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Original research article

Fish red blood cells express immune genes and responses

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ABSTRACT

The erythrocytes or red blood cells (RBCs) and leucocytes or white blood cells (WBCs) are the most abundant cell types in the circulatory system of vertebrates. The RBCs are responsible for gas exchange while WBCs are solely involved with immunity. In this study, we present evidence that fish RBCs despite their respiratory role they also express immune-related genes and responses in the teleost fish, Nile tilapia (*Oreochromis niloticus*). Fluorescent staining identified the presence of numerous mitochondria and lysosomes in tilapia RBCs. RNA sequencing of tilapia RBCs and WBCs led to the assembly and annotation of 20.876 transcripts of which 1017 and 3628 transcripts were specific to RBCs and WBCs, respectively. Pairwise sequence comparisons identified 3251 transcripts that were significantly differential expressed of which 707 (21.75%) were up-regulated and 2544 (78.25%) down-regulated in RBCs when compared to WBCs. 809 transcripts were assigned to 16 immune system pathways, 21 and 163 transcripts were specifically expressed in tilapia RBCs and WBCs respectively, and 23 and 233 transcripts were highly expressed in RBCs and WBCs respectively. Expression of 7 immune genes (interferon regulatory factors (*irf*) *1–5* and *9* and *tlr3*) in both WBCs and RBCs was validated by RT-PCR analysis. Quantitative real-time PCR revealed that all genes were up-regulated in poly(I:C)-challenged WBCs and *irf* (*1, 3, 4,* and *9*) and *tlr3* were also significantly up-regulated in poly(I:C)-challenged RBCs. Therefore, fish RBCs expressed immunity genes and responses and they may play a complementary role in vertebrate immunity.

1. Introduction

In vertebrates, the most abundant cell type in circulation are the erythrocytes or red blood cells (RBC), that are nucleated cells in the majority of vertebrates with the exception of mammals. All nucleated non-mammalian RBC contain organelles in the cytoplasm (Davinia & Mackenzie, 2011) and show different maturation stages including changes in the cytoplasmatic shape, staining, nuclear size and chromatin density. RBC total RNA (tRNA) and organelles content follow an inverse relationship with cellular age and young RBC possess a higher concentration of tRNA while aged cells display loss of cellular organelles including ribosomes and mitochondria (Lund, Phillips, Moyes, & Tufts, 2000). The white blood cells (WBCs) are the immune system circulatory cells and participate in both innate and acquired immune responses by expressing cell-specific immune-relevant genes (Abbas et al., 2005). Extensive studies in fish characterized the immune modulatory mechanisms of different WBCs types (Magnadóttir, 2006; Zapata, Diez, Cejalvo, Gutierrez-de Frias, Cortes, 2006), however, immune-relevant genes can also be expressed in RBCs and still serve functions associated with the immune response. In circulation the major role of RBCs is the transport of gases to cells and tissues, however they seemed to serve other function than oxygen delivery (Morera & MacKenzie, 2011). Progress has been achieved on the characterization of RBCs immune function, from identifying RBC immune adherence function (Fearon, 1980; Nelson, 1953) to the “red cell immune system” hypothesis which claims that RBCs are as important as WBCs in immune defense (Siegel, Liu, & Gleicher, 1981). In fish, some genes including enolase, myxovirus resistance and type I interferon (IFN) genes that are characteristic of WBC are also expressed in RBCs and changes of the gene expression profile with external stimuli has been observed for fish and chicken

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RBC (Passantino et al.,2007; St Paul, Paolucci, Barjesteh, Wood, & Sharif, 2013). several Studies expression of immune-relevant genes have also shown that RBCs constitutively express toll-like receptors (TLRs) in the Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Morera et al.,2011; Workenhe et al., 2008).

An alternative strategy to characterize the function of blood cells is to investigate their entire gene expression profile rather than individual transcripts. Transcriptomic analysis of human RBCs or WBCs have been conducted (Abbas et al., 2005; Kohane & Valtchinov, 2012) and characterization of the mammalian RBCs at different developmental stages (erythroblasts, reticulocytes and mature) indicated a wide spectrum of expressed genes associated with the immune defense (Goh, 2007; Kabanova et al., 2009; Sennikov et al, 2004). Across vertebrates many viral, prokaryotic and eukaryotic pathogens directly target RBCs and a significant number of associated pathologies have been described (Lewis, Hori, Rise, Walsh, & Currie, 2010; Morera et al.,2011). In contrast, fewer studies have described the expression profile of blood cells in non-mammalian vertebrates. In zebrafish comparative analyses evidenced that there is a higher gene turnover of transmembrane proteins in the Natural Killer (NK) cells in relation to T when compared to mammals (Carmona et al., 2017). In the rainbow trout (*O. mykiss*) the genes that are differential expressed in RBCs under different temperatures are involved in stress response, immune response and apoptosis (Lewis et al., 2010; Morera et al., 2011). Major phenotypic changes also occur in fish RBCs when infected with Pseudorabies (PRV) and PRV infection of salmon RBC was found to activate innate antiviral immunity but to suppress other gene expression programs (Dahle et al.,2015). Nucleated RBC from fish and birds express and regulate specific pattern recognition receptor (PRR) and are capable of specific pathogen associated molecular pattern (PAMP) detection that is central to innate immune response. Thus nucleated RBC from non-mammalian vertebrates seem to play a key role in the immune response (Morera et al., 2011) but insufficient systematic and comprehensive data is available. Next-generation sequencing (NGS) is a robust technology to study gene expression due to its high-throughput and accuracy (Metzker, 2010). In the present study we applied NGS technology to characterize the expression profile of RBCs and WBCs of the teleost fish Nile tilapia (*Oreochromis niloticus*). Tilapia is an important economic and commercial teleost fish species and availability of a sequenced and annotated nuclear genome facilitates transcript mapping and annotation (Brawand et al.,2014). The present study elucidates on the expression profile of tilapia blood cells and provides new insights into the evolution of RBC immune function across vertebrates.

