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Immune parameters in the intestine of wild and reared unvaccinated and vaccinated Atlantic salmon (*Salmo salar* L.)

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ABSTRACT

Forming a barrier to the outside world, the gut mucosa faces the challenge of absorbing nutrients and fluids while initiating immune reactions towards potential pathogens. As a continuation to our previous publication focusing on the regional intestinal morphology in wild caught post smolt and spawning Atlantic salmon, we here investigate selected immune parameters and compare wild, reared unvaccinated and vaccinated post smolts.

We observed highest transcript levels for most immune-related genes in vaccinated post smolts followed by reared unvaccinated and finally wild post smolts, indicating that farming conditions like commercial feed and vaccination might contribute to a more alerted immune system in the gut. In all groups, higher levels of immune transcripts were observed in the second segment of mid-intestine and in the posterior segment. In the life stages and conditions investigated here, we found no indication of a previously suggested population of intestinal T cells expressing MHC class II nor RAG1 expression.

1. Introduction

To understand and prevent infections, comprehensive knowledge of mucosal immunology is necessary. In mammals, advanced information has been available for years, as opposed to teleosts such as Atlantic salmon where knowledge within functional anatomy is still in its infancy. Mucosal surfaces, like teleost intestine, gills and skin are important sites of antigen recognition (Gomez et al., 2013). These mucosal surfaces create a barrier to the external environment, and the mucosal immune system must under normal conditions constantly maintain homeostatic balance but be able to react swiftly towards pathogens (Sansonetti, 2004). In the intestinal mucosa, teleosts lack Peyer's patches with lymphoid follicles as in mammals, but have diffuse gut-associated lymphoid tissue (GALT) consisting of scattered immune cells along the intestinal tract (Zapata and Amemiya, 2000). These immune cells include macrophages, granulocytes, lymphocytes, plasma cells and mast cells (Hellberg et al., 2013; Salinas et al., 2011). Rather than immunoglobulin (Ig) A, IgM and IgT are the main immunoglobulins found in the teleost intestine (Salinas et al., 2011), where the latter is thought to have a specific mucosal function (Tadiso et al., 2011; Zhang et al., 2010). In the intestinal epithelium of both mammals and fish, the intraepithelial lymphocytes (IELs) mainly comprise T cells (Hayday et al., 2001; Rombout et al., 2011). In mammals, the intraepithelial T cells have proven to be an extremely heterogeneous group, involving cells expressing γδ or αβ T cell receptor (TCR), where the latter can express co-receptors cluster of differentiation 4 (CD4) or 8αβ (CD8αβ). Additionally, intraepithelial T cells may express CD8αα that act as a TCR repressor rather than a co-receptor (Cheroutre et al., 2011). IELs expressing MCH class II have moreover been described in rat (Kearsey and Stadnyk, 1997). The sequences for the TCRα, β, γ, δ chains and the co-receptors CD4 and CD8 have been identified in teleosts (Castro et al., 2011), however there is still limited knowledge on the teleost distribution of the different intestinal IEL sub-populations (Fischer et al., 2013). Somewhat disputed, a group of IELs of extrathymic origin has been proposed in mouse, developing and undergoing TCR rearrangement locally in the gut (Rocha et al., 1994). Based

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on findings of intestinal transcripts of recombination activating gene 1 (RAG1) that is expressed in immature lymphocytes and involved in recombination of the TCR and immunoglobulin genes (Schatz et al., 1989), such a cell subset has also been suggested for teleosts (Rombout et al., 2011).

We have previously published an article focusing on the intestinal morphology of the different intestinal regions of the wild Atlantic salmon (Løkka et al., 2013). In the present investigation, we are aiming at expanding this work by investigating the immunological state of the wild Atlantic salmon intestine, focusing on possible regional differences. Further, we wanted to address possible differences between wild and reared salmon in the intestinal expression of some key immune parameters. Farmed salmon are exposed to conditions unlike those of wild fish, like high stocking densities, commercially produced feed based on terrestrial plant ingredients and vaccine injections. Several studies have shown intestinal inflammation and morphological changes caused by vegetable ingredients in the feed (Bæverfjord and Krogdahl, 1996; Dale et al., 2009; Moldal et al., 2014; Penn et al., 2011). The standard intraperitoneal injection of oil-adjuvanted vaccine used in the industry today may induce local but also general inflammatory conditions like auto-immunity (Koppang et al., 2008; Haugarvoll et al., 2010). Although intestinal vaccine effects have been examined in some studies (Koppang et al., 1998), possible differences in expression of intestinal immune-related genes between wild and reared Atlantic salmon have to our knowledge not been reported.

We here investigate five different regions of the intestine from wild spawning salmon, wild post smolts and reared unvaccinated and vaccinated post smolts for mRNA levels of selected T cell markers, MHC class II, IgM, IgT, RAG1 and 2 using real-time PCR. Protein expression of CD3ε, constitutively expressed on T cells, major histocompatibility complex MHC class II and Ig was moreover studied by immunohistochemistry and double-labeling of CD3ε and MHC class II by immunofluorescence.

2. Materials and methods

2.1. Animals

Wild Atlantic salmon post smolts and sexually mature spawning salmon in addition to reared vaccinated and unvaccinated post smolts were sampled for this study.

The wild post smolts had a mean weight of 150 g and were collected from the Norwegian Sea using a trawl net in the summer of 2009. All wild post smolts had feed in the stomachs, which indicate that they had been eating up until sampling. The spawning salmon had a mean weight of 10 kg and were sampled by dip net from Hellefossen in River Drammenselva, Buskerud, Norway, in the autumn of 2009. The wild spawning salmon had empty stomachs, and could have stopped eating months before sampling time when they entered the river to spawn (Prof. Trygve Poppe, personal communication). The two groups of wild salmon represent essentially different stages of the wild salmon life cycle, diverging in age, habitat and feeding status (Løkka et al., 2013).

Domesticated Atlantic salmon reared at Matre Research Station, Matredal, Norway, corresponding to the wild post smolt group in size (150 g) and life stage, were sampled. One group remained unvaccinated, while the other was vaccinated approximately four months before sampling by intraperitoneal injection according to standard procedures with an oil-adjuvanted combination vaccine containing inactivated antigens from *Aeromonas salmonicida*, *Vibro salmonicida*, *Vibrio anguillarum*, *Moritella viscosa* and inactivated infectious pancreas necrosis virus (Norvax Minova 6 vet, Intervet/MSD Animal Health, Boxmeer, Netherland). The fish were transferred to sea water at a time corresponding to the natural life cycle and feeding on a standard commercial diet up until sampling. The fish were held at optimal water conditions, ocean water was taken from 90 m depth and the oxygen saturation was 100% in inlet and 80% in outlet flow. All fish were euthanized according to regulations given by the Norwegian Directorate of Fisheries (Forskrift om drift av akvakulturanlegg §34. Avlivning av fisk).

2.2. Tissue sampling

From all fish groups, samples were obtained from five different locations of the intestine including (1) pyloric caeca, (2) first segment of the mid-intestine with pyloric caeca, (3) first segment of the mid-intestine posterior to the pyloric caeca, (4) second segment of the mid-intestine and (5) posterior segment (Fig. 1) (Løkka et al., 2013).

