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ORIGINAL ARTICLE

Intramuscular vaccination of Atlantic lumpfish (*Cyclopterus lumpus* L.) induces inflammatory reactions and local immunoglobulin M production at the vaccine administration site

Toni Erkinharju[1,2] | Guro Strandskog[2] | Øyvind Vågnes[3] | Ivar Hordvik[4] | Roy A. Dalmo[2] | Tore Seternes[2]

[1]Norwegian Veterinary Institute, Harstad, Norway

[2]Norwegian College of Fishery Science, UiT – The Arctic University of Norway, Tromsø, Norway

[3]Vaxxinova Norway AS, Bergen, Norway

[4]Department of Biology, University of Bergen, Bergen, Norway

Correspondence
Tore Seternes, Norwegian College of Fishery Science, UiT – The Arctic University of Norway, Tromsø, Norway.
Email: tore.seternes@uit.no

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Abstract

Atlantic lumpfish were vaccinated by intramuscular (im) or intraperitoneal (ip) injection with a multivalent oil-based vaccine, while control fish were injected with phosphate-buffered saline. Four lumpfish per group were sampled for skin/muscle and head kidney tissue at 0, 2, 7, 21 and 42 days post-immunization (dpi) for histopathology and immunohistochemistry (IHC). Gene expressions of secretory IgM, membrane-bound IgM, IgD, TCRα, CD3ε and MHC class IIβ were studied in tissues by using qPCR. Im. vaccinated fish showed vaccine-induced inflammation with formation of granulomas and increasing number of eosinophilic granulocyte-like cells over time. On IHC sections, we observed diffuse intercellular staining of secretory IgM at the injection site at 2 dpi, while IgM + cells appeared in small numbers at 21 and 42 dpi. Skin/muscle samples from im. vaccinated fish demonstrated an increase in gene expression of IgM mRNA (secretory and membrane-bound) at 21 and 42 dpi and small changes for other genes. Our results indicated that im. vaccination of lumpfish induced local IgM production at the vaccine injection site, with no apparent proliferation of IgM + cells. Eosinophilic granulocyte-like cells appeared shortly after im. injection and increased in numbers as the inflammation progressed.

KEYWORDS

Atlantic lumpfish, immune response, immunoglobulin M, inflammation, intramuscular, oil-based vaccine

1 | INTRODUCTION

Cleaner fish removes and eats dead skin and ectoparasites from other species. Atlantic lumpfish (*Cyclopterus lumpus* L.) is a cleaner fish used to control sea louse infestations in aquaculture production of salmonid fish. As such, lumpfish is currently the second most farmed fish species in Norwegian aquaculture, and nearly 40 million individuals were produced for combating sea lice in 2018 (Hjeltnes, Bang Jensen, Bornø, Haukaas, & Walde, 2019). Disease is a recurring health and welfare challenge for lumpfish, especially infections caused by different types of bacteria. Vaccines against several bacterial pathogens have been developed for lumpfish, but

more work is still needed to provide optimal vaccination strategies (Hjeltnes et al., 2019).

Basic knowledge of the immune system is needed to develop optimal vaccines and vaccination methods for different species of teleost fish. Vaccines improve acquired immunity to particular diseases by stimulating the body's immune system to recognize antigens and fight disease-causing microorganisms through humoral and cell-mediated immune responses. Cellular immunity protects the body through the actions of different immune cells, such as T lymphocytes (T cells), while humoral immunity is mediated by macromolecules found in extracellular fluids, such as antibodies (Tizzard, 2018). Different immune cells are important for these processes, and several have been isolated and identified in lumpfish, including lymphocytes, monocytes, macrophages, neutrophils, basophils, eosinophils and dendritic cells (Haugland, Rønneseth, & Wergeland, 2018). It has been shown that vaccination leads to specific antibody responses and protection against disease in lumpfish (Rønneseth, Haugland, Colquhoun, Brudal, & Wergeland, 2017).

Antibodies, also known as immunoglobulins (Ig), occur in two physical forms, a soluble form secreted by B lymphocytes (B cells) to be free in the body, and a membrane-bound form attached to the cell surface of B lymphocytes (functioning as B-cell receptors). Based on their structure, antibodies can be further categorized into different classes or isotypes. While there are five isotypes of immunoglobulin described in mammals: IgG, IgM, IgD, IgA and IgE, there are only three major types described in teleost fish: IgM, IgD and IgT/IgZ (Mashoof & Criscitiello, 2016). Of these three, soluble IgM is the most prevalent circulatory antibody, although the total concentration can vary between different species (Hordvik, 2015; Uribe, Folch, Enriquez, & Moran, 2011). In lumpfish, the IgM concentration in serum has been estimated to be 1–2.6 mg/ml, almost the same level as Atlantic salmon (*Salmo salar* L.) (Bilal, Lie, Karlsen, & Hordvik, 2016). For young lumpfish, the level of both IgM and IgD, but not IgT/IgZ, in different tissues has recently been described, where IgM was most abundant in spleen and head kidney and IgD in peripheral blood and spleen (Espmark et al., 2019). However, antibodies can also be found in other parts of the fish body, such as in the skin, intestinal tract and gills (Morrison & Nowak, 2002; Sunyer, 2013).

Oil-adjuvanted vaccines are normally administered by intraperitoneal (ip.) injection, while intramuscular (im.) injection is primarily used for DNA-based vaccines (Lillehaug, 2014). However, deposition of oil-adjuvanted vaccines typically causes local inflammatory reactions in the tissue. As such, other possible injection routes have also been tested, such as the dorsal median sinus (dms) in Atlantic salmon, in order to keep economically important tissues (such as reproductive organs) unaffected by these types of reactions (Treasurer & Cox, 2008).

In an earlier study, we studied the systemic antibody response after im. and ip. vaccination of different vaccines (Erkinharju et al., 2017). In the present study, we wanted to investigate the local inflammation process and immune response after im. vaccination at different time points, early and late after vaccine administration. We analysed expressions of several immune gene markers by qPCR, assessed the presence of immunoglobulin M at the vaccination site by immunohistochemistry and used histology with standard haematoxylin and eosin stain to study the local inflammatory cells and tissue reactions.

2 | MATERIALS AND METHODS

2.1 | Fish samples

The lumpfish samples analysed in the present study were material collected from an earlier experiment (Erkinharju et al., 2017). Samples from four lumpfish intramuscularly (im) injected with a mineral oil-based vaccine (VAX) or phosphate-buffered saline (PBS) were collected at 2, 7, 21 and 42 days post-immunization (dpi), and samples from four lumpfish intraperitoneally (ip) injected with a mineral oil-based vaccine or PBS were collected at 21 and 42 dpi. Samples from four non-immunized lumpfish collected at the start of the experiment were used as day 0 samples. Head kidney, spleen and skin/muscle tissue samples were dissected and preserved on RNA*later*® (Ambion) for qPCR and head kidney and skin/muscle samples on 10% formalin for histology/immunohistochemistry. Due to small organ size on the fish, spleen was only sampled for qPCR analysis. Skin/muscle tissue from im. injected fish (Figure 1a) was sampled by combination of a horizontal section along the dorsal column, and three transverse sections around the administration site (Figure 1b). The experiment was approved by the Norwegian animal research authority (ID number 6,843).

