Research paper

Skin immune response of rainbow trout (Oncorhynchus mykiss) experimentally exposed to the disease Red Mark Syndrome

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Abstract

Red Mark Syndrome (RMS) is a skin disease reported from farmed rainbow trout. Since the turn of the millennium it has been spreading through Europe. RMS is probably a bacterial disease caused by a Midichloria-like organism (MLO). It is non-lethal and causes little obvious changes in appetite or behavior but results in red hyperaemic skin lesions, which may lead to economic losses due to downgrading. Here we transfer RMS to naïve specific pathogen free (SPF) fish by cohabitation with RMS-affected seeder fish. During disease development we characterize local cellular immune responses and regulations of immunologically relevant genes in skin of the cohabitants by immunohistochemistry and qPCR. Skin samples from SPF controls and cohabitants (areas with and without lesions) were taken at 18, 61, 82 and 97 days post-cohabitation. Gene expression results showed that lesions had a Th1-type profile, but with concurrent high expression levels of all three classes of immunoglobulins (IgD, IgM and IgT). The marked local infiltration of IgD + cells in the skin lesions as well as a highly up-regulated expression of the genes encoding slgD and mtgD indicate that this immunoglobulin class plays an important role in skin immunity in general and in RMS pathology in particular. The co-occurrence of an apparent B cell dominated immune reaction with a Th1-type profile suggests that the local production of antibodies is independent of the classical Th2 pathway.

1. Introduction

Red Mark Syndrome (RMS) is a skin disease so far only reported from farmed rainbow trout. Affected fish are usually large fish at around market size. Hallmark symptoms are raised haemorrhagic lesions in the skin. These are often associated with scale resorption, lesions in the skin. These are often associated with scale resorption, inflammation (Ferguson et al., 2006; Verner-Jeffreys et al., 2008; Schmidt-Posthaus et al., 2009; Metselaar et al., 2010) and not only skin but also subdermal muscle can be affected by this disorder. Histologically, the inflamed skin includes infiltration of primarily mononuclear cells. The syndrome develops between 2 and 16 °C (Verner-Jeffreys et al., 2008; Schmidt-Posthaus et al., 2009; Metselaar et al., 2010) and not only skin but also subdermal muscle can be affected by this disorder. Histologically, the inflamed skin includes infiltration of primarily mononuclear cells. The syndrome develops between 2 and 16 °C (Verner-Jeffreys et al., 2008) but has been present occasionally at 18 °C (McCarthy et al., 2013) and it has a long latency period, which lasts from weeks to months. Experimental cohabitation (cohab) has shown that RMS can be transferred from infected fish to naïve fish (Verner-Jeffreys et al., 2008).

RMS has been known from the USA as Strawberry Disease (SD) since the 1950s (Metselaar et al., 2010; Lloyd et al., 2008). Apart from one case in the 1980’s (Fleury et al., 1985), the disease appeared in Europe just after the turn of the millennium, where it first spread through Great Britain, but is now found over much of Europe (McCarthy et al., 2013). RMS-like symptoms were first reported in Denmark around year 2010 (Schmidt et al., 2018), and the occurrence of the disease accelerated from 2013. In 2016 close to one third of Danish trout farmers reported observations of RMS-like symptoms. RMS is non-lethal and causes no obvious changes in appetite or behavior but result in severe economic losses due to poor visual appearance and thus downgrading of the product.

Previously Flavobacterium psychrophilum was under consideration as the causative agent of RMS (Ferguson et al., 2006) and also a hypersensitivity type reaction has been suggested as explanation for the lesions (McCarthy et al., 2013). In 2008 Lloyd et al. found 16S rDNA sequences from an unknown Rickettsia-Like Organism (RLO) in SD lesions using PCR (Lloyd et al., 2008) and three years later a severity-
dependent correlation was described (Lloyd et al., 2011). Around the same time Metselaar et al. (2010) found a similar association between RLO and RMS (Metselaar et al., 2010). Montagna et al. (2013) investigated the phylogenetic relationship of the RLO bacterium and placed it within the recently established Midichloriaceae family within the order Rickettsiales (Montagna et al., 2013). The bacterium is thus now also referred to as MLO (Midichloria-like organism). MLO is also found in heart, liver, spleen, intestine and kidney (Cafiso et al., 2016).

