**Identification of Langerhans-like cells in the immunocompetent tissues of channel catfish,
*Ictalurus punctatus***

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ABSTRACT

Dendritic cells (DCs) are the most powerful antigen presenting cells (APCs) that have a critical role in bridging innate and adaptive immune responses in vertebrates. Dendritic cells have been characterized morphologically and functionally in the teleost fish models such as rainbow trout, salmonids, medaka, and zebrafish. The presence of DCs with remarkable similarities to human Langerhans cells (LCs) has been described in the spleen and anterior kidney of salmonids and rainbow trout. However, there is no evidence of the presence of DCs and their role in channel catfish immunity. In this study, we assessed DC-like cells in the immunocompetent tissues of channel catfish by immunohistochemistry (IHC), flow cytometry and transmission electron microscopy (TEM). We identified Langerin/CD207⁺ (L/CD207⁺) cells in the channel catfish anterior kidney, spleen and gill by IHC. Moreover, we described the cells that resembled mammal LC DCs containing Birbeck-like (BL) granules in channel catfish spleen, anterior and posterior kidneys and gill by TEM. Our data suggest that cells with DC-like morphology in the immune related organs of catfish may share morphological and functional properties with previously reported DCs in teleost fish and mammals. More detailed knowledge of the phenotype and the function of catfish DCs will not only help gain insight into the evolution of the vertebrate adaptive immune system but will also provide valuable information for development and optimization of immunotherapies and vaccination protocols for aquaculture use.

Key words: Channel catfish, dendritic cells, Langerhans cells, Birbeck granules

1. Introduction

Dendritic cells (DCs) are the most powerful professional antigen presenting cells (APCs) that bridge, initiate, regulate, and control all innate and adaptive immune responses in vertebrates [1]. Although numerous studies are still mainly focused on mammalian DCs, reports on the

morphological and functional characterization of DCs in teleost fish, the earliest vertebrates to develop functional adaptive immune responses, are scarce. Recently, DC-like cells, with T cell stimulatory capacities that revealed the properties of mature mammalian DCs have been identified in zebrafish (*Danio rerio*) [2, 3]. Furthermore, functional cells with dendritic morphology, motility, phagocytic ability and strong T cell stimulatory properties have been identified in several other teleost fish species such as rainbow trout (*Oncorhynchus mykiss*) and medaka (*Oryzias latipes*) [4, 5]. Co-stimulation is an important function of professional APCs to prime naïve T cells. The major co-stimulatory molecules such as CD80/CD86 and CD83 have been reported in zebrafish [6]. Shao et al. reported the functional conservation of surface phenotypic molecules on DCs in teleost fish as in mammals by demonstrating functional abilities of CD80/86/CD83/CD209⁺ cells in teleost fish to promote CD4⁺ naïve T cell activation [7].

Langerhans cells (LCs) are a distinct population of immature DCs in the epidermis of mammals, and this unique location at a barrier surface provides them with early access to skin pathogens, commensal organisms, foreign chemicals and epidermal self-antigens [8]. Langerhans cells play a sentinel role through their specialized function in antigen uptake and capture, their capacity to migrate to the secondary lymphoid organs and present antigens to specific T cells thus initiating acquired immune responses [9]. Langerhans cells are uniquely characterized by Birbeck granules (BGs), the organelles consisting of superimposed and zippered membranes. It was previously shown that Langerin is constitutively associated with BGs and is a potent inducer of membrane superimposition [10]. Induction of BGs is a consequence of the antigen capture function of Langerin, allowing routing into these organelles and providing access to a non-classical antigen processing pathway [11]. But how BGs influence the processing and presentation of antigens by MHC class I and class II is still not fully understood. A recent report

showed that LCs mediated and enhanced cross-presentation when antigen was delivered through Langerin [12].

