguchi et al., 2018; Haugland et al., 2005). However, adaptive immune responses have mainly been studied several weeks after vaccination, when antigen-specific antibodies and T cells can be detected. Moreover, the majority of published studies of vaccinated salmonid fish have employed limited panels of marker genes, while the use of broader transcriptomic analyses has been infrequent. The profiles of early gene expression, such as those involved in B cell development, are therefore less known.

In recent years, novel analytic techniques have revolutionized the studies of antibodies and the antibody repertoire. Deep sequencing of the variable region of immunoglobulins (Ig-seq) enables a

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comprehensive description of antibody repertoires (Krasnov et al., 2017; Castro et al., 2013). IgM is the key effector of systemic B cell responses in fish, and sequencing of the variable region of IgM heavy chain transcripts including the CDR3 (complementarity determining region 3) enables the examination of repertoires of antibody specificity. Particular CDR3 sequences are referred to as clonotypes, and these are classified as unique when found in one individual, or shared when found in many (or all) individuals (Krasnov et al., 2017; Magadan et al., 2015). Complementing these molecular techniques, new developments in protein level detection such as the xMAP multiplex immunoassay technology enables the detection of systemic antibodies to multiple antigens (Houser, 2012). With this technology, specific antibodies against several vaccine antigens can be analysed simultaneously from the same sample (Morgan et al., 2004). In contrast to a specific antibody response to antigens exposed via vaccine or infection, a non-specific antibody activity can be found in normal serum of most vertebrates (Panda and Ding, 2015), including several fish species (reviewed by (Magnadottir, 2006)), but has commonly been dismissed as background noise in serological assays. Non-specific antibodies (NSAB) are believed to be polyreactive, binding with medium to low affinity to various self-antigens as well as some foreign antigens to which the host has never been exposed. A useful method to estimate the titres of non-specific antibodies is the serological binding to model antigens consisting of hapten-carrier complexes, and the presence of antibodies to model antigens in fish has been reported (Gonzalez et al., 1988; Magnadottir et al., 2009; Sinyakov et al., 2002). However, their functional importance is not yet well-understood (Sinyakov et al., 2002; Magnadottir et al., 1999; Coll, 2018).

Here, we report a time-course study of early immune responses in Atlantic salmon (*Salmo salar*), covering a period from 1 to 35 days post vaccination (dpv). Transcriptome profiling in the head kidney, the primary lymphatic organ, with genome-wide Atlantic salmon DNA microarrays explored processes in both arms of immunity and outlined a number of genes with possible roles in differentiation and maturation of B cells after vaccination. Starting from day 14, an increase in non-vaccine specific antibody titres against a hapten-carrier antigen was observed in vaccinated fish, which coincided with a marked differential expression of multiple genes. Titres of non-vaccine specific antibodies were markedly higher than titres against specific vaccine antigens. An increase in the cumulative frequencies of unique clonotypes in vaccinated fish was accompanied by a decrease in the variance of the nucleotide sequences of CDR3 in vaccinated fish, indicating an expansion of B cells producing specific antibodies.

2. Material and methods

2.1. Fish

The experiment was performed at Veso Vikan Research Facility (Namsos, Norway). Atlantic salmon with a mean weight of 40.5 g (+/-11.1 g) were assigned by random netting to two different treatment groups (vaccinated and saline-injected control). The fish were anesthetized (Metacain, Pharmaq) and i.p. injected with either 0.1 ml Aquavac® PD7 (MSD Animal Health) (hereafter called vaccinated) or 0.1 ml sterile saline (hereafter called saline-injected). Aquavac® PD7 vet is a commercial inactivated, multivalent injection vaccine for immunization of Atlantic salmon. The active components are two inactivated viral antigens, salmon pancreas disease virus (SPDV) and infectious pancreatic necrosis virus (IPNV), and five inactivated bacterial antigens: *Aeromonas salmonicida* subsp. salmonicida, *Vibrio salmonicida*, *Vibrio anguillarum* serotype O1, *Vibrio anguillarum* serotype O2a and *Moritella viscosa* and the oil-based adjuvant. The vaccine is administered to healthy Atlantic salmon of a minimum size of 30 g. The two groups were marked by cutting the left or right maxilla (250 fish per group) under the same anaesthesia and thereafter mixed and kept in a tank supplied with flow through freshwater from a natural water source. The temperature decreased slowly from 11° to 7 °C during the 35 days post vaccination (dpv) sampling regime (7th September to 11th October). In order to induce smoltification, the fish were subjected to 24 h light from 1 dpv. Smoltification status was monitored through seawater exposure test with chlorine testing according to standard procedure at VESO Vikan and the fish were confirmed ready for sea transfer at 43 dpv. Samples were collected 1 day prior to vaccination (0-samples), and thereafter at 1, 3, 7, 14, 21, 28 and 35 dpv. Except for the 0-samples, 10 vaccinated and 5 saline-injected fish were collected at each time-point and subjected to a lethal dose of anaesthetic. Blood was collected from the caudal vein, and plasma collected following immediate spinning of whole blood and frozen at −20 °C until analysis. Head kidney was collected in tubes with RNAlater (Thermo Fisher Scientific), stored for one day in the fridge before storage at −20 °C until analysis. The same type of samples were collected for the 0-samples, taken 1 day prior to vaccination from 10 fish netted from the same fish population. The fish were fed with regular Skretting® Nutura feed according to appetite throughout the freshwater period. Feed was withheld 48 h prior to vaccination and 24 h after vaccination.

