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Antigen Presenting Cells-Mediated Innate and Adaptive Immune Responses to Live Attenuated Edwardsiella Ictaluri Vaccines in Channel Catfish

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Antigen presenting cells-mediated innate and adaptive immune responses to live attenuated Edwardsiella ictaluri vaccines in channel catfish

By

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Vaccination against intracellular pathogens requires generation of pool of memory T cells, which can respond upon infection and mediate immune responses by either killing of infected host cells or induce killing mechanisms in infected cells. T cell-inducing vaccines aim to deliver the antigen to antigen presenting cells (APCs) by presenting on MHC molecules thus bridging innate and adaptive immunity. The intracellular pathogen *Edwardsiella ictaluri* causes enteric septicemia of catfish (ESC), which is a devastating disease in catfish industry. *E. ictaluri* can survive in professional phagocytes and use them as an infection source. Two new live attenuated vaccine (LAV) strains, *Ei*Δ*evpB* and ESC-NDKL, were developed by our group. However, the role of LAVs in phagocytosis, bacterial killing, and antigen presentation is unexplored. Therefore, further research is necessary to determine immune responses in channel catfish against LAVs. The long-term goal of this project is to identify immunological APC-dependent mechanisms that underscore *E. ictaluri* pathogenesis to enable development of effective control strategies for ESC. The overall goal of this project is to assess the role of three professional APCs, dendritic cells (DCs),

macrophages and B cells in the LAV-induced innate and adaptive immune responses in catfish. The central hypothesis is that efficacious LAV strains will enhance phagocytosis and microbial killing, and promote the generation of T cells that regulate and control protective B cell-mediated immunity. The rationale for this research is that more detailed knowledge about phenotype and function of catfish APCs will not only help gain insight into the evolution of vertebrate adaptive immune system but will provide valuable information for development and optimization of immunotherapies and vaccination protocols for aquaculture use.

In this study, we first identified DC-like cells in immune-related organs of catfish and assessed their expression patterns in lymphoid organs of catfish in *E. ictaluri* infection. Although WT strain induces the functional inability of DC-like cells in migration and maturation, LAVs strains promote the migration and maturation of DC-like cells for antigen presentation. Two LAVs enhanced the phagocytosis and killing activity
in catfish macrophages and B cells. Also, LAVs induce high expression of T cell-related genes without causing inflammation.

DEDICATION

I would like to dedicate this research to my family, my husband Sinan Kordon and my son Ahmet Hakan Kordon.

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CHAPTER I

INTRODUCTION

The catfish industry

The channel catfish industry is one of the largest aquaculture industries in the United States, and the majority of catfish production originates from four Southeastern states of Mississippi, Alabama, Arkansas, Louisiana, and Texas (USDA 2009). Catfish production is an important industry in Mississippi which provided $201 million (55.7%) of $361 million in sales in 2015 (USDA 2016). Diseases cause significant losses, and thus therapeutic approaches are needed to control and prevent important channel catfish disease, such as enteric septicemia of catfish (ESC) caused by an intracellular pathogen *Edwardsiella ictaluri*.

Enteric septicemia of catfish (ESC)

Enteric septicemia of catfish (ESC) is one of the most significant diseases of farm-raised channel catfish industry, which causes $50 to $80 million annually (Russo, Shoemaker et al. 2009). ESC occurrence increases in fish under stressful environments such as fluctuating water temperatures, poor water quality (Hawke, Durborow et al. 1998). Enteric septicemia of catfish is a seasonal disease, which is seen in the spring and fall when water temperatures range from 22°C to 28°C (Francis-Floyd 1996). *E. ictaluri* can enter through the gut, gills, skin, and nasal passages to reside in the brain

(Ourth and Chung 2004, Karsi, Menanteau et al. 2006, Griffin and Mitchell 2007). After the entry ofthe bacteria, *E. ictaluri* causes acute and chronic infections in catfish (Shotts, Blazer et al. 1986). The acute form of ESC is characterized with ascites, exophthalmia, red and white ulcers on the skin, and petechial hemorrhages whereas the most characteristic physical sign of chronic form of ESC is ulcers formed between eyes, which is known as "hole-in- head" condition (Hawke, Durborow et al. 1998).

There are several treatment methods of ESC including antibiotics and vaccines. The antibiotics, oxytetracycline, sulfadimethoxine, and florfenicol, are approved by the US Food and Drug Administration (FDA) and administered orally with food (McGinnis, Gaunt et al. 2003). However, the most apparent behavioral sign of channel catfish affected with ESC is reduction of feeding, which reduces the effectiveness of antibiotics. In addition to antibiotics, vaccination is an effective method of providing active acquired immunity through antigen presenting cells (APCs) which activates T cells (Kratky, Reis e Sousa et al. 2011). Several live attenuated vaccines (LAVs) have been developed for effective treatment of ESC. For example, Aquavac-ESC, a modified live *E. ictaluri* vaccine, activated protective immunity in juvenile catfish by bath immersion (Klesius and Shoemaker 1999). Recently, a live attenuated *E. ictaluri* (S97-773) was developed to protect fingerlings by oral vaccination (Wise, Greenway et al. 2015). Moreover, two *E. ictaluri* LAV strains, *Ei*Δ*evpB* and ESC-NDKL1, were developed by our group, which stimulated effective protection in catfish fry and fingerlings (Lawrence and Karsi 2016, Karsi, Lawrence et al. 2017, Nho, Abdelhamed et al. 2017).

Edwardsiella ictaluri

Edwardsiella ictaluri, a Gram-negative, rod-shaped, intracellular, and facultative- anaerobic bacterium, was first isolated from channel catfish in 1976 (Hawke, Kent et al. 2013). The optimal growth temperature of this bacterium is 25°C, and *E. ictaluri* is motile at 25-30°C and nonmotile at higher temperatures (Waltman, Shotts et al. 1986). *E. ictaluri* is a slow-growing bacterium on agar plates as the optimal growth of this pathogen requires 48 hours at 30°C. *E. ictaluri* can spread from fish to fish during infection and encodes a numbers of virulence factors including flagella, type I fimbriae, type III and VI secretion systems, extracellular capsular polysaccharide, lipopolysaccharide (LPS), hemolysins, and outer membrane proteins (OMP) (Weete, Blevins et al. 1988, Newton, Blevins et al. 1990, Vinitnantharat, Plumb et al. 1993, Stanley, Hudson et al. 1994, Lawrence, Banes et al. 2003, Williams and Lawrence 2005, Williams, Gillaspy et al. 2012, Santander, Martin et al. 2013). These virulence factors may be responsible for the disease. *E. ictaluri* can survive in catfish immune cells, such as macrophages and neutrophils (Booth, Beekman et al. 2009, Karsi, Gülsoy et al. 2009).

Innate Immune Responses in Fish

The vertebrate immune system is a large, complex network that includes multiple tissues, organs, cells and molecules focusing on host defense (Zimmerman, Vogel et al. 2010). The cells and molecules of the immune system are equipped to recognize and destroy foreign substances, in particular, pathogens, thus protecting organisms against diseases and maintaining homeostasis (Chaplin 2010). Fish are a

highly diverse group of organisms representing the earliest vertebrates, including the Agnatha (hagfish and lamprey), Chondrichthyes fish (sharks and rays), and Osteichthyes (teleost or bony fish) (Bolis, Piccolella et al. 2001). Similar to higher vertebrates, the immune system of fish is composed of two major components, innate (non-specific) and adaptive (specific) immune responses. However, the innate immune system in fish has fundamental importance in preventing pathogen entry as the adaptive immune responses are less efficient compared to mammals. Therefore, in this review, we aim to highlight recent knowledge, including the data obtained in our laboratory, on the innate immune mechanisms mediated and controlled by professional antigen presenting cells (APCs) in teleost fish.

The fish immune system is composed of innate (non-specific) and adaptive (specific) immune mechanisms. Innate immunity components respond to pathogenic organisms by recognizing pathogen-associated molecular patterns (PAMPs), which are not expressed in host cells. Namely, lipopolysaccharides (LPS) in Gram-negative bacterial cell wall, lipoteichoic acid (LTA) in Gram-positive bacteria, phospholipomannan, beta-glucan, and chitin in fungi, hemagglutinin in viruses are among the most common PAMPs recognized by the innate immune receptors (Mogensen 2009, Silva-Gomes, Decout et al. 2015, Taghavi, Khosravi et al. 2017). Following pathogen recognition, one of the effector mechanisms of innate immunity is the destruction of pathogens by phagocytosis. In contrast, adaptive immunity components recognize pathogens by highly specific receptors generated by V(D)J recombination and somatic hypermutation, resulting in proliferation and differentiation of specific B and T-cells clones (Bonilla 2010).

General Properties of Fish Innate Immunity

Similarly to mammals, the innate immune system in fish is the first line of defense that reacts to pathogens within a very short time and does not provide long-lasting protection (Turvey and Broide 2010). However, unlike in mammals, the innate immune system of fish is a fundamental component in preventing pathogen entry due to the inefficiency of the adaptive immune response (Whyte 2007). The evolutionary position and poikilothermic nature of fish affect the efficiency of adaptive immunity due to slow lymphocyte proliferation and maturation, memory formation and a limited antibody repertoire (Magnadóttir 2006). Fish contain most of the primary and secondary lymphoid organs present in mammals except bone marrow and lymph nodes. However, the structure of these organs in fish lacks the complexity compared to the mammalian counterparts leading to potential limitation and delay in the generation of fully functional adaptive immune responses (Tort L 2003, Firdaus-Nawi and Zamri-Saad 2016).