Several morphological studies identified the cell type strikingly resembling mammalian LCs within inflammatory gill lesions and the spleen of healthy fish based on the presence of BGs by using a commercial polyclonal antibodies (pAbs) developed against human Langerin [13-15]. In particular, Langerin / CD207⁺ (L/CD207⁺) cells were found in the cytoplasm of the spleen and anterior kidney of Atlantic salmon (*Salmo salar*) and rainbow trout [14, 15]. Interestingly, BG-like granules were observed in the thymus and anterior kidney followed by the appearance within cells of the newly developed spleens in rainbow trout and Atlantic salmon [15]. Langerin-positive cells in healthy rainbow trout were seen predominantly in the spleen however, during microsporidial gill disease, the number of L/207⁺ cells has been significantly increased in the spleen and anterior kidney [14].

Channel catfish (*Ictalurus punctatus*) is the most important aquaculture species in the United States, accounting for \$352 million in 2014 [16]. Vaccination against catfish pathogens requires generation of T and B cell-mediated immunity controlled by professional APCs to eliminate infection and induce protective memory immune responses. Comprehensive understanding and knowledge of putative DC populations in catfish is not only important for health assessment and vaccine development, but also to study the evolution of the immune system. However, there is no evidence of the existence of DCs and their role in orchestrating innate and adaptive immunity in channel catfish. Therefore, the purpose of our research was to conduct a morphological assessment of DCs in immunocompetent organs of channel catfish. Here, for the first time, we report the presence of DC-like cells with the striking similarities to human LCs in the immune related organs of catfish. The identification of DC-like cells in catfish suggests that specialized

APCs might share properties of the mammalian DCs to initiate and orchestrate innate and specific immune responses.

2. Materials and Methods

2.1. Animals

Specific pathogen free (SPF) channel catfish fingerlings (five-six month old) and fry (one month old) were acquired from the fish hatchery at the College of Veterinary Medicine, Mississippi State University and maintained into flow-through tanks at 25-28 °C. Fish were fed with a floating catfish feed to satiety and acclimated for one week. In this present study, all fish experiments were carried out by a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Mississippi State University. The fish were euthanized in water containing 100 mg/L tricaine methanesulfonate (MS-222, Western, Chemical, Inc.). Samples were obtained as described below.

2.2. Cell Preparation

Peripheral blood, anterior kidney, and spleen cell separation was performed as described previously [17]. Briefly, peripheral blood was collected from the caudal vein followed by centrifugation at 500g and resuspended in Phosphate-buffered saline (PBS). Anterior kidney and spleen were dissected from the fish and placed in a sterile culture dish containing PBS. To obtain a single-cell suspension, tissue were minced with sterile forceps, repeatedly aspirated using a 1 ml syringe and passed through cell dissociation sieves (Sigma, St. Louis, MO). The resulting cell suspensions were washed and resuspended in PBS. Cell suspensions and peripheral blood were

layered over Histopaque 1077 (Sigma) and centrifuged at 500g for 30 min. Cells then were collected from the interface, washed three times in PBS, counted and assessed for viability by trypan blue exclusion.

2.3. Cell Staining and Flow Cytometry

Monoclonal antibody specific to human L/CD207 conjugated with fluorochrome (R-PE) (PE Mouse Anti-human L/CD207, clone no: 2G3, BD Biosciences) and isotype-matched controls (Ig Lambda, clone no: 1-155-2, eBioscience) were used to identify the intracellular staining of L/CD207 in the immune related organs of catfish. Cells from peripheral blood, spleen and anterior kidney were fixed and permeabilized with BD Perm/Wash buffer (BD Biosciences) on ice for 20 min, and washed twice with BD Perm/Wash™ buffer (BD Biosciences). Following fixation and permeabilization, cells were re-suspended with Streptavidin/Biotin (Vector Laboratories) and incubated for 15 min in the dark and washed with BD Perm/Wash™ buffer. After that, cells were incubated with R-Phycoerythrin-conjugated mAbs to human L/CD207 on ice for 30 min in the dark, and washed with BD Perm/Wash™ buffer. Finally, cells were incubated with Streptavidin R-Phycoerythrin Conjugate (Life Technologies) on ice for 30 min in the dark and washed with BD Perm/Wash™ buffer.