2.2. Microarrays

RNA was extracted with Biomek NXP Laboratory Automation Workstation robot (Beckman Coulter) using the RNAdvance Tissue Kit Total RNA isolation (Agencourt) according to the manufacturer's instruction. The RNA concentration and quality were determined using the NanoDrop 8000 Spectrophotometer (Thermo Scientific) and 2100 Bioanalyzer (Agilent Technologies), respectively. RNA was stored at -80 °C until use. In all samples used for gene expression analyses RIN was higher than 8. Nofima's genome-wide Atlantic salmon microarray Salgeno with 44k 60-mer oligonucleotide probes was used. The platform was annotated with bioinformatics package STARS (Haugland et al., 2005). Samples included for microarray analysis were: 1 day prior to vaccination (0-samples, n = 3), vaccinated fish at 1, 3, 7, 14, 21, 28 dpv (n = 5) and 35 dpv (n = 3), and saline injected fish at 1, 7, 14 and 28 dpv (n = 4). Data from 28 dpv and 35 dpv were combined since they were nearly identical. Microarrays were manufactured by Agilent Technologies, and the reagents and equipment were purchased from the same provider. RNA amplification and labelling were performed with a One-Color Quick Amp Labelling Kit, and a Gene Expression Hybridization kit was used for fragmentation of labelled RNA. Total RNA input for each reaction was 500 ng. After overnight hybridization in an oven (17 h, 65 °C, rotation speed 0,01 g), arrays were washed with Gene Expression Wash Buffers 1 and 2 and scanned with Agilent scanner. Subsequent data analyses were carried out with STARS. Global normalization was performed by equalizing the mean intensities of all microarrays. Next, the individual values for each feature were divided to the mean value of all samples producing expression ratios (ER). The $\log_2$-ER were calculated and normalized with the locally weighted non-linear regression (Lowess). Differentially expressed genes (DEG) were selected by the following criteria: expression ratio > 1.75-fold and $p < 0.05$. Effects of vaccination were assessed by comparison between vaccinated and saline injected salmon. Samples collected before vaccination were included in analyses to examine the temporal changes. Enrichment analysis was carried out for the functional categories of GO, KEGG pathways and STARS (Krasnov et al., 2011) annotations. Numbers of genes associated with the terms were compared in the list of DEG and the entire microarray platform. Significance of enrichment was assessed by Yates' corrected chi test.

2.3. RT-qPCR

cDNA was synthesized from 1 μg of RNA using QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's recommendations and stored at -20 °C until use. The RT-qPCR was performed on three to five individuals per group and time-point. Samples were

Table 1
PCR primers.

Gene	Accession no.	Sequence (5′–3′)	PCR eff.
EF1A	X70165.1	F – CCCCTCCAGGACGTTTACAAA R – CACACGGCCCACAGGTACA	107%
mIgM	BT059185	F - CCTACAAGAGGGAGACCGA R - GATGAAGGTGAAGGCTGTTTT	108%
sIgM	Y12457	F - CTACAAGAGGGAGACCGGAG R - AGGGTCACCGTATTATCACTAGTTT	107%
IgT	ACX50290	F - CAACACTGACTGGAACAACAAGGT R - CGTCAGCGGTTCTGTTTTGGA	107%

analyzed in duplicates in 20 ul reactions, and each plate included a negative control lacking the template and a positive control/calibrator for plate to plate variations. The RT-qPCR was performed using Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) according to the manufacturer's description and with the following set-up: 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 1 min at 60 °C. Samples were analyzed for transcripts of secreted immunoglobulin M (*sigm*), membrane bound IgM (*migm*) and *igt*. Elongation factor 1α (*ef1α*) was used as the reference gene. The primers used are listed in Table 1. The RT-qPCR was carried out with cDNA corresponding to 15 ng of RNA. Primer optimization was performed and a concentration of 450 nM F/R for each primer was used. PCR reaction efficiency for each gene assay was determined using 5-fold serial dilutions of randomly pooled cDNA in triplicates.

2.4. Bead coupling and multiplex immunoassay

For detection of specific vaccine-relevant antibodies, the A-layer protein from *Aeromonas (A.) salmonicida* (Stromsheim et al., 1994), in-house produced), and whole cell sonicate from *Moritella (M.) viscosa* (kindly provided by Liv-Jorun Reitan, Norwegian Veterinary Institute) were included. For detection of antibodies not specific to the vaccine components, the hapten-carrier DNP-keyhole limpet hemocyanin (DNP-KLH) (Calbiochem, Merck, Darmstadt, Germany) was used. Antigens were coupled to distinct MagPlex® -C Microspheres (Luminex Corp. Austin, TX, USA) of different bead regions and according to the manufacturer's protocol using the Bio-Plex amine coupling kit (Bio-Rad) as previously described (Teige et al., 2017). The DNP-KLH antigen was used at an amount of 10 µg per 1x scale coupling reaction, and A-layer and *M.viscosa* sonicate at an amount of 12 µg and 7 µg, respectively. Stock uncoupled beads and beads coupled with the protein carrier KLH alone (Sigma-Aldrich, Oslo, Norway) were also included. For the immunoassay, Bio-Plex Pro™ Flat Bottom Plates were used. Beads were diluted in assay buffer consisting of PBS with 0,5% BSA (Rinder-albumin; Bio-Rad Diagnostics GmbH, Dreieich, Germany) and 0,05% azide (Merck, Darmstadt, Germany), and 2500 beads per region were added to each well. Beads were washed three times with 200 µl assay buffer per well for 30 s in the dark and on a shaker at 800 rpm, then kept for 120 s in a Bio-Plex handheld magnetic washer before the supernatant was poured off. Plasma samples were diluted 1:200 in assay buffer, before 50 µl sample was added to each well in duplicates. The plate was incubated for 30 min at RT in the dark and on a shaker at 600 rpm. All subsequent washing –and incubation steps were performed similarly. Following incubation, beads were washed and all wells were added 50 µl of Anti-Salmonid-IgH monoclonal antibody (1:400, clone IPA5F12, Cedarlane, Burlington, Ontario, Canada). After incubation and washing, each well was added biotinylated goat Anti-Mouse IgG2a antibody (1:1000, Southern Biotechnology Association, Birmingham, AL, USA), and finally, after incubation and washing, Streptavidin-PE (1:50, Invitrogen). After the final incubation, beads were washed and resuspended in assay buffer. Plates were analyzed using a Bio-Plex 200 in combination with Bio-Plex Manager 5.0 software (Bio-Rad). The reading was carried out using a low PMT target value, the DD-gate was set to 5000–25000, and 100 beads from each region were read in each well. Each bead is classified by its signature fluorescent pattern and then analyzed for the mean fluorescence intensity (MFI) of the reporter molecule.