The fish skin is the outer protective barrier against environmental challenges and RMS is primarily affecting this barrier. Fish skin consists of several layers where the epidermis represents the border between the fish and the environment. The epidermal layer includes goblet cells, which produce mucus that contain polysaccharides, glycoproteins and antimicrobial components. Mucus is secreted to the outer surface of the epidermis and constitutes the first line of defense between the fish and the environment (Elliott, 2000). The dermis comprising two layers, is located below the epidermis. Closest to the epidermis is the stratum spongiosum, which consists of a loose network of connective tissue and contains reticulin fibers, fibroblasts, pigment cells and leukocytes. The scales are found in this layer (Elliott, 2000; Ingerslev, 2010). The other dermal layer is the dermis compactum. The present study investigates the immunopathological reactions in the different skin layers in RMS-affected rainbow trout skin from a controlled experimental infection model, which allows us to pinpoint prominent innate and adaptive immune elements at exact time-points after exposure to RMS. Affected skin is compared with apparently un-affected skin from the same individual throughout disease development. We looked at the changes in the presence of IgM, IgT, IgD, CD8 and MHC II in lesions in situ with immunohistochemistry (IHC). These proteins are mainly associated with adaptive immunity and antigen presentation. Furthermore, we analyzed the expression of a panel of immune-relevant genes using real-time qPCR. Gene expression levels for cell markers (CD4, CD8, IgDm, IgT, MHC I, MHC II) were analyzed mainly to investigate the involvement of B- and T cells. Regulations in the expression of cytokines (IFN, IL-1β, IL-4/13 A, IL-8, IL-10 A, IL-17 A/F1, IL-17-C1, IL-17-C2) and three transcription factors (GATA3, ROR γ and T-bet) were examined to elucidate which T cell lineages were involved. The regulation of genes encoding acute phase components (C3, C5 and SAA) was also investigated to understand their involvement in disease progression. To substantiate that the disease is caused by MLO, amount of MLO 16 s rDNA was correlated to the observed immune responses for each lesion.

2. Materials and methods

2.1. Ethics

The experiment was conducted in agreement with animal experiment permit number 2013-15-2934-00976 issued by The Animal Experiments Inspectorate of Denmark, and was additionally approved by the internal coordination committee for animal experiments at DTU. There are no in vitro alternatives to experimental animals for RMS, but number of animals was kept to the estimated necessary minimum. In addition, all individuals were PIT-tagged for individual recognition, thus further reducing the number of experimental animals. All procedures were performed under anesthesia.

2.2. SPF fish

Rainbow trout eyed eggs were purchased from a Danish commercial hatchery certified free of the fish-pathogenic viruses IPN, IHN, VHS as well as bacterial kidney disease (BKD). In addition, the fish were later tested free from MLO and the virus PRV-3. Upon arrival to the clean section of the high-contained aquarium facilities at DTU the eggs were disinfected with iodine, hatched in trays and then transferred to 180 L cylindrical clear plastic (PETG) tanks supplied with recirculated (filtered and UV disinfected) municipal tap water. The fish were kept at 12 ± 1 °C and at 12:12 h light:dark cycle. They were fed commercial fish pellets (BioMar A/S, Brande, Denmark) of appropriate size. Two days prior to the start of the cohabitation experiment 110 fish were anesthetized in benzocaine (80 mg/L, Sigma-Aldrich, Brøndby, Denmark) and injected with a passive integrated transponder (PIT) tag. They were also weighed, measured and photographed during anesthesia. The fish weighed 86.9 ± 17.0 g and measured 18.7 ± 1.4 cm.

2.3. Seeders

Rainbow trout with RMS-like lesions were purchased from a modern fish farm with concrete raceways and a high degree of recirculation. The farm is declared free of the viral diseases VHS, IHN and ISA, and generally had few disease problems. In addition, they were screened for common fish pathogens. Forty fish were PIT tagged as described for the SPF fish. The fish were 173.3 ± 26.9 g and 24.1 ± 1.2 cm at the start of the experiment.

2.4. Experimental set-up for cohabitation challenge

An experimental cohabitation model was used to transfer RMS to naive SPF fish. On the morning of the start of the experiment the PIT tagged seeder fish were distributed with 10 into each of four 180 L tanks. The fish in two of these tanks were treated with formalin by closing the tank off (i.e. no water recirculation through external filters or fresh flow) and adding 37% formalin to the water at a ratio of 1:5000. After 30 min half of the volume of water was changed with fresh water and the filter and fresh flow was re-opened. After three hours the PIT tagged SPF fish were distributed with 20 SPF fish in each of the four tanks with 10 seeder fish and 30 SPF fish in a separate negative control tank. Light:dark regime was 12:12 and temperature 12 ± 1 °C. The fish were hand-fed 3 mm pellets (BioMar A/S, Brande, Denmark) at 1.1% body weight daily during the experiment.

