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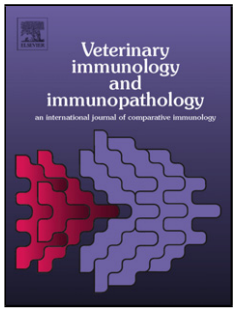

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Highlights

- Red Mark Syndrome (RMS) was transferred to naïve specific pathogen free fish
- Lesions in the skin showed a Th1-type profile using gene expression
- The same lesions showed a high expression of IgD, IgM and IgT
- Results support that MLO causes RMS
- Fish overcome the infection with a Th1-type response supplemented by antibodies

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 sIgD and mIgD 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.

Keywords: Red Mark Syndrome; MLO; Th1-type response; Antibodies; IgD

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, lichenoid dermatitis and acute inflammation [1-4] 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 [2] but has been present occasionally at 18 °C [5] 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 [2].

RMS has been known from the USA as Strawberry Disease (SD) since the 1950s [4,6]. Apart from one case in the 1980's [7], 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 [5]. RMS-like symptoms were first reported in Denmark around year 2010 [8], 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 [1] and also a hypersensitivity type reaction has been suggested as explanation for the lesions [5]. In 2008 Lloyd *et al*. found 16S rDNA sequences from an unknown *Rickettsia*-Like Organism (RLO) in SD lesions using PCR [6] and three years later a severity-dependent correlation was described [9]. Around the same time Metselaar *et al*. found a similar association between RLO and RMS [4]. Montagna *et al*. investigated the phylogenetic relationship of the RLO bacterium and placed it within the recently established Midichloriaceae family within the order Rickettsiales [10]. The bacterium is thus now also referred to as MLO (*Midichloria*-like organism). MLO is also found in heart, liver, spleen, intestine and kidney [11].

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 [12]. 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 [12,13]. 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, IgDs, IgM, IgT, MHC I, MHC II) were analyzed mainly to investigate the involvement of B- and T cells. Regulations in the expression of cytokines (IFN γ, IL-1β, IL-4/13A, IL-8, IL-10A, IL-17A/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 16s rDNA was correlated to the observed immune responses for each lesion.

Materials and methods

Ethics

The experiment was conducted in agreement with animal experimentation 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.

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.

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.

Experimental set-up for cohabitation challenge

An experimental cohabitation model was used to transfer RMS to naïve 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 minutes 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.

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.

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.

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. 12812474) and rehydrated from a graded series of ethanol (99 %, 96 % and 70 %) to Tris-buffered saline (TBS) pH7.5. Slides were subsequently immersed in 1.5 % H_2O_2 in TBS for 10 min to quench endogenous peroxidase activity and were then heat-incubated in citrate buffer (1mM Sodium citrate, pH6.0) 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 [14], MHC II 1:300 [14], IgD 1:10.000 [15] IgM 1:3000 [16] and IgT 1:200 [17]. The slides were incubated with Primary Antibody Amplifier Quanto (AH diagnostics as, Denmark, cat. no. TL-125-QPB) for 10 min and then with HRP Polymer Quanto (AH diagnostics as, Denmark, cat. no. TL-125-QPH) for 10 min avoiding exposure to light. Lastly, the slides were incubated with DAB Quanto Chromgen (AH diagnostics as, Danmark, cat. no. TA-004-QHCX) to produce a color reaction for 5 min, which was followed by a de-ionized water wash step and nuclear counter staining with Mayer haematoxylin Lillie's modified (Dako, Denmark, cat. no. S3309) for 40 seconds. Slides were mounted with Microscopy Aquatex MERCK HC 380763 (VWR, Denmark, cat. no. 1085620050). IHC staining against IgT differed in the following respects: 1) xylene (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.

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 up as outlined in supplementary material S1 using the software Image J [18]. 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 mm^2. Thus, the density of cells could be estimated as

$$\frac{Cells}{mm^2} = \frac{Number\ of\ cells * 100}{\%\ \text{coverage x } 0.581\ \text{mm}^2}$$

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.

qPCR for immune relevant genes and MLO

qPCR samples

Samples for qPCR were placed into RNAlater (Sigma-Aldrich, Brøndby, Denmark), placed at 4 °C for 1-4 days and stored at -20 °C until further processing. Prior to nucleic acid extraction the tissue pieces were split in half into narrow strips (1-1.5 mm wide). RNA was extracted from one piece and DNA from the other.

