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Interleukin gene expression is strongly modulated at the local level in a fish–parasite model

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ABSTRACT

The goal of this work was to identify interleukin (IL)-related genes in the gilthead sea bream (GSB) (*Sparus aurata* L.) and how they are modulated by the parasite *Enteromyxum leei,* a myxozoan that causes severe enteritis with a strong inflammatory response. A Blast-X search of our transcriptomic GSB database (www.nutrigroup-iats.org/seabreamdb) identified 16 new sequences encompassing seven ILs (IL-7, IL-8, IL-10, IL-12β, IL-15, IL-18, and IL-34), the interleukin enhancer-binding factor 2 (ILF2), and eight IL receptors (IL-R); IL-R1, IL-6RA, IL-6RB, IL-8RA, IL-10RA, IL-10RB, IL-18R1, and IL-22R. Except for ILF2, their expression, plus that of IL-1β, IL-1R2, IL-6, and TNF-α (from public repositories), were analysed by 96-well PCR array of samples of blood, spleen, head kidney, and intestine of GSB that were anally intubated with *E. leei* (recipient group, RCPT). Only the expression profile of the intestine of RCPT fish showed significant difference as compared to samples from PBS-inoculated fish. At 17 days post intubation (dpi), the expression of key pro-inflammatory ILs, such as IL-8, IL-8R, IL-12β, and TNFα was significantly up-regulated, whereas at 64 dpi, anti-inflammatory IL expression (IL-6, IL-6RB, IL-7, IL-10, IL-10RA, and IL-15) was predominant. These results indicate a modification of the IL expression at late times post infection, probably to protect the fish intestine from the parasite and damage inflicted by an excessive inflammatory response. Furthermore, the response is mainly mediated at the local level as no significant changes were detected in blood, spleen and head kidney.

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1. Introduction

The gilthead sea bream (GSB) (*Sparus aurata* L.), a teleostean sparid fish, is currently the main cultured species in the Mediterranean basin, with a total production increasing each year [1]. An understanding of the biology of fish species and of their immune system, in particular, is essential for improving fish health and wealth management [2], as well as for increasing fish farming production, which is currently the most important animal production industry capable of meeting the food demands of the constantly increasing human population. Intensive fish farming practices can also have an increasing effect on the evolution of pathogens, potentially contributing to the development of more virulent pathogens [3]. Among the different pathogens found in sparid fish, Myxozoa represent some of the most dangerous parasites threatening Mediterranean fish farms [4,5]. *Enteromyxum leei*, in particular, dwells in the paracellular space in the intestine of several fish species, causing weight loss, delayed growth, reduced marketability, and even massive mortalities in some hosts [6].

Interleukins (ILs) are a group of cytokines that play a major regulatory role in the immune system. It was initially thought that ILs signalled only between leucocytes, but now it is known that they are produced by and target a wide variety of cells and comprise a complex system of cell-signalling within the immune system. Cytokines in general are produced at the site of entry of a pathogen and drive inflammatory signals that regulate the capacity of resident and newly arrived phagocytes to destroy the invading pathogen. They also regulate antigen presentation function in dendritic cells, and their migration to lymph nodes to initiate the adaptive immune response in mammals [7]. Since the first description of ILs more than 30 years ago [8], 37 ILs have been discovered. While most studies have been performed in mammals, they have recently been extended to lower vertebrates, including fish. In fact, since the adaptive immune system developed in jawed

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vertebrates, studies of fish ILs may shed light on the evolution of these proteins and the immune system, in general [9]. The development of genomic and EST databases, and PCR-based homology cloning has recently led to the description of several key T cell markers, including CD4, CD8, CD3, CD28, and CTLA4, as well as important cytokines in fish, suggesting the existence of different T helper (Th) subtypes, similar to the mammalian Th1, Th2, and Th17 [10,11]. These Th subsets are associated with certain specific cytokine profiles and differentiated functions. Currently, 20 genes encoding teleostean ILs have been identified and characterized [12–15]. However, global analyses of the dynamics of fish IL-related gene expression during parasitic infections are very scarce. To address this, we performed extensive Blast-X searches of the IATS updated transcriptomic GSB database (www.nutrigroup-iats.org/seabreamdb) and as result, we identified 16 new IL-related genes in GSB. This allowed us to develop an IL PCR-array of 19 genes, including tumour necrosis factor alpha (TNF-α), to measure the transcriptomic response of GSB exposed to *E. leei*.

2. Material and methods

2.1. Animal care, experimental design and sampling procedure

Clinically healthy juvenile GSB were obtained from a commercial fish hatchery. Upon arrival to the facilities of the Instituto de Acuicultura Torre de la Sal (IATS), they were checked for the absence of *E. leei* and grown in an open flow system with 5 μm-filtered and UV-irradiated sea water (37.5‰ salinity). Day length corresponded to the natural changes at our latitude (40°5′N; 0°10′E), and water temperature was maintained between 18 °C and 25 °C. The oxygen content of water was kept above 85% saturation, and unionized ammonia remained below toxic levels (<0.02 mg/l). Fish were fed *ad libitum* a commercial diet (BioMar, Palencia, Spain).

