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The development of cellular immune defence in marine medaka *Oryzias melastigma*

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ABSTRACT

Environmentally induced alterations of the immune system during sensitive developmental stages may manifest as abnormalities in immune organ configuration and/or immune cell differentiation. These not only render the early life stages more vulnerable to pathogens, but may also affect the adult immune competence. Knowledge of these sensitive periods in fish would provide an important prognostic/diagnostic tool for aquatic risk assessment of immunotoxicants. The marine medaka *Oryzias melastigma* is an emerging seawater fish model for immunotoxicology. Here, the presence and onset of four potentially sensitive periods during the development of innate and adaptive cellular immune defence were revealed in *O. melastigma*: 1.) initiation of phagocyte differentiation, 2.) migration and expansion of lymphoid progenitor cells, 3.) colonization of immune organs through lymphocyte progenitors and 4.) establishment of immune competence in the thymus. By using an established bacterial resistance assay for *O. melastigma*, larval immune competence (from newly hatched 1 dph to 14 dph) was found concomitantly increased with advanced thymus development and the presence of mature T-lymphocytes. A comparison between the marine *O. melastigma* and the freshwater counterpart *Oryzias latipes* disclosed a disparity in the T-lymphocyte maturation pattern, resulting in differences in the length of T-lymphocyte maturation. The results shed light on a potential difference between seawater and freshwater medaka in their sensitivity to environmental immunotoxicants. Further, medaka immune system development was compared and contrasted to economically important fish. The present study has provided a strong scientific basis for advanced investigation of critical windows for immune system development in fish.

1. Introduction

Even though it is widely recognized that embryonic, larval and juvenile stages in fish are more susceptible to toxicant exposure than adult individuals, safety limits for environmental risk assessment rely mainly on adult toxicity data. It is therefore questionable if sufficient protection is provided for developing fish (Barrett, 2012). Environmental agent induced toxicity has been shown to interfere with various body systems at specific sensitive developmental periods: critical windows. In fish, the critical windows concept so far has been employed in the assessment of reproductive system development (Ankley et al., 2009; Maack and Segner, 2004), whereas the application of this concept for immune system development has been overlooked. The critical windows of developmental immunotoxicity (DIT) are defined as the sensitive stages of early immune system development, during which any induced malformation/impairment may result in long-term impacts in adulthood (Burns-Naas et al., 2008; Dietert and Holsapple, 2007; Holsapple and O'Lone, 2012). In fish, chemical-induced DIT may render the larvae more vulnerable to pathogens, and potentially weaken immune competence in adulthood. The knowledge of DIT is particularly important for the understanding of immune related diseases in adult feral fish (Burns-Naas et al., 2008; DeWitt et al., 2012; Holsapple and O'Lone, 2012).

Previous research in juvenile sea bass highlighted the existence of sensitive periods during the primary immune organ maturation, head kidney and thymus, in response to exogenous 17β-estradiol (Seemann et al., 2016, 2015). As for most other economically important marine fish species, maturation times are rather long for sea bass. Immune maturity is achieved about 4–5 months after hatching and immune organ development is completed in 8–12 month old individuals (dos Santos et al., 2000). Adulthood is reached at an age of about 4–5 years, rendering the tracking of impaired immune competence in adults due to early developmental exposure very costly and time-consuming (Pickett and Pawson, 1994). In this study, the marine medaka *Oryzias melastigma* is proposed as alternative seawater fish model for studying DIT. The small size *O. melastigma* offers many advantages: (i) its whole life

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cycle is completed in seawater under optimal laboratory conditions, (ii) short generation time (3 months), which allows for time- and cost-effective assessment of lifelong impacts, (iii) high fecundity, (iv) transparent embryos and larvae, and (v) availability of multiple tissue omics data (miRNAs, mRNAs and proteins) (Lai et al., 2015; Kim et al., 2016; Chen et al., 2015). Furthermore, *O. melastigma* has gained increasing recognition as a model fish for marine ecotoxicology and immunotoxicology (Dong et al., 2014; Li et al., 2014; Rhee and Rhee, 2016; Kong et al., 2008; Ye et al., 2016). However, its immune system development and the existence of critical windows during immune system development remain poorly understood. So far, only a few studies reported *O. melastigma* immune system development. Bo et al. (2011) investigated the developing immune system in regard to the antimicrobial peptide hepcidin gene expression until hatching. Huang et al. (2012) and Fang et al. (2013) reported changes in the inflammation response in embryos until hatching after exposure to bisphenol A and in 1-month old marine medaka after embryonic exposure to PFOS, respectively. An in-depth knowledge of immune system development and associated DIT is most needed for *O. melastigma*. On the other hand, its freshwater counterpart, the Japanese medaka *Oryzias latipes*, has been more thoroughly investigated for cellular immune system development (staging after Iwamatsu, 2004). Briefly, hematopoiesis and phagocyte development take place between stage 21–24 (Moriyama et al., 2010, Grabher et al., 2007; Aghaallaei et al., 2010), providing the baseline for fish innate immune defence. The thymic primordium is colonized by the lymphoid progenitor cells between the stages 30–32 (Bajoghli et al., 2015; Li et al., 2007a). A medullary-like thymus compartment containing mature T-lymphocytes, which was not described histologically before 14 days post hatching (dph) (Li et al., 2007a), has been recently evinced by the medulla-specific *aire* gene expression at 7 dph (14 days post fertilisation (dpf)) in *O. latipes* (Bajoghli et al., 2015). The head kidney, considered as the principal hematopoietic site in teleost, begins to develop from stage 19–20 on in *O. latipes* (Fedorova et al., 2008).