DNA extraction

Skin samples (maximum 50 mg) were transferred to 2 ml Eppendorf tubes and 200 µl of lysozyme mixture (20 mM Tris-HCl (pH 8), 2 mM EDTA, 1.2 % Triton X, 10 mg/ml collagenase and 25 mg/ml lysozyme) was added and then samples were incubated for 30 min at 37 °C. Subsequently, 350 µl lysis buffer and one 5 mm Ø stainless steel bead (Qiagen GmbH, Hilden, Germany) was added to the samples followed by shaking on a Qiagen TissueLyser II (Qiagen GmbH, Hilden, Germany) for 1

min at 30 Hz. Samples were then incubated for 1 h at 56 °C with 30 µl proteinase K, 20 mg/ml. The DNA was then extracted on a Maxwell®16 Research Instrument System (Promega Corporation, Wisconsin, USA) according to the manufacturer's instructions. The concentration of the DNA was quantified on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

RNA purification

RNA purification was conducted using the GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich, cat. no. RTN350). In brief, skin tissues were incubated in a lysis buffer containing beta-mercaptoethanol and subsequently sonicated on ice (Sonicator Ultrasonic Liquid Processor Model XL 2020, Heat Systems). Purified RNA was DNase-treated (Fermentas, cat.no. EN052), quantified (Nanodrop 2000 (Saveen & Werner APS)) and quality assessed on a 2 % agarose gel containing ethidium bromide.

cDNA synthesis

In a 20 µl reaction, 1000 ng of RNA was converted to cDNA using the TaqMan® Reverse Transcription Reaction Kit (Thermo Fisher Scientific, cat.no. N8080234) according to the manufactures instructions and using a T3 Thermocycler (Biometra, In Vitro, Denmark). Negative controls without the enzyme reverse transcriptase and negative controls using H_2O as template were included.

Real-time PCR using cDNA

Real-time PCR was run in an Agilent Technologies AriaMX Real-Time PCR system (AH diagnostics as, Denmark) using a 3 min denaturation step at 95 °C, 40 cycles of 5 seconds at 95 °C followed by

a combined step of annealing and elongation for 10 seconds 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.

Real-time PCR using DNA

MLO 16S rDNA was quantified from extracted DNA of skin samples following the protocol published by Cafiso *et al*. (2016) [11], 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'- TGCGACACCGAAACCTAAG -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).

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 100% ± 5% the simplified $2^{-\Delta\Delta Cq}$ method [19] was suitable for quantitative analysis using the ELF1α as reference gene [20]. The two RMS groups (RMS-, RMS+)

were compared to specific pathogen-free fish (SPF). Furthermore, the RMS+ group with samples from RMS lesions was compared to the RMS- group with samples from un-affected skin. Results were only considered significant when $P < 0.5$ (Student's t-test) and regulation was more than two fold. The $\Delta\Delta$Cq values represent log-transformed folds (exponential data) and were used when the Student's t-test was performed. In four cases (C5, IL-17A/F1, IL-17C1 and IL-17C2) sufficient numbers of valid Cq values (>3) were not obtained. In these cases, a qualitative approach using the presence/absence of valid Cq values and the nonparametric Mann-Whitney test ($P<0.5$) 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

Results

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 $2^{-\Delta Cq}$ 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/mm^2 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. 4). 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-10A, IL-8 and IFN γ 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 ROR γ and GATA3 were un-regulated 61 dpco. The expression of the gene encoding the T cell marker CD4 increased 7 fold.

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, ROR γ, SAA and T-bet remained at the same level as day 61 post cohabitation whereas IFN γ, IL-10A, MHC I and MHC II increased in level in the RMS lesions. Three genes encoding C3, GATA3 and ROR γ 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.

qPCR of MLO DNA

The amount of MLO DNA was high in lesion areas, whereas only low amounts of MLO DNA was detected in non-lesion areas of RMS-affected fish. No MLO was detected in uninfected controls (Fig. 5).

Correlation analysis

Amount of MLO (or to be exact: The number of detected MLO 16S rDNA copy numbers) was correlated to host gene expression profiles (Fig. 6). All genes except C3, GATA3, ROR γ and MHCI showed a strong positive correlation (between 0.5 and 1) and the five strongest correlations were found for the genes CD4, IgDm, IgDs, IgM, IL-1β and IL-10A ($r = 0.76, 0.73, 0.65, 0.75, 0.72$ and 0.75, respectively) with P values of < 0.01 (Fig. 6, Supplementary material S2).

Discussion

RMS in rainbow trout has previously been characterized as an immunopathological syndrome [5] based on clinically 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.