2.2 | Preparation of samples for histology and immunohistochemistry

Formalin-fixed skin/muscle and head kidney tissues from im. injected fish were prepared for histological and immunohistochemical staining using standard procedures (Suvarna, Layton, & Bancroft, 2019). Tissue samples were processed, embedded in paraffin, sectioned at 2 μm thickness and mounted on either regular glass slides or poly-L-lysine-coated slides (Thermo Fisher Scientific). Unstained slides were stored at room temperature until further processing.

2.3 | Immunohistochemistry (IHC) for IgM and PCNA

The antibodies used for IHC analysis in the present study were polyclonal rabbit anti-lumpfish IgM (α-IgM) (Bilal et al., 2016), and monoclonal mouse anti-proliferating cell nuclear antigen (α-PCNA) (Dako (now part of Agilent), CAT# M0879, RRID: AB_2160651). The α-IgM antibody has previously been used in other experimental work (Erkinharju, Dalmo, Vågsnes, Hordvik, & Seternes, 2018; Erkinharju et al., 2017). Anti-PCNA antibodies have been used for detection of proliferating cells in a number of fish species, including zebrafish (*Danio rerio*) (Diotel et al., 2017), marine medaka (*Oryzias melastigma*) (Kong et al., 2008), lake trout (*Salvelinus namaycush*) (Lee,

Jaroszewska, Dabrowski, Czesny, & Rinchard, 2012), Atlantic salmon (Yousaf, Koppang, Zou, Secombes, & Powell, 2016) and Atlantic lumpfish (Ahmad, Paradis, Boyce, McDonald, & Gendron, 2019).

Prior to staining, slides were deparaffinized by heat treatment at 60°C for 15 min followed by Histolab Clear washing (Histolab Products AB) and rehydrated in increasing ethanol dilutions and water. After rehydration, slides were transferred to a preheated sodium citrate solution (pH 6.0) and incubated at 90°C for 10 min in a heating cabinet for heat-induced epitope retrieval. This proved to be the most optimal method, since lumpfish skin appeared to be vulnerable to damage and tissue loss during direct boiling using autoclave and/or microwave heating. After cooling for 15 min, slides were washed three times in Tris-buffered saline (TBS) for 5 min, and a PAP pen (VWR International) was used to create a hydrophobic barrier around the slide specimen.

Immunohistochemical staining was carried out using the UltraVision Quanto Detection System AP kit (Thermo Fisher Scientific) according to the manufacturer's descriptions. Sections were transferred to a humidity chamber and blocked by incubating with Ultra V Block (Thermo Fisher Scientific) for 5 min. Subsequently, the sections were dried in air, followed by incubation with 100–200 µl of primary antibody for 1 h at room temperature. Primary antibodies used were α-IgM and α-PCNA, diluted 1:7,500 and 1:200 in 2.5% bovine serum albumin (BSA) in TBS, respectively. Sections were then washed three times in TBS, incubated for 10 min with primary antibody Amplifier Quanto (Thermo Fisher Scientific), washed again and incubated with AP polymer Quanto (Thermo Fisher Scientific) for 10 min in darkness. After washing, staining was performed using ImmPACT™ Vector Red (Vector Laboratories) chromogen and haematoxylin counterstaining. Finally, the sections were dehydrated in graded ethanol dilutions and coverslips mounted on the slides with Pertex® mounting medium (Histolab Products AB).

2.4 | Histological staining and analysis

Lumpfish tissue sections were stained with haematoxylin and eosin (HE) according to standard histological instructions (Suvarna et al., 2019) and examined with a DM 2000 light microscope (Leica). Photomicrographs were prepared using a BX43 light microscope with a SC100 colour camera attached (Olympus) and the Stream start ver. 2.3 (Olympus) image software.

2.5 | RNA isolation, cDNA synthesis and quantitative PCR (qPCR)

RNA was isolated from head kidney and spleen tissue using RNeasy® 96 BioRobot® 8,000 (Qiagen) by cutting out 2×2×2 mm size pieces, transferring the pieces to a TRIzol solution (Thermo Fisher Scientific), and extracting the RNA as described by the manufacturer. Initial analysis of muscle samples produced low RNA yield (resulting in several non-detects), and as such, RNA was manually isolated from muscle tissue using RNeasy Mini Kit (Qiagen) by homogenizing tissue in 1 ml RLT buffer containing 20 µl of 2M DTT using TissueLyser II (Qiagen). RNA was quantified using NanoDrop ND 1,000 Spectrophotometer (Thermo Fisher Scientific). No RNA was isolated from muscle samples of two fish, one VAX at 7 dpi and one PBS at 21 dpi. cDNA was synthesized with the QuantiTect Reverse Transcription Kit (Qiagen) starting with 500 ng total RNA in a 20 µl reaction following the manufacturer's protocol. The kit

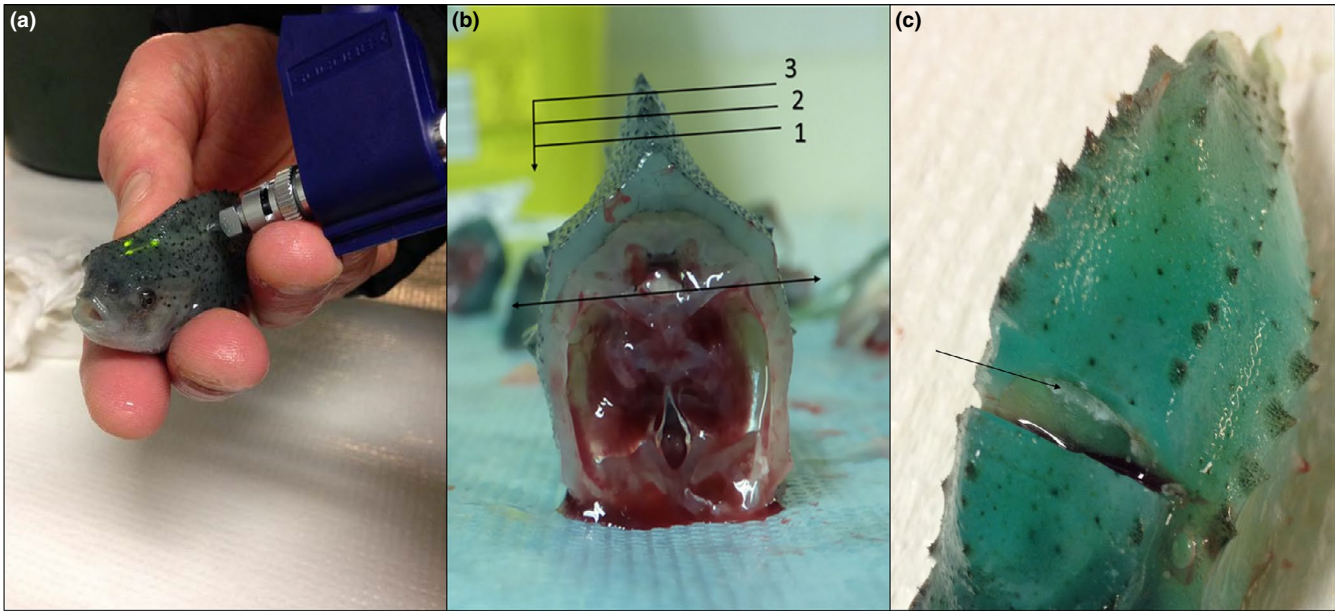

FIGURE 1 Intramuscular vaccination of lumpfish (a). Cross section of lumpfish caudal to the head (b), showing the direction of one horizontal section (horizontal double-headed arrow, ↔) and three transverse sections (vertical arrows, ↓ 1, 2 and 3) for tissue sampling. Upon cross section of immunized fish (c), administered vaccine was located in between the skin and muscular layer of the lumpfish (arrow, →). Photograph: Martin R. Lundberg