2.3. Gene expression studies

Tissue segments (5 × 5 mm) from the five intestinal regions ($n = 9$ for wild post smolts, reared unvaccinated and vaccinated post smolts; $n = 5$ for wild spawning salmon) were carefully dissected to remove all connective tissue at the serosal surface. Samples were immediately fixed in RNA later® and stored at 4 °C. RNA extraction was performed using the TRIzol treatment (Invitrogen, California, USA) and the NucleoSpin® RNAII (Macherey & Nagel, Düren, Germany) including the integrated DNase step. Extracted RNA was quantified spectrophotometrically using a Biospec-Nano (Shimadzu Corporation, Kyoto, Japan) and stored in −80 °C. Reverse transcription was performed using ~1 μg RNA/reaction and M-MLV reverse transcriptase (Promega, Madison, WI, USA) or SuperScript III reverse transcriptase (Invitrogen). The synthesis was prepared with oligo(dt), random hexamer and RNase inhibitor to prevent RNA degradation.

Quantitative real-time PCR was performed in a 7900HT fast real-time PCR system (Applied Biosystems Carlsbad, CA, USA) according to the producer's instructions and running 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 15 s at 58 °C and 60 s at 60 °C. Reactions were run in triplicates using 13 μl of reaction volume including TaqMan Gene Expression Master Mix (Applied Biosystems) and cDNA template corresponding to ~10 ng RNA. A control sample lacking the template was included for each master mix in the experiments. Genes analyzed were CD3ζ, CD4-2a, CD8α, TCRα, TCRδ, secretory and membrane bound IgM (sIgM, mIgM), IgT, major histocompatibility complex (MHC) class II, RAG1 and RAG2. The level of expression was normalized using elongation factor $1A_B$ ($EF1A_B$) as a reference gene (Olsvik et al., 2005). Sequences of primers and probes are listed in Table 1. When possible, primers and probes were designed to span across intron sections. The data was analyzed using Sequence Detection Systems Software v2.3 (Applied Biosystems).

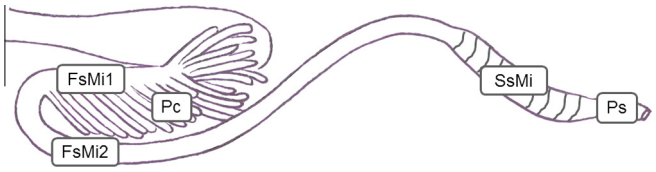

Fig. 1. Tissue samples from the Atlantic salmon intestine. Tissue samples from five different regions of the intestine including pyloric caeca (Pc), first segment of mid-intestine with pyloric caeca (FsMi1), first segment of mid-intestine posterior to the pyloric caeca (FsMi2), second segment of mid-intestine (SsMi) and posterior segment (Ps).

Table 1
Primers and probes used for real-time PCR.

Genes GenBank accession No.	Gene sequence (5′ → 3′)	Relative transcript levels wild post smolt – SsMi
$EF1A_B^*$	F-TGCCCCTCCAGGATGTCTAC	
57 bp	R-CACGGCCCACAGGTACTG	
BG933897	P-FAM-AAATCGGCGGTATTGG-MGB	
CD3ζ	F-AACAGGGATCCAGAGAGTGCTG	2.7×10^{-3}
72 bp	R-AAGGGACGTGTAAGTGTCGTCA	
BT060238	P-FAM-ACGGCACGCGATAATCGCAGGA-BHQ	
CD4-2a	F-GCCCCTGAAGTCCAACGAC	3.0×10^{-3}
79 bp	R-AGGCTTCTCTCACTGCGTCC	
EU409792	P-FAM-CCGCACACTAGAGGGTCCACCACG-BHQ	
CD8α	F-ACTTGCTGGGCCAGCC	2.0×10^{-3}
96 bp	R-CACGACTTGGCAGTTGTAGA	
NM_001123583	P-CGACAACAACAACCACCACG-BHQ	
TCRα	F-AACTGGTATTTTGACACAGATGC	7.2×10^{-3}
101 bp	R-ATCAGCACGTTGAAAACGAT	
AY552002	P-FAM-ACCATTCTGGGCCTGAGAATTCTGT-BHQ	
TCRδ	F-AGCGTTGTGAGATGGATGGC	0.5×10^{-3}
94 bp	R-GCAGTTGTAGCCGTGGTGTTATAG	
EG821964	P-FAM-AGCCGCCCGTTGATGATAAAGATGATTC-BHQ	
MHC class II	F-CCACCTGGAGTACACACCCAG	417.0×10^{-3}
96 bp	R-TTCCTCTCAGCCTCAGGCAG	
X70165	P-FAM-TCCTGCATGGTGGAGCACATCAGC-BHQ	
RAG1	F-GAGGCCATGATGCAAGGC	–
114 bp	R-CTTGACGGTGCGGTACATCT	
NM_001124737	P-FAM-ATCCTGCTGTGTGTCTGGCCATC-BHQ	
RAG2	F-GTTCTTCGAGACGTTCAAACAG	0.035×10^{-3}
89 bp	R-TTCACTGCAGTCAGTGGTTG	
DY697789	P-FAM-ACGTTAGCTACTTGAGCAGGAGCCAC-BHQ	
sIgM	F-TGCGCTGTAGATCACTTGGAA	15.2×10^{-3}
101 bp	R-GCCAGCAGAAAGACAGATGGA	
S48652	P-FAM-AGGGAGACCGGAGGAGATCCACAGC-BHQ	
mIgM	F-TGCGCTGTAGATCACTTGGAA	0.2×10^{-3}
104 bp	R-ATGGTGTTGCTGCATGGACA	
S48658	P-FAM-AGGGAGACCGAGTGTCTCGTGTTGACTG-BHQ	
IgT	F-CAGCAGTCTGCTGAAGGTC	0.5×10^{-3}
111 bp	R-GGTTCTGTTTTGGAGATCG	
GQ907003/4	P-FAM-CTGCACCACACAGCTGTACTTGACC-BHQ	

Gene assays used for quantitative real-time PCR with accession numbers. Primers and probes were designed to span intron sections and used in a concentration of 200 nM (primer) and 50 nM (probe). Star * indicates assays designed by others, $EF1A_B$ (Olsvik et al., 2005). Average transcript levels relative to the $EF1A_B$ are listed in the right column for the second segment of the mid-intestine in the wild post smolt group. SsMi, second segment of the mid-intestine.

2.4. Immunohistochemistry (IHC)

From the five intestinal regions (n = 5 for each fish group), tissue segments (1 × 1 cm) were fixed in 10% buffered formalin for 24 h, routinely dehydrated and embedded in paraffin. 3 μm sections were cut, mounted on glass slides (Superfrost®; Mentzel, Braunsweig, Germany), incubated for 24 h at 37 °C, de-waxed in xylene and rehydrated in graded alcohol baths. Sections were subsequently de-masked by heat-treatment in 0.01 M citrate buffer, pH 6.0, at 120 °C for 15 min followed by treatment with phenylhydrazine (0.05%; Sigma–Aldrich, St. Louis, MO, USA) for 40 min at 37 °C to inhibit endogenous peroxidase.