2.5. Ig-seq

Libraries for sequencing were produced with PCR of fragments containing CDR3. The cDNA was synthesized using SuperScript II (Thermo Fisher Scientific) and IGMR1 primer to the constant region of Atlantic salmon IgM heavy chain (TAAAGAGACGGGTGCTGCAG). Two consecutive PCR amplifications were performed in a 50 µl volume with Platinium Taq DNA polymerase (Thermo Fisher Scientific). The first PCR was performed with primers containing *igm* sequences and Illumina adaptors (underlined):

IGV5 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGARGAC-WCWGCWGTGTATTAYTGTG

IGC3 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGAACAA AGTCGGAGCAGTTGATGA.

IGV5 was designed to the 3'-ends of fifteen highly similar Variable regions of Heavy chain (VH) segments, which are present in more than 60% *igm* transcripts (Krasnov et al., 2017), and the reverse primer matched the 5'-end of the constant region. The second PCR (10 cycles) amplified 5 µl of PCR1 product with primers from Illumina Nextera Index kit. DNA concentrations were determined using Qubit fluorometer (Thermo Fisher Scientific). Aliquots from all samples were combined and purified twice with Qiagen PCR cleanup kit. Sequencing with Illumina MiSeq Reagent Kit v2 (read one, 200 cycles from the 3'-end) was performed according to manufacturer's instructions. After trimming primers, sequences were filtered (Illumina $q > 20$) and the J segments were identified using Smith-Waterman algorithm. Sequences were translated in three frames and CDR3 were determined by guidelines of IMGT (Brochet et al., 2008; Lefranc, 2014). Clonotypes were assigned by the amino acid sequences of CDR3 and the numbers of transcripts per clonotype (frequencies) were assessed. In each sample, clonotypes represented with at least two transcripts were used for further analyses. Clonotypes were denoted as unique if transcripts were detected in a single fish, or shared if transcripts were found in at least two individuals with a transcript frequency of at least 1 per 10,000. The hundred most abundant clonotypes were selected in each individual and cumulative frequencies of unique and shared clonotypes were calculated. Further, sequences were submitted to IMGT HighV-QUEST server (Brochet et al., 2008). Nucleotide sequences corresponding to each CDR3 were determined and the numbers of nucleotide substitutions were calculated.

2.6. Statistics

Data produced with RT-qPCR and Ig-seq methods were analysed with ANOVA followed with post hoc tests using Statistica 13.

3. Results

3.1. Microarrays and RT-qPCR

A total of 4928 genes of 44 k presented on the array were differentially expressed on at least one time-point in the head kidney of vaccinated and saline-injected control fish. The number of differentially expressed genes (DEG) was 836 at 1 dpv, increased to a maximum level of 3033 at 14 dpv, and decreased afterwards but remained relatively high to the end – 703 genes at the last time-point (Fig. 1). An overview of enriched functional categories of DEG is shown in Table 2.

Genes of innate immunity were divided into three groups based on their expression profiles after vaccination; constantly up-regulated, early up-regulated, and late up-regulated (Fig. 2). The constantly up-regulated group included genes with diverse immune functions, such as

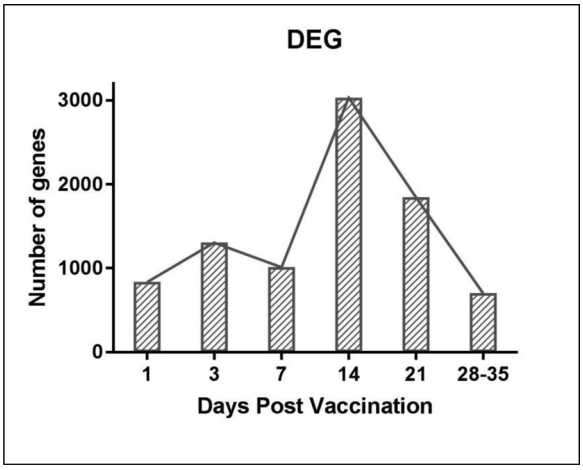

Fig. 1. Numbers of differentially expressed genes (DEG) in the head kidney between vaccinated and saline-injected fish (microarray analyses).

Table 2
Enrichment of functional categories and pathways.

Group_Name	DEG[1]	ALL	Annotation
Innate immune response	137	627	GO
Inflammatory response	102	544	GO
Cytokine-cytokine receptor interaction	68	380	KEGG
Chemokines	25	71	STARS
Eicosanoid metabolism	26	106	STARS
Lectins	46	200	STARS
Acute phase proteins	28	114	STARS
Complement classical pathway	31	80	GO
Scavenger receptor activity	42	209	GO
Neutrophil chemotaxis	28	89	GO
Cell adhesion molecules	65	388	KEGG
Hematopoietic cell lineage	44	133	KEGG
Myeloid cell differentiation	23	71	GO
T cells	54	282	STARS
B cell receptor signaling	50	230	KEGG
Lymphocytes	41	199	STARS

[1] For each term, the number of differentially expressed genes in all time-points (DEG) was compared with the total number of genes presented on the microarray platform (ALL) and enrichment was assessed with Yates' corrected chi square test.

antimicrobial and acute phase proteins (*saa, cath, differentially regulated trout protein, irg1*), genes involved in eicosanoid metabolism (*alox, loxe3, aloxe3*), pathogen recognition (*tlr8*) and regulation of signalling (*socs3b*). Strong expression changes were exhibited by genes with poorly explored or unknown (*l-serine dehydratase, saitotoxin binding protein*) immune roles. In the early up-regulated group, genes encoding for acute phase proteins, lectins and complement factors had increased expression already at 1 dpv and remained up-regulated until 14 dpv. Several chemokine genes showed an increase in expression from 1 dpv to 7 dpv. The late up-regulated genes, which had an increase from 14 dpv, include leukocyte markers, cytochromes and enzymes producing free radicals (*cyba, cybb, mpo, ncf2, ncf4*).