2.5. Disease monitoring

Two weeks after cohabitation the cohabitants started showing signs of infection with Flavobacterium psychrophilum. Between 18 days and 33 days post-cohabitation (dpc) a total of 12 cohabitants were diagnosed with a severe F. psychrophilum infection and thus terminated. Approximately 30 dpc infection with Ichthyophthirius multifiliis (Ich) was observed. Following this observation all fish were kept in 1% salt water from 34 to 59 dpc. Four cohabitants were euthanized due to heavy infection, and one died – presumably due to Ich infection. All cohabitation tanks were affected to similar extents. F. psychrophilum was also diagnosed from the negative control tank, but Ich was not.

2.6. Sampling

Four control fish and 8 cohabitants (4 each from a tank with formalin-treated and non-treated seeders) were sampled at 18 dpc. Due to loss of fish to F. psychrophilum and Ich this was scaled down to three control fish and 6 cohabitants (three each from a tank with formalin-treated and non-treated seeders) on 61, 82 and 97 dpc (Fig. 1).

Fish were euthanized in an overdose benzocaine. Control and lesion sites were chosen (all cohabitants had multiple lesions sites) for histological and qPCR sampling. Samples were full-thickness skin samples including muscle.

2.7. Immunohistochemistry (IHC)

The samples for immunohistochemistry were placed in 4% neutral-buffered formaldehyde at 4 °C for 2–3 days before being trimmed to size, processed routinely and cast in paraffin wax. Sections of 2–4 μm were placed on SuperFrost® glass slides coated with a tissue capture pen
(Sigma-Aldrich, Brøndby, Denmark). Slides were deparaffinised with Histo-Clear II (Fisher Scientific, Denmark, cat. no. 12954900) and rehydrated from a graded series of ethanol (99%, 96% and 70%) to Tris-buffered saline (TBS) pH 7.5. Slides were subsequently incubated in 1.5% H₂O₂ in TBS for 10 min to quench endogenous peroxidase activity and were then heat-incubated in citrate buffer (1 mM Sodium citrate, pH 6.0) for 2 h at 80 °C for antigen retrieval. The slides were left to cool at room temperature (RT) for 15 min and then incubated with 2% bovine serum albumin (BSA) in TBS for 10 min at RT to block non-specific binding of antibodies. Sections were incubated with primary antibodies at 4 °C overnight followed by three washes of 1 x TBS. The following primary antibodies were used (monoclonal against Atlantic salmon or rainbow trout): CD8+ 1:100 (Hetland et al., 2010), MHC II 1:300 (Jørgensen et al., 2011) and IgT 1:200 (Olsen et al., 2011). The binding of antibodies. Sections were incubated with primary antibodies for 30 min with an AEC Staining Kit (Sigma-Aldrich, Denmark, cat. no. 534056) was used instead of Histo-Clear II; 2) incubation times for Primary Antibody Amplifier Quanto and HRP Polymer Quanto were increased to 15 min; and 3) Slides were incubated for 30 min with an AEC Staining Kit (Sigma-Aldrich, Denmark, cat. no. AEC101) to develop a color reaction.

In the case of IgM, the percentage of IgM staining in a specific layer was measured instead of the numbers of cells due to the widely dispersed non-cellular associated staining.

2.7.1. Evaluation of immunohistochemical staining

In order to calculate the number of stained cells in tissue sections, images were obtained at 100 x magnification using a Leica DMLB microscope (Leica Microsystems, Denmark) and The Leica software Application Suite v4 (Leica Microsystems, Denmark). Layers were marked as outlined in supplementary material S1 using the software Image J (Rueden et al., 2017). Within every layer, cells were counted using the multi point counting function of Image J. Subsequently, it was calculated how much the layer in question covered of the whole image (in percent) using Image J. At 100 x magnification, the area of a full image represented 0.58100 mm². Thus, the density of cells could be estimated as

\[ \text{Cells/mm}^2 = \frac{\text{Number of cells} \times 100}{\% \text{ coverage} \times 0.581 \text{ mm}^2} \]

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at 95°C followed by a combined step of annealing and elongation for
10 s at 60°C with Brilliant III Ultra-fast qPCR Master Mix (AH
diagnostics as, Denmark, cat. no. 600880). Primers and probes
(labelled with FAM at the 5′ end and with BHQ1 at the 3′ end) (1
μM and 0.5 μM final concentrations, respectively) are shown in
Supplementary material S2.