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	% Efficiency
EF1α	GGCCAGATCAATGCCGGATA	CTCCACAACCATGGGCTTCT	105.4
sIgM	AGAACCAGTATGGGACGGGA	ACACTGACGGTCGTTGAGTC	99.9
mIgM	ACGAATGGAACAAGGGGACA	AGCAGTGGTTCCAATGGTGA	93.9
IgD	GCACGCATTTGAACCAGTGT	AGGGAGTCTCTGGCCATTCT	91
TCRα	AGCCGTCCTTCTACAAGCTG	GGTCCATGTCCAGGTTCAGG	92.1
CD3ε	AACTGTTACGAGGTGGACGC	GTGAGGGCTCAGTGGTTCAT	90.1
MHCIIβ	ACTGATGAGTTGGCAGACGG	AGGTCAGACCCAGGATCAGT	91.9

TABLE 1 Primers with sequences and percentage efficiencies for SYBR Green qPCR assay

included removal of genomic DNA with the integrated component gDNA Wipeout Buffer.

Primers were designed for genes encoding elongation factor 1 alpha (EF1α), secretory IgM (sIgM), membrane-bound IgM (mIgM), IgD, T-cell receptor alpha (TCRα), cluster of differentiation 3 epsilon (CD3ε) and major histocompatibility complex class II beta (MHCIIβ) (Table 1). Target lumpfish mRNA sequences were identified by local BLAST in a spleen transcriptome database, derived from sequencing of cDNA from an adult wild-caught lumpfish (data not shown). qPCR was performed using 4.0 µl 1:5 dilution of cDNA in a 15 µl reaction mixture containing 7.5 µl Fast SYBR® Green Master Mix (Thermo Fisher Scientific) and 300 nM forward and reverse primers.

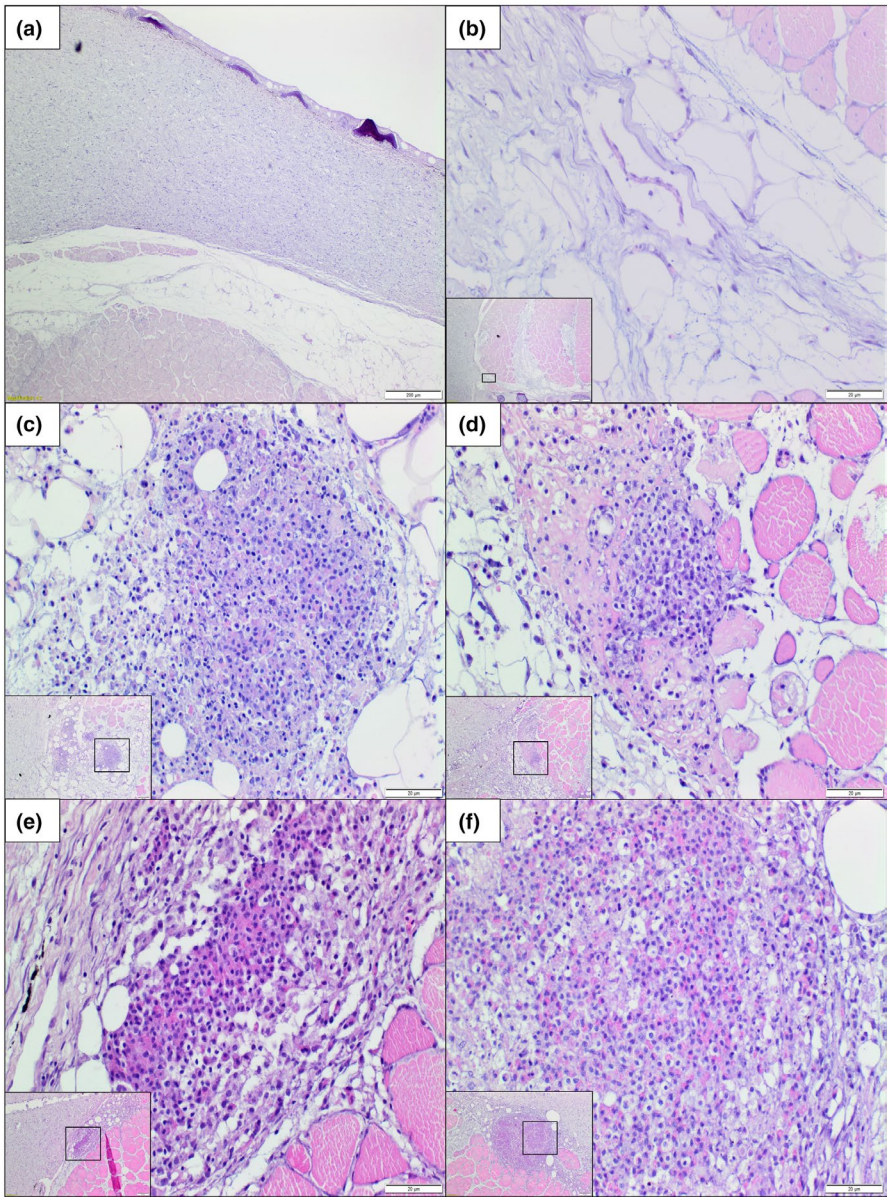

FIGURE 2 Haematoxylin and eosin (HE)-stained skin/muscle sections of im. vaccinated lumpfish at 2 (c), 7 (d), 21 (e) and 42 (f) days post-immunization (dpi). Images show high magnification (40×) of area indicated by black squares in inserts, showing the same section at low magnification (10×). Unvaccinated fish (a and b), day 0 samples, included for reference (4× magnification in a), b) shows 40× magnification of area indicated by black square in insert [10×]). Note the presence of numerous eosinophilic granulocyte-like cells within the inflammatory lesions. Scale bar is 200 µm for (a) and 20 µm for (b–f)

Each sample was run in duplicate wells on a 7,500 Fast Real-Time PCR System (Applied Biosystems). The mixtures were incubated at 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. This was followed by melting curve analysis (65°C–95°C: increment 0.5°C for 5 s) to verify the amplification of a single product. Amplicon efficiency of each gene was determined from qPCR data generated by twofold serial dilutions of cDNA, synthesized from RNA pools of head kidney samples from eight lumpfish (Table 1). The cycle threshold (Ct) data were then used to calculate the individual fold change for each gene by using the double delta Ct ($2^{-\Delta\Delta Ct}$) method. Data from unvaccinated lumpfish (day 0 samples) were used as calibrator, and EF1α was used as reference gene.

Some individual samples did not produce measurable data for some of the analysed genes, possibly due to low amount of detectable material for the specific gene in the sample. As such, these measuring points are lacking in the result section. For muscle samples, there were two points for IgD (VAX (1) and PBS (1) at 21 dpi) and two points for CD3ε (PBS (2) at 2 dpi). For head kidney samples, there was one point for TCRα (IP PBS (1) at 21 dpi) and four points for CD3ε (IM PBS (1) and IP PBS (1) at 21 dpi, IP VAX at 21 (1) and 42 (1) dpi). For spleen samples, there was one point for sIgM (IP VAX at 21 dpi), three points for IgD (IP VAX at 21 (2) and 42 (1) dpi), three points for TCRα (IP VAX (1) at 21 dpi, IP VAX (1) and PBS (1) at 42 dpi), three points for CD3ε (IM PBS (1) at 21 dpi, IP VAX (1) and PBS (1) at 42 dpi) and one point for MHCIIβ (IP VAX at 21 dpi).