While innate responses were characterized by constant or prolonged changes in vaccinated fish, this was less common for genes that are associated with B cells (Fig. 3). Only *cd72-like* and two *blimp-like* genes had an increased expression level until 14 dpv. Several genes showed biphasic responses and a large group of genes were up-regulated only at 1 dpv or not later than 3 dpv. Among these, multiple structurally divergent genes from different orthology groups (Zdobnov et al., 2017) are denoted as *cd22-like* by the nearest matches. A suite of genes that can take part in B cells differentiation including *rag1*, *rag2* and *cd5* were up-regulated from 14 dpv. Of note is that multiple genes were differentially expressed only at 14 dpv, where up- and down-regulation was observed in respectively 438 and 654 genes. A notable feature was transient reduced abundance of genes associated with T cells, including *cd3*, *cd8* and *cd28*.

Expression of immunoglobulins (*ig*, 30 probes to different segments) changed in both the vaccinated and saline-injected group (Fig. 4A). Expression levels were higher in the vaccinated fish already at 1 dpv, and the difference reached a maximum at 14 dpv followed by a gradual decrease. At the end of the study, the *ig* levels were equalized. Microarray analysis detected changes of the entire pool of *ig* transcripts. The qPCR analysis separated *s (soluble)igm*, *m (membrane)igm* and *igt*, which comprised respectively 90.7%, 8.2% and 1.1% of total *ig*. Only *sigm* showed higher expression in vaccinated salmon, and the greatest difference between the groups was observed at 14 dpv (Fig. 5A). At the end of the trial the expression levelled out between the groups in concordance with the microarray results. Marked difference between the vaccinated and control group was shown by the *sigm / sigt* ratio suggesting a shift to production of IgM in immunized salmon (data not shown). The reduction of *ig* transcripts in the saline-injected control group was found in parallel with an increased expression of erythrocyte markers (Fig. 4B). This set, which was previously identified in a study of red blood cell differentiation in Atlantic salmon (Krasnov et al., 2013),

Gene	D1	D3	D7	D14	D21	D28-35
Constant up-regulation						
Serum amyloid A (saa)	3.7	11.2	12.6	18.1	1.9	3.0
Arachidonate 15-lipoxygenase B-like (alox15)	1.9	2.7		4.9	2.8	2.7
Carcinoembryonic antigen-related cell adhesion 20 (ceacam	9.0	8.4	3.3	6.2	3.0	2.6
Cathelicidin antimicrobial peptide (cath, 2 genes)	11.4	17.3	14.8	21.6	9.8	3.9
Differentially regulated trout protein (3 genes)	2.4	5.2	4.0	2.8		1.8
Epidermis-type lipoxygenase 3 (loxe3)		1.9		3.5	2.2	2.0
Hydroperoxide isomerase ALOXE3-like	2.0	2.9	2.0	5.0	2.9	2.6
Immunoresponsive 1 homolog (mouse) (irg1)	18.0	20.3	8.1	3.9	3.1	2.3
L-selectin-like (cd62l)	2.2	2.0	2.3	4.1	3.2	2.5
L-serine dehydratase (2 genes)	3.5	15.0	11.4	4.3	2.1	
Suppressor of cytokine signaling 3b (socs3b)	12.0	6.1	2.3	2.1		1.8
TNF receptor member 11B (tnfr11b)	15.0	22.8	10.1	3.9	2.3	
Toll-like receptor 8 (tlr8)	2.2	2.7	2.3	2.3	1.8	1.9
Early up-regulation						
Saxitoxin and tetrodotoxin-binding protein (psbp1)	5.8	10.9	9.3	1.9		
Complement component 3a receptor (c5ar)	2.2	4.2	3.4	2.3		
Leukemia inhibitory factor receptor-like (cd118)	3.4	5.0	3.9	1.8		
C type lectin receptor B (2 genes)	2.4	7.2	5.1	2.0		0.7
C type lectin receptor A	3.8	3.7	2.0	1.9		
C-type lectin domain family 4 member E	1.9	1.8		1.9		
Complement component C8 gamma chain (c8g)		2.2	2.7	1.9		

Gene	D1	D3	D7	D14	D21	D28-35
Early up-regulation						
C-C motif chemokine 4-like	18.6	36.4	14.6		0.7	
C-C motif chemokine 19 -1	2.9	3.6	2.3			
C-C motif chemokine 4-like	2.8	2.4	1.9			
Haptoglobin-like (5 genes)	4.8	5.3	7.3			
Late up-regulation						
Angiogenin-1 / RNase ZF3 (rnh1, 4 genes)		0.6			2.7	2.8
Arachidonate 5-lipoxygenase-activating (alox5ap, 2 genes)				2.0	1.8	
CD209 antigen-like protein C				2.5	2.4	2.3
C-type lectin domain family 4 member E-like (clec4e)				2.7		2.2
C-type lectin domain family 4 member F-like (clec4f)				1.8	2.2	2.2
C-type lectin domain family 4 member M (clec4m, 2 genes)				3.0	2.2	2.6
C-X-C chemokine receptor type 4				2.5	2.3	2.0
Cytochrome b-245 light chain (cyba)				2.4	2.1	1.8
Cytochrome b-245, beta polypeptide (cybb)				2.6	2.5	1.8
Leukotriene A-4 hydrolase (lta4)				2.4	2.3	2.0
Leukotriene B4 receptor 1 (ltb4r)				2.2	1.8	2.1
Myeloperoxidase (mpo)		0.7		2.4	2.2	
Neutrophil cytosolic factor 2 (ncf2)				2.3	1.8	
Neutrophil cytosolic factor 4 (ncf4)				1.8	1.8	
Ornithine aminotransferase, mitochondrial (oat)				2.3	2.1	
P-selectin-like (selp)				2.3	1.9	