IHC was performed according to an indirect immunoperoxidase method (EnVision™+ System-HRP; Dako, Glostrup, Denmark) as described elsewhere (Løkka et al., 2013), using polyclonal rabbit antisera recognizing salmon CD3ε (dilution 1:600, (Koppang et al., 2010)), MHC class II β-chain (dilution 1:1600, (Koppang et al., 2003)) and IgM (dilution 1:20,000, (Håvarstein et al., 1988)). In addition, a commercially produced anti-salmonid Ig monoclonal mouse antibody (dilution 1:500; No. IPA-3DI, ImmunoPrecise Antibodies, Vancouver, Canada) not verified for IHC previously was tested. Color development for CD3ε, MHC class II and pAb IgM was performed by 3-amino-9-ethylcabazole (AEC; Dako) and for mAb Ig by 3,3′-diaminobenzidine (DAB; Dako). Sections were investigated using a Leica DMLB microscope and pictures were taken using a Leica DFC295 camera (Leica Microsystems, Wetzlar, Germany). Number of MHC class II^+ cells was blindly counted in the epithelium of five folds from each region in each individual, starting from the fold base and 400 μm up the fold, ensuring an area as similar as possible between the intestinal regions. Counting was performed using Adobe® Photoshop count tool. MHC class II^+ cells in the lamina propria were scarce in all sections and hence not counted. The CD3ε stained sections were scored semi-quantitatively as high density and diffuse outline of immunopositive cells made cell counting impossible. 0 = no immunopositive cells, 1 = few positive cells; 2 = several positive cells and 3 = considerable amount of immunopositive cells. The CD3ε score was performed blindly and made separately for both epithelium and lamina propria.

2.5. Immunofluorescence (IF) double-labeling

To address the possibility of IELs expressing both CD3ε and MHC class II, one individual from each fish group was investigated

by IF double-labeling. Paraffin sections from the five intestinal regions were de-waxed and de-masked as described in the first section of paragraph 2.4. Unspecific binding was prevented by blocking with 10% normal goat serum in phosphate buffered saline (PBS)/0.5% Tween®80 (Sigma–Aldrich) for 30 min. A mix of two antibodies were added and incubated at 4 °C overnight, comprising polyclonal rabbit antisera against salmon MHC class II β-chain (dilution 1:1400, (Koppang et al., 2003)) and a monoclonal mouse antibody recognizing CD3ε in rainbow trout (*Oncorhynchus mykiss*) cross-reacting with salmon CD3ε (Lindholm et al., 2013) (dilution 1:100, (Boardman et al., 2012)). Sections were incubated with a mix of secondary antibodies Alexa Fluor® 488 goat anti-mouse IgG and Alexa Fluor® 594 goat anti-rabbit IgG (dilution 1:400, Molecular Probes, Inc., OR, USA) for 1 h and mounted with polyvinyl alcohol media (pH 8) (Ullevål Apotek, Oslo, Norway). Section investigations were performed using a Zeiss Axio Imager M2 microscope (Carl Zeiss, Oberkochen, Germany) and pictures were taken with a Zeiss Axiocam 506 mono using software Zen Pro 2012.

2.6. Statistical analyses

Statistical analyses were performed using Prism 6.0 (GraphPad Software, San Diego, CA, USA). For the real-time PCR results, a Kolmogorov Smirnov test was used to test data for normal distribution and a Brown–Forsythe test for homogeneity of variance. Log transformation was performed when data were not-normally distributed and/or with non-homogenous residuals. Additionally, visualization of data distribution was performed to ensure normal distribution using JMP 10 (SAS Institute Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) was used to compare transcript levels. Pearson correlation analysis was performed to measure association between genes, and positive correlation was considered as weak when $r = 0–0.3$, moderate when $r = 0.3–0.7$ and strong when $r = 0.7–1$. For the IHC results, due to non-normally distributed data, a non-parametric Kruskal–Wallis test was used to test for differences between regions and groups. Differences were considered statistical significant when $P < 0.05$.

3. Results

3.1. Real-time PCR

3.1.1. T cell markers and MHC class II

In all fish groups, the transcript levels of the investigated T cell markers and MHC class II were highest in the second segment of the mid-intestine or the posterior segment (Fig. 2A–D). Whether there was a significant difference between these two segments and the other regions did however fluctuate, as variation within the groups concealed the moderate differences between the regions.

Among the three post smolt groups, we found gradually rising levels of CD3ζ, CD4-2a, TCRα and MHC class II from wild to reared unvaccinated to vaccinated post smolts (Fig. 3A–D), with significant differences between wild post smolts and reared vaccinated post smolts in all intestinal regions. The differences were most pronounced for MHC class II. There was a strong positive correlation between these four genes ($r = 0.7–0.86$; Fig. 4A–F). CD8α and TCRδ had however lowest transcript levels in the reared unvaccinated group (Fig. 3E and F) and correlation between CD8α and TCRδ was only weak to moderately positive ($r = 0.32$; Fig. 4G).

The ratios of CD4-2a/CD8α and TCRα/TCRδ were significantly higher in the reared unvaccinated and vaccinated post smolts than in the wild post smolt group (Fig. 3G and H).

3.1.2. Immunoglobulins

For all regions and fish groups, the transcript levels of sIgM were about 75 times higher than mIgM and 50 times higher than IgT (Table 1). In the wild spawning salmon, there was no significant