3 | RESULTS

3.1 | General inflammatory lesions in tissue of im. immunized lumpfish

We used histological staining with haematoxylin and eosin for microscopic assessment of inflammatory reactions in lumpfish skin/musculature after im. vaccination, in addition to macroscopic evaluation upon sampling. The fish displayed normal behaviour prior to sampling. In vaccinated fish, residual vaccine was observed in between the skin and muscular layer, evenly distributed from the point of injection and along the dorsal side of the fish, without any clear signs of necrosis or discoloration (Figure 1c).

In HE-stained histologic sections, inflammatory cells appeared in the subcutaneous tissue (hypodermis) at 2 dpi, increasing in numbers as the inflammation progressed (Figure 2c-f) and forming well-defined granulomas around vaccine vacuoles located in the tissue (Figure 2f). Clearly noticeable eosinophilic granulocyte-like cells appeared in small numbers both in between and within granulomas at 2 dpi (Figure 2c), and increased in numbers as the inflammation progressed, nearly becoming the dominating cell type of granulomas at 42 dpi (Figure 2f). Similar cells were also observed in head kidney sections of unvaccinated fish (day 0 samples), vaccinated fish and control fish (PBS), without any apparent difference in cell number during the course of the study (not shown). Formation of connective tissue was observed in increasing levels at the site of inflammation as time progressed, eventually encapsulating granulomas at late stages (Figure 2f). The number of im. skin/muscle samples with eosinophilic granulocyte-like cells and connective tissue in inflammatory lesions is summarized in Table 2. A few eosinophilic granulocyte-like cells were observed in a small inflammatory focus at 7 dpi in one skin/muscle sample among the control fish.

3.2 | Presence of IgM, IgM-positive cells and PCNA-positive cells at the injection site of im. immunized lumpfish

We have previously shown that im. vaccination with oil-formulated bacterial antigens of lumpfish led to a systemic antibody response (Erkinharju et al., 2017). To investigate local distribution of IgM and presence of proliferating cells, we performed IHC on tissue sections obtained from samples taken at the administration site in skin/muscle tissue of im. injected lumpfish. In general, diffuse intercellular red staining was observed in inflamed hypodermal tissue at the site of inflammation at 2 dpi (Figure 3b). While the inflammation foci progressed into granulomas, intensity of the staining was generally reduced (Figure 3c–e).

IgM^+ cells were observed at the site of inflammation in small numbers at 21 and 42 dpi, located in between, but not within, granulomas (Figure 3d and 3e). IgM^+ cells were also observed in head kidney sections of unvaccinated fish (day 0 samples), vaccinated fish and control fish, without any apparent difference in cell number (not shown). A few proliferating cells ($PCNA^+$ cells) started appearing at 2 dpi (Figure 4b)

TABLE 2 Summary of histological and immunohistochemical assessment of skin/muscle sections from im. vaccinated lumpfish at 0, 2, 7, 21 and 42 days post-immunization (dpi). Sections were analysed for the presence of eosinophilic granulocyte-like cells and connective tissue formations, and for positive staining of IgM and PCNA, in inflammatory lesions. Vaccinated fish (VAX), control fish (PBS). The total number of samples (*n*) per group is *n* = 4

	Samples with eosinophilic granulocyte-like cells		Samples with connective tissue formations		Samples positive for secreted IgM		Samples with IgM^+ cells		Samples with $PCNA^+$ cells	
dpi	PBS	VAX	PBS	VAX	PBS	VAX	PBS	VAX	PBS	VAX
0	0		0		0		0		0	
2	0	3	0	3	0	4	0	0	0	2
7	1	4	0	4	0	4	0	0	0	3
21	0	4	0	4	0	3	0	4	0	4
42	0	4	0	4	0	3	0	4	0	4

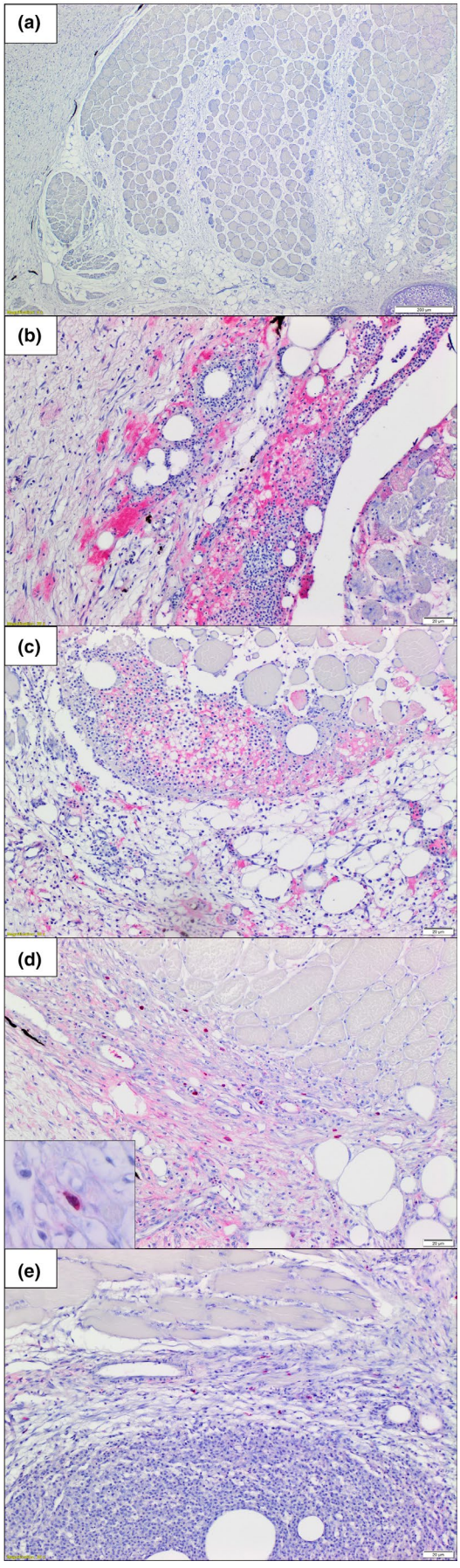

FIGURE 3 Immunohistochemistry sections of im. vaccinated lumpfish at 2 (b), 7 (c), 21 (d) and 42 (e) days post-immunization (20× magnification). Insert in (d) shows a positive cell at higher magnification (60×). Unvaccinated fish (a), day 0 samples, included for reference (4× magnification). Red colour indicates the presence of immunoglobulin *M* (IgM). Haematoxylin was used as counterstain. Scale bar is 200 μm for a) and 20 μm for b–e)

in some skin/muscle sections, and increased in numbers as the inflammation progressed, located both in between and within granulomas in the inflamed tissue (Figure 4). Compared to the HE-stained tissue sections and based on the staining pattern, it appeared that some of the proliferating cells might be eosinophilic granulocyte-like cells. IgM^+ cells did not show any clear indication of undergoing proliferation. The number of im. skin/muscle samples with positive IHC staining is summarized in Table 2. None of the control fish demonstrated vaccine-induced inflammatory lesions and granulomas.