Fig. 2. Differentially expressed innate immunity genes in the head kidney. Data are vaccinated (n = 5) to saline-injected (n = 4) expression ratios (folds), all shown values are significantly different (t test, $p < 0.05$). Up and down-regulated genes are highlighted with respectively red and green colours (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Gene	D1	D3	D7	D14	D21	D28-35
B-cell lymphoma 3 protein (bcl3)	3.4					
B-cell receptor CD22-16	3.0					
B-cell receptor CD22-17	1.8					
B-cell receptor CD22-3	2.3	2.0				
B-cell receptor CD22-4	2.6	1.9				
B-cell receptor CD22-5	2.1	1.8				
B-cell receptor CD22-6 (2 genes)	1.8					
B-cell receptor CD22-9	1.8					
Phosphatidylinositol 4,5-bisphosphate 3-kinase (p13kca)	1.9					
Phosphoinositide 3-kinase regulatory subunit 5 (pi3krs6)	2.1					
PR domain containing 1a, with ZNF domain (blimp1-like)	2.5			1.9		
B-cell differentiation antigen CD72-like	*2.3*		*1.9*	*2.0*		
B-cell antigen receptor complex-associated (2 genes)	1.9				1.8	
B-cell CLL/lymphoma 6a (bcl6a, 6 genes)	1.9			1.8		1.8
Phosphoinositide 3-kinase regulatory subunit 5 (pi3krs5)	2.5			1.7	1.8	
PR domain containing 1a, with ZNF domain (blimp1, 3 genes)	2.5			1.9		
B-cell receptor CD22-10				1.8	1.8	
B-cell receptor CD22-8	0.7	0.6			1.8	1.8
DNA cross-link repair 1C (dclre1c)	0.7	-5.2	-3.2	2.9	1.9	1.9
Hematopoietic lineage cell-specific protein (hcls1)				*1.9*	*1.9*	
Phosphatidylinositol 4,5-bisphosphate 3-kinase (pi3kca, 3 genes)		0.7		2.2	2.0	
Phosphoinositide 3-kinase regulatory subunit 5 (pi3krs6)				2.0	2.0	
Rag1	0.7	0.5		1.9	1.7	
Rag2		0.7		2.6	2.1	
CD5 antigen-like				1.9	1.8	1.0
CD3gammadelta-B			0.6	-2.2	0.7	0.7
CD28				-1.9	0.6	0.7
CD8 alpha		0.6	0.7	-1.9		

Fig. 3. Differentially expressed genes involved in development of B cells. Data are vaccinated (n = 5) to saline-injected (n = 4) expression ratios (folds), all shown values are significantly different (t test, $p < 0.05$). Up and down-regulated genes are highlighted with respectively red and green colours (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

contains 52 erythrocyte related genes including *hemoglobins*, multiple components of the cytoskeleton and membrane, transporters, enzymes and transcription factors. Expression of these genes in vaccinated salmon was reduced to the minimum level at 3 dpv and slowly increased afterwards. Difference between the vaccinated and saline-injected group was greatest at 14 dpv and completely disappeared at 28 dpv.

3.2. Multiplex immunoassay

Titres of specific antibodies against two bacterial components of the vaccine, the A-layer of *A. salmonicida* (Fig. 6A) and *M. viscosa* whole-cell sonicate (Fig. 6B), were significantly different in vaccinated Atlantic salmon compared to the saline-injected controls from 14 dpv and their titres reached top levels at 28 dpv. Specific antibody titres in the saline-injected group remained low throughout the observation period. Both vaccinated and saline-injected fish showed high titres of non-vaccine specific antibodies, recognizing the hapten-carrier antigen DNP-KLH (Fig. 6C). Vaccinated salmon showed increased titres of non-vaccine specific antibodies compared to the saline-injected group from 14 dpv, and significant differences in titres from 21 dpv and until the end of trial. The overall dynamic range in MFI values varied depending on the coating antigen, with the greatest median MFI values observed for antibody titres against DNP-KLH, showing more than 30-fold higher titres than the titres against A-layer at 14 dpv. Comparison between titres of non-vaccine specific antibodies and A-layer specific antibodies (Fig. 7A) and between the two vaccine-specific antibodies (Fig. 7B) showed very low correlation: Pearson r respectively 0.1 and 0.19.

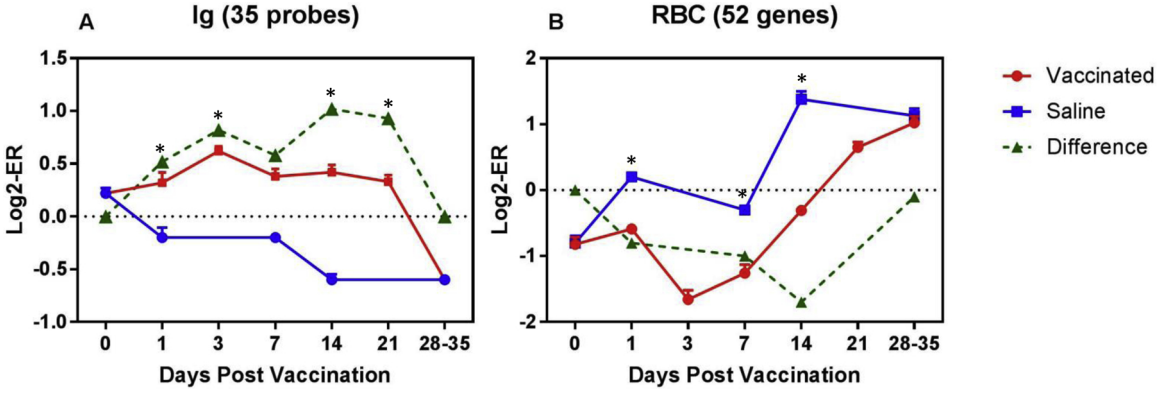

Fig. 4. Expression of *ig* (A) and markers of red blood cells (B), microarray data. Data are mean $\log_2$-ER ± SE. Significant differences between vaccinated (n = 5) and saline-injected fish (n = 4) (t test, $p < 0.05$) are indicated with asterisks.