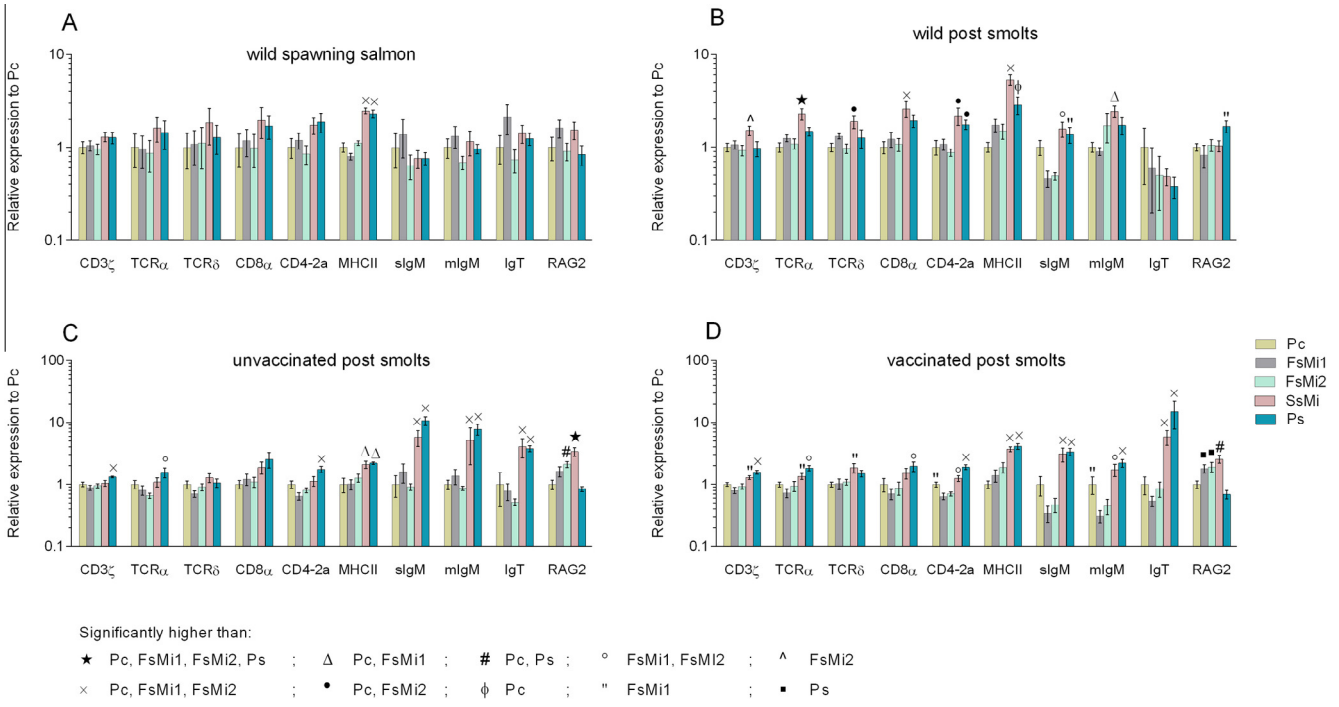

Fig. 2. Transcript levels of selected immune-related genes comparing intestinal regions. Higher transcript levels are observed for most genes in the second segment of mid-intestine (SsMi) and posterior segment (Ps) compared with pyloric caeca (Pc), first segment of mid-intestine with pyloric caeca (FsMi1) and first segment of mid-intestine posterior to the pyloric caeca (FsMi2). (A) Wild spawning salmon, (B) wild post smolts, (C) reared unvaccinated post smolts and (D) reared vaccinated post smolts. Error bars represent standard error of the mean (SEM) and transcript levels are presented relative to $EF1A_B$ and to the pyloric caeca within the group for each gene.

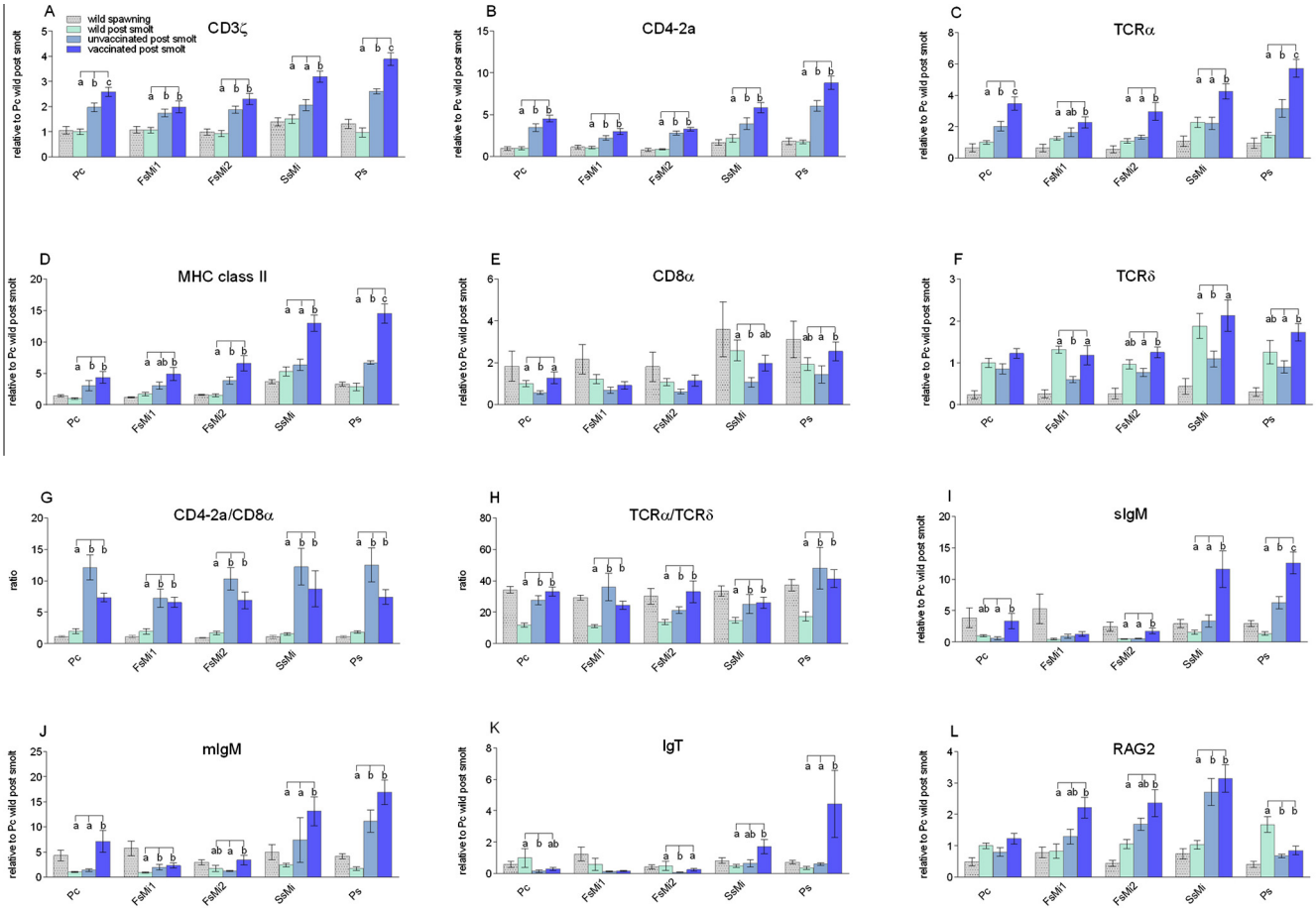

Fig. 3. Transcript levels of selected immune related genes comparing groups. Gradually higher transcript levels from wild to reared unvaccinated to vaccinated post smolts are observed in all intestinal regions for CD3ζ (A), CD4-2a (B), TCRα (C) and MHC class II (D), while CD8α (E) and TCRδ (F) have lowest levels in the reared unvaccinated group. sIgM (I), mIgM (J), IgT (K) and RAG2 (L) have rising levels from wild to reared unvaccinated to vaccinated post smolts in some regions. The ratio of CD4-2a/CD8α (G) and TCRα/TCRδ (H) is higher in the reared than in the wild post smolts. Bars indicating transcript levels for wild spawning salmon are included, but comparison between spawning salmon and post smolts is not performed due to different developmental stages. Error bars represent standard error of the mean (SEM) and transcript levels are presented relative to $EF1A_B$ and to the pyloric caeca for the wild post smolt group. ^{a,b,c}Significant differences between groups are denoted by different letters (ANOVA, $P < 0.05$). Pc, pyloric caeca; FsMi1, first segment of mid-intestine with pyloric caeca; FsMi2, first segment of mid-intestine posterior to the pyloric caeca; SsMi, second segment of mid-intestine; Ps, posterior segment.

difference in transcript levels of IgM and IgT between the different intestinal regions, although the levels were slightly higher in the first segment of the mid-intestine (Fig. 2A). In the wild post smolts, sIgM and mIgM had highest levels in the second segment of the mid-intestine and/or the posterior segment, but with varying significance compared with the other regions (Fig. 2B). In reared unvaccinated and vaccinated post smolts, both IgM genes and IgT were significantly higher in the second segment of the mid-intestine and the posterior segment compared with the other intestinal regions (Fig. 2C and D).