3.3 | Immunoglobulin and immune cell marker gene expression in head kidney, spleen and skin/muscle tissues of immunized lumpfish

qPCR was used to measure mRNA expression of sIgM, mIgM, IgD, TCRα, CD3ε and MHCIIβ genes in samples from im. and ip. vaccinated lumpfish and control fish from the different sampling points. Skin/muscle, spleen and head kidney were included in the study. No statistical tests on differences in expression level were performed due to the low number of individuals included. For skin/muscle samples from im. vaccinated lumpfish, we found a trend of increased expression for sIgM and mIgM at 21 dpi and 42 dpi for some of the vaccinated fish compared with the control fish (Figure 5). The differences in expression levels of the other genes between skin/muscle from vaccinated fish and skin/muscle from controls (IgD, TCRα, CD3ε and MHCIIβ) were negligible. Head kidney and spleen samples demonstrated small changes for all selected genes—in samples from vaccinated fish compared to controls, irrespective of tissues, except for a possible increase in mIgM and IgD expression for ip. vaccinated fish at 42 dpi (Figs 6 and 7).

4 | DISCUSSION

In this study, we have investigated local immune responses and inflammatory processes in skin/muscle tissue of lumpfish intramuscularly injected with a polyvalent oil-based vaccine, at early and late time points post-immunization. We assessed the general inflammatory reactions at the administration site with histology/histopathology, observed the presence and distribution of IgM (both intercellular and positive-staining cells) and PCNA-positive cells in the tissue by immunohistochemistry and analysed the gene expressions of sIgM, mIgM, IgD, TCRα, CD3ε and MHCIIβ in skin/muscle, head kidney and spleen samples of immunized fish by qPCR. Tissue samples of

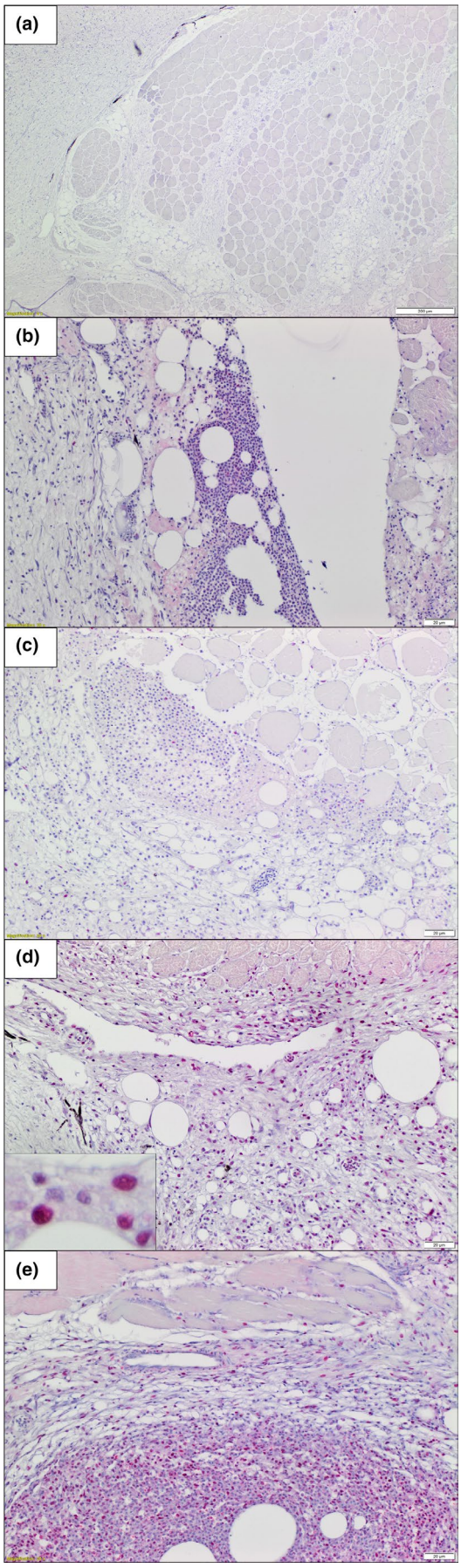

FIGURE 4 Immunohistochemistry sections of im. vaccinated lumpfish at 2 (b), 7 (c), 21 (d) and 42 (e) days post-immunization (20× magnification). Insert in (d) shows positive cells at higher magnification (60×). Unvaccinated fish (a), day 0 samples, included for reference (4× magnification). Red colour indicates the presence of proliferating cell nuclear antigen (PCNA). Haematoxylin was used as counterstain. Scale bar is 200 μm for (a) and 20 μm for (b–e)

lumpfish in the present study were sampled material from an earlier immunization trial with an oil-based vaccine containing formalin-inactivated atypical *Aeromonas salmonicida* A-layer types V and VI, *Vibrio anguillarum* serotype O1 and *Moritella viscosa* sp. (Erkinharju et al., 2017). It needs to be emphasized that results in the present study are based on analysis of lumpfish with a sample size of four fish per group ($N = 4$). Consequently, we have not performed statistical analysis of the quantitative qPCR data presented in the results.

It has been shown that the extracellular A-layer protein (A-protein) of *Aeromonas salmonicida* binds to rabbit IgG and human IgM under certain conditions (Phipps & Kay, 1988). Sinyakov, Dror, Zhevelev, Margel, and Avtalion (2002) observed groups with low or high antibody activity levels against the A-protein in farmed goldfish (*Carassius auratus* L.); however, the authors argued that the high activity in some fish probably stemmed from antibodies developed from natural immunization with the pathogen in the holding pond. However, little is known of the "non-specific" binding capacity of lumpfish antibodies to the A-protein. Bilal et al. (2016) observed very strong binding activity of lumpfish IgM to magnetic protein A beads, which is a different surface antigen, originally found in the cell wall of the *Staphylococcus aureus* bacteria. We have previously shown in an immunization study with *Aeromonas salmonicida* that the majority of both vaccinated lumpfish and unvaccinated control lumpfish displayed no or low IgM response to purified A-protein by ELISA (Erkinharju et al., 2018). A characteristic feature of oil-adjuvanted vaccines is the slow release of antigen from inside the vaccine vacuoles to the surrounding tissue environment, thus creating a long-lasting depot effect (Herbert, 1968). This might lead to positive staining of residual antigens, along the rim and within the lipid droplet vacuoles when using specific antibodies against the vaccine antigens (Afonso, Gomes, Silva, Marques, & Henrique, 2005; Coscelli, Bermúdez, Losada, Santos, & Quiroga, 2015; Haugarvoll, Bjerkås, Szabo, Satoh, & Koppang, 2010; Mutoloki, Alexandersen, Gravningen, & Evensen, 2008). We did not observe any positive staining of this kind with the α-IgM antibody in any of the im. vaccinated lumpfish, which would be expected if the A-protein antigen bound unspecifically to lumpfish IgM. As such, we interpret the diffuse intercellular staining in the immunohistochemistry assay (Figure 2), as positive staining of soluble or secretory IgM present within the tissue due to local immune responses to the injected vaccine.

