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Short communication

Characterization of surface phenotypic molecules of teleost dendritic cells

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ABSTRACT

Dendritic cells (DCs) are among the most important professional antigen-presenting cells (APCs) that participate in various biological activities in mammals. However, evidence of the existence of DCs in teleost fish and other lower vertebrates remains limited. In this study, phenotypic and functional characteristics of teleost DCs were described in a zebrafish model. An improved method to efficiently enrich DCs was established. Immunofluorescence staining revealed that the surface phenotypic hallmarks of mammalian DCs, including MHC-II, CD80/86, CD83, and CD209, were distributed on the surfaces of zebrafish DCs (*Dr*DCs). Functional analysis results showed that *Dr*DCs could initiate antigen-specific $CD4^+$ T cell activation, in which MHC-II, CD80/86, CD83, and CD209 are implicated. Hence, teleost DCs exhibit conserved immunophenotypes and functions similar to those of their mammalian counterparts. Our findings contributed to the current understanding of the evolutionary history of DCs and the DC-regulatory mechanisms of adaptive immunity.

1. Introduction

Dendritic cells (DCs), originally described by distinct dendritic morphological characteristics, are among the most important professional antigen-presenting cells (APCs) (Steinman, 2012; Steinman and Cohn, 1973). As important components of innate immunity and medium bridging innate and adaptive immunities, DCs participate in various biological activities, such as naive T cell response activation, immune tolerance and escape, and tumor and autoimmune disease development (Lee and Iwasaki, 2007; Steinman, 2012). In a steady state, immature DCs express low levels of MHC-II and co-stimulatory molecules but possess high levels of pattern recognition receptors (Chow et al., 2002; Kadowaki et al., 2001). Furthermore, DCs can recognize and ingest invading pathogens; these cells can also present exogenous antigens or peripheral self-antigens to induce clonal deletion of autoreactive T cells (Bonasio et al., 2006; Wong-Baeza et al., 2010). Once a pathogen infects a host, immature DCs alter chemokine receptor expression, migrate into secondary lymphoid tissues via lymphangion, and develop into mature DCs. The maturation of DCs leads to a dramatic transformation of the phenotype and function, including the upregulation of MHC-II complexes, costimulatory molecules and adhesion lectins at DC surface which are utmost equipped to activate T cells (Reis e Sousa, 2006).

Although numerous studies have focused on mammalian DCs, studies on the presence and the functional characteristics of DCs in teleost fish and other ancient vertebrates remain limited. Recently, several studies have revealed that DC-like cells are possibly present in teleost fish. In one study, a population of DC-like cells has been identified in zebrafish on the basis of peanut agglutinin (PNA) lectin-binding affinity, dendritic morphology, enzyme activity, and stimulatory effects on T cell activation (Lugo-Villarino et al., 2010). A double reporter transgenic zebrafish line has also been generated to show that kidney $MHC\text{-}II^{hi}CD45^{hi}$ cells are exclusively mature DCs and monocytes/macrophages, which can phagocytose exogenous antigens and induce inflammatory cytokine expression upon bacterial challenge (Wittamer et al., 2011). Furthermore, functional DC-like cells have been found in several other fish species, such as rainbow trout (*Oncorhynchus mykiss*) and medaka (*Oryzias latipes*); these cells can strongly stimulate a mixed lymphocyte reaction (MLR) similar to that of mammalian DCs (Aghaallaei et al., 2010; Bassity and Clark, 2012). These results provided preliminary observations that DC-like subset and its involvement in adaptive immunity may have originated from teleost fish during early

Abbreviations: APCs, antigen-presenting cells; *Dr*DCs, *Danio rerio* DCs; KLH, keyhole limpet hemocyanin; PNA, peanut agglutinin; $CD4^+$ T_{KLH}, the KLH-stimulated $CD4^+$ T cells; HK, head kidney.

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vertebrate evolution. Nevertheless, further studies should be conducted to elucidate phenotypes, functions, and regulatory mechanisms of teleost DCs.

Among surface phenotypic molecules, co-stimulatory molecules (CD80, CD86, and CD83) and a C-type lectin molecule (CD209) are considered as important hallmarks of DCs in mammals, which play essential roles in DC-mediated adaptive immunity (Colmenares et al., 2002; Fujii et al., 2004; Orabona et al., 2004; Zhou and Tedder, 1995). CD80 and CD86 are type I membrane glycoproteins expressed on the surface of APCs, providing an important co-stimulatory signals to augment and sustain a T-cell response via an interaction with CD28. Although the CD80 and CD86 independently exist in mammals, only a single copy (CD80/86) exists in teleost fish (Orabona et al., 2004; Sharpe and Freeman, 2002). CD83 is a cell surface membrane glycoprotein expressed on mature DC. It is necessary for effective DC-mediated activation of naive T-cells and T-helper cells (Lechmann et al., 2002). CD209 is a type II membrane-associated C-type lectin that functions as cell adhesion molecules and pathogen receptors. In mammals, it is expressed on phagocytic cells such as dendritic cells and macrophages, which plays important roles in establishing interactions between APCs and resting T cells and participating in the activation of T and B lymphocytes (Colmenares et al., 2002). Our previous studies successfully identified CD80/86, CD83, and CD209 in zebrafish (Lin et al., 2009; Zhu et al., 2014). Thus, whether these molecules also act as surface phenotypic hallmarks on fish DCs and play roles similar to those of their mammalian counterparts has yet to be clarified. In our study, phenotypic and functional characteristics of CD80/86, CD83, and CD209 in *Dr*DCs were reported. *Dr*DCs exhibited a phenotypic pattern with MHC-II$^+$CD80/86$^+$CD83$^+$CD209$^+$ on their cellular surfaces. *Dr*DCs could present antigens to stimulate CD4$^+$ T cell activation. CD80/86, CD83, and CD209 molecules were also implicated in this process. To the best of our knowledge, this study is the first to report the functional conservation of surface phenotypic molecules of DCs between teleost fish and mammals. Our study could not only enrich the knowledge of fish immunology, but also provide insights into the evolutionary history of DCs and DC-regulatory mechanisms such as co-stimulatory signals underlying adaptive immunity for over 450 million years.