<u>B cell-related responses</u>

All three immunoglobulins (Igs), which are present in rainbow trout (IgD, IgT and IgM) were involved in the immune response against RMS. IgD+ cells infiltrated the epidermis/*stratum spongiosum* (ESS) and the hypodermis to a great extent (Fig. 2) and across the layers the number of IgD+ cells increased from 5 to 327 cells/mm^2 in RMS-affected skin areas, which corresponds well to the high increase in expression level observed using qPCR. This suggests that IgD plays a major role in the mucosal immune response against the causative agent of RMS. At 97 dpco IgD+ cells disappeared from the lesions almost entirely, whereas IgT+ cells and IgM changed little from 82 to 97 dpco.

In mammals, naïve mature B-cells are IgD+IgM+ double positive, and after activation they lose IgD. However, a small fraction of anergic and autoreactive IgM$^{-/low}$IgD^{high} B cells can be found in the periphery (esp. in the upper respiratory tract), and secreted IgD apparently has a homeostatic function in mucosal tissues by arming myeloid effector cells [21]. IgD has been understudied in general [21], and very little is known about this Ig isotype in fish, and since IgD – although ancient – displays considerable variance between taxa care should be taken when extrapolating from mammals to fish.

Nonetheless, the few IgD studies that have been performed in fish, point in the same direction: In channel catfish myeloid cells are also armed with IgD [22], and in rainbow trout a subpopulation of IgD$^+$IgM$^-$ B cells have been described from the gills [23]. Also, IgD has previously been found to be up-regulated after vaccination in rainbow trout and channel catfish (*Ictalurus punctatus*) [24,25] and following challenge with viruses, bacteria and parasites in rohu (*Labeo rohita*) [26] confirming the role as immunologically important especially at mucosal surfaces.

Very recently IgD expression was also found to be upregulated and IgD+ cells infiltrating skin ulcers caused by *F. psychrophilum* [27]. 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 [28,29] and is involved in mucosal immune responses against viruses, bacteria and parasites [30,31]. 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 [32]. The tissue-specific locations of MHC II has been investigated in some species *e.g.* Atlantic salmon, [33], 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 [34], 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 [35]. B-1 B cells are IgM^{high}/IgD^{low} in mammals and generate antibody responses mainly towards polysaccharide antigens and produce antibodies of the IgM class without help from T cells in mammals [35,36] representing a "bridge between innate and adaptive responses" [37]. 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 on 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 subpopulations of mammalian innate-like lymphocytes [37,38]. 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*.

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 (Th)1- 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 [35,39] whereas the Treg-type pathway suppresses adaptive immune responses [35].

We found that in lesions Th2-type associated cytokines are down-regulated (down-regulation of the GATA3 transcripts and no regulation of IL-4/13A transcripts) while Th1-type associated cytokines are up-regulated (IFN γ and T-bet). The down-regulation of ROR γ 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-17A/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 ROR γ 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 [5] and indicate that the immune response in RMS lesions are skewed towards a Th1-type response with a suppression of theTh2-, 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 Igs 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 extracellular 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 [40]. Nonetheless, with the involvement of Igs 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 Igs 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 [41]. 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.

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, IgD, 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 [42] or the expression level of the CD4 co-receptor. The amount of MLO is also directly linked to the numbers of IgD+ B cells infiltrating the skin. The amount of IgM 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 [43]. 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 [24,44,45].

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*. [5] 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 [5]. 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.

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 sIgD and mIgD 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 unaffected 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.

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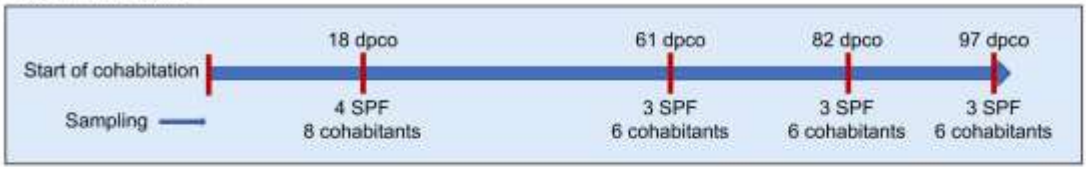
Experiment timeline



Experimental tanks

Tank type	Cohabitation tanks				Control tank
Pre-treatment	No treatment		Seeders pre-treated with formalin		No treatment
Seeder fish	10	10	10	10	0
SPF fish	20	20	20	20	30