We observed diffuse intercellular staining of IgM at the site of inflammation in vaccinated lumpfish, first at 2 days post-immunization, followed by 7, 21 and 42 dpi. In our previous study, we demonstrated high specific IgM responses against atypical *A. salmonicida*

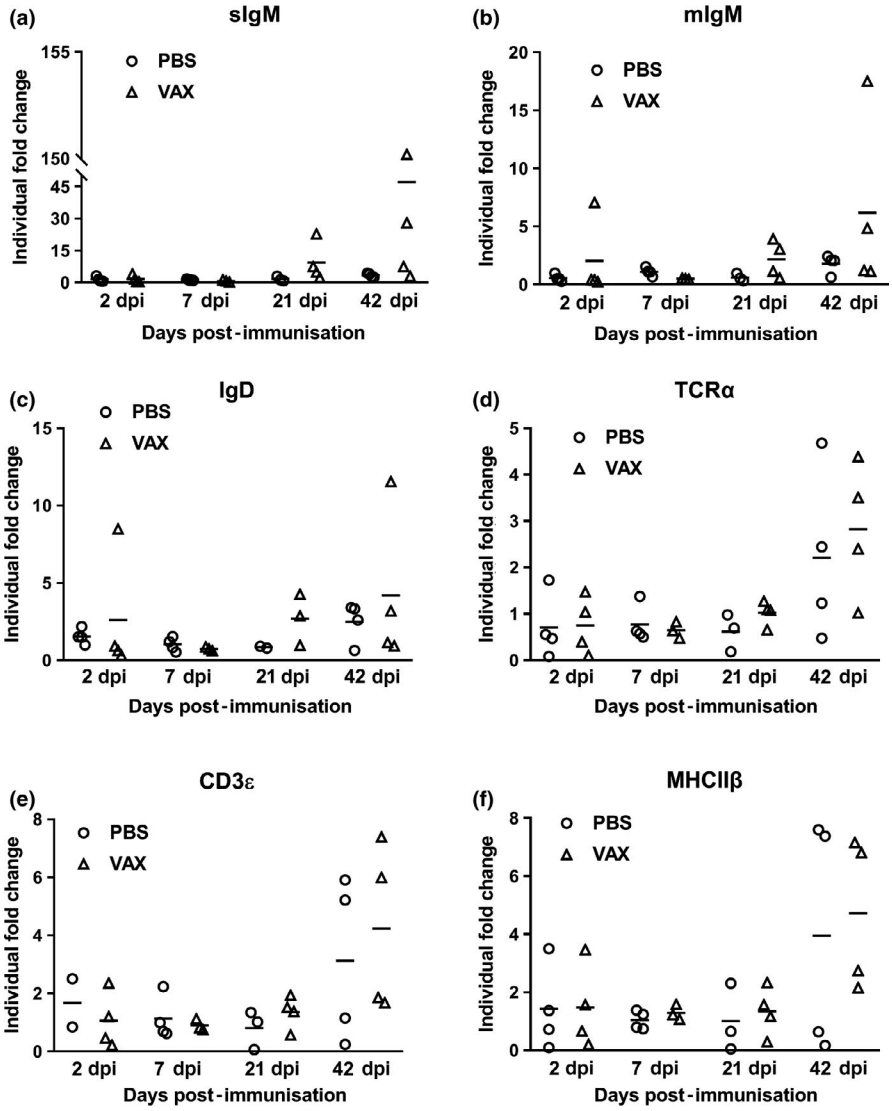

FIGURE 5 Fold change of immune genes sIgM (a) and mIgM (b), IgD (c), TCRα (d), CD3ε (e) and MHCIIβ (f) in skin/muscle samples obtained from im. injected lumpfish at 2, 7, 21 and 42 days post-immunization (dpi), when compared to reference gene EF1α. The number of individual data plots (*n*) per group is $n = 4$, except for VAX at 7 dpi (sIgM, mIgM, IgD, TCRα, CD3ε, MHCIIβ) and 21 dpi (IgD), and PBS at 21 dpi (sIgM, mIgM, TCRα, CD3ε, MHCIIβ) where $n = 3$, and PBS at 2 (CD3ε) and 21 dpi (IgD) where $n = 2$. Mean indicated by black line (-). Vaccinated fish (VAX), control fish (PBS)

A-layer types V and VI in serum samples of lumpfish both im. and ip. vaccinated with the same vaccine as in the present study, at 42 and 84 dpi in addition to low responses at 21 dpi (Erkinharju et al., 2017). This indicated that the presence of IgM within the vaccine administration site at early time points might likely be natural IgM antibodies present in the fish, before an acquired specific IgM response had been developed. Natural antibodies are secreted immunoglobulins mainly produced by a subset of B lymphocytes (termed B-1 cells) in the absence of prior immune activation by stimulation of exogenous antigenic exposure, which may be present already at early developmental stages and are often seen in relatively high levels in fish blood (Grönwall, Vas, & Silverman, 2012; Uribe et al., 2011; Whyte, 2007). Such antibodies have shown to provide both antibody activity and/or protection against fish pathogenic bacteria (Magnadottir, Gudmundsdottir, Gudmundsdottir, & Helgason, 2009; Sinyakov et al., 2002; Wang, Ji, Shao, & Zhang, 2012), viruses (Gonzalez, Matsiota, Torchy, Kinkelin, & Avrameas, 1989) and parasites (Katzenback, Plouffe, & Belosevic, 2013). Teleost IgM is mainly found in the blood circulation and has been suggested to function as a form of backup defence system that triggers when the primary defence barrier (e.g. skin and mucosal surfaces) fails to prevent invading pathogens to enter the systemic environment (Munang'andu, Mutoloki, & Evensen, 2015). This may be the case in our study where the vaccine was delivered intramuscularly by injection, potentially bypassing the first line of defence in the fish's immune system, and thereby inducing an early IgM response from the accumulation of vaccine antigens at the injection site.

At 21 and 42 dpi, we observed cellular IgM (membrane-bound) in the form of IgM^+ cells spread around in small numbers within the inflammatory lesion of vaccinated lumpfish. IgM can be expressed on the surface of B lymphocytes (B cells), and Rønneseth, Ghebretnsae, Wergeland, and Haugland (2015) reported peripheral blood and spleen as the sites with the highest fractions of IgM^+ B cells among isolated lumpfish leucocytes. The role of B cells in teleost immunity has traditionally been perceived as producers of immunoglobulins for specific adaptive immune responses, up until it was discovered that they also possess potent phagocytic capabilities (Li et al., 2006), which also recently have been reported in lumpfish (Rønneseth et al., 2015). Other IgM^+ cells than B cells have been reported in vaccine-induced granulomatous reactions in fish,