The components of the innate immune system in fish are commonly divided into three compartments: physical parameters, humoral parameters, and cellular factors (Uribe, Folch et al. 2011) (Figure 1). The physical barriers include fish scales, a mucus layer, and epithelial cells, which line the skin, gills, and alimentary tract, providing a crucial role in combating infection (Ellis 2001, Magnadottir 2010). Goblet cells produce a mucus layer in which antimicrobial substances trap pathogens and inhibit the spread of infection (Harris and Hunt 1975, Alexander and Ingram 1992). Fish mucus contains a wide range of immune substances, in particular, lectins, pentraxins, lysozyme,

complement proteins, antibacterial peptides and IgM), which are capable of inhibiting pathogen entry or elimination of the pathogen (Rombout, Taverne et al. 1993, Aranishi and Nakane 1997, Boshra, Li et al. 2006, Saurabh and Sahoo 2008). In addition to the mucus barrier, the epidermis, composed of epithelial cells, prevents the entry of foreign materials and is populated with effector cells, such as macrophages and lymphocytes (Fischer, Utke et al. 2006, Esteban 2012). Both mucus and epithelial cells act as physical and chemical barriers against microorganisms and foreign agents (Firdaus-Nawi and Zamri-Saad 2016).

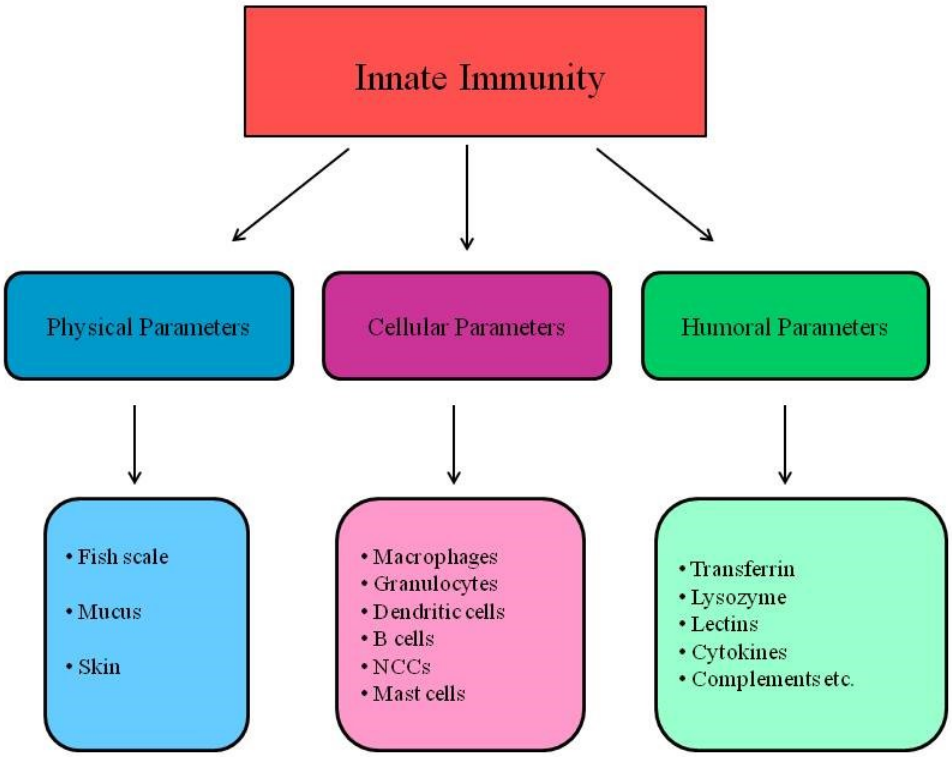

Figure 1 The components of the fish innate immune system.

The fish innate immune system is composed of three main parameters: 1) Physical parameters, 2) Cellular parameters, and 3) Humoral parameters.

Among the humoral immune parameters that include cell receptors or soluble molecules, teleost fish contain a large number of non-specific defense substances, such as transferrin, antimicrobial peptides, lysozyme, lectins, natural antibodies, cytokines, and complement components, which are able to kill microorganisms or inhibit their growth (Magnadóttir 2006, García-Fernández, Sánchez et al. 2011). The function of these humoral factors was reviewed and discussed in multiple studies previously (Uribe, Folch

et al. 2011, Firdaus-Nawi and Zamri-Saad 2016). In this review, we will focus on the role of cellular parameters and receptors of innate immunity.

Innate Immune Receptors in Teleost Fish

The innate immune response to non-self or self-antigens relies on the germline-encoded pattern recognition receptors (PRRs) that recognize PAMPs or danger-associated molecular patterns (DAMPs) (Santoni, Cardinali et al. 2015). Non-self or exogenous antigens are not expressed in the host, and PAMPs are derived from diverse pathogens. In contrast, DAMPs are intracellular molecules which are secreted to the extracellular environment from apoptotic cells or damaged extracellular matrices, such as host DNA and RNA, high-mobility group box 1 (HMGB1) protein, S100 proteins, and heat-shock proteins (HSP) (Rosin and Okusa 2011, Land 2015).

In mammals, PRRs can be divided into five groups based on their protein domain homology as follows: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding domain (NOD), leucine-rich repeat (LRR)-containing (NOD-like) receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), and the absent in melanoma-2 (AIM-2)-like receptors (ALRs) (Brubaker, Bonham et al. 2015). These receptors can be localized at the different compartments of cells; for example, TLRs and CLRs are found at the cell surface or endocytic compartment whereas NLRs, RLRs, and ALRs are located in the cytoplasm (Takeuchi and Akira 2010, Thompson, Kaminski et al. 2011). The cell surface classes of PRRs sense the extracellular environment for the presence of pathogen ligands, while the cytoplasmic PRRs survey the presence of intracellular pathogens. Importantly,

endosomal types of PRRs detect pathogens which are engulfed into the phagolysosome (Kawai and Akira 2009).

Following the recognition of PAMPs or DAMPs, PRRs induce signaling cascades, which lead to the NF-κB and interferon (IFN) response factor (IRF) transcription factors activation, thus triggering the production of pro-inflammatory cytokines, chemotactic cytokines, and interferons (Li, Li et al. 2017). Furthermore, activation of PRRs also leads to the non-transcriptional innate immune responses, such as phagocytosis, autophagy, cell death, and cytokine processing (Drummond and Brown 2011, Deretic, Saitoh et al. 2013, Lamkanfi and Dixit 2014).

Similar to the mammalian innate immune system, teleost fish have multiple PRRs to recognize non-self and self-antigens. Surprisingly, fish possess higher numbers of different TLRs compared to mammals (Takano, Kondo et al. 2011). Currently, at least 17 or 18 TLRs have been identified in teleost fish, and the following TLRs are conserved in both mammals and fish: TLR1, TLR2, TLR3, TLR5, TLR7, TLR8, and TLR9, whereas mammalian TLR4 has been detected only in zebrafish (Takano, Kondo et al. 2011). Several novel TLR genes, such as TLR14, TLRs 21-23 have been identified in fugu, while TLRs18-22 have been identified in zebrafish (Oshiumi, Tsujita et al. 2003, Jault, Pichon et al. 2004, Meijer, Gabby Krens et al. 2004, Roach, Glusman et al. 2005). Furthermore, 20 different TLRs have been described in channel catfish, including the unique TLRs such as TLR25 and TLR26, which were not found in other vertebrates, and TLR26 was found to be a channel catfish-specific (Quiniou, Boudinot et al. 2013).

Multiple CLR genes have been identified in fish, namely, mannose receptor in seabream and grass carp, and CLR-like protein A, B, and C genes in Atlantic salmon (Rodrıguez, Esteban et al. 2003, Soanes, Figuereido et al. 2004, Wang, Liu et al. 2014). Furthermore, a lectin-like receptor gene was described in zebrafish, and a CLR-like protein gene was detected in ayu (Panagos, Dobrinski et al. 2006, Chen, Lu et al. 2010). Recently, a novel CLR gene has been identified and tentatively named PaCD209L in ayu (Yang, Lu et al. 2015).

Teleost fish also contain the NLR family of PRRs. For example, the members of NLR family, NOD1, NOD2, and NOD3 that are conserved between zebrafish and mammals have been identified in zebrafish (van der Vaart, Spaink et al. 2012). NOD1 was also detected in rainbow trout, channel catfish, and goldfish, and its expression was increased in goldfish infected with *Aeromonas salmonicida* or *Mycobacterium marinum* (Li, Wang et al. 2012, Xie, Hodgkinson et al. 2013, Jang, Kim et al. 2016). Moreover, the mammalian analog of the NLR subfamily member NALP was detected in zebrafish (Stein, Caccamo et al. 2007, Laing, Purcell et al. 2008). On the other hand, no counterparts of members of ALR family have been detected in teleost fish species yet (Aoki, Takano et al. 2015).