Comparing the three post smolt groups, sIgM and mIgM generally had rising transcript levels from wild to reared unvaccinated to vaccinated post smolts with most pronounced differences in the second segment of the mid-intestine and the posterior segment (Fig. 3I and J). IgT showed a similar pattern in the second segment of the mid-intestine and the posterior segment, while in the remaining regions the transcript level was highest in the wild post smolt group and lowest in the reared unvaccinated post smolts (Fig. 3K). For all Ig genes, differences were significant between wild post smolts and reared vaccinated post smolts in the second segment of the mid-intestine and the posterior segment. There was a strong positive correlation between sIgM and mIgM ($r = 0.71$; Fig. 4H), while the correlation to IgT was only moderately positive ($r = 0.42$ for sIgM; Fig. 4I and $r = 0.36$ for mIgM; not shown).

3.1.3. Recombination activating genes

For all fish groups and all intestinal regions, RAG1 gene expression was not detected within 40 cycles by real-time PCR while RAG2 had relatively low transcript levels (Table 1). In the wild spawning salmon, the transcript levels of RAG2 were approximately equal in the first mid-intestinal segment with pyloric caeca and the second segment of the mid-intestine (Fig. 2A). In the wild post smolt group, the level of RAG2 was highest in the posterior region, while in contrast this region had the lowest level in reared unvaccinated and vaccinated post smolts, where the second segment of the mid-intestine had the highest transcript level (Fig. 2B–D).

Comparing the three post smolt groups, there was rising transcript levels of RAG2 from wild to reared unvaccinated to vaccinated post smolts in both first segments and the second segment of the mid-intestine, with significant differences between wild and reared vaccinated post smolt groups. The pyloric caeca and the posterior segment RAG2 levels were lowest in the reared unvaccinated post smolts (Fig. 3L).

3.2. Immunohistochemistry

3.2.1. CD3ε

As shown in a previous publication (Koppang et al., 2010), $CD3\varepsilon^+$ cells were mainly found in intraepithelial locations and

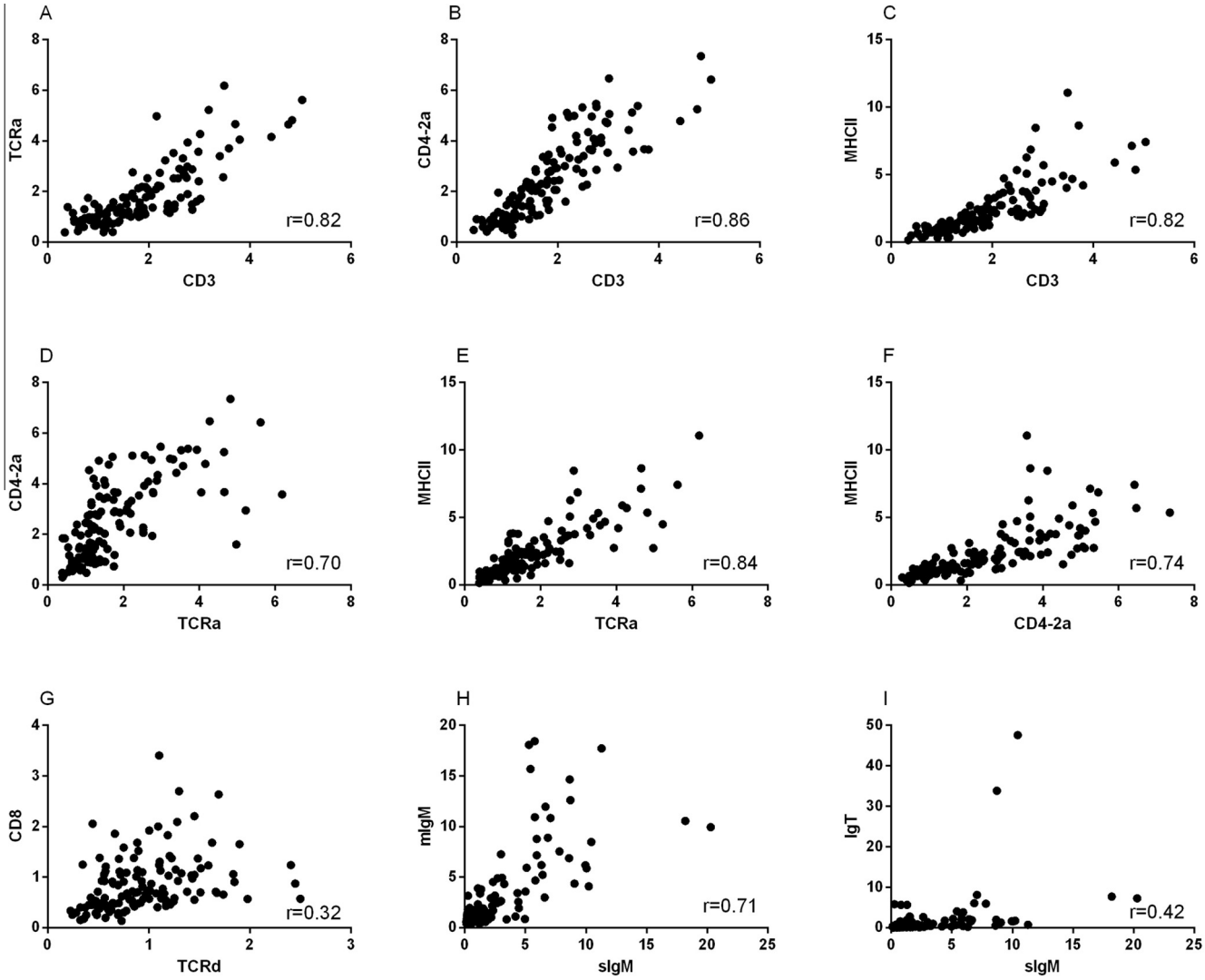

Fig. 4. Correlation between genes. Strong positive correlation is found between CD3ζ, CD4-2a, TCRα and MHC class II (A–F), while only weak to moderate positive correlation between CD8α and TCRδ (G). For the immunoglobulins, there is strong positive correlation between sIgM and mIgM (H), but only moderate positive correlation between sIgM and IgT (I).

basal to the enterocyte nuclei (Fig. 5A and B). Occasional cells were however observed at more luminal positions (Fig. 5A). The CD3ε$^+$ cells had round or irregularly formed nuclei, while the shape of the cytoplasm was difficult to define due to diffuse outline and high density of immunopositive cells (Fig. 5B). The density of CD3ε$^+$ cells in the epithelium fluctuated, where both areas with a coherent layer and areas with dispersed immunopositive cells were observed. The distribution was seemingly independent of the epithelial folding except in the second segment of the mid-intestine, where CD3ε$^+$ cells decreased towards the apical end of the complex folds. Semi-quantitative scoring of CD3ε$^+$ cells in the epithelium revealed no significant difference in density of immunopositive cells between the intestinal regions or between the fish groups (not shown).

Scattered CD3ε$^+$ cells were observed in the lamina propria (Fig. 5A), and in some sections this location was more heavily infiltrated by immunopositive cells. Although not significant, semi-quantitative scoring revealed that the wild spawning salmon and the reared unvaccinated and vaccinated post smolts had a slightly higher density of lamina propria CD3ε$^+$ cells in the two posterior-most regions compared with the wild post smolts and compared with the more anterior regions within the own group (Fig. 6A).