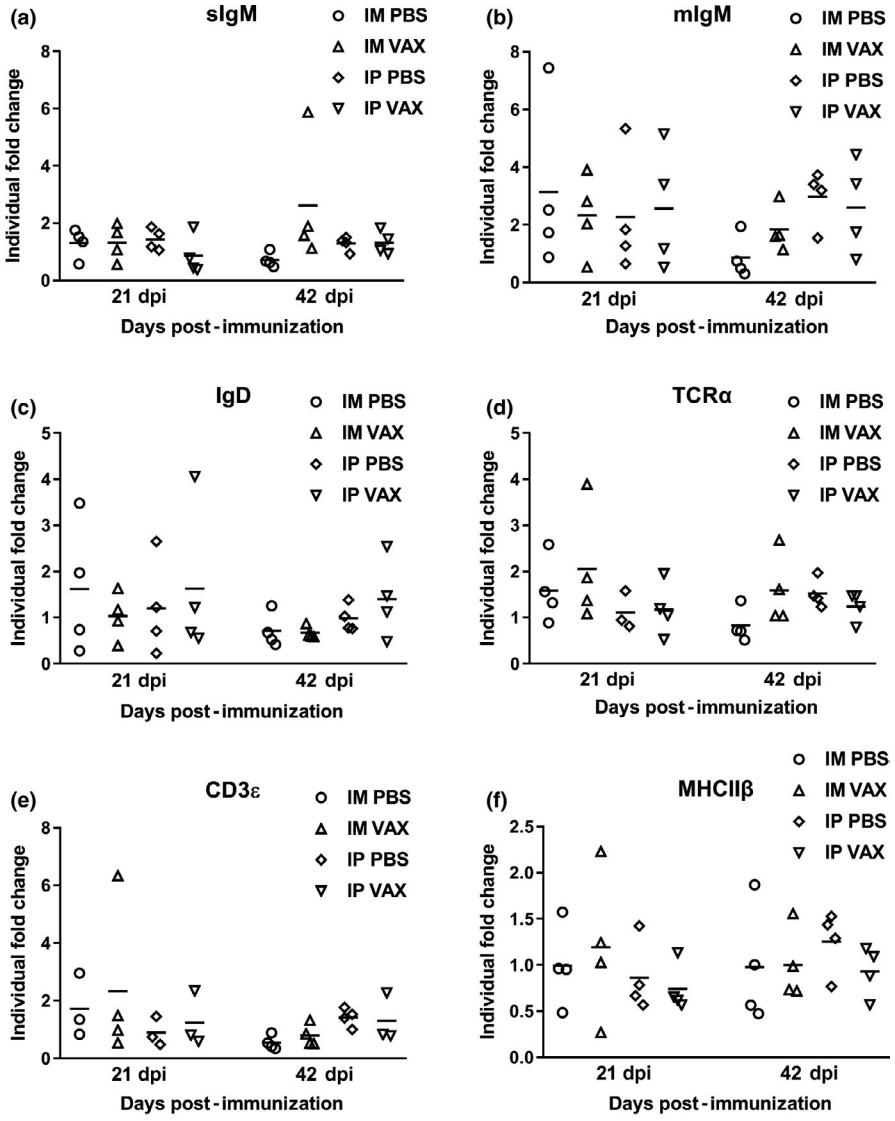

FIGURE 6 Fold change of immune genes sIgM (a), mIgM (b), IgD (c), TCRα (d), CD3ε (e) and MHCIIβ (f) in head kidney samples obtained from im. and ip. injected lumpfish at 21 and 42 days post-immunization (dpi), when compared to reference gene EF1α. The number of individual data plots (n) per group is $n = 4$, except for IP PBS at 21 dpi (TCRα, CD3ε), IM PBS and IP VAX at 21 dpi (CD3ε), and IP VAX at 42 dpi (CD3ε), where $n = 3$. Mean indicated by black line (-). Vaccinated fish (VAX), control fish (PBS)

such as Mott cells in ip. vaccinated Atlantic salmon (Haugarvoll et al., 2010). These cells are described as a dysfunctional form of the plasma cell, distinctly identifiable in histological sections, and displayed variable staining for IgM, possibly due to either intracellular accumulations of IgM or expression of IgM molecules on the cell surface. Such cells were not observed in our sections and have not been described in lumpfish.

Few studies have investigated the role of lymphocytes in granulomatous inflammatory reactions in vaccinated fish, and the ones that are available focus primarily on injection into the abdominal cavity. IgM^+ cells in vaccine-induced granulomas were first reported by Haugarvoll et al. (2010) in ip. vaccinated Atlantic salmon several months after vaccination. They observed scattered cells embedded in the fibrous tissue formed within the abdominal cavity, some located in the periphery of granulomas, but rarely in the centre. In a different study, Gjessing, Falk, Weli, Koppang, and Kvellestad (2012) discovered two different types of inflammatory patters in peritoneal tissue of Atlantic cod (*Gadus morhua* L.) ip. injected with incomplete Freund's adjuvant, where the second type consisted of four stratified layers at 7 days post-injection, and part of the outermost fourth layer was dominated by mononuclear cells and some lymphocytes. Noia et al. (2014) tested several adjuvant-containing vaccines by different injection directions into the abdominal cavity of turbot (*Scophthalmus maximus* L.), and lymphocytes were only occasionally observed in the granulomatous reactions, up to five weeks after injection. Afonso et al. (2005) observed a steady increase in lymphocyte numbers throughout the sampling period of ip. vaccinated sea bass (*Dicentrarchus labrax* L.), which reached high values at both 30 and 60 days after injection. However, no further characterization of the lymphocytes registered in these studies was reported. In addition, the type of vaccine used for injection might also be of significance, as reported by Castro et al. (2014), where intramuscularly immunized rainbow trout (*Oncorhynchus mykiss* L.) showed early recruitment of high levels of B lymphocytes (both IgM^+ and IgT^+) into the muscle, when injected with a DNA vaccine against VHSV, while fish injected with complete Freund's adjuvant mainly showed