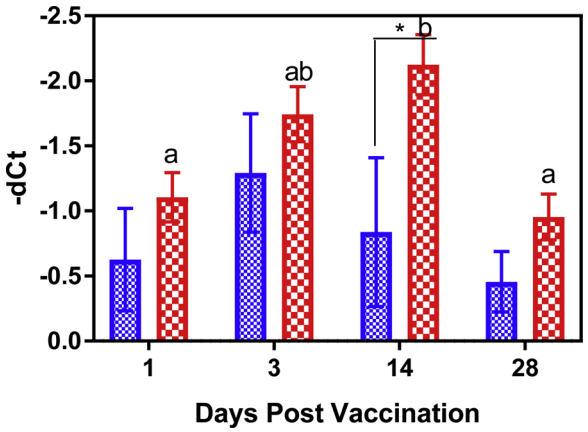

Fig. 5. RT-qPCR. Expression of secretory immunoglobulin M – *sigm*. Three to five fish were analyzed per group (vaccinated and saline) and time-point. Significant difference between the time-points within each group is indicated with letters (omitted when no significant difference was found), and significant difference between groups at given time-points is indicated with asterisks (ANOVA, Tukey test, $p < 0.05$).

3.3. Ig-seq

A total of 15 M high quality immunoglobulin sequences were produced. Two metrics were applied to characterize the IgM repertoire and the status of unique and shared clonotypes: cumulative frequencies of the most abundant clonotypes (CF) and variance of the CDR3 region sequences. The most abundant clonotypes (leaders), i.e. clones that encode a large fraction of antibodies, represent the greatest interest. Exposure to pathogenic antigens induces proliferation of B cells clones and / or increases their production of antibodies. Either way, a response to a recent immunization can be detected as an increase of CF due to enhanced transcription of the leaders. Here, CF were calculated for the hundred most abundant clonotypes and further divided into two fractions, shared or unique. CF of shared clonotypes showed no difference between vaccinated fish and saline-injected fish (not shown), while CF of unique clonotypes were significantly higher in vaccinated salmon at two time-points (Fig. 8A). Difference between vaccinated and saline-injected fish in the variance of the nucleotide sequences of CDR3 of unique clonotypes was observed at 14 dpv (Fig. 8B).

4. Discussion

The aim of this study was to expand the knowledge of immune processes taking place in Atlantic salmon shortly after vaccination, by analysing the production of circulating antibodies, and the IgM repertoire and gene expression in the head kidney. Vaccination induced a powerful and multifaceted immune reaction, which included communication and signalling via chemokines, cytokines and lipid mediators, activation of innate factors (acute phase proteins, complement, scavengers, lectins) and genes associated with both innate and adaptive cells. A synopsis of results produced with three independent methods pointing to 14 days post vaccination as the critical time-point is presented in Fig. 9.

Microarray results showed an upregulation of a number of genes with diverse immune roles as well as genes with unknown functions, which can be ranked by the magnitude and stability of expression changes after vaccination. Constant up-regulation was shown by

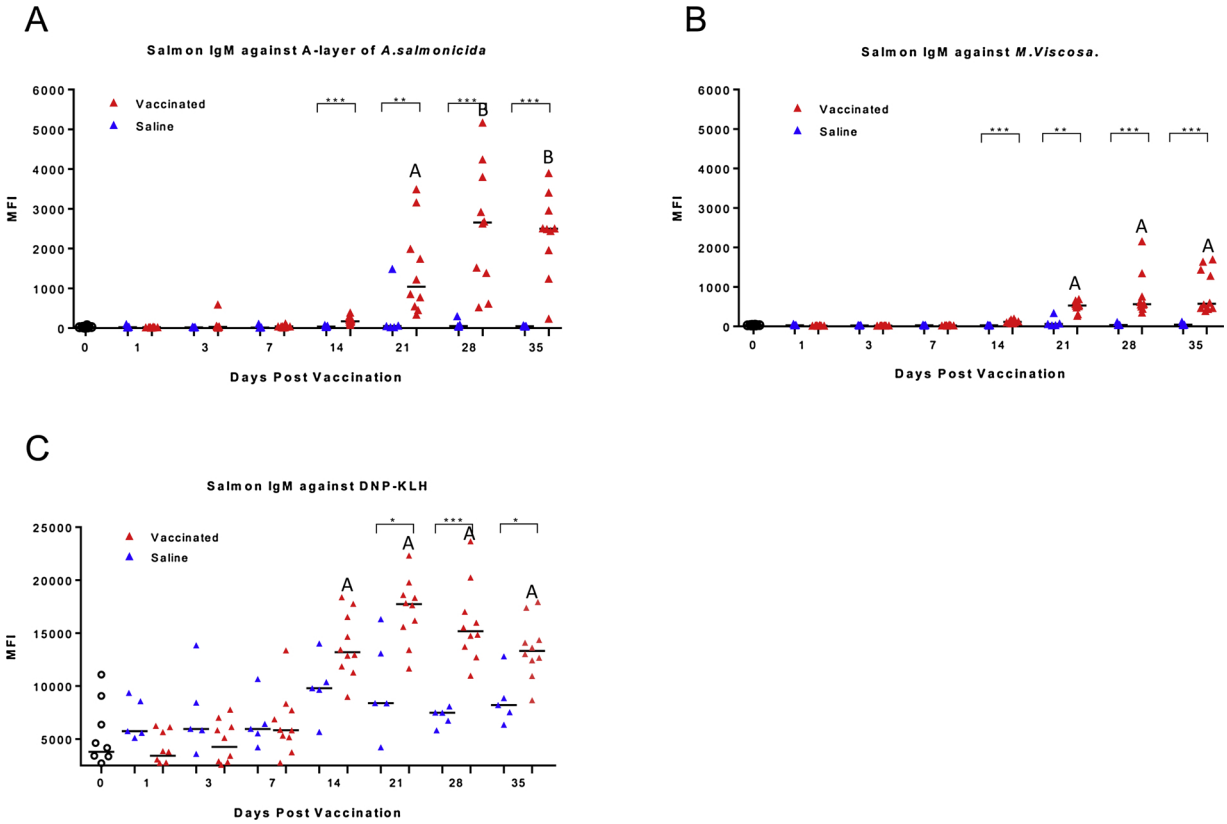

Fig. 6. Multiplex antibody assays. Salmon IgM antibodies against A-layer of *A. salmonicida* (A), *M. viscosa* (B) and DNP-KLH (C). All individual values of vaccinated (red triangles, n = 10), saline-injected (blue triangles, n = 5) and pre-vaccinated fish (black open circles, n = 10) are shown. The median value of each sample group is shown as a black line. Significant difference between the time-points and treatment groups are indicated with respectively letter and asterisks (ANOVA, Newman – Keuls test). MFI = Mean Fluorescent intensity (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