2.7.2.6. Real-time PCR using DNA. MLO 16S rDNA was quantified from
extracted DNA of skin samples following a previously published
protocol (Cafiso et al., 2016), but with some modifications. First of
all, there was a typing error in the article (Cafiso pers. comm.). The
correct reverse primer (and the one used here as well as by Cafiso) is 5′-
TGCGACACCGAAAACCTAAG -3′. Secondly, we used Brilliant II SYBR
Green QPCR Master mix (Agilent Technologies, Santa Clara, CA, USA)
and the following cycling conditions: 10 min at 95°C followed by 40
cycles of 30 s at 95°C and 60 s at 60°C, after which a melt curve
analysis was performed (1 min at 95°C, 30 s at 55°C, and incremental
temperature increase to 95°C).

2.7.2.7. Data analyses
Cell count data obtained by IHC for 18 days post cohabitation
(dpco) (only two groups to compare) were analyzed statistically by a
Mann Whitney test. For the three other time points, cell counts and
percentage measurements of IgM were analyzed by a One-way ANOVA
(Kruskal-Wallis test and a Dunn’s posttest). Results were considered
statistically signiﬁcant when P < 0.05.

As all qPCR assays had efﬁciencies of at least 100% ± 5% the simpliﬁed
ΔΔCq method (Livak and Schmittgen, 2001) was suitable for quanti-
tative analysis using the ELF1α as reference gene (Ingerslev et al.,
2006). The two RMS groups (RMS-, RMS+) were compared to spe-
ciﬁc pathogen-free (SPF). Furthermore, the RMS+ group with samples
from RMS lesions was compared to the RMS- group with samples from
un-aﬀected skin. Results were considered statistically signiﬁcant when
P < 0.05 (Student’s t-test) and regulation was more than two fold. The
ΔΔCq values represent log-transformed folds (exponential data) and
were used when the Student’s t-test was performed. In four cases (C5,
IL-17 A/F1, IL-17C1 and IL-17C2) suﬃcient numbers of valid Cq values
(> 3) were not obtained. In these cases, a qualitative approach using

Fig. 2. Levels of IgM staining and number of IgD+, IgT+, CD8+ and MHC II+ cells in different tissue layers of skin aﬀected by RMS in rainbow trout
(Oncorhynchus mykiss). Fish were experimentally infected with RMS by cohabitation. Areas with RMS lesions (RMS+) and areas without lesions (RMS-) were
analyzed using monoclonal antibodies targeting IgD, IgT, IgM, CD8 and MHC II. Pathological lesions were visible at 61, 82 and 97 days post cohabitation (dpco).
However, at 97 dpco lesions were more or less healed macroscopically. Only little swelling and haemorrhaging remained and lesions were mainly visible due to an
absence of scales. The skin was divided into three layers: 1) The epidermis plus the stratum spongiosum, 2) the stratum compactum and 3) the hypodermis. The ﬁrst row
represents a summary of all tissues. The number of stained cells/mm² is shown on the left Y-axis for all antibodies except anti-IgM, which is shown as percentage
staining in the tissues on the right Y-axis. # = signiﬁcantly diﬀerent from SPFs (P < 0.05), * = signiﬁcantly diﬀerent from RMS- (P < 0.05).
the presence/absence of valid Cq values and the nonparametric Mann-Whitney test (P < 0.05) was performed. In the case of MLO, an absolute quantification using plasmids as standards was performed. These data were log-transformed before a statistical analysis was performed (Student’s t-test). Correlations between the expressions of the genes of interest and MLO were generated by the nonparametric Spearman test (S2 Table). All statistics was done in GraphPad Prism v 7.00

3. Results

3.1. IHC and qPCR

IHC and qPCR were conducted in order to examine immune reactions during RMS. Full-thickness skin was sampled from 1) SPF; 2) RMS-exposed fish in areas without lesions (RMS-); and 3) RMS-exposed fish in areas with pathology (RMS+). Gene expression analyses were conducted for 22 immunologically relevant genes of which only significantly regulated genes in RMS-affected fish are discussed. A comprehensive overview of the gene expression results can be found as supplementary material S2. Likewise, the general expression levels as $2^{-\Delta\Delta Cq}$ are reported in supplementary material S3.