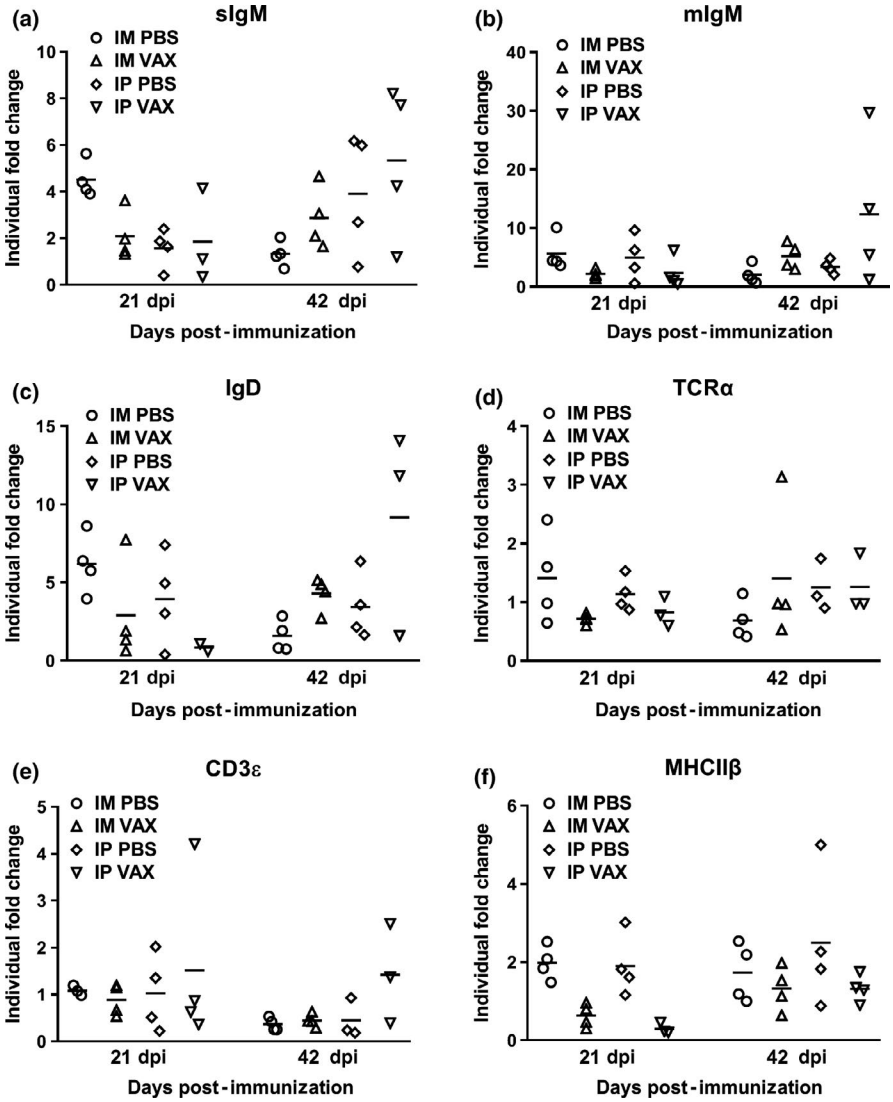

FIGURE 7 Fold change of immune genes sIgM (a), mIgM (b), IgD (c), TCRα (d), CD3ε (e) and MHCIIβ (f) in spleen samples obtained from im. and ip. injected lumpfish at 21 and 42 days post-immunization (dpi), when compared to reference gene EF1α. The number of individual data plots (n) per group is $n = 4$, except for IP VAX at 21 dpi (sIgM, TCRα, MHCIIβ) and 42 dpi (IgD, TCRα, CD3ε), IM PBS at 21 dpi (CD3ε) and IP PBS at 42 dpi (TCRα, CD3ε), where $n = 3$, and IP VAX at 21 dpi (IgD) where $n = 2$. Mean indicated by black line (-). Vaccinated fish (VAX), control fish (PBS)

infiltration of granulocyte-/monocyte-type cells. Our results are in agreement with several of these authors' observations, although it is uncertain whether the amount of IgM^+ cells would remain stable or increase/decrease as the inflammation progressed further beyond the time range of this study.

The qPCR results displayed an increase in mRNA expression for sIgM and mIgM in skin/muscle tissue of some im. vaccinated lumpfish at 21 and 42 dpi, and only small differences between vaccinated and control fish in head kidney and spleen samples, except for mIgM in the spleen of ip. vaccinated fish at 42 dpi. These results are in accordance with our observations of IgM^+ cells at 21 and 42 dpi in im. vaccinated skin/muscle sections, and with our observations of no apparent differences in cell numbers of IgM^+ cells in head kidney of both vaccinated and control fish. Unfortunately, as stated in the Materials and methods section, we did not have enough material for both qPCR and IHC analyses of the spleen and were therefore unable to look closer into the presence and distribution of IgM^+ cells within this organ. This indicates that there might have been local production of IgM within the skin/muscle of im. vaccinated lumpfish, without clear signs of corresponding immunological activity in the head kidney/spleen.

As for the other genes analysed in our study, we detected a difference for IgD between ip. vaccinated and control fish in spleen at 42 dpi. The tissue distribution of IgD in juvenile lumpfish was recently reported, and high gene expression numbers were demonstrated in peripheral blood and spleen (Espmark et al., 2019). Membrane-bound form of IgD has also been identified in lumpfish (Haugland et al., 2018). We only observed small changes between vaccinated and control fish for the T-cell marker genes (TCRα, CD3ε) and MHC class IIβ (MHCIIβ). Both T cells and MHC class II are important parts of the adaptive immune system for ensuring proper exogenous antigen presentation and development of specific antibody responses (Tizzard, 2018). However, there is still a need for more basic knowledge regarding these factors in the lumpfish immune system, in order to better understand their exact functions during vaccination of this fish species.

Im. injection of oil-based vaccine into lumpfish skin/muscle leads to infiltration of inflammatory cells that increased in numbers over time, eventually encompassing and developing granulomas around vaccine droplets within the tissue. Such reactions are a distinctive form of chronic inflammation, where granuloma

formations within different organs are regarded as a hallmark of several infectious disorders, in addition to non-infectious causes such as foreign bodies (Shah, Pritt, & Alexander, 2017). Late-stage granulomas were clearly encapsulated by fibrous-looking connective tissue, which is often seen as part of prolonged inflammatory vaccine reactions in fish (Poppe & Koppang, 2014). As mentioned elsewhere, different types of leucocytes participate in the inflammatory response, especially neutrophilic granulocytes (neutrophils), macrophages and lymphocytes. Currently, there is a lack of specific cell markers for properly identifying most of the lumpfish leucocytes within histological sections. Identification based on morphological characteristics might also be challenging for certain cell types, for example lumpfish neutrophils, which do not display the typical multilobulated nucleus found in salmon and trout neutrophils (Haugland et al., 2018). Haugland et al. (2012) and Rønneseth et al. (2015) applied different cytochemical stains to sorted lumpfish leucocytes and observed positive staining of neutrophils with myeloperoxidase (MPO) stain, and strong positive staining of monocytes/macrophages, in addition to positive staining of IgM^+ B cells, with acid phosphatase (AcP) stain. We tried applying these stains to our formalin-fixed, paraffin-embedded lumpfish tissue sections, but the procedures resulted in either too much background stain (with MPO) or no noticeable staining at all (with AcP) (results not shown).

In vertebrates, including teleost fish, influx of neutrophilic granulocytes during the initial inflammatory response is an essential part of the body's first line of defence against invading pathogens (Havixbeck & Barreda, 2015). However, in our study we observed increasing numbers of granular cells within the inflammatory lesions over time, which stained strongly eosinophilic and, as such, closely resemble the eosinophilic granulocytes (eosinophils). Such cells have not been identified among isolated lumpfish leucocytes, but have been observed in tissue sections from lumpfish (Haugland et al., 2018). Eosinophils are usually described as leucocytes with a basophilic bean-shaped or lobulated nucleus, a lightly stained cytoplasm with acidophilic or eosinophilic granules, and may aid the body's defence system by combating invading parasites or modulating allergic reactions. In lower vertebrates, both immature and mature variants of the cells might be observed, as parts of the cell development take place in the blood circulation, and in many fish species, they are also the most abundant type of granulocyte (McMillan & Harris, 2018). As a side note, there have also been descriptions of a different eosinophilic cell type in teleosts, bearing structural and functional resemblance to mammalian mast cells (MCs), termed eosinophilic granular cells (EGCs), which have caused some confusion within the scientific literature. Such cells are primarily located in the digestive tract and gills and have been observed in low-to-high numbers as part of the host inflammatory response to ip. injected vaccines, parasite infestations or different types of noxious stimuli in several fish species (reviewed in Reite & Evensen, 2006). It would be beneficial to follow-up with supplementary analysis, such as direct study of cellular morphology through electron microscopy, or IHC with specific cell markers, for a more thorough characterization of the cells observed in our study.

To conclude, im. vaccination with the oil-based vaccine induced local inflammatory reactions that during the first days after immunization showed recruitment of inflammatory cells into the injection site, which over time formed well-defined granulomas surrounding vaccine oil droplets. Especially prominent were the eosinophilic granulocyte-like cells that increased in cell numbers over time, indicating that they might have an important function in the lumpfish immune system. There was a presence of secretory intercellular IgM during early time points, while membrane-bound cellular IgM was first seen at 21 days post-immunization. The qPCR analysis showed indicative increased expression of secretory and membrane-bound IgM mRNA in the skin/muscle at 21 and 42 days post-immunization, but no such development was seen in the head kidney or spleen. These findings suggest that there might be local immunoglobulin M production at the administration site of im. vaccinated lumpfish. Future work should look closer into these factors, in order to gain better understanding of the relationship between local and systemic immune responses in Atlantic lumpfish after vaccination.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ORCID

Roy A. Dalmo https://orcid.org/0000-0002-6181-9859
Tore Seternes https://orcid.org/0000-0001-5010-5095

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