Catfish mononuclear cells were gated based on their relative size and granularity using forward and side scatters (FSC and SSC, respectively) with a FACSCalibur Flow Cytometer (Becton Dickinson). Immunofluorescent staining was analyzed using FlowJo 7.6.4 Software (Tree Star Inc.). The intensity of L/CD207 staining in catfish mononuclear cells was analyzed by using single histogram statistics.

2.4. Tissue Preparation for Immunohistochemistry

Spleen, anterior kidney, gill, and skin were isolated from the euthanized channel catfish and immediately fixed in 10% neutral buffered formalin for 24 h. Tissues were embedded in paraffin wax after the dehydration with a graded series of ethanol, and 5 or 6 sections were cut for per slide as described [14].

2.5. Immunohistochemistry (IHC)

Immunohistochemical staining of fingerling catfish spleen, anterior kidney, skin and gill paraffin slides for the presence of L/CD207⁺ cells was performed with two different antibodies: purified human CD207-specific pAbs (R&D Systems, Inc.) and R-Phycoerythrin-conjugated mAbs specific to human L/207. Immunohistochemical staining with purified pAbs was conducted as described elsewhere with minor modifications [15]. In this study, antigen retrieval was examined by incubation of sections in target retrieval solution (DAKO) for 40 min at 100°C. Following this incubation, sections were incubated in protein block (DAKO) for 1 h. After that, primary antibody was diluted at a concentration of 0.2 mg/ml (1:500 from stock solution), and sections were incubated in primary antibody for overnight at room temperature in a humid chamber. We used rainbow trout spleen as a positive control for L/CD207-specific staining. Also, negative control that was normal goat IgG (Vector Laboratories) was applied at the same concentrations as the primary antibody. After overnight incubation, streptavidin/biotin blocks were applied for 15 min. Finally, sections were incubated in Streptavidin-HRP (Vector Laboratories) for one hour. Then, slides were analyzed and photographed with OLYMPUS BX43 microscopy and cell Sens standard software program.

Immunohistochemistry with conjugated mAbs was performed as described for purified pAbs. However, there were some differences such as: 0.3% hydrogen peroxide in 100% methanol were not used in alcohol serial dilutions, and sections were incubated with primary conjugated antibody for four hours and washed in PBS for 10 min. Then, slides were analyzed by Immunofluorescent Microscopy (CYTATION 5 imaging reader, BioTek).

2.6. Transmission Electron Microscopy (TEM)

Sample preparation for TEM was carried out as described [14]. Spleen and posterior kidney samples were fixed in McDowell's fixative. After primary fixation, samples were rinsed and postfixed in 2% buffered Osmium tetroxide, dehydrated through a graded ethanol series, and embedded in Suprr's resin. Ultra-thin sections were cut on a Reichert-Jung Ultracut E ultramicrotome, and then sections were stained with uranyl acetate and lead citrate. Sections were viewed using a JEOL JEM1230 Transmission Electron microscope at 80kv equipped with an AMT XR50 camera.

2.7. Statistical analysis

Analysis of variance (ANOVA), followed by Fisher's LSD multiple comparison post hoc test was used to evaluate differences in mean fluorescence intensity (MFIs). The level of significance for all tests was set at $P < 0.05$.

3. Results

3.1. Assessment of Langerin/CD207⁺ cells in immunocompetent tissues of channel catfish by flow cytometry

To identify the presence of L/CD207⁺ LC-like cells in the immunocompetent organs of channel catfish mononuclear cells derived from spleen, anterior kidney, and peripheral blood were analyzed for the intensity of staining with human L/CD207⁺- specific mAbs. The levels of L/CD207 expression in mononuclear cells obtained from 7 fingerling catfish were low to negative in anterior kidney and spleen, and virtually negative in the peripheral blood (data not shown). There were no statistically significant differences in the intensity of L/CD207 staining of the spleen and anterior kidney mononuclear cells compared to the isotype-matched controls (P>0.05).