2. Materials and methods

2.1. Fish and blood cells purification

Experiments with fish were performed in agreement with the guidelines for the care and use of animals for scientific purposes approved by the Committee on the Ethics of Animal Experiments of Shanghai Ocean University. Fish were sacrificed, and all efforts were exerted to minimize suffering. Clove oil (30–40 mg/L) was used for anesthesia. Nile tilapia (*O. niloticus*) were maintained in outdoor breeding pond of 5 m wide and 10 m long at the Xinchang Aquafarm of Shanghai Ocean University. The strain “New GIFT” has been selected for more than 20 years. Blood samples were collected from the caudal vein of six fish with a heparinized syringe and RBCs and WBCs were separated by gradient centrifugations. Anticoagulant blood was carefully layered over the separation medium (1.080 g/mL) (Beijing Propbs Biotechnology company, China) and centrifuged at 400×*g* and 4 °C for 20 min. RBCs were collected from the bottom and WBCs that are localized in the middle were treated with RBC lysis solution (Beijing Propbs Biotechnology Company, China) to eliminate potential RBCs before collection. RBCs and WBCs samples were washed twice with 1 × PBS and stored at −80 °C for RNA isolation.

2.2. Cells staining and microscopy

Smears of separated RBCs and WBCs samples were Giemsa-stained for standard light microscopy. The purity of separated RBCs and WBCs were examined by counting 10 fields of smears based on cell morphology. For fluorescent staining of mitochondria and lysosomes, live RBCs were immediately washed with 1 × PBS after separation and incubated with MitoTracker Red CMXRos and LysoTracker® Green DND-26 (Invitrogen, USA) in 200 nmol/L Dulbecco's Modified Eagle Medium at 28 °C for 10 min. RBCs were washed with 1 × PBS and used to prepare smears. All microscope observations and photographs were carried out using a Nikon Eclipse Ni-E microscope.

2.3. Polyinosinic: Polycytidylic acid [Poly(I:C)] challenge

Fish of 100 ± 10 g body weight were acclimatized under a standard photoperiod of 12 h light/12 h dark for a period of 2 weeks at 28 °C and were fed with fish bait daily at 5% body weight. Healthy fish were randomly selected and distributed in two groups ($n = 5$ individuals each group). The fish of the control group were intraperitoneally injected with sterile 1 × PBS and the experimental fish group was injected with poly(I:C) (0.5 μg/g body weight) (Sigma-Aldrich) dissolved in sterile 1 × PBS. Poly(I:C) is an immunostimulant and was used in the form of its sodium salt to simulate a viral infection. After 24 h post injection, blood samples from the control and experimental groups were collected and RBCs and WBCs were isolated as described above and subsequently used for RNA isolation and for Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) analysis.

2.4. RNA isolation and RNA-Seq

tRNA was extracted from RBCs and WBCs of six fish using TRIzol reagent according to the manufacturer's instructions (Invitrogen, USA) and RNA quantity and quality were determined using the NanoDrop Spectrophotometer 2000 and 2100 Bioanalyzer (Agilent, USA). Two micrograms of tRNA from RBCs and WBCs per fish were mixed and pools of control and experimental fishes were used to produce two libraries for sequencing. tRNA samples were treated with DNase I (TaKaRa) to eliminate contaminating genomic DNA and Poly(A) mRNAs was purified using Dynabeads Oligo $(dT)_{25}$ (Life Technologies, USA) and subsequently broken into short fragments for the synthesis of first- and second-strand cDNA using the Ultra™ RNA Library Prep Kit (NEB, USA). End-repaired fragments were linked with adapters and purified to obtain cDNAs of desirable lengths, followed by PCR amplification and purification using the AMPure XP beads. After PicoGreen staining and fluorospectrophotometry checking, 10 ng of cDNA libraries were used for cluster generation with TruSeq PE Cluster Kit (Illumine, USA) and sequencing analysis on an Illumina HiSeq2500 platform.

2.5. Transcriptome data analysis

Raw reads of 150 bp in length were filtered into clean reads using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Reads of adaptor sequences, low-quality bases and read lengths <40 bp were removed. Clean reads of RBCs and WBCs samples were