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Which Th pathway is involved during late stage amoebic gill disease?

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ABSTRACT

Amoebic gill disease (AGD) is an emerging disease in North European Atlantic salmon (*Salmo salar* Linnaeus 1758) aquaculture caused by the amoeba *Paramoeba perurans*. The host immune response to AGD infection is still not well understood despite past attempts to investigate host-pathogen interactions. With the significant increase in our knowledge of cytokine genes potentially involved in Th responses in recent years, we examined their involvement in this disease using Atlantic salmon post-smolts sampled 3 weeks after exposure to either 500 or 5000 cells/l *P. perurans*. Gene expression analysis of cytokines potentially involved in the different Th pathways was performed on the first gill arch including the interbranchial lymphoid tissue (ILT). Th1, Th17 and Treg pathways were found to be significantly down regulated, mainly in samples from fish given the higher dose. In contrast, the Th2 pathway was found to be significantly up regulated by both infection doses. Correlation analysis of the gene expression data and the *P. perurans* load, assessed by real time RT-PCR of the 18S rRNA, was also performed. In humans, Th2 driven responses are characterized by the production of IgE, which in the majority of worm infections results in the generation of a Th2-mediated response and directs the immune system away from a Th1 inflammatory response. The present results seen during late stage AGD suggest that either an immune evasion strategy, similar to the responses driven by helminthic parasites to avoid cell-mediated killing mechanisms, or an allergic reaction caused by the parasite, is occurring.

1. Introduction

Amoebic gill disease (AGD) is an ectoparasitic infection that affects marine fish species farmed in sea net cages [1]. The causative agent of this disease in Atlantic salmon (*Salmo salar* Linnaues 1758) is *Neoparamoeba perurans* [2], an amphizoic amoeba that has successfully fulfilled the Koch's postulates [2,3]. Recently, nuclear small subunit (SSU) rDNA phylogenetic analysis has shown that the genera *Neoparamoeba* and *Paramoeba* are phylogenetically inseparable and, therefore, *Neoparamoeba* can be used as a junior synonym of *Paramoeba* [4]. AGD has been reported from numerous countries worldwide: South Eastern Australia (Tasmania), Ireland, Japan, New Zealand, Portugal, Norway, USA, Chile, South Africa [5–11] and, since 2011, AGD has been an issue for Scottish Atlantic salmon farms, mainly in summer periods.

The host immune response to AGD infection in Atlantic salmon is still not well understood despite past attempts to investigate host-pathogen interactions. Early studies on transcriptional responses to AGD have shown no differences in the gill tissue expression of tumour necrosis factor (TNF)-α1, TNF-α2, interleukin (IL)-1β, inducible nitric oxide synthase (iNOS), and interferon (IFN)-γ mRNAs compared to tissue from healthy fish, during the early onset of the disease in Atlantic salmon [12]. With the progression of the disease, IL-1β mRNA level was found to be up regulated and lesion-restricted in numerous studies [1,12–14]. Gene expression profiling using a 16K salmonid microarray has also been performed [15,16]. In AGD-affected tissue, significant, coordinated down regulation of the major histocompatibility complex (MHC) class I (MHC-I) pathway-related genes occurred during the later stages of

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infection and appeared to be mediated by down regulation of interferon regulatory factor (IRF)-1, independent of type I interferon, IFN-γ and IRF-2 expression [16]. However, anterior gradient-(AG)-2, involved in inhibiting the tumour suppressor protein p53 and required for mucin (MUC) 2 post-transcriptional synthesis and secretion, was up regulated in AGD-affected gill tissue, while p53 tumour suppressor protein mRNA was concurrently down regulated in AGD lesions, suggesting a role for AG-2 and p53 in AGD pathogenesis [17]. MHC class II$^+$ cells, considered to be antigen-presenting cells and B cells, were found within gill lesions by immunohistochemistry and it was shown that these cells exhibited variable levels of expression [17].

A recent study showed that mRNA expression level of pro-inflammatory cytokines (IL-1β, TNF-α), cellular markers of cell-mediated immunity (T cell receptor (TCR)-α chain, cluster of differentiation (CD) 4, CD8, MHC-I, MHC-IIα), and antibody-mediated immunity (IgM, IgT) is correlated with a classical inflammatory response in the gills of AGD-affected Atlantic salmon at 10 days post-infection [1]. Moreover, in the same study, it was found that mRNA expression levels of these genes within gill lesions were different to the mRNA expression levels of the same genes in parts of the gill without lesions during AGD, suggesting that there are differences in the transcriptional response to AGD between areas of the gill with lesions and without lesions [1].

In 2008, a novel lymphoid tissue called the interbranchial lymphoid tissue (ILT) was discovered in the gills of salmonids and the ILT together with leucocytes in the gill filaments constitute the gill associated lymphoid tissue (GIALT) [18–20]. This novel lymphoid tissue is located at the base of the caudal edge of the interbranchial septum between the gill filaments [18] and is visible as a greyish structure by the naked eye [19]. The ILT consists largely of T-cells embedded in a meshwork of epithelial cells [19] and few B-cells [21]. Abundant MHC class II$^+$ cells are also detectable in the epithelium on the caudal rim of the interbranchial septum and in the epithelium covering the gill filaments [18]. Functional investigations of the ILT have been performed in fish infected with infectious salmon anaemia (ISA) virus and *Neoparamoeba perurans* [21–23]. In ISA virus infected fish, there is a small delayed increase in IgT transcripts [21] and a decrease in size of the ILT compared with healthy fish [22]. Fish affected by AGD show an increased length of the ILT 28 days post exposure in the dorsal area of the gill arch, with a peak of lymphocyte density 7 days post exposure [23].