3.2. Identification of Langerin/CD207⁺ cells in immunocompetent tissues of channel catfish by IHC

To confirm our data on a possible presence of LC-like L/CD207⁺ cells in the immunocompetent organs of catfish by flow cytometry, we have adapted the IHC protocol that was previously described for identification of the L/CD207⁺ cells in rainbow trout and salmonid [15]. Visually strong L/CD207 cytoplasmic expression was observed in the spleen and anterior kidney tissues compared to virtually undetected reaction in the negative controls (Fig. 1 A, B, C {Panels I, II}). Interestingly, the L/CD207⁺ cells in the anterior kidney were more abundant compared to their counterparts in the spleen and relatively rare in the gill (Fig. 1 D {Panels I, II}). Notably, the numbers of L/CD207⁺ varied significantly in pathogen free fingerlings, from

just a few cells to the cell numbers shown in Figure I C. However, the skin of channel catfish was constantly negative for L/CD207 (data not shown).

Spleen, anterior kidney, gill and skin tissues were examined by IHC using R-PE conjugated mAbs specific to human L/CD207 and the isotype-matched control mAbs to document the presence of L/CD207⁺ cells in the tissues of catfish. Langerin-positive cells have been identified mostly in catfish anterior kidney confirming our findings obtained by flow cytometry and immunohistochemistry using purified polyclonal L/CD207-specific Abs (Fig. 2). Importantly, the L/CD207⁺ cells in the spleen were concentrated around blood vessels or sinusoids (Fig. 2). Contrary, gill and skin tissues showed the background intensity of L/CD207 staining comparable with negative controls (data not shown).

3.3. Identification of Dendritic-like cells in lymphoid tissue of channel catfish by TEM

Dendritic-like (DL) cells were observed in the immunologically important organs of non-vaccinated catfish fingerling and fry, and the ultrastructure of these DL cells was analyzed by TEM (Fig. 3A, B). Dendritic-like cells contained an unsegmented nucleus and did not have long cytoplasmic processes. These cells possessed Birbeck-Like (BL) granules with tennis-racket or rod-shaped morphology. Birbeck-Like (BL) granules that were not a continuation of the cell membrane were found surrounding the centriole in the cytoplasm of the DL cells.

4. Discussion

The aim of the current research was to assess morphologically the most powerful professional APCs, DCs in immune related organs of channel catfish. We identified L/CD207⁺

235 cells in the spleen, anterior kidney, and gill of channel catfish. Importantly, the L/CD207⁺ DCs
236 visually prevailed in the catfish anterior kidney compared to their counterparts in the spleen.
237 Also, the L/CD207⁺ cells were virtually absent in the skin and rare in the gill of catfish.
238 L/CD207⁺ were firstly described in the spleen and anterior kidney of Atlantic salmon and
239 rainbow trout with remarkably similarities to human LCs [15]. Unlike in channel catfish,
240 L/CD207⁺ were observed in a fewer numbers in the anterior kidney compared to the spleen of
241 Atlantic salmon and rainbow trout and like in catfish, were not detected in the skin [15]. These
242 results show that anterior kidney and spleen in channel catfish serve as important organs for
243 antigen presentation as was described previously for rainbow trout, salmon and zebrafish [2, 5,
244 15]. Interestingly, L/CD207⁺ cells in teleost fish were found predominantly in the spleen and
245 anterior kidney as LCs in mammals populate mainly the epidermis of skin and in the process of
246 maturation migrate to the secondary lymphoid organs [18]. In particular, we were able to
247 document the presence of L/CD207⁺ DCs in the fingerling spleen located in close proximity to
248 sinusoids or blood vessels suggesting that catfish LCs can sample and capture antigens
249 trafficking through the blood with subsequent maturation and presentation of processed antigens
250 to immune competent T cells in the spleen.. Interestingly, a single L/CD207⁺ identified in
251 catfish gill was located near the blood vessel suggesting possible sentinel role of fish DCs in
252 sampling antigens in the periphery and trafficking them to the lymphoid organs. As fish lack
253 lymph nodes, the migration pattern and maturation of DCs might be different from humans and
254 other mammals. Dendritic-like populations identified in the immunocompetent organs of catfish
255 may share morphological and functional properties with previously reported DCs in teleost fish
256 and mammals.