2. Materials and methods

2.1. Experimental fish

Zebrafish (*Danio rerio*) of both sexes, aged 3–12 months with approximately 3–4 cm in body length, were raised in a circulating water bath at 28 °C under standard laboratory conditions. All of the fish used in the experiments were offspring of a single AB-strain parent pair after five generations of partial inbreeding (Hohn and Petrie-Hanson, 2012; Monson and Sadler, 2010; Zhu et al., 2014). Only healthy fish, as determined by their general appearance and level of activity, were used in the study.

2.2. Antibodies

Rabbit or mouse polyclonal antibodies against zebrafish MHC-II, mIgM, CD80/86, CD83, CD209 and CD4 were previously prepared in our laboratory with high specificities as described (Gong et al., 2009; Lin et al., 2009; Zhu et al., 2014). Normal rabbit IgG, mouse IgG, FITC-conjugated anti-rabbit IgG and PE-conjugated anti-mouse IgG were purchased from Chemicon.

2.3. Leukocyte isolation

Blood, spleens, and head kidneys (HKs) from at least 40 fish were collected in ice-cold Ca^{2+}/Mg^{2+}-free HBSS with heparin (10 U/mL). Whole blood cell suspensions were obtained using heparinized capillary tubes. Single-cell suspensions of spleens and HKs were prepared by gently teasing the tissues through an 80 μm nylon mesh filter. Leukocytes were enriched from the cell suspensions by Ficoll–Hypaque (1.080 g/mL) centrifugation at 2500 rpm for 25 min at room temperature; afterward, leukocytes were collected from the interface layer. Cellular viability was determined by trypan blue (0.4%) exclusion assay.

2.4. DC sorting

To enrich DCs, we performed immunomagnetic sorting (MACS), as previously described (Wen et al., 2010; Zhu et al., 2014). In brief, leukocyte concentrations were adjusted to approximately 2×10^8 cells/mL. After the cells were blocked with 5% normal goat serum, the cell suspension was incubated with rabbit anti-mIgM and mouse anti-CD209 Abs for 15 min at 10 °C, washed with MACS buffer (PBS containing 2 mM EDTA and 0.5% BSA), and incubated with anti-rabbit IgG magnetic beads (Miltenyi Biotec) for 15 min at 10 °C to exclude mIgM$^+$ B cells. Unconjugated cells were subsequently incubated with anti-mouse IgG magnetic beads for 15 min at 10 °C; these cells were then passed through an LS separation column according to the manufacturer's instructions. Afterward, positive cells were cultured in L-15 medium (Gibco) supplemented with 10% FBS (Gibco), 1% L-glutamine, 1.5% HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin at 28 °C. After 6 h of cell culture, non-adherent lymphocytes were removed, and the remaining adherent cells were sequentially cultured for 24 h. Cell culture flasks were then agitated to suspend non-adherent cells, and the culture medium was collected and pooled. The purity of the sorted DCs was determined by flow cytometry, Wright–Giemsa staining, and RT-PCR. According to previous observation that PNA preferentially binds to zebrafish DC cells, the biotinylated PNA (Sigma-Aldrich) was used for flow cytometry as previously described (Lugo-Villarino et al., 2010). The markers of B cells (mIgM), αβ-T cells (TCRα and TCRβ), DCs (MHC-II, CSF-1R, and IL-12p40), monocytes/macrophages (mpeg1), eosinophils (Gata2), neutrophils (mpx), mast cells (cpa5), and erythrocytes (Gata1) were examined by RT-PCR.

2.5. Flow cytometric analysis

Cell suspensions were blocked with 5% normal goat serum for 1 h at 4 °C, incubated with biotinylated peanut agglutinin (Sigma–Aldrich), rabbit anti-CD83, rabbit anti-CD80/86 or normal rabbit IgG (as an isotype control) for 1 h at 4 °C, and washed. The samples were then incubated with secondary FITC-conjugated streptavidin (Sigma–Aldrich) or FITC-conjugated anti-rabbit IgG (Chemicon) for 1 h at 4 °C. After the samples were washed, fluorescence signals were determined immediately by using a FACScan flow cytometer (BD Biosciences) equipped with an argon laser with emission at 488 nm. At least 10,000 events were collected from the myeloid cell gate (Traver et al., 2003). CellQuest (BD Biosciences) was used for flow cytometric analyses, and ModFit LT was used for T cell proliferation assays.

2.6. Real-time PCR analysis

Total RNA was extracted from the sorted DCs by using TRIzol reagent, evaluated on 1.5% (w/v) agarose gel, and reverse transcribed into cDNA by using an RNA PCR kit according to the manufacturer's instructions (AMV version 3.0, TaKaRa). PCR was performed on a Mastercycler ep realplex machine (Eppendorf, Germany) by using a SYBR Premix Ex *Taq* kit (TaKaRa) in a total reaction volume of 10 μL. The following PCR program was used: 94 °C for 2 min, followed by 40 cycles at 94 °C for 20 s, 55 °C for 20 s, and 68 °C for 20 s. Relative gene expression levels were calculated using