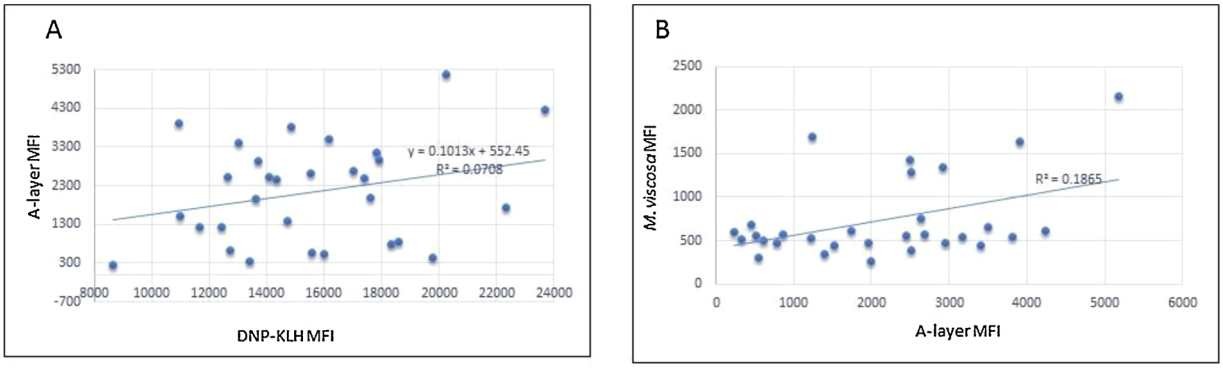

Fig. 7. Relationship between titres of antibodies to different antigens determined in multiplex assays. A: DNP-KLH and A-layer. B: A-layer and *M. viscosa*.

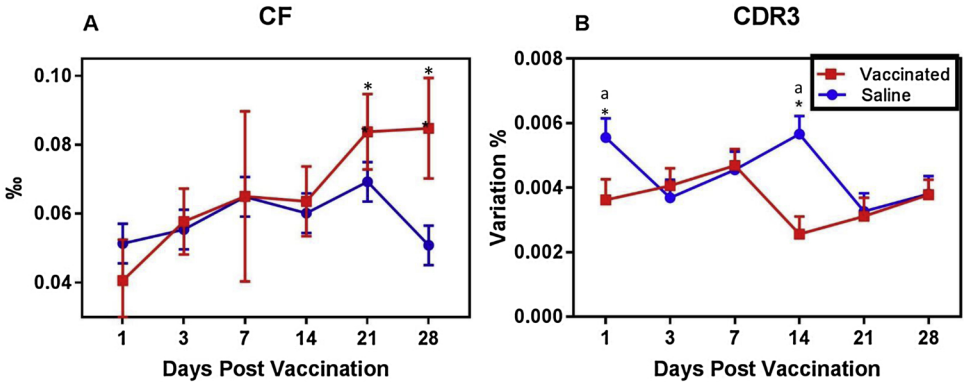

Fig. 8. Deep sequencing of the variable regions of IgM. Results for unique clonotypes are shown.
A: Cumulative frequencies (CF) of most abundant unique clonotypes in vaccinated and saline-injected fish (N = 5). B: Variance of nucleotide sequences of Complementarity Determining Region 3 (CDR3). Differences between vaccinated and saline injected fish are indicated with asterisks (ANOVA, LSD test, $p < 0.05$).

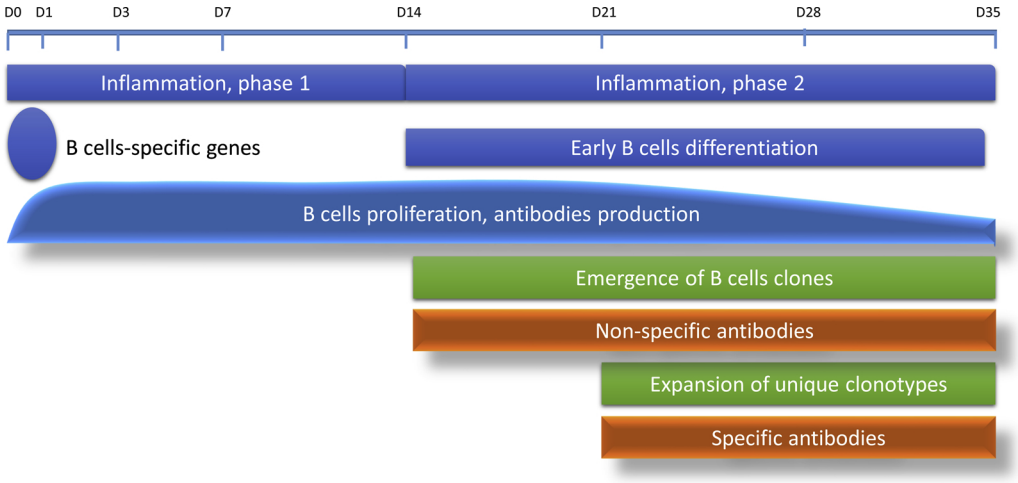

Fig. 9. Schematic presentation of processes taking place in the head kidney and plasma of Atlantic salmon after vaccination. Results produced with microarrays (blue), Ig-seq (green) and multiplex antibody assays (orange) are presented in different colours (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

cathelicidin and *serum amyloid A*, generic inflammatory markers with anti-microbial roles in Atlantic salmon (Chang et al., 2006; Jensen et al., 1997; Lee et al., 2017). *Irg1* or *cis-aconitate decarboxylase* contributes to the antimicrobial activity of macrophages (Michelucci et al., 2013), and mammalian *tnfrsf11b* is a regulator of lymphocyte development (Kong et al., 1999). The increased expression of innate response genes in vaccinated fish persisted for at least four weeks and many genes maintained an activated status to the end of observations. This immune stimulation is likely caused by the oil adjuvant providing prolonged stimulation and exposure of the bacterins in the vaccine, and is a wanted effect of immunization to enhance the innate immune response to the vaccine. A novel finding here, was switch of the character of inflammation after 14 dpv, which coincided with other events including changes in the population of B cells.