At the pre-pathological stage 18 dpco, lesions were not visible. IHC results are therefore only presented for SPF and RMS- fish. Gene expression was not investigated for this time-point as the fish were affected by F. psychrophilum, and this pathogen was suspected to also affect gene expression. The IHC results showed little change at this time-point. Only the number of MHC II+ cells in the stratum compactum (SC) showed a significant difference (down-regulation) for the RMS- fish (Fig. 2) but in one fish a few IgD+ cells were observed in the hypodermis (Fig. 3).

Two months (61 dpco) following cohabitation the classical red inflammatory lesions were clearly visible and results obtained by IHC and qPCR are presented for SPF fish, RMS- and RMS+ samples. A significant increase in both IgD+ cells and gene expression of sIgD and mIgD was observed in all skin layers (epidermis plus stratum spongiosum (ESS), SC, hypodermis (HYP) (Fig. 2, 3) in lesion areas both compared to SPFs and RMS- fish. The HYP of RMS+ samples reached 589 IgD+ cells/mm² compared to 0 in SPFs and RMS- samples (Fig. 2). The number of CD8+ cells by means of IHC was significantly higher in ESS and positive cells were also found in HYP (Fig. 3), while qPCR results showed a significantly higher gene expression of CD8 across all layers (Fig. 2). IHC results showed that the IgT+ cell number was significantly higher in ESS and SC of RMS+ compared to SPF and RMS- (Fig. 2), while lesion skin IgT gene expression was 47-fold increased compared to SPF (Fig. 4). Some cohabitants had increased anti-IgM staining at lesion sites (Fig. 3) especially in ESS, whereas the gene expression in all layers combined was significantly higher in lesions compared to both SPF fish (105 fold higher) and RMS- samples (5 fold higher). qPCR results further revealed that the expression of complement factor C3 was only slightly elevated in RMS- areas and that the acute phase reactant SAA increased 77 fold in the RMS+ areas. The expression of the cytokines IL-1β, IL-10 A, IL-8 and IFNγ increased significantly in lesions compared to SPF and RMS- (Fig. 4, Supplementary material S2). Transcription factor T-bet expression was up-regulated 5 fold and RORγ and GATA3 were un-regulated 61 dpco. The expression of the gene encoding the T cell marker CD4 increased 7-fold.

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**Fig. 3.** Immunohistochemical detection of IgD+ and CD8+ cells and of IgM in skin from rainbow trout (Oncorhynchus mykiss) experimentally infected with red mark syndrome (RMS). Skin from RMS lesions (RMS+) and from areas without lesions (RMS-) were stained with monoclonal antibodies specific for IgD, IgT, IgM, CD8 and MHC II. The most significant results were obtained for IgD, IgM and CD8. Representative images of RMS lesions are shown in the figure (or non-lesion site in the case of the pre-pathological stage 18 dpco). All panels are oriented with skin surface up. Scale bars are 100μm and apply to all images of the same magnification.
Macroscopically lesion severity peaked between 61 and 82 dpco. While there were early signs of healing, lesions appeared more severe 82 than 61 dpco. The numbers of IgD+ cells had decreased by 82 dpco (especially in the ESS) and only IgM and CD8 showed a significant elevation by IHC and qPCR analyses (Figs. 2 and 3) and some lesions were heavily infiltrated by IgM (Fig. 3). Expression of the genes encoding CD4, IgT, IL-8, RORγ, SAA and T-bet remained at the same level as day 61 post cohabitation whereas IFNγ, IL-10 A, MHC I and MHC II increased in level in the RMS lesions. Three genes encoding C3, GATA3 and RORγ were down-regulated. IL-1β transcripts were less up-regulated compared to 61 dpco.

Three months after initial exposure (97 dpco) RMS pathology was less visible and the fish were recovering. IgM was still significantly elevated in SC and HYP of lesion areas, but also in HYP of non-lesion areas there was a high amount of IgM. CD8 and MHC II+ cells were significantly elevated in SC of RMS+ fish. qPCR was not performed on samples from this day, as lesions were in the healing phase, and expected to have a more general wound healing profile not particularly related to RMS.

Fig. 4. Gene expression analysis by qPCR from skin tissues of rainbow trout (Oncorhynchus mykiss) experimentally infected with red mark syndrome (RMS). Expression analysis was conducted for skin sections with and without RMS lesions (RMS+ and RMS-, respectively) from cohabitated fish and a range of immune-relevant genes was investigated. Pathological lesions were visible at both time points. Macroscopically lesion severity peaked between the two time-points, but slightly closer to the later. Expression was analyzed as relative fold change where RMS- and RMS+ samples are related to basal expression of SPF fish.
affected fish collected from farms. The present study is the first investigation of immunopathological reactions in experimentally RMS infected rainbow trout, and this allowed us to monitor the immune reactions at controlled time-points during disease development.