We described BL granules in DC-like cells of catfish spleen and posterior kidney by transmission electron microscopy. The granules had a rod portion and were vacuolated at one end that gave the organelles a tennis-racket morphology that was described previously in Atlantic salmon and zebrafish strikingly resembling human LCs [2, 13, 14]. Birbeck-like granules with rod-shaped and tennis-racked morphology were observed for the first-time in fish within the diseased gills of Chinook salmon (*Oncorhynchus tshawytscha*) and lymphoid organs of salmonids [13, 14]. Lately, BL granules were also detected in the DCs isolated from the skin of zebrafish [2]. Importantly, the unique location of these granules surrounding the centriole in the cytoplasm of the DL cells in the spleen of catfish was very similar to the location in salmonids [13, 14]. Furthermore, BGs in LCs were commonly found near centrioles and microtubules in patients with ‘atypical hyperplasia’ in humans [19]. It was suggested that BGs in humans have a function of a loading compartment for antigens before LCs undergo maturation and migration to the secondary lymphoid organs [20]. Therefore, centrioles and microtubules are necessary during antigen processing in immature DCs [21]. Despite of the lacking information on the ultrastructural details of BL granules and accumulation of Langerin in their close proximity, our study identified BL granules in catfish that are virtually identical to BGs described in Dc-like cells of salmonids and zebrafish, and also LCs in humans.

Langerin protein and BL granules, the hallmarks of mammalian LCs were detected in the immunocompetent organs of catfish, suggesting that teleost fish, the earliest vertebrates that develop adaptive immunity possess LC-like cells. The LC-like cell populations in catfish may represent an earlier lineage to Langerhans cells that are found within the epidermis in humans and other mammals. In conclusion, we have identified LC-like cells in catfish based on the expression of Langerin by immunohistochemistry and flow cytometry and their unique

morphology by TEM. However, some questions still remain unanswered. Is the Langerin identified by using human-specific antibodies the only type of protein present in fish or do other isoforms that are fish-specific and could not be detected by human-specific antibodies exist? We also cannot rule out that several LC-like populations in catfish are present. More detailed knowledge about the functional properties of the catfish Langerin⁺ cells, their role as professional APCs in innate and adaptive immunity will not only help gain insight into the evolution of the vertebrate adaptive immune system but will provide valuable information for development and optimization of immunotherapies and vaccination protocols for aquaculture use.

Conflict of Interest Statement

The authors have declared no competing interests.

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Figure legends

Fig. 1. Assessment of L/CD207⁺ cells in the spleen, anterior kidney, and gill of catfish fingerling by immunohistochemical staining with purified pAbs specific to human Langerin. Photomicrographs (600X). Panels (I) show L/CD207-specific staining with pAbs in different concentrations: A) Catfish spleen, Abs dilution 1:50; B) Catfish spleen, Abs dilution 1:500; C) Catfish anterior kidney, Abs dilution 1:500; D) Catfish gill, Abs dilution 1:500; E) Rainbow trout spleen (positive control), Abs dilution 1:50. Panels (II) show negative controls (normal goat IgG) catfish spleen, anterior kidney, gill, and rainbow trout spleen, respectively. Insets on the left show magnified images of the selected areas.

Fig. 2. Assessment of L/CD207⁺ cells in the anterior kidney of catfish fingerling by immunohistochemical staining with R-PE-conjugated mAbs specific to human Langerin. Photomicrographs (600X). Panel (I) shows Langerin-specific staining. Panel (II) shows tissue staining with matching R-PE-conjugated isotype control mAbs (negative control). Panel (III) shows magnified images of the selected areas. Arrows identify Langerin-specific binding with red fluorescence.

Fig. 3. Dendritic-Like (DL) cell in the immune-related organs of *Ictalurus punctatus* by electron microscopy. DL cell with unsegmented nucleus (N) and Birbeck-like (BL) granules (solid arrow) in the cytoplasm surrounding the centriole (dotted arrow). (A) Spleen, fingerling; (B) Anterior kidney, fry; (C) Posterior kidney, fry; (D) Gill, fry.

390 Figure 2.

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393 Figure 3

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