Microarray results highlighted a number of genes whose mammalian orthologues are known for major roles in differentiation and maturation of B cells. Biphasic or early responses were found in genes encoding B cell markers expressed on mature B cells (*cd22, cd72*) that are lost with differentiation into plasma cells (Poe and Tedder, 2012; Li et al., 2006; Wu and Bondada, 2009). Their early rise and subsequent decline may thus reflect B cell proliferation followed by differentiation into plasma cells. *Blimp1* is an essential regulator of plasma cell functions (Tellier et al., 2016), and accordingly, we find expression sustained for a longer period. Of special interest was the late up-regulation of *rag1* and *rag2* genes (Hansen and Kaattari, 1996; Hansen, 1997). In mice, late *rag* expression in lymphoid tissues was observed following immunization, representing newly recruited immature but non-proliferating B cells mainly assigned to the adjuvant (Nagaoka et al., 2000). *Rag* expressing B cells outside germinal centres cells were later shown to edit their receptors and concomitantly express *cd5* which are hallmarks of B1 cells in mammals and likely also in fish (Abos et al., 2018; Hillion et al., 2005). In the present study, *cd5* was moderately

upregulated at day 14–21, along with *rag1* and *rag2*, and might suggest a presence of cells of this type that could be a cellular source of non-specific antibodies, discussed below. However both *cd5* and *rag* are also expressed in T-cells, and although *cd3* and *cd8* were downregulated at this time, the contribution of T-cells cannot be excluded.

Changes in the antibody repertoire were analysed by multiplex immunoassay and immunoglobulin sequencing. Antibodies in sera of naïve fish are often referred to as natural by analogy with natural antibodies of mammals. Another frequently used term is polyreactive antibodies, which strictly would require the demonstration of multiple binding capacities. For the present study, we prefer an assumption-free term: non-vaccine specific antibodies or non-specific antibodies (NSABs) due to profound differences in observations made in fish and mammals. *Sensu stricto*, natural antibodies are found in the absence of antigenic challenge, and their presence should be demonstrated in a germ-free animal. Natural antibodies are produced by specialized populations of B cells, such as murine B1, are characterized with a close to germline structure, and their responses to antigens are rapid (reviewed by (Panda and Ding, 2015)). In contrast, NSABs are commonly found in relatively high titres in naïve fish that have been exposed to diverse endogenous and environmental microflora (Kachamakova et al., 2006). In a number of independent studies we have observed a strong increase in NSAB titres following immunization. In mammals, natural or non-specific IgM has been shown to contribute to antimicrobial defence in part by complement activation, and is regarded to be important in early antiviral defence, especially prior to establishment of specific immune responses (reviewed in (Baumgarth, 2013; Racine and Winslow, 2009)). In fish, the mechanisms of production of NSABs are still poorly understood, and their role in immunity to vaccination and infection needs to be further elucidated.

The presence of shared clonotypes with complex structure in multiple individuals and their structural features (Krasnov et al., 2017) suggest selection of B cell clones by exposure to antigens, as part of an antigen-driven response. Until now, we have not found highly abundant clonotypes with a simple structure, as all CDR3 have insertions and deletions (Krasnov et al., 2017). In the present study, we found an increased production of NSABs binding to the hapten-carrier antigen DNP-KLH from 14 days after vaccination. Increase in the cumulative frequency of immunoglobulin repertoire was found only for unique clonotypes simultaneously with an increase in systemic specific antibodies to the bacterial antigens delivered with the vaccine. In parallel, a decrease of CDR3 sequence variance coinciding with the increase in the expansion of unique clonotypes suggests a rapid expansion of recently emerged B-cell clones. The short-term character of the observed decrease may indicate egress of B cells to spleen, a secondary lymphatic organ of Atlantic salmon (Ma et al., 2013; Bromage et al., 2004), and migration of cells might also explain decrease of *ig* transcripts starting from 28 dpv. Of note is that the approach used in this study underestimates divergence, since only silent nucleotide substitutions are taken into account.

Large parts of the mechanisms of initiation of adaptive responses in fish remain unclear. S. Kaattari et al. described seven cell populations corresponding to the stages of B cells differentiation, each characterized with a set of gene markers (Ye et al., 2011; Bromage et al., 2004; Zwollo et al., 2005). We here observed marked stimulation of NSAB in vaccinated salmon, which preceded detection of markedly elevated levels of specific antibodies against A-layer and *M. viscosa*. Increase of NSABs titres could be due to an indirect stimulation of multiple B cell clones via the cytokine and chemokine network. It is plausible to think that at least part of these NSABs are encoded with highly abundant shared clonotypes, which have been selected by recognition of common endogenous or external antigens (Krasnov et al., 2017). However, if these NSAB producing cells are not proliferating and start editing their receptors upon stimulation (Hillion et al., 2005) as the late *rag* expression might suggest, they may appear as unique clonotypes, thus explaining the lack of vaccination effect on frequency of shared clonotype in the present study. Perhaps independently of this, the vaccine antigen gives rise to a slower and much more specific B-cell expansion and production of antigen-specific antibodies and unique clonotypes. However, it is important to emphasize that trends observed in the current study regarding the maturation and trafficking of B cells should be taken with caution. Subsequent analysis into this field should include the analysis of gene expression and immunoglobulin repertoire in additional lymphatic organs. Moreover, there is an urgent need for antibodies that can target key cell markers of B cells in salmon.

In summary, this study presented parallel analyses of multiple gene expression, immunoglobulin sequencing and plasma antibody titers in the first few weeks after injection of a commercially used multivalent vaccine to Atlantic salmon. Particularly intriguing are findings pointing to a contribution of non-specific antibodies in the early vaccine response. Further studies are needed to reveal the origin and functions of such antibodies, including their role in homologous and heterologous disease protection.

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