Antigen Presenting Cells

In mammals, there are three professional antigen presenting cells (APCs) described, that have crucial roles in recognition of pathogens by PRRs and activation of adaptive immunity by the presentation of antigens to naïve T cells. As professional APCs, dendritic cells (DCs), monocyte/macrophages and B cells, engulf pathogens and

process antigen to peptides. Following antigen processing, APCs present peptides by major histocompatibility (MHC) molecules (Drutman and Trombetta 2010). For example, intracellular antigens are presented by MHC class I molecules to cytotoxic T cells (CD8+T cells), while extracellular antigens are presented by MHC class II molecules to helper T cells (CD4+ T cells) (Vyas, Van der Veen et al. 2008, Keech, Pang et al. 2010) (Figure 2). Upon activation, another critical feature of APCs is the expression of co-stimulatory molecules, such as B7-1 (CD80), and B7-2 (CD86), which are necessary to prime naïve T cells and induce the differentiation of T cells by producing cytokines (Chen and Flies 2013). Recently, the fish professional APCs received more attention resulting in the increased numbers of studies on their morphology and function. In this review, we discuss the data obtained in our and other studies on the important functions of teleost fish APCs to uptake and process antigens and provide three crucial signals (antigen- specific, co-stimulatory, and cytokine) to naïve T cells.

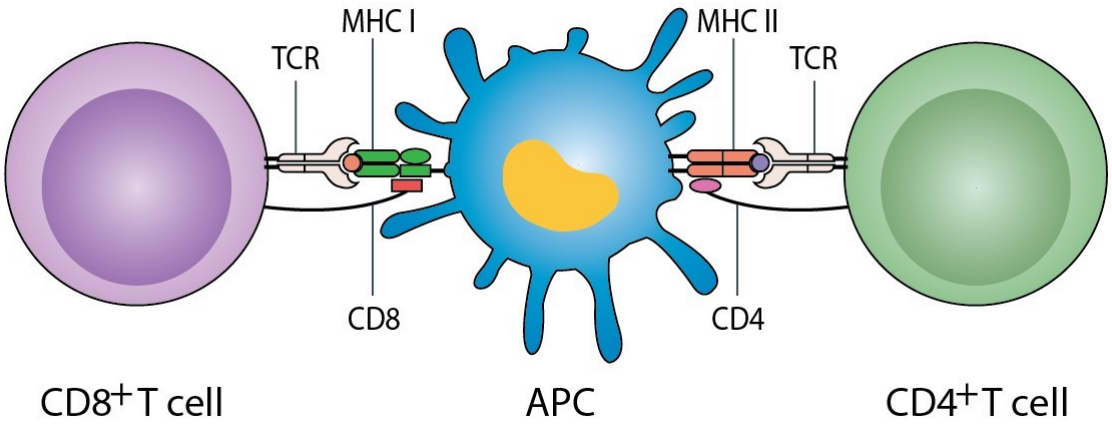

Figure 2 APC-T cell interactions.

Intracellular antigens are presented by MHC class I molecules to $CD8^+$ T cells. The TCRs on $CD8^+$ T cells recognize the antigens, and CD8 molecules interact with MHC I molecules. On the other hand, extracellular antigens are presented by MHC class II molecules to $CD4^+$ T cells. TCRs recognize the antigens, and CD4 molecules interact with MHC class II molecules.

Dendritic Cells

Dendritic cells (DCs) are the universal and powerful APCs that are critical players in bridging and shaping both innate and adaptive immune responses in vertebrates (Mildner and Jung 2014). They are present in most tissues and sense the environment for foreign materials and self-antigens, and can capture pathogens or antigens and travel to the secondary lymphoid tissues for presentation to T cells to regulate adaptive immune responses (Alvarez, Vollmann et al. 2008, Summerfield, Auray et al. 2015). Recently, DCs have been characterized in several teleost fish based on their morphology and function. For example, DC-like cells with mammalian DC morphology were identified in cell cultures from whole kidney marrow of zebrafish, and T-cell stimulatory capability of these cells have been documented in zebrafish

(Lugo-Villarino, Balla et al. 2010, Wittamer, Bertrand et al. 2011). Moreover, non-adherent cells with dendritic morphology in rainbow trout were identified in hematopoietic cultures including anterior kidney (AK), the anterior portion of trunk kidney, and spleen (Bassity and Clark 2012). In addition to morphology, DCs in rainbow trout showed active motility, and their migration *in vivo* to mucosal and lymphoid tissues were determined by flow cytometry (Bassity and Clark 2012). Also, the same study reported that DCs in rainbow trout could phagocytize fluorescent beads, and these cells are activated by TLR-ligands (imiquimod, Poly I:C, ssRNA, and flagellin) and showed aggregation (Bassity and Clark 2012). Furthermore, DCs in barramundi were identified in hematopoietic cell cultures, and these cells have with many large dendrites which extend from the cell body (Zoccola, Delamare-Deboutteville et al. 2015). Also, migration and phagocytic abilities of DCs in barramundi were demonstrated by the same study. After exposure to TLR ligands (LPS and PTG) and two strains of barramundi primary bacterial pathogens, *Streptococcus iniae (*strains QMA0076 and QMA0248), DCs actively migrated towards PTG and both *S. iniae* strains (Zoccola, Delamare-Deboutteville et al. 2015). Barramundi DCs were also able to uptake micro-beads and two strains of *S. iniae* after two hours incubation, and the proliferation assay showed that DCs in barramundi capable of stimulating the proliferation of T cells (Zoccola, Delamare-Deboutteville et al. 2015). Another study reported DCs with long cytoplasmic extensions and an eccentric nucleus in medaka (Aghaallaei, Bajoghli et al. 2010).

Professional APCs in mammals express co-stimulatory molecules which play a crucial role in priming naïve T-cells (Chen and Flies 2013). A previous study reported the

presence of the major co-stimulatory molecules (e.g., CD80/CD86 and CD83) in zebrafish (Lin, Xiang et al. 2009). Furthermore, a recent study showed that the surface molecules of zebrafish DCs (CD80/86/83/CD209^{+}) could promote CD4^{+} naïve T cell stimulation as in mammals (Shao, Zhu et al. 2015). Moreover, the expression of CD83 in rainbow trout DCs significantly increased after the TLR-ligands treatment, and also, MHC II expression on their surface was higher than macrophages and B cells (Bassity and Clark 2012). Furthermore, DC-SCRIPT, a specific molecular marker for DCs, and MHC II were expressed by barramundi DCs (Zoccola, Delamare-Deboutteville et al. 2015). Also, PTG and LPS were injected to barramundi, and DC-SCRIPT expression increased in spleen and AK at the beginning of infection, and then its expression decreased in both organs after 3 days post injection, but the expression of DC-SCRIPT increased again in spleen at 7 days post-injection (Zoccola, Delamare-Deboutteville et al. 2015). This study showed that immature DCs migrated from AK and spleen to the site of infection, and migrated back the spleen for antigen presentation (Zoccola, Delamare- Deboutteville et al. 2015).

Dendritic cells in mammals have different subsets, and the Langerhans cell (LC) is a unique subset of DCs present in the epidermis of the skin. This unique location provides LCs with the ability to recognize pathogens, foreign chemicals, and self-antigens soon after invasion (Igyártó and Kaplan 2013). Langerhans cells can engulf antigens and migrate to the secondary lymphoid tissues to present the antigen to T cells, thus initiating adaptive immune responses (Sugita, Kabashima et al. 2007). Langerhans cells are characterized by the presence of Birbeck granules (BGs), which are rod-shaped organelles consisting of superimposed and zippered membrane (Mc Dermott, Ziylan et al. 2002). Langerin is a type II transmembrane C-type lectin and specific marker for

LCs, also associated with the formation of BGs (Valladeau, Ravel et al. 2000, Lau, Chu et al. 2008). The antigen capture function of Langerin triggers the induction of BGs by allowing routine antigens into BGs, thus providing non-classical antigen processing pathway and cross-presentation (Valladeau, Ravel et al. 2000, Fehres, Bruijns et al. 2015).

Several studies identified cells with mammalian LCs morphology in teleost fish. In particular, Langerin/CD207$^+$ (L/CD207$^+$) cells were described in the AK and spleen of Atlantic salmon and rainbow trout (Lovy, Wright et al. 2008). Also, L/CD207$^+$ cells have been identified in the gills of Chinook salmon during *Loma salmonae* infection (Lovy, Wright et al. 2006). Furthermore, BG-like granules were observed in the lymphoid organs (spleen and AK) of Atlantic salmon and rainbow trout (Lovy, Wright et al. 2008). In addition, BG-like granules in the cytoplasm of DCs were observed in the skin of zebrafish (Lugo-Villarino, Balla et al. 2010). Recently, our group identified L/CD207$^+$ cells in the AK, spleen, and gill of channel catfish by immunohistochemistry (Kordon et *al*., 2016). Additionally, BG-like granules in our study were observed in the DC-like cells of the spleen, anterior and posterior kidneys, and gill by transmission electron microscopy of channel catfish (Kordon, Scott et al. 2016).