In this study, the early life stage development of immune organs (thymus, head kidney, spleen) in *O. melastigma* were characterized and contrasted with the freshwater *O. latipes*. The findings are essential for defining potentially sensitive immune system developmental stages in marine medaka and necessary for comparison of seawater and freshwater fish sensitivity to environmental immunotoxicants. Furthermore a possible extrapolation of findings from this study to the immune system development of economically important fish species and mammals was discussed.

2. Material and methods

2.1. Animal origin and maintenance

The orange-yellow Japanese medaka strain (*O. latipes*), originally provided by the Molecular Aquatic Toxicity Laboratory, Duke University (NC, USA), has been maintained at the City University of Hong Kong since 2008. The marine medaka strain (*O. melastigma*) was purchased from Interocean Industries (Taiwan) and kept in the State Key Laboratory in Marine Pollution in Hong Kong for 30 generations. Both strains are kept in optimal rearing conditions (Ye et al., 2016; Gopalakrishnan et al., 2013). Fertilized eggs were collected on a daily basis and reared in an optimal environment (*O. latipes*: Kinoshita et al., 2009; *O. melastigma*: 26 °C, 25‰, 12:12 h light:dark cycle). The fresh/seawater was changed daily and dead eggs/larvae discarded. The embryos and larvae were sampled daily from 0 days post fertilisation (dpf) to 28 days post hatching (dph). After hatching larvae were fed two times per day with dry food and once per day with *Artemia* nauplii. The embryo stages were defined after Iwamatsu (2004). Animal maintenance and the experiments described below were approved by the animal ethics committee from the City University of Hong Kong.

2.2. Detection of first appearance of phagocytes with Sudan Black

Six embryos and larvae of each species (*O. melastigma* and *O. latipes*) with ages from zero dpf to one dph (0, 1, 2, 3, 4, 5, 6, 7 dpf and 0, 1 dph), were fixed in 4% paraformaldehyde overnight at 4 °C after manual dechorionation. The next day, the samples were washed with PBS/Tween-20 and stained with a 0.03% Sudan Black/Ethanol solution for 30 min at room temperature. After washing the fish with 70% ETOH and rehydration with PBS/Tween-20, the embryos/larvae were incubated with a 1% KOH/H_2O_2 solution at room temperature for 15 min to clear the samples. Pictures of the embryos/larvae were taken with the microscope imaging system Nikon-Eclipse 90i. Sudan black, was used to identify the appearance of myelomonocytic cell lineage, indicative for the onset of phagocyte (neutrophil and monocyte) differentiation (Le Guyader et al., 2008).

2.3. Immune cell specific gene expression by WISH

Whole mount in situ hybridization (WISH) was conducted in developing embryos (*O. melastigma* and *O. latipes*) sampled daily from two dpf to 28 dph ($n = 6$ per probe) to localize the expression of *recombinant activation gene 1* (*Rag1*), a marker for lymphocyte differentiation and thymus maturation, and the *T-cell receptor β* (TCR-β) and *T-cell receptor* γ (TCR-γ) genes, which indicate the maturation of T-lymphocytes (Hunt and Rice, 2008; Huttenhuis et al., 2005; Willett et al., 1997). The probe-containing plasmids were kindly provided by B. Bajoghli (EMBL, Heidelberg). The plasmids were transformed into competent *Escherichia coli* cells (DH5α strain) via heat-shock at 42 °C, multiplied on ampicillin-agar plates, and afterwards extracted with the PureYield Plasmid Miniprep System (Promega) following the manufacturers description. The so obtained plasmids were then digested with the respective enzymes and the quality of the digestion was controlled through gel-electrophoresis. After Phenol-chloroform precipitation the DNA quality was again controlled through gel-electrophoresis and the quantity determined using Nanodrop measurement. The DNA was transcribed into digoxigenin-labelled RNA using the DIG RNA labeling kit (SP6/T7, Roche) following the suppliers description for 2 h at 37 °C. The whole mount in-situ hybridization was conducted manually following Quiring et al. (2004). All stages of both species were processed at the same time for the same probe. Six individuals were used per stage. Images were taken with the microscope-camera system Nikon-eclispe 90i.

2.4. Histological determination of growth and compartmentalisation of immune organs

To associate the gene expression with the morphology and structure of the immune organs in *O. melastigma* head kidney, thymus and spleen, four larvae at 0, 7, 11, 14 and 17 dph were fixed in paraformaldehyde overnight, embedded in an agarose gel as described by Cheung et al. (2012), gradually dehydrated and embedded in paraffin. 5 µm thick Sections were cut with a microtome (Leica). The sections were allowed to adhere to the glass slides and dry overnight at 37 °C. Subsequently, the microscope slides were stained with Haematoxylin–Eosin and mounted. The volume of the organs and their compartments were measured as described in Seemann et al. (2015) using the microscope imaging system Olympus BX51and the software CAST (Olympus). Even though present at 0 dph, the spleen was too small for volume determination; therefore, data are presented from 7 dph onwards.

2.5. Larval host resistance assay (larval HRA)

To assess resistance to pathogens at different stages of thymocyte maturation, *O. melastigma* (0, 7 and 14 dph) were exposed to *Edwardsiella tarda*, a widely distributed pathogenic bacterium for marine and freshwater fish (Mohanty et al., 2007). An *E. tarda* stock