3.2.2. MHC class II

MHC class II$^+$ cells had a similar localization as the CD3ε$^+$ cells, but fewer in number; mainly in the epithelium basally to the enterocyte nuclei (Fig. 5C) and occasionally at more luminal positions in the epithelium. Corresponding to previous observations (Koppang et al., 2003), MHC class II$^+$ cells included small round cells with a round nucleus (lymphocyte-like) (Fig. 5D), larger, more irregular shaped cells with an ovoid or irregular shaped nucleus (macrophage-like) (Fig. 5D) and large cells with ovoid or irregular shaped nuclei and with dendrites or a "tail" (dendritic cell-like) (Fig. 5E). Occasional MHC class II$^+$ cells were additionally present in the lamina propria. Faint staining of the enterocytes was observed in several sections (not shown), but this could not be attributed to region or group.

Although not significant, counting of cells showed a trend in the wild spawning salmon, wild post smolts and reared unvaccinated post smolts showing higher number of MHC class II$^+$ cells in the epithelium in the second segment of the mid-intestine and the posterior segment compared with the other intestinal regions (Fig. 6B). In the reared vaccinated post smolts, the number of MHC class II$^+$ cells was however highest in the pyloric caeca, followed by the second segment of the mid-intestine. The wild

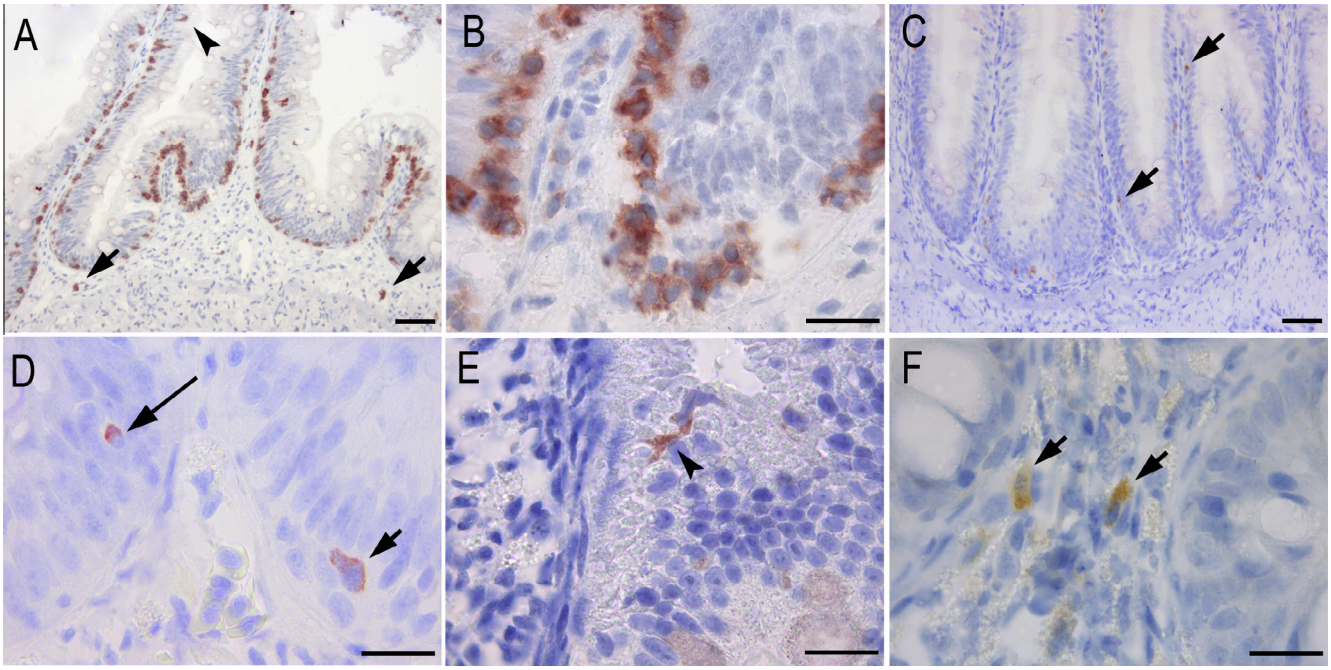

Fig. 5. Representative microphotographs of immunohistochemical stainings of CD3ε, MHC class II β-chain and Ig. (A) CD3ε$^+$ cells (red) are located as a more or less coherent layer basally in the epithelium, although occasional cells are observed at more luminal positions (arrowhead). Scattered immunopositive cells are observed in the lamina propria (arrows). (B) Round or irregular nuclei of CD3ε$^+$ cells, while outlines are diffuse and hard to identify. (C) MHC class II$^+$ cells are located basally in the epithelium (arrows), although much fewer in number than cells positive for CD3ε. (D, E) MHC class II$^+$ cells are identified as lymphocyte-like (long arrow), macrophage-like (short arrow) or with dendrites (arrowhead). (F) Ig$^+$ cells (short arrows), stained by a mAb mouse antibody are strictly located in the lamina propria. Scale bars = 20 μm.

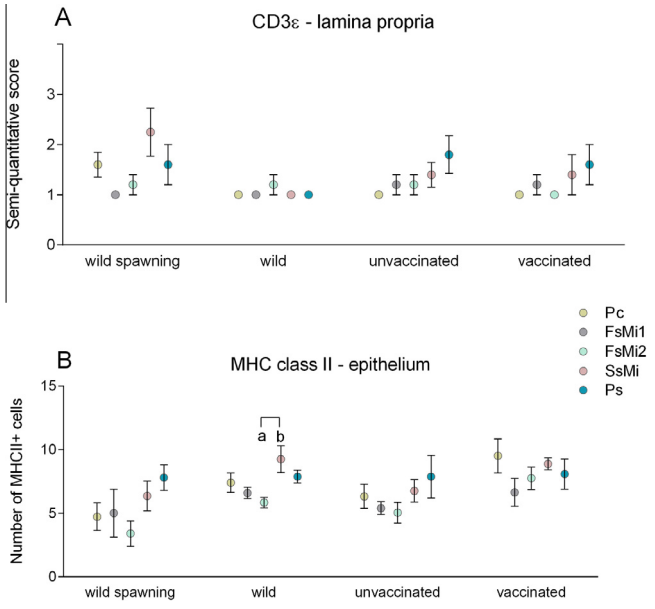

Fig. 6. Scoring of CD3ε$^+$ and counting of MHC class II$^+$ cells. (A) No significant differences are found in scoring of CD3ε$^+$ cells in the lamina propria between intestinal regions or between post smolt groups, but in wild spawning salmon and reared unvaccinated and vaccinated post smolts, there are slightly higher scoring of SsMi and Ps compared with the wild post smolts and compared with the more anterior regions within the same group. (B) Number of MHC class II$^+$ cells are counted to be slightly higher in the SsMi and Ps than in the other regions in wild spawning salmon, wild post smolts and reared unvaccinated post smolts, although significant difference only between FsMi and SsMi in wild post smolts. ^{a,b,c}Significant differences between groups are denoted by different letters (ANOVA, $P < 0.05$). Pc, pyloric caeca; FsMi1, first segment of mid-intestine with pyloric caeca; FsMi2, first segment of mid-intestine posterior to the pyloric caeca; SsMi, second segment of mid-intestine; Ps, posterior segment.

spawning salmon had slightly lower number of MHC class II$^+$ cells than the three post smolt groups in all intestinal regions, except for the posterior segment (Fig. 6B).

3.2.3. Ig

Scattered immunopositive cells were recognized in the lamina propria in addition to staining of the blood serum by both the polyclonal IgM antisera and the monoclonal Ig antibody (Fig. 5F). In all regions and groups, immunopositive cells were restricted to the lamina propria and not found in the epithelium.