Macrophages

Macrophages are large mononuclear cells present in virtually all animal tissues. Until quite recently, according to the mononuclear phagocytes system theory, peripheral blood monocytes in vertebrates were considered the progenitors for the tissue macrophages (van Furth, Cohn et al. 1972). However, recent evidence demonstrates that self-maintaining resident populations of tissue macrophages were derived from different

sources, such as yolk sac and fetal liver during embryonic development of mammals (Ginhoux and Jung 2014, Perdiguero and Geissmann 2016). In addition, blood monocytes contribute to tissue-resident macrophage populations during inflammatory conditions and the depletion of resident macrophages in their environment (Serbina, Jia et al. 2008, Hashimoto, Chow et al. 2013, Varol, Mildner et al. 2015). The similar developmental pattern has also been determined in teleost fish. In zebrafish, macrophages are produced for the first time from lateral plate mesoderm during primitive hematopoiesis at the 12– 24 h post-fertilization period (Herbomel, Thisse et al. 1999, Rombout, Huttenhuis et al. 2005). Furthermore, another study showed that during the absence of definitive hematopoiesis, tissue macrophages had been observed in zebrafish lacking *c-myb*, which is the transcription factor and the key regulator of definitive hematopoiesis in mice (Soza- Ried, Hess et al. 2010). Like mammals, monocytes give rise to macrophages in fish during inflammation (Hodgkinson, Grayfer et al. 2015).

Macrophages are professional phagocytes and possess germline-encoded PRRs which recognize the PAMPs, such as LPS from the Gram-negative bacterial cell wall, peptidoglycan, and LTA from the gram-positive bacterial cell wall (Akira, Uematsu et al. 2006, Gordon 2007, Silva and Correia-Neves 2012). Several PRRs that are described in fish are TLRs, RIG-I-like receptors (RLRs), Fc receptors, complement receptors, and scavenger receptors which recognize different PAMPs (Smith, Smythies et al. 2011, Meng, Zhang et al. 2012, Poynter, Lisser et al. 2015). Following the recognition of pathogens via different PRRs, macrophages attach and ingest pathogens into vesicles known as phagosomes that fuse with lysosomes to form phagolysosomes (Esteban,

Cuesta et al. 2015). Macrophages produce antibacterial substances, such as reactive oxygen and nitrogen species which kill and destroy the pathogen in the phagolysosomes (Sharp and Secombes 1993, Esteban, Cuesta et al. 2015) (Figure 3). Therefore, macrophages have a crucial role in innate immunity for the clearance of pathogens and infections.

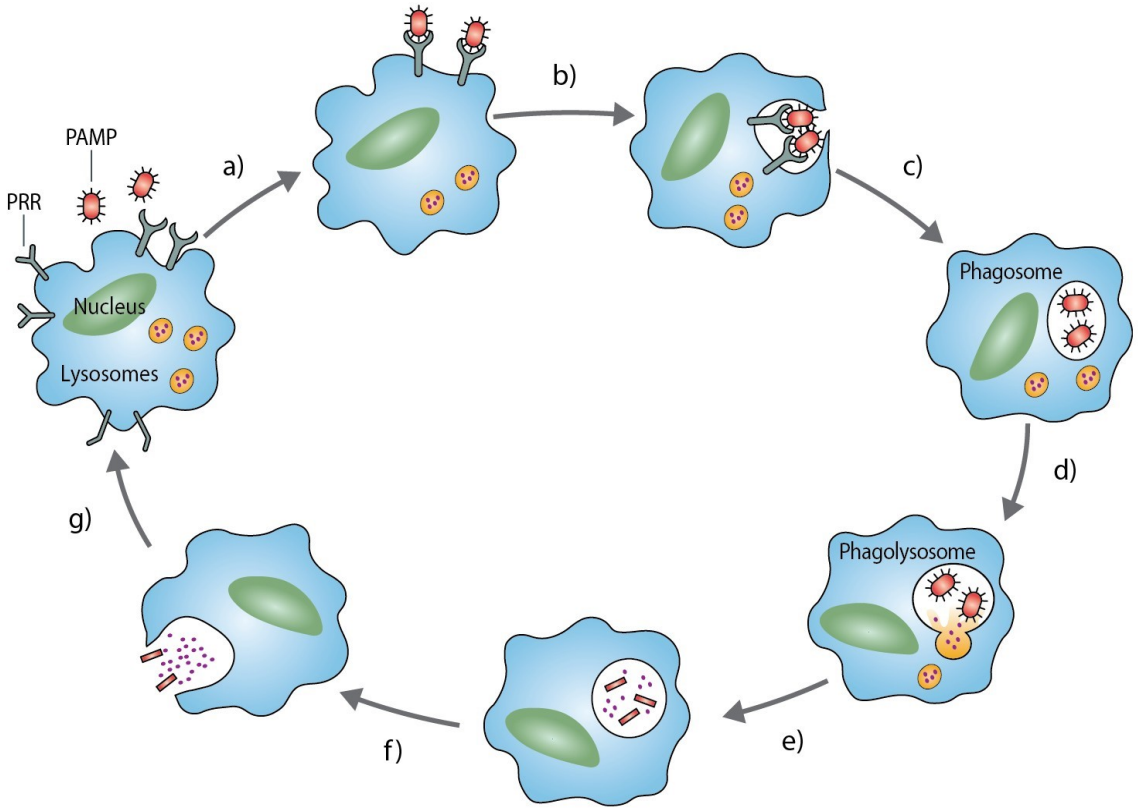

Figure 3 Antigen uptake and processing by phagocytosis in macrophages.

a) Recognition: macrophage contains PRRs which can recognize and interact with PAMPs; b) Engulfment: macrophage engulfs the pathogen via endocytic PRRs; c) Phagosome formation: phagosome is formed in the cytosol; d) Phagolysosome formation: Phagosome fuses with lysosome to form phagolysosome; e) Pathogen destruction and elimination: pathogen is destroyed and killed by enzymes in phagolysosomes; f) Discharge, macrophage discharges the waste of pathogen.

Multiple studies reported that AK macrophages in teleost fish showed robust phagocytic capability and bactericidal activity against intracellular pathogens, such as parasites, yeast, and bacteria (Bennani, Schmid-Alliana et al. 1995, Dieter and Katharina 1997, Esteban, Mulero et al. 1998, Muñoz, Álvarez-Pellitero et al. 2000, Qiu, Liu et al. 2016). In addition to AK macrophages, a peritoneal macrophage separation method has been established for *in vitro* immunologic studies in catfish (Jenkins and Klesius 1998). Seabass peritoneal macrophages showed significantly higher phagocytic activity against *Escherichia coli and Salmonella typhimurium* than monocytes and macrophages which were obtained from blood and AK (Esteban and Meseguer 1997). Moreover, phagocytosis of *Yersinia ruckeri* and *Photobacterium damselae piscicida* by peritoneal macrophages was significantly greater than other phagocytic cells in the peritoneal cavity of sea bass and rainbow trout (António, Susana et al. 1998, Do Vale, Afonso et al. 2002). Also, the phagocytic and microbicidal activity of peritoneal macrophages were described in many fish species, such as rohu, walking catfish, and flounder (Bodammer and Robohm 1996, Rashid, Sardar M. et al. 2002, Awasthi, Rathore et al. 2015).

Several studies from humans and other mammals documented the high-intensity antigen uptake at 37^{o} C; however, the antigen uptake intensity was low at background levels of endocytosis at 4^{o} C in professional APCs (Boyd, Lee et al. 2004, Ammari, Harris et al. 2014). Interestingly, our research group observed active uptake of *Edwardsiella ictaluri* at 32^{o} C and 4^{o} C in catfish peritoneal macrophages, but uptake intensity of this pathogen was significantly higher at 32^{o} C (Kordon, Abdelhamed et al. 2018). Several factors may be necessary for antigen uptake at low temperatures. The

poikilothermic nature of fish may play an important role in endocytosis, and there are phenotypic and functional interspecies differences in particular macrophages, between APCs.

In mammals, macrophages have distinct subsets based on their functions, classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages) (Hodgkinson, Grayfer et al. 2015). M1 macrophages mediate host defense against a diverse group of pathogens, such as bacteria, viruses, and protozoa, and are activated by PAMPs and the cytokines TNF-α or IFN-γ (e.g., LPS) (Murray and Wynn 2011, Italiani and Boraschi 2014, Zhou, Huang et al. 2014). Activated M1 macrophages produce pro-inflammatory cytokines (e.g., TNFs, IL1, and IL-6) and secrete nitric oxide (Arango Duque and Descoteaux 2014). Therefore, M1 macrophages facilitate antimicrobial and antitumor activity and also prevent cell proliferation (Mills 2012). On the other hand, M2 macrophages have anti-inflammatory function and produce growth factors (such as transforming growth factor-beta (TGF-β)) and ornithine instead of nitric oxide, thus promoting cell proliferation and repair, wound healing, and fibrosis (Mills 2012, Martinez and Gordon 2014). Furthermore, M2 macrophages are divided into three subclasses: M2a, M2b, and M2c which are activated by different chemokines (Hodgkinson, Grayfer et al. 2015). Macrophages are able to switch from one functional phenotype to another type in response to environmental signals (Murray and Wynn 2011). In teleost fish, M1 and M2 macrophages are characterized by their similarity to mammalian macrophage function and phenotype (Neumann, Stafford et al. 2000). Recent research demonstrated the polarization of M1

macrophages into M2 macrophages in zebrafish in response to environmental cues (Nguyen-Chi, Laplace-Builhe et al. 2015).

