Local immune responses to two stages of Ichthyophthirius multifiliis in ginbuna crucian carp

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Short communication

Local immune responses to two stages of *Ichthyophthirius multifiliis* in ginbuna crucian carp

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Abstract

Ichthyophthirius multifiliis is a ciliated protozoan parasite and is known to infect many freshwater teleosts. Characterizing the immune system in epithelial tissues, where the parasites penetrate and settle, is key to understanding host–parasite interactions. This study examined local immune responses *in vivo* to the infective stage (theront and trophont) of the parasites using intra-fin administration, which has been developed to analyze *in vivo* immune responses using fish fin. $CD8\alpha^+$ and $CD4^+$ T-cell compositions were increased significantly in the fin cavity injected with theront or trophont antigens. The expression of GATA-3 and T-bet mRNA, which regulate differentiation of helper T-cells, was upregulated significantly in leukocytes from the trophont antigen–injected site. In contrast, the percentages of macrophages and neutrophils, which are innate immunity components, were decreased significantly in the injection sites. These results suggest that *I. multifiliis* antigens inhibit the migration of macrophages and neutrophils, and T-cells are the first responders to *I. multifiliis*. Thus, to better understand the interaction of host immunity and *I. multifiliis*, further studies should focus on exploring the inhibitory factors from *I. multifiliis* or examining innate functions of teleost T-cells.

Key words: local immune response, parasites, intra-fin administration, ginbuna crucian carp

1. Introduction

Ichthyophthirius multifiliis is a ciliated protozoan that infects many freshwater fish species, causing white spot disease, and often leads to significant economic losses in the edible and ornamental fish industry [1,2]. The *I. multifiliis* life cycle consists of four developmental stages: theront (free-swimming infective stage), trophont (stage that feeds on fish tissue), protomont (stage just after being detached from hosts after full development), and tomont (cystic stage producing and releasing theronts) [1]. The infective theront stage of the parasite invades the skin and gills of fish, penetrates the epidermis, settles above the basal lamina, and immediately transforms into the trophont. Thus, it is important to understand how host leukocytes recognize these stages of the parasites and eliminate them during the theronts' transformation to mature trophonts.

I. multifiliis provides an optimal model to study immunity against extracellular parasites [3-5]. Although information on immunity against *I. multifiliis* is gradually accumulated in several fish species, the *in vivo* immune responses to the stages from theronts to trophonts still remain unclear. Recently, a novel method to analyze *in vivo* immune responses using fish fin has been established in ginbuna crucian carp, and has shown *in vivo* immune functions, such as phagocytosis [6] and lymphoblasts [7]. As this technique provides a unique tool that could help better understand the mechanisms of local immune responses to antigens in epithelium tissues, we considered it necessary to monitor *in vivo* the immune responses to *I. multifiliis*. Therefore, this study identified leukocytes that respond to *I. multifiliis* antigens using the intra-fin administration method.

2. Materials and Methods

2.1. Fish

The OB1 strain of the clonal ginbuna crucian carp, *Carassius auratus langsdorfii*, was maintained at a temperature of 25°C and fed daily with commercial food pellets [8]. The fish weighing approximately 10 g were used in this study. The spontaneous occurrence of white spot disease was not observed in these experimental fish. The fish were anesthetized with 25 mg/L quinaldine when injected or dissected. All experiments using fish were performed following the guidelines of the Animal Experiments Committee at Kyushu University.

2.2. Preparation of antigens

The preparation of theronts followed the method of Sukeda et al. (2021), with minor modifications [8]. Briefly, the parasite strains used in this study originated from infected fish obtained from Kyorin Co., Ltd. (Himeji city, Hyogo prefecture) and ornamental fish shops in Fukuoka city. Live theronts were collected from water in an aquarium with *I. multifiliis*–infected fish. To remove large impurities, theronts were passed through a sieve with a pore size of 37 μm (ITOH SEISAKUSHO CO., Tokyo, Japan). The theronts collected were concentrated by centrifugation at 100 × g for 5 min and were identified as *I. multifiliis* by microscopic observations and polymerase chain reaction (PCR) using *I. multifiliis*–specific primers (data not shown) [9]. Theronts were counted using an optical plastic plankton counter (MATSUNAMI, Osaka, Japan). Some of them were killed and disrupted by sonication. The live and sonicated theronts were used as antigens for intra-fin administration.