3.3. Double-labeling for CD3ε and MHC class II

By IF double-labeling, we observed CD3ε$^+$ and MHC class II$^+$ cells located next to each other both in the intraepithelial compartment and in the lamina propria, but we could not identify double immunopositive cells in any of the intestinal regions in any of the fish investigated (Fig. 7).

4. Discussion

Following up a previous study by our group focusing on intestinal morphology (Løkka et al., 2013), we here addressed the immunological state of the different intestinal regions in wild post smolts and spawning Atlantic salmon. The two fish groups represent different stages of the anadromous wild salmon life cycle, where the young post smolts are feeding in the ocean while the sexually mature salmon stop eating when they enter the river to spawn (Mills, 1991). Further, possible immunological differences between wild caught post smolts and reared unvaccinated and vaccinated post smolts were investigated.

In all fish groups, there was a trend of higher transcript levels in the second segment of the mid-intestine and the posterior segment compared with the pyloric caeca and the first segments of the mid-intestine for most of the investigated immune-related genes.

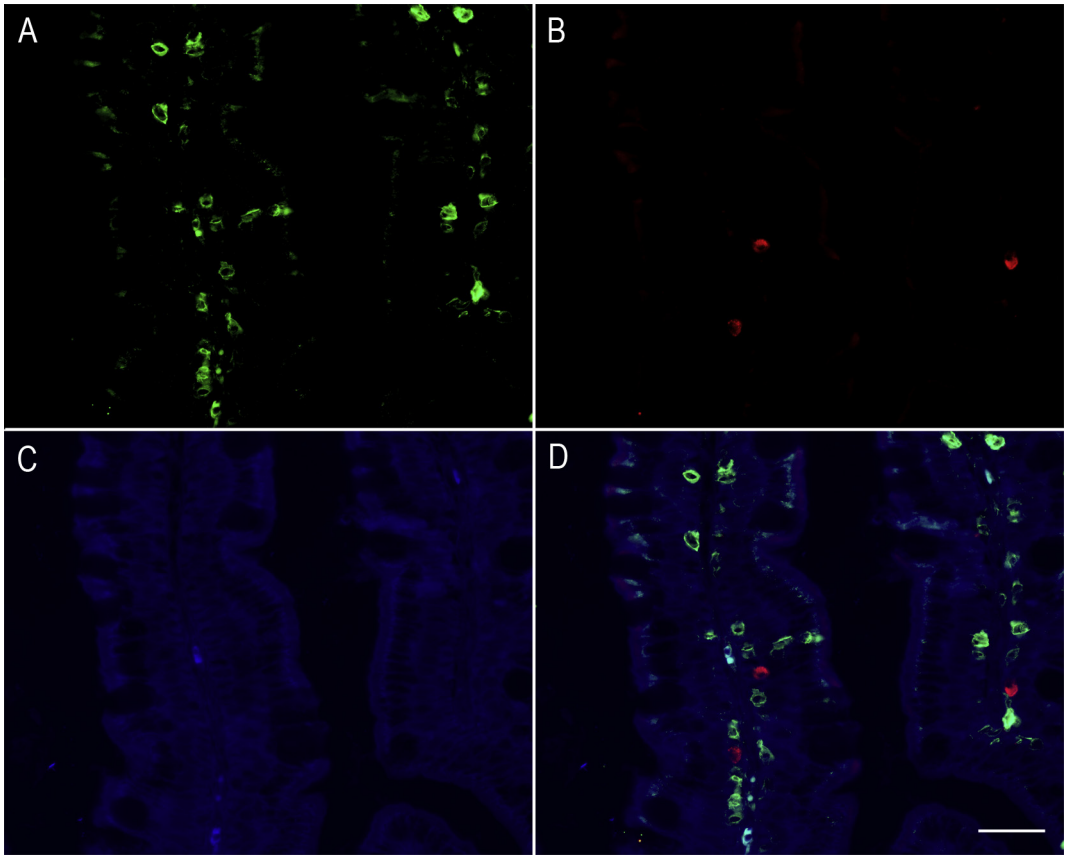

Fig. 7. Immunofluorescence double-labeling for CD3ε and MHC class II. (A) CD3ε$^+$ cells in green. (B) Lymphocyte-like MHC class II$^+$ cells in red. (C) Blue filter not detecting signals from the secondary antibodies to illustrate autofluorescence. (D) Merged image. CD3ε and MHC class II expression are restricted to different cells and do not overlap. Scale bar = 40 μm.

Furthermore, between the three post smolt groups the differences in transcript levels were most pronounced in the same two regions. By IHC, these segments scored slightly higher on the density of lamina propria CD3ε$^+$ cells than the other investigated regions in reared unvaccinated and vaccinated post smolt groups. Moreover, the same two regions had the highest number of epithelial MHC class II$^+$ cells in wild spawning salmon, wild post smolts and reared unvaccinated post smolts. Combined, these results suggest that the two most distal segments of the salmon intestine might be more involved in immunological functions and thus subjected to immune regulation than the anterior regions. In salmon, higher levels of MHC class II in hindgut than foregut have previously been reported (Harstad et al., 2008; Koppang et al., 1998), and special immunological functions of the second segment of the mid-intestine have been suggested (Fuglem et al., 2010; Georgopoulou et al., 1988; Petrie and Ellis, 2006). Also, soy-bean meal induced enteritis particularly strikes this region (van den Ingh et al., 1991). Interestingly, in the reared unvaccinated and vaccinated post smolt groups, the posterior segment had the highest transcript levels for most of the investigated genes. Although it has been reported a numerical increase in T cells and IgM$^+$ cells in the anterior-posterior direction of the gastrointestinal tract of sea bass (*Dicentrarchus labrax*) (Abelli et al., 1997), this region has been considered as more important for osmoregulation (Rombout et al., 2011). As Rombout et al. (2011) points out, the posterior segment has often been overlooked as a separate region although in salmon it clearly differs from the second segment of the mid-intestine both in intestinal folding and histological characteristics (Løkka et al., 2013).

Comparing the three post smolt groups, gradually rising transcript levels were observed from wild to reared unvaccinated to vaccinated post smolts for the majority of the investigated genes in all intestinal regions. In addition, the two reared post smolt groups scored slightly higher, although not significant, on CD3ε$^+$ cells in the lamina propria of the two most posterior regions than the wild post smolts. Combined, the results may indicate a small immune stimulation or a more alerted immune system in the intestine of the two reared salmon groups. The reared post smolts used in the study were from standard strains used in the Norwegian farming industry, genetically selected for better pathogen resistance and feed utilization (AquaGen strain); hence these individuals might differ in their immune response from the wild post smolts. The feed used in the trial was a standard diet used for salmon in the period of transfer to sea water that according to the producer triggers the immune system to a more alert state (Anonymous, 2012). In contrast to a wild salmon diet commercially produced feed contains components including terrestrial plant ingredients that are not natural food sources for wild salmon. Terrestrial protein components may act as inflammation inducers (Buddington et al., 1997) and lead to infiltration of T cells in the lamina propria, as described in soybean meal-induced inflammation previously (Bakke-McKellep et al., 2007). Apart from that, one group was given a standard oil-adjuvanted vaccine by intraperitoneal injection, hence creating a depot of vaccine fluid in the peritoneum and directly exposing antigens to the intestinal serosa, which makes up the outer border between the intestinal wall tissue and the peritoneal cavity. The vaccine might for this reason cause changes in local intestinal immune reactions in

addition to systemic effects and possibly autoimmunity (Haugarvoll et al., 2010).