In addition to innate immunity, macrophages are essential in initiation of adaptive immunity as other professional APCs. Like DCs, macrophages sense signals from the microenvironment to orchestrate innate and adaptive immune responses. In mammals, intestinal macrophages recognize pathogens during inflammation, and inflammatory monocytes ($Ly6C^+$ monocytes) are recruited to the site of infection and differentiate into inflammatory macrophages which present antigens to T cells, thus promoting the differentiation of Th1 and Th17 cells (Flannigan, Geem et al. 2015). Macrophages are capable of expressing MHC Class I, MHC Class II and co-stimulatory molecules; however, the expression level of these molecules is markedly lower in macrophages compared to DCs (Wu and Kaiser 2011). In zebrafish, macrophages expressing MHC class II molecules showed a similar role to their mammalian counterparts (Lieschke, Oates et al. 2001, Wittamer, Bertrand et al. 2011). Furthermore, piscine macrophages also express MHC class II molecules and have a role as an APC (Vallejo, Miller et al. 1992, Brubacher, Secombes et al. 2000). Moreover, monocytes in fugu are APCs as they expressed B7 molecules which regulate T cell responses (Sugamata, Suetake et al. 2009).

B cells

B cells in mammals are composed of different subsets with different functions and locations; B-2 cells (or follicular B cells), B-1 cells, and marginal zone (MZ) B cells (Zhang 2013). B-2 cells respond to thymus-dependent antigens and require the

cooperation of T helper cells in germinal centers; therefore, they produce high-affinity antibodies with precise antigen specificity (Tafalla, González et al. 2017). However, B-1 and MZ B cells are responsible for immune responses against thymus-independent antigens and do not need help from T helper cells, thus producing low-affinity antibodies with broad reactivity which provide fast protection, in particular at the mucosal interfaces (Zhang 2013, Tafalla, González et al. 2017). Furthermore, B-1 cells are subdivided into two classes; B-1a cells, that express CD5, a surface marker, and B-1b cells that lack the expression of CD5 (Rothstein, Griffin et al. 2013). Although the primary function of B cells is to produce antibodies, they also have a role in innate immunity. The long-held paradigm dictates that B cells are unable to uptake large particles; however, it has been broken by the recent discovery that human primary B cells engulfed live *Salmonella typhimurium* in B cell receptor (BCR)-mediated manner (Souwer, Griekspoor et al. 2009). Another study showed the phagocytic ability of murine B cells from bone marrow, spleen, blood, and the peritoneal cavity where the largest percentage of phagocytic B cells (~10-17%) were present in the peritoneal cavity, while other tissues contained B cells with phagocytic capacity less than 1.6% (Parra, Rieger et al. 2012). Interestingly, phagocytic B cells in the peritoneal cavity were B-1 cells, which had engulfed latex beads and bacteria, and the phagocytic ability of B-1a subset (14-17%) was significantly higher than the other subset, B-1b cells (8.6-11.4%) (Parra, Rieger et al. 2012). Moreover, both subsets of B-1 cells were capable of maturation of their phagosome into phagolysosomes and also killed internalized bacteria, and they presented ingested antigen to CD4+ T cells (Parra, Rieger et al. 2012).

Similar to B-1 cells of mammals, teleost B cells are capable of ingesting particles and intracellular killing of ingested microbes (Li J, DR. et al. 2006, Sunyer 2013). For example, B cells in zebrafish have strong phagocytic ability for both soluble and particulate antigens (Zhu, Nie et al. 2013, Zhu, Lin et al. 2014). Moreover, phagocytic ability of B cells was described in rainbow trout, and the phagolysosome formation was observed; therefore, B-cells in rainbow trout contribute the bacterial killing (Li J, DR. et al. 2006, Sunyer 2012). Furthermore, in Atlantic salmon and Atlantic cod, B cells were also able to phagocytize fluorescent beads and also showed higher phagocytic capacity than neutrophils in cod (Øverland, Pettersen et al. 2010). Our research showed that B cells in AK of channel catfish were able to uptake and destroy *E. ictaluri* (Kordon et al., unpublished observation).

Like mammals, teleost B cells serve as a professional APC. Zebrafish B cells can present both soluble and particulate antigens to prime naïve $CD4^+$ T cells (Zhu, Nie et al. 2013). This study showed that the expression of MHC class II molecules and co-stimulatory molecules (CD86 and CD83) was upregulated in B cells (Zhu, Nie et al. 2013). Other *in vivo* and *in vitro* studies have demonstrated that B cells in teleost fish are important APCs which activate T cells and initiate adaptive immunity (Lewis, Del Cid et al. 2014, Zhu, Lin et al. 2014).

Adaptive Immune Responses in Fish

If innate immunity is insufficient to clear the infection, adaptive immunity is activated by non-specific immunity. The adaptive immune system is composed of specialized cells and proteins which eliminate and inhibit the growth of invaders. In adaptive immunity, diverse antigen receptors are generated from a small number of

genes because of somatic hypermutation and V(D)J recombination mechanisms; therefore, the adaptive immune system recognizes pathogens in highly specific manner. Another key property of adaptive immunity is memory because adaptive immune system provides long-lived specific immunity due to the development of memory cells.

The adaptive immune system is divided into two main components: the lymphocytes (effector cells), also called cell-mediated responses, and antibodies, also known as humoral-mediated responses (Biller-Takahashi J. D. and C. 2014). Cells of the adaptive immune system are the lymphocytes, T- and B-cells. Like mammals, T cells develop and mature in the thymus of teleost fish (Nakanishi, Shibasaki et al. 2015). After activation of naïve T cells, they differentiate into cytotoxic T-cells (CTLs) or helper T (Th) cells (Nakanishi, Shibasaki et al. 2015). B-cells are produced mostly in the anterior kidney of teleosts and produce antibodies (Zwollo P., Cole S. et al. 2005). Similar to higher vertebrates, fish antibodies have high specificity and affinity to antigens and result in neutralization and opsonization of pathogens and also activate the complement system (Mashoof and Criscitiello 2016).

Cell-mediated responses

Lymphocytes, T-and B- cells, play a vital role in cell-mediated responses of adaptive immunity. Similar to humans, T-and B-cells in teleost fish rearrange antigen receptors by the recombination-activating gene (RAG) (Greenhalgh and Steiner 1995). Therefore, they use common molecular pathway for the regulation of lymphocytes production. T cells help the regulation of other leukocytes, or they kill directly infected host cells. On the other hand, B cells secrete antibodies and contribute to clearing of infections.

T cells

In mammals, T cells are produced with diverse antigen repertoire of cell-surface receptors by the RAG proteins, and these receptors are called T cell receptors (TCR) which recognize peptide of antigens presented by MHC (Langenau and Zon 2005). Like mammals, T cells in teleost fish contain TCR genes, which may encode the TCR α, β, γ, and δ chains (Nam, Hirono et al. 2003). Moreover, T cell development in teleost fish seems to be similar with mammals because their transcription factors are similar, such as ikaros (expressed by early T-cells) and lck (expressed later in development) (Willett, Kawasaki et al. 2001). T cells develop in the thymus; therefore, they are also called thymocytes.

T cells are divided into two major populations in higher vertebrates based on their function, cytotoxic T cells (CTLs) and helper T (Th) cells (Nakanishi, Shibasaki et al. 2015). CTLs express CD8 molecules on their surface which interact with MHC class I molecules while helper T cells express CD4 molecules that interact with MHC class II molecules (Fig. 4) (Andersen, Schrama et al. 2006). Furthermore, naïve helper T-cells are differentiated into several populations of effector T cells including Th1, Th2, Th17, Th9, follicular helper T-cells (Tfh), and regulatory T cells (Tregs) which have different functions in immune responses (Dardalhon, Awasthi et al. 2008, Annunziato and Romagnani 2009, Wan and Flavell 2009). Both types of T cells, CTLs and helper T cells, also differentiate into memory T cells which involve long term protective immunity (Tanel, Fonseca et al. 2009, MacLeod, Kappler et al. 2010).

T cell-related genes, such as CD3, CD4, CD8, MHC class I and II, have been identified in teleost fish, and these studies showed that the presence of $CD4^+$ helper T

cells and $CD8^+$ CTLs in fish are similar to those of higher vertebrates (Toda, Shibasaki et al. 2009, Koppang, Fischer et al. 2010, Toda, Saito et al. 2011, Fischer, Koppang et al. 2013). CTLs in fish are primarily crucial in virus infection because CTLs lead to the lysis of virus-infected host cells as in higher vertebrates (Somamoto, Nakanishi et al. 2002). On the other hand, helper T cells are involved in the regulation of the immune responses by recruitment and activation of cell-mediated immunity, including $CD8^+$ T cells, B cells, macrophages, and granulocytes in mammals (Castro, Bernard et al. 2011). For example, like mammals, Th1 cells activate macrophages against intracellular bacterial and viral infections in fish (Zou, Carrington et al. 2005, Díaz-Rosales, Bird et al. 2009, Mitra, Alnabulsi et al. 2010). In mammals, Th2 cells have a role in parasitic infections, and studies teleost fish showed that the expression of Th2 markers (*IL-4/13A and GATA-3)* in the gills and skin against parasites (Takizawa, Koppang et al. 2011). Another Th cell lineage, Th17, involves in the immune response against extracellular bacterial and fungal infections by recruiting neutrophils (Martinez, Nurieva et al. 2008, de Jong, Suddason et al. 2010). Several studies showed the presence of all cytokines involved in the differentiation of Th17 pathway and cytokines secreted by Th17 cells in teleost fish (Gunimaladevi, Savan et al. 2006, Flores, Hall et al. 2007). Recently, Th17-like immune response has been identified in zebrafish mucosal tissues after live attenuated *Vibrio anguillarum* infection (Zhang, Shen et al. 2014). In mammals, Treg cells play a vital role in maintaining tolerance self-antigens and suppressing excessive immune responses which are deleterious to the host health (Sakaguchi, Yamaguchi et al. 2008). Transcriptional factor, FOXP3, and cytokines related to development of Treg cells have been characterized in teleost fish (Bird, Zou et al. 2006, Mitra, Alnabulsi et

al. 2010). A recent study suggested that Treg cells in the skin of rainbow trout maintain peripheral tolerance (Leal, Granja et al. 2016).