To prepare the trophont antigen, the fish infected with mature parasitic trophonts were anesthetized with 25 mg/L quinaldine and rinsed with water. The anesthetized fish were placed on Petri dishes containing pure water, and the skin was scraped gently using a cell scraper (Zellschaber; Techno Plastic Products AG, Trasadingen, Switzerland) to collect the trophonts. The trophonts were separated gently from the mucosa by pipetting and washed with pure sterile water; then, they were disrupted by sonication. As the trophonts could not be injected because of their size, only sonicated (killed) trophonts were used as antigens for intra-fin administration.

To visualize *I. multifiliis*, the live theronts and trophonts were incubated with 2 μg/mL Calcein-AM (Dojindo, Kumamoto, Japan) at room temperature for 30 min. After washing them twice with pure water, the calcein-labeled parasites were observed under a fluorescence stereomicroscope (MZ10F; Leica, Germany) (Fig. 1A and B). The cell volumes of trophonts and theronts were approximately compared by estimating the calcein released from them. Theronts were serially diluted and sonicated, and the released calcein fluorescence was subsequently quantified using Nanodrop 3300 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The standard curve was constructed according to the cell number of theronts and their intensities, and the intracellular concentration of calcein from trophonts was compared with that from theronts.

2.3. Intra-fin administration

Intra-fin administration of the antigens was conducted according to the method described by Matsuura et al. [6], with slight modification for a parasite antigen. Briefly, the theronts were suspended and adjusted to 10,000 cells/200 μL in phosphate-buffered

saline (PBS). The live and killed theronts were injected into the base of one compartment of the dorsal fin (Fig. 1C) in a volume of 20 μL (1,000 cells/fish). The trophont antigen was adjusted to an amount equivalent to 1,000 cells of theronts, as described above. The control fish were administered the same volume of PBS.

To confirm the administered theronts, calcein-labeled live theronts, which were injected into the fin, were visualized using a fluorescence stereomicroscope (Fig. 1D and E).

2.4. Preparation of fin leukocytes and flow cytometry analyses

Three fish were sampled at 3, 12, 24, and 48 h after intra-fin administration. Tissues, including the administration site, were cut from the fin base and disaggregated by pressing through a 150-gauge mesh stainless steel sieve in OPTI-MEM medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (Biowest, Nuaillé, France). The cells were washed with OPTI-MEM. $CD8^+$ T, $CD4^+$ T, IgM^+, and macrophages/granulocytes were detected by flow cytometry (FCM) following methods reported previously [6, 10, 11]. $CD8^+$ T, $CD4^+$ ($CD4\text{-}1^+$) T, and IgM^+ cells were stained with monoclonal antibodies (mAb; rat mAb, 2C3, and 6D1; and mouse mAb, B12, and GB21, respectively). Donkey anti-rat IgG (H+L) and goat anti-mouse IgG (H+L) conjugated with Alexa Fluor 488 (Thermo Fisher Scientific) were used as secondary antibodies. T cells, B cells, macrophages and neutrophils were identified as positive cells in lymphocyte, macrophage, and granulocyte gate, respectively (Fig. S1). The percentages of the cells were calculated as positive cells per cell in forward and side scatter (FSC and SSC) gating, which excluded cells in lower FSC and SSC than lymphocytes, and higher ones than granulocytes.