Local cellular immune responses and regulations of immunologically relevant genes were investigated with IHC and qPCR. Gene expressions were furthermore correlated to the infection levels with the putative causative agent of RMS, namely MLO. Correlation analyses showed that a series of innate and adaptive elements could be correlated with MLO load. Additional evidence of the involvement of adaptive elements was found using IHC.

At the pre-pathological stage (18 dpco) reactions were almost absent. Externally visible RMS-related skin changes appeared around 45–50 dpco and lesion severity peaked around 30 days later. From 61 dpco a severe immune reaction was observed in the skin. The reactions involved a series of humoral and cellular elements.

4. Discussion

RMS in rainbow trout has previously been characterized as an immunopathological syndrome (McCarthy et al., 2013) based on clinically

Fig. 6. Correlation analysis of the MLO measurement and the expression of significantly regulated immune relevant genes. Expression analyses were conducted in skin of rainbow trout (Oncorhynchus mykiss) infected with RMS. Expression in areas with RMS lesions was compared to areas without visible lesions and significantly regulated genes were correlated to the amount of MLO measured. A correlation factor r from 0.5 to 1 is considered a strong positive correlation. * indicates statistical significance (P < 0.01).
IgD+ cells infiltrating skin ulcers caused by *F. psychrophilum* (Muñoz-Atienza et al., 2018). The lesions resulting from *F. psychrophilum* and RMS are very different, as the former are typically ulcerative with a large degree of tissue proteolysis and necrosis, and the latter are not. Nonetheless, comparing the results of Muñoz-Atienza et al. (24) with the present study the two pathogens appear to produce surprisingly similar immune responses. Since we know *F. psychrophilum* is present in our infection model, this raises the question of whether the responses observed in our study can be partially ascribed to this pathogen. We believe not, as 1) control fish also contracted *F. psychrophilum*, but not RMS; 2) symptoms of *F. psychrophilum* disappeared before the appearance of RMS symptoms; and 3) *F. psychrophilum* did not correlate with lesions (manuscript in prep.). Instead the responses may reflect an overlap in biology of the two pathogens. However, apart from this observation, local infiltration of IgD+ cells has – to our knowledge – not previously been seen at this high level. Our study thus corroborates previous indications that the immunoglobulin IgD and IgD+ cells perform important functions at mucosal surfaces, and RMS thus provides an interesting model to further study IgD function in fish.

IgT is a fairly well described immunoglobulin of rainbow trout (Hansen et al., 2005; Xu et al., 2016) and is involved in mucosal immune responses against viruses, bacteria and parasites (Xu et al., 2013; Castro et al., 2013). In this study IgT gene expression was significantly up-regulated in RMS lesions 61 dpco and IHC showed that IgT+ cell infiltration was specifically seen in the ESS and SC layers.

The most abundant immunoglobulin in the blood of fish is IgM. This isotype plays a major role in immune responses due to its ability to agglutinate and assist complement guided killing of invading pathogens. IgM gene expression was also highly up-regulated in skin of trout with active RMS lesions – even at apparently unaffected sites. In comparison IgD was upregulated only in lesions. However, a significant increase in IgM+ cells outside of lesion sites could not be shown with IHC. In lesions, staining for IgM was diffuse and the far majority of the staining likely derived from secreted IgM from serum trapped in the lesion, rather than membrane-bound IgM on B cells, which illustrates that IgM is a systemic molecule and that staining may not represent a local reaction from IgM+B cells to a very large extent. IgM staining was only significantly increased at the advanced stages of lesion development when the lesion had developed and was oedematous.

To sum up the results for the immunoglobulins, there is a tendency towards the mucosal immunoglobulins IgD and IgT reacting first with a subsequent increase of the systemic IgM. IgD seems more specific for the lesions compared to IgT and IgM, and IgD+ cells are the most abundant Ig-bearing cells in early stage lesions. The present results do not allow much to be deduced on the function of IgD, but IHC and qPCR results both show quite different patterns of IgM and IgD distribution, and thus elevated levels of these Igs are not a result of infiltration of IgM+IgD+ double positive cells. Our results indicate that all three immunoglobulins have important roles to play in the immune response against RMS.