Mature T cells are present in lymphoid tissues of teleost fish, such as thymus, kidney, and spleen (Araki, Suetake et al. 2005, Picchietti, Guerra et al. 2009). Recently, the presence of T cells has been detected in mucosal tissues of fish including the intestine, gill, and skin (Rombout, Joosten et al. 1998, Bernard, Six et al. 2006, Haugarvoll, Bjerkås et al. 2008, Rombout, Abelli et al. 2011).

B cells

Second cell type of cell-mediated responses in adaptive immunity is B cells which are developed in the anterior kidney of fish (Zapata, Diez et al. 2006). B cells are activated in the spleen, which is considered secondary lymphoid tissue (Bromage, Kaattari et al. 2004). In mammals, mature B cells generate the plasmablasts (precursor cells) and differentiate into plasma cells which produce antibodies (Dörner and Radbruch 2005). A similar differentiation pattern of B cells to plasma cells are seen in teleost fish (Zwollo, Cole et al. 2005, Ye, Kaattari et al. 2011). The plasma cells of teleost fish are divided into two populations, short-lived plasma cells (SLPC) and long-lived plasma cells (LLPC), and major differences between these two populations are their lifespan and distribution in the body since SLPCs are formed in the spleen, while LLPCs are located only in the anterior kidney of fish (Kaattari, Bromage et al. 2005, Ye, Kaattari et al. 2011). Similar to T cells, B cells also differentiate into memory B cells after activation by antigenic interaction; therefore, teleost fish are protected by memory responses for several years post-immunization (Findly R. C., Zhao X et al. 2013, Firdaus-Nawi and Zamri-Saad 2016).

In addition to antibodies production, B cells have innate features, such as natural antibody secretion, phagocytic capability, and intracellular killing (Li J, DR. et al. 2006, Sunyer 2013). Phagocytic ability of B-cells is described in the trout, and the phagolysosome formation was observed in these fish; therefore, B cells in trout contribute the bacterial killing (Deluca, Wilson et al. 1983, Li J, DR. et al. 2006). Moreover, phagocytosis of bacteria and soluble antigens by zebrafish B cells was studied, and like mammals, B-cells in zebrafish are important APCs which activate T cells and initiate the adaptive immunity (Zhu, Nie et al. 2013).

In addition to lymphoid tissues, B cells are found in distinct tissues, such as intestine, skin, and gill (Salinas, Zhang et al. 2011, Salinas 2015). B cells are mainly found in the lamina propria of anterior and posterior intestine, and also present in a small number in the epithelium (Salinas, Zhang et al. 2011). Furthermore, B cells are detected in the epithelium of skin and gills (Rombout J. H. W. M., Taverne-Thiele A. J. et al. 1993, Grove, Johansen et al. 2006).

Significance of research and objectives

The effects of efficacious *E. ictaluri* LAVs on innate and adaptive immune responses specifically, phagocytosis, microbial killing, and APCs antigen presentation, are still unexplored. APCs initiate effective and protective innate and adaptive immune responses by presenting the antigens to T cells resulting in generation of memory cells, which can prevent disease or at least reduce severity of disease during re-infection. Therefore, there is a critical need to identify the roles of LAVs in the APC-dependent immune responses in channel catfish.

The overall objective of this project is to assess the role of three professional APCs, dendritic cells (DCs), macrophages, and B cells in the LAV-induced innate and adaptive immune responses in channel catfish. The central hypothesis is that efficacious LAV strains generate protective immunity by enhancing the phagocytosis and microbial killing, which promotes the generation of T cells that will regulate and control protective B cell-mediated immune responses. The rationale for the proposed research is that more detailed knowledge about the phenotype and the function of catfish APCs 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. With this new knowledge, we can elucidate the development of adaptive immunity and prevent ESC disease in catfish. Therefore, the specific objectives of this study were:

1. Identification and morphological assessment of dendritic cells (DCs) in the immunocompetent tissues of channel catfish.

2. Evaluation of DC-dependent immune responses to Live Attenuated *Edwardsiella ictaluri* Vaccines in Channel Catfish.

3. Assessment of Live Attenuated Vaccine-Induced Sera Effects on Phagocytic Uptake and Bacterial Killing in Channel Catfish Peritoneal Macrophages.

4. Assessment of Live Attenuated Vaccine-Induced Sera Effects on Phagocytic Uptake and Bacterial Killing in Channel Catfish B cells.

5. Evaluation of T Cell-mediated Immune Responses to Live Attenuated *Edwardsiella ictaluri* Vaccines in Channel Catfish.

CHAPTER II

IDENTIFICATION OF LANGERHANS-LIKE CELLS IN THE IMMUNOCOMPETENT TISSUES OF CHANNEL CATFISH

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. In addition, we found L/CD207^{+} cells in the catfish anterior kidney and spleen by flow cytometry. Moreover, we described the cells that resembled mammalian LC DCs containing Birbeck-like (BL) granules in channel catfish spleen 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

thephenotype 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.

Introduction

Dendritic cells (DCs) are the most potent professional antigen presenting cells (APCs) that bridge, regulate, and control all innate and adaptive immune responses in vertebrates (Mildner and Jung 2014). 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*) (Lugo-Villarino, Balla et al. 2010, Wittamer, Bertrand et al. 2011). 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*) (Aghaallaei, Bajoghli et al. 2010, Bassity and Clark 2012). 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 (Lin, Xiang et al. 2009). 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 (Shao, Zhu et al. 2015).

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 (Igyarto and Kaplan 2013). 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 (Sugita, Kabashima et al. 2007). 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 (Valladeau, Dezutter-Dambuyant et al. 2003). 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 (Valladeau, Ravel et al. 2000). However, 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 (Fehres, Bruijns et al. 2015).

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 commercial polyclonal antibodies (pAbs) developed against human Langerin (Lovy, Wright et al. 2006, Lovy, Wright et al. 2008, Lovy, Savidant et al. 2009). 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 (Lovy, Wright et al. 2008, Lovy, Savidant et al. 2009). 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 (Lovy, Savidant et al. 2009). 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 (Lovy, Wright et al. 2008).

Channel catfish (*Ictalurus punctatus*) is the most important aquaculture species in the United States, accounting for $352 million in 2014 (USDA 2014). Vaccination against catfish pathogens requires the 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.

Materials and Methods

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 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.

Cell Preparation

Peripheral blood, anterior kidney, and spleen cell separations were performed as described previously (Zhao, Findly et al. 2008). Briefly, peripheral blood was collected from the caudal vein followed by centrifugation at 500*g* 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, tissues 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 500*g* for 30 min. Cells then were collected

from the interface, washed three times in PBS, counted and assessed for viability by trypan blue exclusion.

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/WashTM 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/WashTM 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/WashTM 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/WashTM 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.

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 (Lovy, Wright et al. 2008).

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 (Lovy, Savidant et al. 2009). 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 one h. After that, primary antibody was diluted to a concentration of 0.2 mg/ml (1:500 from stock solution), and sections were incubated with 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).

Transmission Electron Microscopy (TEM)

Sample preparation for TEM was carried out as described (Lovy, Wright et al. 2008). 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 Spurr'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.

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 $P<0.05$.

Results

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 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 ($P>0.05$).

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 (Lovy, Savidant et al. 2009). Visually strong L/CD207 cytoplasmic expression was observed in the spleen and anterior kidney tissues compared to the virtually undetected reaction in the negative controls (Figure 4A, B, and C). 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 (Figure 4D). However, the skin of channel catfish was constantly negative for L/CD207 (data not shown).