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152 ***2.5. Gene expression analyses of immune-related genes***

153 Three fish in each group were sampled 3 and 48 h after intra-fin administration. The
154 cells from the fin cavity were collected as described above. Extraction of total RNA and
155 real-time PCR were conducted according to a previous study [11]. Briefly, total RNA
156 was extracted from 1.0×10^6 cells of the gill and kidney leukocytes using the
157 NucleoSpin RNA II (Macherey-Nagel GmbH & Co. KG, Duren, Germany), according
158 to the manufacturer's protocol. First-strand cDNA was synthesized from total RNA
159 using the Moloney-murine leukemia virus reverse transcriptase (Invitrogen) with an
160 oligo (dT) primer, according to the manufacturer's instructions. Primers used for
161 real-time PCR were previously designed to amplify cDNA fragments encoding the
162 following T-bet, GATA-3, and IgM to evaluate T- and B-cells [11]. For normalization,
163 EF-1α served as an internal control for the normalization [10]. The primer sets and
164 thermal cycling are indicated in the reference articles. The expression of inducible nitric
165 oxide (iNOS) was evaluated for inflammation in the antigen-injection site. The primer
166 sequences for IL-10 were as follows: forward primer
167 (5′-TTGGTACATGGGCACTGAGATT-3′) and reverse primer
168 (5′-CCAACCCGCTCAAGAACATT-3′). The relative quantitative value of each gene
169 was calculated according to the standard curve from a serial dilution of a reference
170 cDNA in the same plate and normalized with the level of EF1α. Data from the three fish
171 were shown as the mean fold change in mRNA expression relative to the
172 non-administered fish.

173

174 ***2.6. Statistical analyses***

The statistical significance was compared between the control (PBS-administered fish in each time point) and experimental groups by one-way analysis of variance, followed by the Dunnett test. The significance level was set at 0.05.

3. Results and Discussion

Intra-fin administration has been developed as a simple and noninvasive method to analyze local immune responses *in vivo* [6]. We considered this a suitable method to analyze local immune responses to the infective stage of the parasites, which settles in the epithelial tissue. First, the administered *I. multifiliis* were visually observed in the fin cavity, indicating that this method can be used to administer the parasites and monitor local immune responses *in vivo* (Fig. 1). The fish injected with the antigens or PBS did not die and did not show disease symptoms during the experiment.

The percentage of $CD8\alpha^+$ and $CD4^+$ T-cells in the killed-theront-injected fish was significantly higher than those in the control fish sampled 12 and 48 h after administration (Fig. 2A). Similarly, the trophont antigen induced increases of $CD8\alpha^+$ and $CD4^+$ T-cell compositions at injection sites, but not at 12 h after administration (Fig. 2B). GATA-3 and T-bet, which regulate the differentiation of helper T-cells, were upregulated significantly by administration with only trophonts (Fig. 3A and B), suggesting that trophont antigens induced T-cell differentiation at the injection sites. Alternatively, the composition of IgM^+ cells and expression of the IgM gene were unregulated in all groups (Fig. 2C and Fig. 3C). The cell composition profile suggests that T-cells migrate initially to the *I. multifiliis* infectious site, whereas B-cells do not.

Contrary to T-cells, neutrophils and macrophages, which are important components of innate immunity, seem to be inhibited by *I. multifiliis*. The percentages of

macrophages from fish sampled 24 h after killed-theront administration were significantly lower than those from the control fish (Fig. 2D). Neutrophil compositions from fish were collected and analyzed 3, 12, 24, and 48 h after theront antigen administration, or 12 and 48 h after trophont antigen administration, and were significantly lower than those from the control fish (Fig. 2E). These results suggest that neutrophils and macrophages, especially neutrophils, are inhibited from migrating by *I. multifiliis* antigens. *I. multifiliis* has been reported to express a protein similar to chlamydial polymorphic repeat-containing outer membrane protein, which can suppress the innate immune response, suggesting that theronts can escape from the host immune system [12-14].