In mammals MHC molecules present peptides to T cells. Cytosolic peptides are presented in class I molecules and peptides from intracellular vesicles in class II. All nucleated cells display MHC I, whereas MHC II is restricted to antigen-presenting cells such as macrophages, B cell and dendritic cells. MHC molecules likely function in the same basic way in fish, although at the genetic level huge differences are observed – with the complete lack of MHC II in Atlantic cod as an extreme example (Star et al., 2011). The tissue-specific locations of MHC II has been investigated in some species e.g. Atlantic salmon, (Koppany et al., 2003), but little is known about what specific cell types express MHC II. In mammals MHC II is strongly expressed in B cells.

In the light of the observed B cell and Ig responses we see relatively little increase in MHC II in RMS lesions with respect to transcripts as well as MHC II+ cells. This could indicate that B cells do not express MHC II to a very large extent, and that these are perhaps not primary antigen-presenting cells in rainbow trout. Since B cells (and in fish in particular) have been shown to be highly phagocytic (Parra et al., 2015), the presence of B cells in the lesion could mainly be to clean up cell debris and thus reduce inflammation. In RMS lesions we observe most of the MHC II+ cells in the stratum compactum and fewest in hypodermis. The opposite is true for IgD+ cells. Also, the only statistically significant increase in MHC II+ cells is at 97 dpco in the stratum compactum. At this time-point IgD+ cells are almost entirely absent.

The cytokine and transcription factor profile indicates that the high increase of immunoglobulins in RMS areas is induced either by Th1-like cells as can be found in mammals, through a non-local reaction or through a T-cell independent pathway (Murphy et al., 2008). B-1 B cells are IgMhigh/IgDlow in mammals and generate antibody responses mainly towards polysaccharide antigens and produce antibodies of the IgM class without help from T cells in mammals (Murphy et al., 2008; Haas et al., 2005) representing a “bridge between innate and adaptive responses” (Scapigliati et al., 2018). If what we see is a B-1 B cell-like response to polysaccharides from a member of the Rickettsiales order adaptive memory in the classical sense is not generated. Experimental studies in acquired protection following RMS has however not yet been conducted, but observations from fish farms indicate that some kind of protection exists following an RMS outbreak. Fish B cells have similar features to mammalian B-1 B cells and it has been hypothesized and demonstrated that most fish lymphocytes behave like subpopulation of mammalian innate-like lymphocytes (Scapigliati et al., 2018; Abós et al., 2018). However, to what extent the Ig classes are “natural” antibodies (produced by B-1 B cell-like cells) or specific for the causative agent of RMS (putatively MLO) is something we are not presently able to determine, as we are currently unable to isolate or propagate MLO in vitro.

### 4.2. T cell-related responses

When an infection (as the case with RMS) evades the innate defense mechanisms of the skin, an adaptive immune response is induced. The adaptive immune response can be skewed towards different effector T cells by signals from the innate immune response. In this study we distinguish between T helper (Th1)- Th2- Th17- and T regulatory (Treg)-type responses even though these response pathways are less clearly described in fish compared to mammals. A Th1-type response is classically aimed at intracellular bacteria, whereas the function of a Th2-type response mainly is to neutralize extracellular pathogens with generation of pathogen-specific antibodies. There is some evidence that the Th17-type response has a role in the fight against extracellular pathogens at mucosal sites (Murphy et al., 2008; Khader et al., 2009) whereas the Treg-type pathway suppresses adaptive immune responses (Murphy et al., 2008).

We found that in lesions Th2-type associated cytokinins are downregulated (down-regulation of the GATA3 transcripts and no regulation of IL-4/13A transcripts) while Th1-type associated cytokinins are upregulated (IFNγ and T-bet). The down-regulation of RORγ is an indication that the Th17 pathway is suppressed. IL-1β is a chemoattractant for leucocytes in fish, induces inflammation and was found to be up-regulated when the lesions were severe. Expression of Th17-type cytokines (IL-17 A/F1, IL-17-C1 and IL-17-C2) was low (undetectable in several samples), and together with a down-regulation of the associated transcription factor RORγ this indicates that this pathway is if not suppressed then at least not activated during the course of RMS. These findings correlate with a former study, which investigated Th profiles from RMS fish sampled from fish farms (McCarthy et al., 2013) and indicate that the immune response in RMS lesions are skewed towards a Th1-type response with a suppression of the Th2-, Treg- and Th17-type responses. Therefore, our gene expression profile results support that an intracellular bacterium is the likely causative agent of this disease. It does, however, not explain the significant involvement of lgs and B cells that we have described from lesions, since a long-standing immune system paradigm has stated that antibody responses are not associated...
with intracellular pathogens. However, all intracellular pathogens must have an intracellular phase unless they are transmitted by close contact between a transmission vector and a host cell, and accordingly this paradigm far from always holds true (Casadevall et al., 2006). Nonetheless, with the involvement of Ig2 one might expect upregulation of markers for a Th2 response. However, either 1) Th2 responses could have been detected in immune organs such as spleen, thymus or kidney instead of skin, or at an earlier time-point; or 2) Th2-type responses were not involved. The observed Ig2 could thus be mainly “natural” and produced by B-1 B cell-like cells as described above.