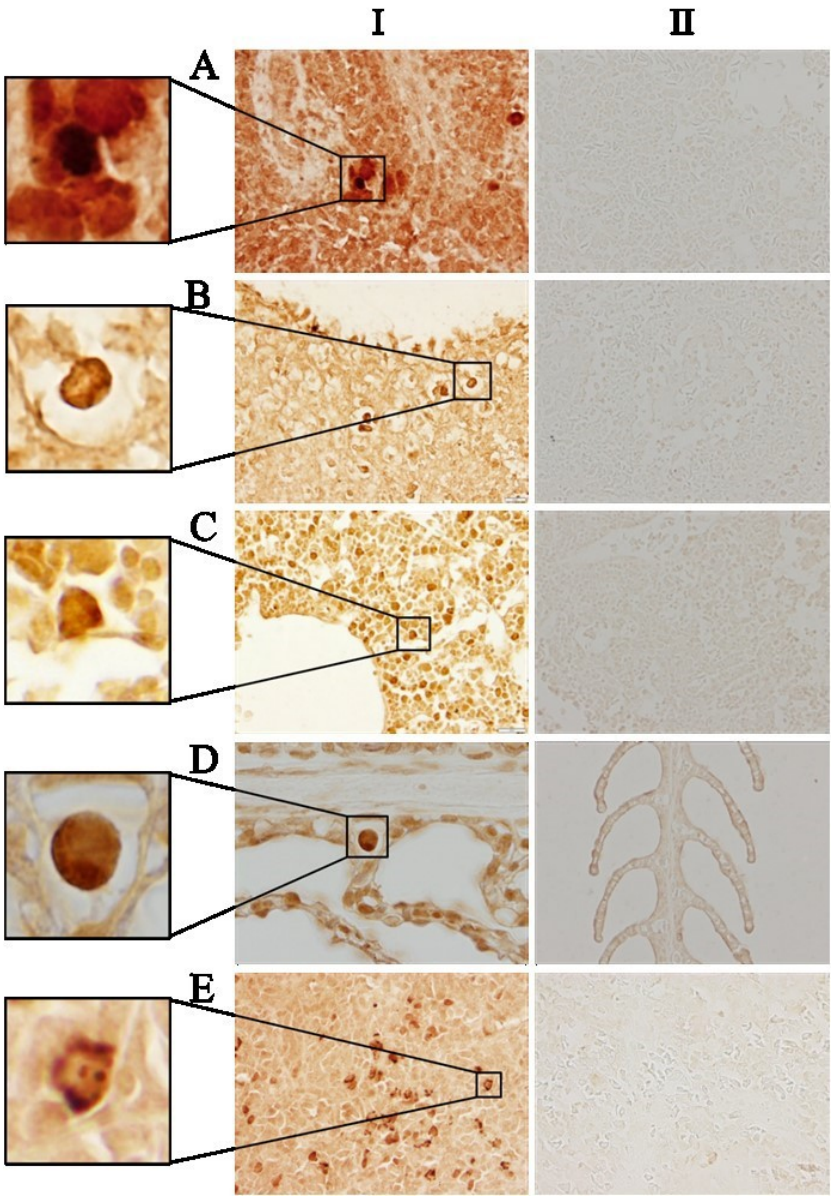

Figure 4 Assessment of L/CD207^{+} cells in the spleen, anterior kidney, and gill of catfish by immunohistochemical staining with purified pAbs specific to human Langerin.

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 dilution1:500; 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. Excerpt images on the left show magnified images of the selected areas.

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 (Figure 5). Contrary, gill and skin tissues showed the background intensity of L/CD207 staining comparable with negative controls (data not shown).

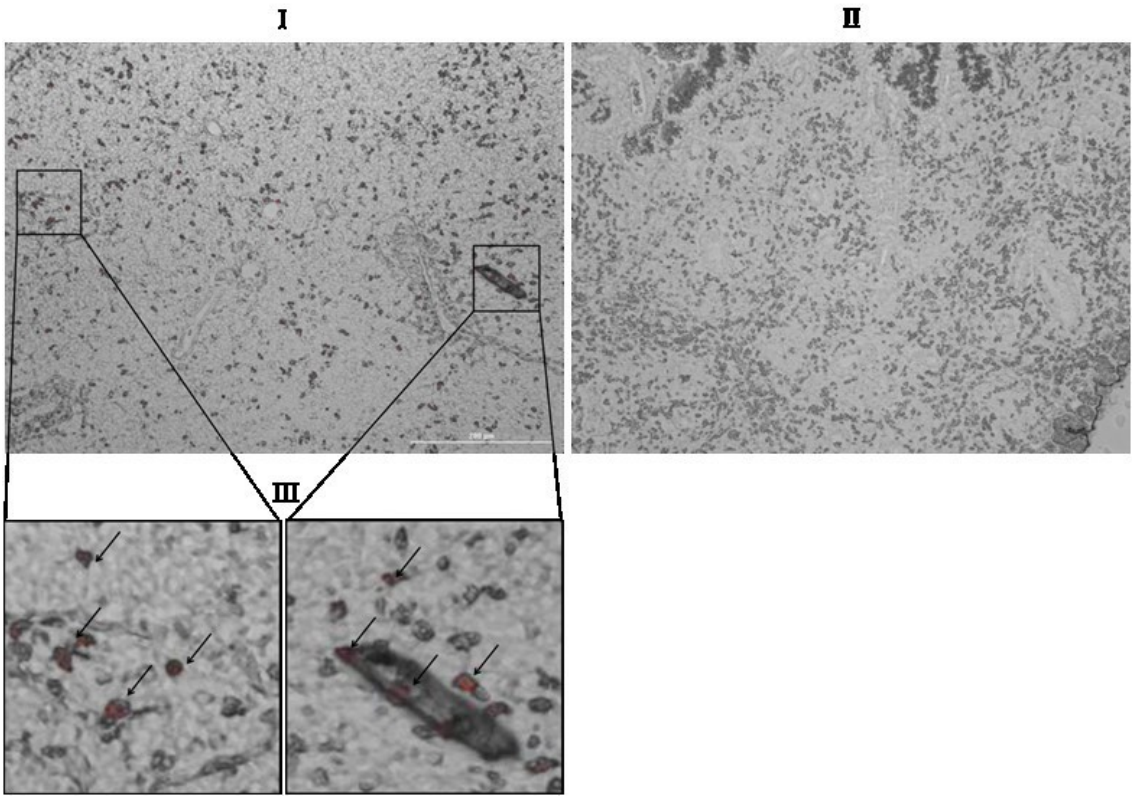

Figure 5 Assessment of L/CD207^{+} cells in the anterior kidney of catfish by immunohistochemical staining with R-PE-conjugated mAbs specific to human Langerin.

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

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

Dendritic-like (DL) cells were observed in the spleen and posterior kidney of channel catfish fingerlings and fry, respectively, and the ultrastructure of these DL cells was analyzed by TEM (Figure 6). 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.

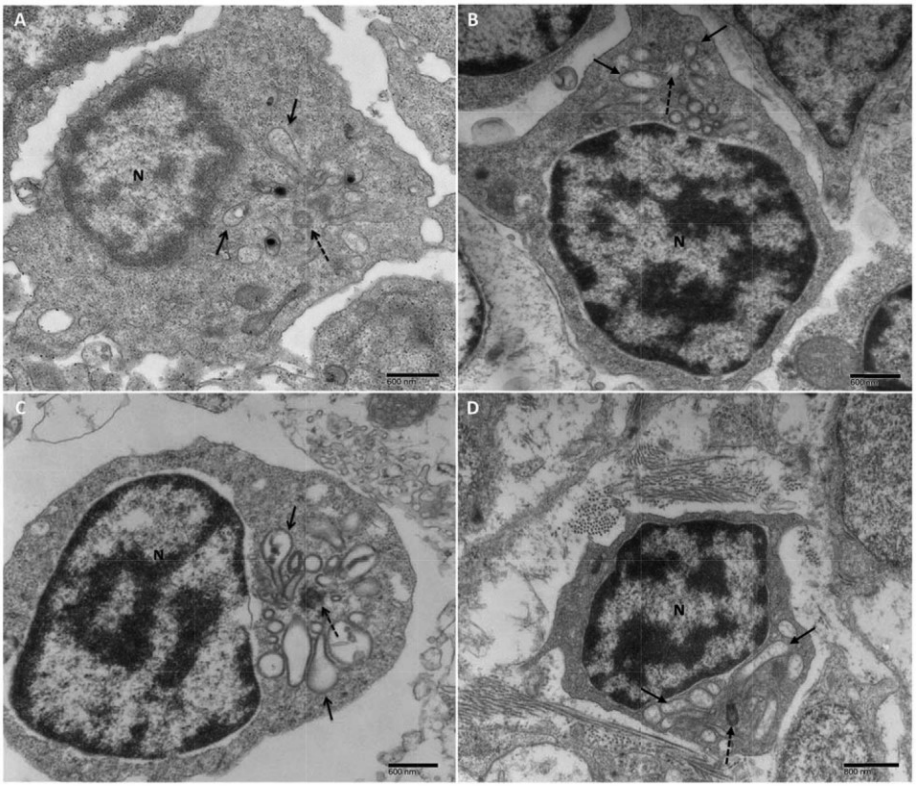

Figure 6 Dendritic-Like (DL) cell in the immune-related organs of Ictalurus punctatus by electron microscopy.

DL cell with an 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.

Discussion

The current research aimed to morphologically assess the presence of DCs, the most powerful professional APCs, in immune-related organs of channel catfish. We

identified L/CD207$^+$ cells in the spleen, anterior kidney, and gill of channel catfish. Importantly, the L/CD207$^+$ DCs visually prevailed in the catfish anterior kidney compared to their counterparts in the spleen. Also, the L/CD207$^+$ cells were virtually absent in the skin and rare in the gill of catfish. L/CD207$^+$ were firstly described in the spleen and anterior kidney of Atlantic salmon and rainbow trout with remarkable similarities to human LCs (Lovy, Savidant et al. 2009). Unlike in channel catfish, L/CD207$^+$ were observed in fewer numbers in the anterior kidney compared to the spleen of Atlantic salmon and rainbow trout and like in catfish, were not detected in the skin (Lovy, Savidant et al. 2009). These results show that anterior kidney and spleen in channel catfish serve as important organs for antigen presentation as was described previously for rainbow trout, salmon, and zebrafish (Lovy, Savidant et al. 2009, Lugo-Villarino, Balla et al. 2010, Bassity and Clark 2012). Interestingly, L/CD207$^+$ cells in teleost fish were found predominantly in the spleen and anterior kidney as LCs in mammals populate mainly the epidermis of the skin and in the process of maturation migrate to the secondary lymphoid organs (Geissmann, Dieu-Nosjean et al. 2002). As fish lack lymph nodes, the migration pattern and maturation of DCs might be different from humans and other mammals. Dendritic-like populations identified in the immunocompetent organs of catfish may share morphological and functional properties with previously reported DCs in teleost fish 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

(Lovy, Wright et al. 2006, Lovy, Wright et al. 2008, Lugo-Villarino, Balla et al. 2010). 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 (and lymphoid organs of salmonids (Lovy, Wright et al. 2006, Lovy, Wright et al. 2008). Lately, BL granules were also detected in the DCs isolated from the skin of zebrafish (Lugo-Villarino, Balla et al. 2010). 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 (Lovy, Wright et al. 2006, Lovy, Wright et al. 2008). Furthermore, BGs in LCs were commonly found near centrioles and microtubules in patients with 'atypical hyperplasia' in humans (Shamoto, Hoshino et al. 1976). 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 (Mc Dermott, Ziylan et al. 2002). Therefore, centrioles and microtubules are necessary during antigen processing in immature DCs (Peachman, Rao et al. 2004). Despite the lacking information on the ultrastructural details of BL granules and accumulation of Langerin in their 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 in the epidermis in humans and other mammals. 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.