The expression of iNOS mRNA was upregulated significantly 48 h after trophont administration (Fig. 3D), suggesting that inflammation is induced at the injection site. As the macrophages and neutrophils were not increased after administration, this inflammation may be induced by cells other than these leukocytes. Immunohistochemical analyses have shown that iNOS is expressed in the epidermis and outer layers of gill filaments and lamellae in angler catfish [15]. This finding speculates that trophont antigens trigger iNOS production from their epithelial cells in the fin.

We did not directly compare the values between the theront- and trophont-administered fish because there is no way to correctly adjust their antigen doses. However, the comparison between the controls suggests that theront antigens induced more rapid migration of T-cells than trophont antigens. Trophont antigens induce the upregulation of GATA-3 and T-bet, but not theront antigens, suggesting that the differentiation of T-cells occurs at the trophont antigen injection site. Live theronts did not induce the migration of leukocytes and regulation of the immune-related genes.

Although they were observed in the fin cavity under the fluorescent microscope 4 h after administration (data not shown), they were also scattered in the cavity, and seemed to have decreased. Thus, some of the theronts may migrate into other tissues within 4 h after administration, and they did not induce the migration of leukocytes in the fin cavity. Alternatively, there is a possibility that live theronts inhibit host immune responses more efficiently than killed *I. multifiliis* antigens. The *in vitro* cultivation of *I. multifiliis* using an Epithelioma Papulosum Cyprini (EPC) cell line showed that theronts transformed within 2 days into the trophont stage [16]. However, in our experiment, neutrophils and macrophages were not increased at the injection sites within 48 h, suggesting that both theronts and trophonts efficiently inhibited the activity of host innate immune cells. Therefore, to better understand host immunity against the parasites, further investigations are required to identify the inhibitory factors involved in the interaction between hosts and *I. multifiliis*.

In conclusion, theront and trophont antigens induce T-cell migration, but not neutrophil and macrophage migration. This fact is consistent with a previous study on the *in vitro* immune response to theronts [8]. $CD8^+$ T-cells are innate cytotoxic cells against theronts in ginbuna crucian carp and contribute to innate immunity against the parasites. As teleost T-cells are abundant in epithelial tissues [17], they may act as important initiators in the first line of defense, not only as adaptive immune cells. Mucosal-associated invariant T-cells (MAIT cells), identified as unconventional T-cells in mammals, have also been reported to contribute to innate immunity in the mucosal tissues of mammals [18]. Thus, further studies should focus on elucidating the functions of “innate T-cells” in the mucosal tissues of teleost fish.

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Conflicts of Interest: The authors declare no conflict of interest.

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Figure Legends

Fig. 1. Administration of *I. multifiliis* in the fin cavity. Images of calcein-labeled theronts (A) and a trophont (B). *I. multifiliis* antigens were injected into one dorsal fin compartment (white arrow) (C). Observation of live theronts administered in the fin cavity (D: bright field; E: dark field).

Fig. 2. Percentages of $CD8\alpha^+$ T-cells (A), $CD4^+$ T-cells (B), IgM^+ B-cells (C), macrophages (D), and neutrophils (E) from the fin cavity. Open, shaded, dotted, closed, and hatched bars indicate the percentages from non-administered, PBS-, live theront-, killed-theront-, and trophont-administered fish, respectively. Results are presented as the means of three individual fish, and error bars indicate the standard deviation (SD). The asterisks indicate significant differences between the control (PBS-administered fish) at each time point (*$P < 0.05$).

Fig. 3. Quantitative expression profiles of T-bet, GATA-3, IgM, and iNOS in cells from the fin cavity. Open, shaded, dotted, closed, and hatched bars indicate the percentages from non-administered, PBS-, live theront-, killed-theront-, and trophont-administered fish, respectively. Results are presented as the means of three individual fishes, and error bars indicate the SD. The asterisks indicate significant differences between the control (PBS-administered fish) at each time point (*$P < 0.05$).