CD8 is a cell marker for cytotoxic T cells, which act directly on altered self cells, i.e. cancer cells, infected cells or damaged cells. From what is known so far CD8+ cells function quite similar in fish and mammals (Nakanishi et al., 2018). MLO is likely an intracellular bacterium and the increase of CD8+ cells in RMS areas found both 61 and 82 dpco using IHC and qPCR may indicate a host reaction towards an intracellular organism.

4.3. Correlation of MLO to the immune response

The correlation analyses point towards a relationship between the amount of MLO and most of the gene transcription levels confirming that MLO likely is the causative agent of the disease. The strongest correlation is found between MLO expression and CD4, IgG, IgM, IL-1b and IL-10. This result indicates that the pathogen may directly or indirectly influence the number of CD4+ immune cells – such as macrophages and T cells (Takizawa et al., 2016) – or the expression level of the CD4 co-receptor. The amount of MLO is also directly linked to the numbers of Ig5B+ B cells infiltrating the skin. The amount of IgG found in RMS-affected skin can be considered a consequence of haemorrhaging in areas with high MLO due to inflammatory reactions. IL-1β and IL-10, which are both correlating to the amount of MLO, induce counteracting immune effects thus while IL-1β induce inflammation IL-10 reduce inflammatory. In mammals IL-10 is mainly produced by some subsets of CD4+ T cells, but after innate activation B-1 B cells are also known to produce high amounts of IL-10 (Geherin et al., 2016). If we have similar cells in the skin of rainbow trout with RMS this may partly explain the strong correlation between the pathogen and IL-10. The specific role of SAA is relatively unknown in fish but it is often found to be highly upregulated, like in this study, during inflammatory responses, vaccination and parasite infections (Skov et al., 2018; Gonzalez et al., 2007; Roy et al., 2017).

By visual inspection of the IHC slides the epidermis is much less affected than the stratum spongiosum, which confirms the argument of McCarthy et al. (2013) that RMS is an endogenously generated disorder rather than a direct reaction to invasion from the exterior environment. It is often noted (including in this study) that the reaction apparently starts in the scale pockets (McCarthy et al., 2013). Even though the infection pathway is unknown for the causative agent of RMS it is thus tempting to suggest that the disease pathogen primarily is restricted to mucosal surfaces. All skin layers examined in our study are affected by RMS but it is notable that IgD+ cells and IgM are significantly regulated in the hypodermis, which is an adipose tissue between the skin and muscle or bone. Immunological functions of this layer have not to our knowledge been described in fish.

5. Conclusion

Immune responses in macroscopically unaffected and affected skin of rainbow trout with RMS revealed a Th1-type profile in the lesions. Interestingly, this occurred together with a high production of the immunoglobulins IgD, IgM and IgT, which is usually coupled to a Th2-type response. A significant local infiltration of IgD+ cells in the lesions as well as a highly up-regulated expression of the genes encoding IgD and mlgD was observed and this relatively undescribed immunoglobulin is suggested to play an important role in the immune response in RMS lesions. The suspected causative agent of RMS (the putative intracellular bacterium MLO) was found in skin lesion areas and to a lesser degree in uninfected skin areas of RMS-affected fish, but never in uninfected control fish. Our results support that MLO probably is the causative agent of RMS and that the fish overcome the infection by a Th1-type response supplemented by a possible T cell independent production of antibodies.

Acknowledgements

Dr. Erin Bromage (University of Massachusetts, Dartmouth, USA) is acknowledged for supplying the anti-IgD antibody. Alessandra Caíso and Chiara Bazzocchi (University of Milan, Italy) kindly supplied the MLO and IgF plasmids. Lone Madsen (DTU Aqua) is thanked for performing Psychrophilum diagnostics and Tine M. Iburg (DTU Aqua) for histopathological assessment. This study was financed by the European Maritime and Fisheries Fund through the project “Vetelasing” (grant no. 33111-1-16-009/-010) and by Henrik Henriksen’s Fond.

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