Comparisons of transcript levels between the spawning salmon and the post smolts were not performed as the proportions of the muscularis layer of the intestinal wall are fundamentally different between the two life stages and would therefore influence the observed transcript levels (Løkka et al., 2013). Immunohistochemical results could however be matched, indicating a slightly higher score of CD3ε$^+$ cells in the lamina propria of the wild spawning salmon compared with the three post smolt groups. This might be explained by the starving of the spawning individuals, as starvation has been described to induce processes similar to feed-induced enteritis (Bæverfjord and Krogdahl, 1996).

The rising transcript levels of CD3ζ, CD4-2a and TCRα from wild to reared unvaccinated to vaccinated post smolts showed a strong positive correlation. Moreover, the TCRα/TCRδ ratio and CD4-2a/CD8α ratios were higher in both reared post smolt groups than in the wild post smolts. Combined, these results indicate that the difference in T cell expression between the groups was mainly due to differences in the CD4-2a$^+$TCRα$^+$ population. CD3ζ, CD4-2a and TCRα were additionally strongly positively correlated with MHC class II expression, which agrees well with the fact that antigen presenting cells expressing MHC class II are known to interact with CD4$^+$TCRαβ$^+$ cells (Mayer et al., 1991). Counting of MHC class II$^+$ cells did however not reveal significant differences between the three post smolt groups, indicating that the higher transcript levels were not caused by a higher number of MHC class II$^+$ cells. The difference could rather originate from higher transcript levels within the already present MHC class II$^+$ cells. Enterocytes expressing MHC class II have been described in mammals (Glimcher and Kara, 1992; Stokes and Waly, 2006) and salmon (Romarheim et al., 2013) and faint MHC class II enterocyte staining was observed in the present study, although not attributed to certain intestinal regions or fish groups. It has been speculated of a possible population of intraepithelial T cells expressing MHC class II in salmon (Koppang et al., 2003) as found in rat (Kearsey and Stadnyk, 1997). We observed MHC class II$^+$ cells resembling lymphocytes by IHC, but IF double-labeling for CD3ε and MHC class II did not reveal any co-expression, thus at least the majority of these cells are not T cells.

CD8α and TCRδ had lower transcript levels in all intestinal regions in the reared unvaccinated post smolts than in the wild post smolt group, and also the vaccinated group occurred to have lower or similar transcript levels of these genes than the wild post smolts. This might be explained by different antigen load in the water for wild and reared groups (Bergh et al., 1989). Inclusion of different levels of bacterial meal in the feed has been shown to alter CD8α$^+$ IELs previously (Romarheim et al., 2013), but commensal bacteria have also been shown to influence MHC class II expression of the enterocytes and IELs (Goto and Ivanov, 2013). Corresponding responses would hence be expected also for more of the investigated genes, but other factors might hide this environmental effect.

In the teleost gut, cells expressing IgM and IgT are mainly known to be located in the lamina propria (Bakke-McKellep et al., 2000; Salinas et al., 2011), which corresponds with our observations by IHC detecting no Ig$^+$ cells in the epithelium. However, IgM$^+$ cells in the epithelium have been reported in Atlantic halibut (*Hippoglossus hippoglossus*), particularly in the posterior intestine (Grove et al., 2006). Both IgM$^+$ and IgT$^+$ cells were moreover described in the epithelium of rainbow trout recently, with increased amounts in the pyloric caeca upon oral vaccination (Ballesteros et al., 2013). Our findings of higher transcript levels of sIgM than mIgM and IgT in all intestinal segments and all fish groups are in agreement with a previous publication (Tadiso et al., 2011). The percentages of IgM$^+$ and IgT$^+$ cells are reported to be about equal in the rainbow trout intestine (Ye et al., 2013), however the amount of IgM in the gut mucus and serum is much higher than IgT (Salinas et al., 2011; Tadiso et al., 2011), which can explain our results.

RAG1 transcripts were not detected in any of the intestinal regions by real-time PCR. Similar or barely detectable expression has previously been reported in Atlantic salmon smolts (Takizawa et al., 2011) and adult zebrafish (*Danio rerio*) (Willett et al., 1997). On the contrary, in immature sea bass and carp (*Cyprinus carpio*) larvae, intestinal RAG1 expression has been found (Huttenhuis et al., 2006; Picchietti et al., 2011), leading to the idea of a teleost population of intestinal T cells developing extrathymically in a similar fashion as proposed in mouse (Rocha et al., 1994). Extrathymic T cell development in the African clawed frog (*Xenopus laevis*) has however been rejected (Gravenor et al., 1995). Interestingly, RAG1 expression in the intestine of carp was decreasing in juvenile and adult stages (Huttenhuis et al., 2006), which is consistent with rainbow trout intestinal RAG1 expression found in young individuals (Hansen, 1997), but not in 1–2 years old fish (Takizawa et al., 2011). RAG2 was, in contrast to RAG1, expressed in all intestinal regions and fish groups in the present study although with a relatively low expression. RAG2 without RAG1 expression has previously been reported from oocytes of African clawed frog (Greenhalgh et al., 1993) and the bursa of Fabricius in chicken (Carlson et al., 1991), but the functional aspect of RAG2 expression alone is unclear. In maturing T and B lymphocytes RAG1 and 2 are expressed together (Oettinger et al., 1990; Schatz et al., 1989), indicating that RAG2 cannot induce recombination single-handedly. Hence, our results suggest that somatic recombination by RAG genes does not take place in the intestine in post smolts and sexually mature Atlantic salmon.

5. Conclusions

Only scattered populations of immune cells were observed along the intestinal tract, in contrast to the organized GALT of mammals, which is in co-ordinance with previous descriptions.

Measured by real-time PCR, the highest transcript levels of immune-related genes in all fish groups were found in the second segment of the mid-intestine and the posterior segment, compared with the more anterior regions, which points to special immunological function towards the posterior part of the salmon gastrointestinal tract. Comparing wild and reared post smolts, highest transcript levels were generally found in reared vaccinated post smolts, followed by the reared unvaccinated post smolts and finally the wild post smolt group, indicating that rearing conditions including commercial feed and vaccination by injection might lead to a more reactive GALT. RAG1 was not found to be expressed in the intestine of neither wild nor reared post smolts or in spawning salmon, indicating that extrathymic differentiation of T cells in the intestine does not occur in these life stages. We could not identify cells expressing both CD3ε and MHC class II within the epithelium in any of the intestinal regions, which points to no intraepithelial T cell subset expressing MHC class II in Atlantic salmon.

Authors' contribution

G.L. contributed to study design, collection of material, performing real-time PCR, IHC and IF, statistical analyses, interpreting results and writing of manuscript. L.A. to study design, designing of TaqMan assays, statistical analyses, interpreting results and editing of manuscript. K.F. to study design and obtaining polyclonal IgM antisera. E.B. to obtaining monoclonal CD3 antibody. P.G.F. and T.H. to study design and supervision of experimental design at Matre Research Station. I.H. to competence

in molecular immunology and obtaining polyclonal antisera towards CD3 and MHC class II. E.O.K. to study design, polyclonal antisera towards CD3 and MHC class II, interpreting results, editing of manuscript and supervising the study. All authors read and commented on the manuscript.

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