Further investigation of the T and B cell responses in the ILT is needed to clarify the function of this novel lymphoid tissue during AGD infection in Atlantic salmon. With the increasing number of cytokine genes that are now known, many of which may have a role in adaptive immune responses, the aim of this study was to perform an analysis of T helper (Th) type responses to AGD. For this purpose, gene expression profiles of signature cytokines produced by Th subsets (Th1, Th2, Th17, regulatory T cells – Treg) were screened in the ILT of Atlantic salmon infected with amoeba at two different concentrations (500 and 5000 cells/l), to determine their potential role in host defences against this parasite.

2. Materials and methods

2.1. Amoeba culture

The amoebae were cultivated at 15 °C in small petri dishes containing a 5 ml underlay of malt yeast agar (MYA) (0.05 g malt extract, 0.05 g yeast extract, 10.00 g bacteriological agar, 500 ml of 35 ppt filtered seawater), with approximately 7 ml overlay of 35 ppt filtered sterilized seawater. Stericup® Filter Units (© EMD Millipore Corporation, Billerica, MA, USA, 2014) with a 0.22 μm pore size were used to filter the seawater coming from the North Sea (ca. 35 ppt salinity). Cultures were maintained in a non-axenic environment containing different bacterial strains isolated with amoebae from gills during culture establishment.

2.2. In vivo challenge and sampling of ILT

Amoebae were cultivated to reach a concentration of 500 cells/l and 5000 cells/l for the *in vivo* challenge. The neutral red (NR)-assay was used to determine cell viability. Briefly, 0.35 μl of neutral red (Sigma–Aldrich, Germany) was added to a 100 μl aliquot of amoeba cultures. After 30 min to allow NR uptake, amoebae were centrifuged at 2200 × g for 10 min, the supernatant was removed and amoebae resuspended in 100 μl of sterilized seawater (35 ppt). Counts were performed in triplicate in 96-well plates.

The experiment was designed to establish an AGD challenge with a type I error of 5% assuming a success rate of 70% (power analysis). Atlantic salmon were taken through smoltification in aquarium facilities at the Marine Scotland Science Marine Laboratory in Aberdeen, UK. Fish were held at 12 °C in full-strength seawater (ca. 35 ppt) and fed daily to 1% body weight using the Skretting Atlantic Smolt diet. Two groups of 5 fish (ca. 400 g) were exposed to 500 cells/l and 5000 cells/l in a total volume of 120 l of seawater (ca. 33–35 ppt) and held in this static volume, with aeration, for 4 h. The same procedure was applied for a negative control where 5 fish were exposed to the medium used for amoeba culture, which was filtered with a 3.0 μm pore size Cyclopore™ Track Etched Membrane (GE Healthcare, Whatman, UK), in order to separate out the amoebae but retain the culture bacteria. No signs of distress were observed in fish during this period. After 4 h, the water volume was increased to 350 l and exchanged in a flow through system at a rate of 3 l/min. Fish were fed daily to satiation. At 3 weeks post-exposure, fish were anaesthetised with 0.3 g/l of ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma–Aldrich, Germany) and killed. Tissue samples from the first gill arch were collected, primarily to include the interbranchial lymphoid tissue (ILT) avoiding the gill arch and the end of the gill filaments (Fig. 1). Gill samples were stored in RNA*later* (RNA*later*® Stabilization Solution, Ambion®) at – 80 °C for gene expression and *P. perurans* load analyses.

For histological analysis and assessment of the pathology associated with AGD, samples from the first gill arch were fixed in 10% buffered neutral formalin solution for a minimum of 24 h, washed in 100% ethanol (EtOH), and then stored in 70% EtOH until processing. Samples were then washed three times in 100% EtOH, in xylene (3 dips) and embedded in paraffin wax. Sections (3 μm) were stained with haematoxylin and eosin (H&E stain) and scored

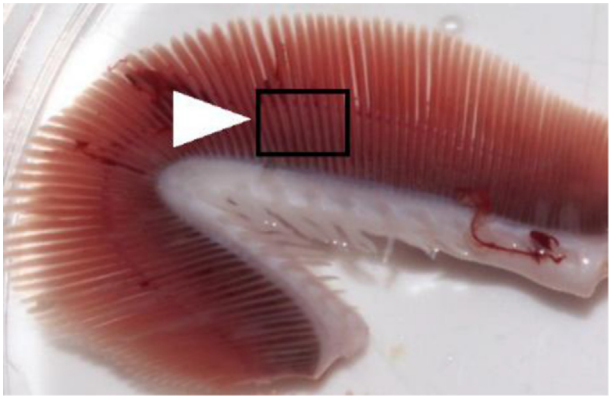

Fig. 1. Localization of the gill samples taken from the first gill arch for gene expression analysis, which included primarily the interbranchial lymphoid tissue (ILT) avoiding the gill arch and the end of the gill filaments (© Marine Scotland Science).