The infection was performed by anal intubation as previously described [16]. Briefly, 36 GSB (average initial weight = 60.5 g) were intubated with 0.5 ml of *E. leei* infected-intestinal scrapings (recipient group, RCPT) and 36 fish (average initial weight = 58.7 g) were intubated with the same volume of PBS (control group, CTRL). At 17 (time point = t1) and 64 (time point = t2) days post intubation (dpi), seven fish from both the CTRL and RCPT group were sacrificed by overexposure to the anaesthetic MS-222 (Sigma, St. Louis, MO, USA) and pieces of head kidney (HK), spleen (SP), and posterior intestine (PI), were rapidly excised, frozen in liquid nitrogen and stored at −80 °C. Tissue samples of anterior (AI), middle (MI), and posterior (PI) intestine were also taken for parasite diagnosis. Blood was collected from the caudal vein with EDTA-treated syringes and 150 μl were transferred to cooled eppendorf tubes with 500 μl lysis solution until RNA extraction.

The experiment was carried out in accordance with the principles published in the European animal directive (86/609/EEC) for the protection of experimental animals, and was approved by the Consejo Superior de Investigaciones Científicas (CSIC) ethics committee and IATS Review Board, with permits associated to project AGL2009-13282-C02-01.

2.2. Diagnosis of the infection

Parasite diagnosis was performed on AI, MI, and PI intestine samples fixed in 4% paraformaldehyde, processed following routine histological procedures, embedded in paraffin, and stained with Giemsa. Infection intensity in each organ was semiquantitatively evaluated in histological sections following a conventional scale from 1+ to 6+, with the following ranges: 1+ = 1–5; 2+ = 6–10; 3+ = 11–25; 4+ = 26–50; 5+ = 51–100; 6+ >100 per microscope field observation at 120×. *E. leei* stages were classified as spores, sporoblasts, and proliferative stages, the latter corresponding to stages one to three described in Ref. [17]. A fish was considered positive for infection, when the parasite was found at least in one intestinal segment.

2.3. RNA extraction and reverse transcription

Total RNA from target tissues was extracted using the MagMAX™-96 total RNA isolation kit (Applied Biosystems, Foster City, CA, USA). RNA from blood samples was extracted and purified using the real total RNA spin blood kit (Durviz SL, Valencia, Spain). The RNA yield was 30–50 μg with absorbance measures (A260/280) of 1.9–2.1. Reverse transcription (RT) of 500 ng total RNA with random decamers was performed with the High-Capacity cDNA Archive kit (Applied Biosystems) and negative control reactions were run without reverse transcriptase.

2.4. Gene expression analysis

Real-time quantitative PCR was carried out with the CFX96 Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), using a 96-well PCR array layout designed for simultaneously profiling a panel of 19 genes from 4 individuals under uniform cycling conditions. The array included 18 IL-related genes, the cytokine TNF-α, the peroxisome proliferator-activated receptor-gamma (PPARγ) as a standard gene, and β-actin as a housekeeping gene. The genes of interest included members of the IL-1, IL-6, IL-10, IL-12, common γ-chain cytokine, CXC chemokine, and other undetermined families, as well as some of their receptors. The liquid manipulations required to perform the PCR array were performed by means of the EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany). Briefly, for each RT reaction, 660 pg of total input RNA was diluted to a 25 μl volume for each PCR reaction. PCR-wells contained a 2× SYBR Green Master Mix (Bio-Rad) and specific primers at a final concentration of 0.9 μM were used to obtain amplicons of 50–150 bp in length (Table 1). The PCR reaction was run under the following conditions: an initial denaturation step was carried out at 95 °C for 3 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 60 s at 60 °C. The efficiency of the PCR reactions was always higher than 90% (amplification factor >1.90) and similar for all genes. Negative controls without sample templates were routinely performed for each primer set. The specificity of the reactions was verified by analysis of melting curves (ramping rates of 0.5 °C/10 s over a temperature range of 55–95 °C), the linearity of serial dilutions of RT reactions, and electrophoresis and sequencing of the amplified products. Fluorescence data acquired during the PCR extension phase were normalized by the delta–delta Ct method [18]. Inter-assay variation was corrected using the serial dilutions of the standard gene as common reference values among plates. Technical replicates of the samples were run initially to test the reproducibility of the method. As the obtained data had a very high reproducibility score, technical replicates were finally omitted.

2.5. Statistics

Gene expression data are represented as the mean ± SEM of seven fish for each group shown in Fig. 2 (intestine). In Supplementary Table 1 (HK, SP, blood) only the averages are shown for visual simplification. For each gene from the different tissues, the effects of pathogen exposure were analysed by the two-tailed Student's *t* test. When the test of normality or equal variance failed, a Mann–Whitney Rank Sum test was applied instead. The significance level was set at P <0.05. All statistical

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