CHAPTER III

DISTRIBUTION PATTERNS OF LANGERHANS-LIKE CELLS IN THE IMMUNE-RELATED ORGANS OF CATFISH VACCINATED WITH LIVE ATTENUATED AND WILD-TYPE *EDWARDSIELLA ICTALURI* STRAINS

Abstract

Several studies identified the cells with mammalian Langerhans cells (LCs) morphology in teleost fish. Langerin/CD207$^+$ (L/CD207$^+$) cells were described in the anterior kidney (AK) and spleen of Atlantic salmon, rainbow trout, and channel catfish. In addition, our group has identified LC-like cells in the gill of channel catfish. However, there is no evidence of the role of the powerful dendritic cell (DC) subset in catfish immune responses. In this study, we assessed the numbers of L/CD207$^+$ cells in the immune-related tissues of channel catfish fingerlings challenged with two novel *E. ictaluri* live attenuated vaccines (LAVs), *Ei*Δ*evpB* and ESC-NDKL1, and wild-type (WT) strain by immunohistochemistry (IHC). Importantly, in the vaccinated catfish, the increased numbers of L/CD207$^+$ cells in the AK and spleen present at the early stage of infection suggest their involvement in the initiation of innate immune responses and migration/maturation from AK and spleen to the site of infection, gill after 3 d post-challenge. Finally, at 7 d post-challenge, the increased numbers of L/CD207$^+$ cells in the spleen suggest possible migration back to the spleen for initiation of adaptive immune responses. In the WT *E. ictaluri*-infected catfish, the DC-like cell distribution

patterns at 6 h and 1 d post-challenge resembled the patterns in the vaccinated fish in all three immune-related organs. However, significant declines in the numbers of L/CD207^{+} cells in the AK and spleen at 3 and 7 d post-challenge comparable to the uninfected controls imply possible functional inability of the WT-induced DCs of migration/maturation and initiation of adaptive immune responses. Taken together, our data suggest that both LAVs targeted catfish DC-like cells for bridging innate and adaptive immunity thus completely satisfying the stringent requirements for successful LAVs.

Introduction

Dendritic cells (DCs) are the most powerful antigen-presenting cells (APCs) that are critical players in bridging and shaping of all innate and adaptive immune responses in vertebrates (Mildner and Jung 2014). Dendritic cells are recognized as the APCs present in lymphoid and non-lymphoid tissues that sense the environment. The powerful APCs recognize foreign materials and self-antigens by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), CD206, and DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN/CD209) and are capable of stimulating naïve T-cells (Diebold 2009, Kawai and Akira 2009, Summerfield A. 2015). Immature DCs capture antigens, process them into peptides and then transport to the secondary lymphoid tissues while undergoing the process of maturation. Mature DCs present antigens to naïve T cells to induce adaptive immune responses (Alvarez, Vollmann et al. 2008).

Recently, DCs have been characterized in several teleost fish based on their morphology and function. For example, DC-like cells with mammalian DC morphology

and T-cell stimulatory capability have been described in zebrafish (Lugo-Villarino, Balla et al. 2010, Wittamer, Bertrand et al. 2011). Moreover, DCs are also identified in rainbow trout, barramundi, and medaka based on their morphology, motility, phagocytic ability, and T-cell activation properties (Aghaallaei, Bajoghli et al. 2010, Bassity and Clark 2012, Zoccola, Delamare-Debouteville et al. 2015).

Upon activation, another critical feature of APCs in mammals is the expression of co-stimulatory molecules, such as CD28, B7-1 (CD80), and B7-2 (CD86), which are necessary to prime naïve T cells and induce the differentiation of T cells by producing cytokines (Chen and Flies 2013). A previous study reported the presence of the major co- stimulatory molecules (e.g., CD80/CD86 and CD83) in zebrafish (Lin, Xiang et al. 2009). Furthermore, similarly to mammals, a recent study showed that the surface molecules of zebrafish DCs (CD80/86/83/CD209^{+}) could promote CD4^{+} naïve T-cell stimulation (Shao, Zhu et al. 2015).

Dendritic cells in mammals have multiple subsets, and Langerhans cells (LCs) are the distinct subset of DCs. Langerhans cells are present in the epidermis, and this unique location provides LCs with early recognition of pathogens, foreign chemicals, and self- antigens (Igyártó and Kaplan 2013). Langerhans cells can engulf antigens and migrate to the secondary lymphoid tissues to present the antigen to naïve T-cells, thus initiating adaptive immune responses (Sugita, Kabashima et al. 2007). Langerhans cells are uniquely characterized by Birbeck granules which are rod-shaped organelles consisting of the superimposed and zippered membrane (Mc Dermott, Ziylan et al. 2002). Langerin is a type II transmembrane C-type lectin, which is a specific marker for LCs and associated with the formation of Birbeck granules (Valladeau, Ravel et al.

2000, Lau, Chu et al. 2008). The antigen capture function of Langerin triggers the induction of Birbeck granules by allowing routing antigens into Birbeck granules, thus providing non-classical antigen processing pathway and cross-presentation (Valladeau, Ravel et al. 2000, Fehres, Bruijns et al. 2015).

Several studies identified the cells with mammalian LC morphology in teleost fish. In particular, Langerin/CD207^{+} (L/CD207^{+}) cells were described in the AK and spleen of Atlantic salmon and rainbow trout (Lovy, Wright et al. 2008, Lovy, Savidant et al. 2009). Furthermore, Birbeck -like granules were observed in the lymphoid organs (spleen and AK) of Atlantic salmon and rainbow trout (Lovy, Wright et al. 2008). Also, the same research group observed DC-like cells containing Birbeck -like granules in the gills of Chinook salmon during *Loma salmonae* infection (Lovy, Wright et al. 2006). Recently, our group identified L/CD207^{+} cells in the channel catfish AK, spleen, and gill by IHC. Moreover, we described the cells that resembled mammal LC DCs containing Birbeck-like granules in channel catfish spleen, anterior and posterior kidneys and gill by transmission electron microscopy (Kordon, Scott et al. 2016).

Edwardsiella ictaluri is a Gram-negative facultative intracellular fish pathogen that causes enteric septicemia (ESC) of channel catfish, which is one of the most devastating diseases in the US catfish industry (Hawke 1981, Miyazaki and Plumb 1985, Wagner, Wise et al. 2006, Zhang and Arias 2007). A live *E. ictaluri* vaccine (Aquavac- ESC) against ESC was developed by Klesius and Shoemaker in 1999, and this vaccine enabled to protect juvenile catfish (Klesius and Shoemaker 1999). Then, immersion studies demonstrated that *E. ictaluri* LAVs stimulated the protective immunity in catfish fry, fingerlings, and eyed catfish eggs (Shoemaker, Klesius et al.

1999, Wise, Klesius et al. 2000, Shoemaker, Klesius et al. 2002, Shoemaker, Klesius et al. 2007). Recently, a live attenuated *E. ictaluri* isolate (S97-773) was developed by Wise, and oral vaccination with this live attenuated isolate protected in fingerlings (Wise, Greenway et al. 2015). *Edwardsiella ictaluri* can survive and replicate in channel catfish macrophages, and *E. ictaluri* LAVs induced cell-mediated immunity to protect catfish against ESC (Shoemaker and Klesius 1997, Ellis 1999, Booth, Elkamel et al. 2006). Also, catfish vaccinated with LAVs triggered humoral immune responses which augmented the bacterial killing activity of macrophages (Shoemaker and Klesius 1997, Kordon, Abdelhamed et al. 2018).

Our research group has developed two novel *E. ictaluri* LAV strains (*Ei*Δ*evpB* and ESC-NDKL1), which provided significant protection against ESC in both catfish fry and fingerlings (Lawrence and Karsi 2013, Karsi, Lawrence et al. 2017, Nho, Abdelhamed et al. 2017). Recently, we demonstrated the phagocytic and killing properties of catfish peritoneal macrophages induced by LAV vaccines (Kordon, Abdelhamed et al. 2018). However, the roles of these LAVs on DC-dependent innate and adaptive immune responses in catfish are still unexplored. Therefore, we aimed to evaluate the distribution patterns of L/CD207^{+} LC-like cells in different immune-related tissues, such as AK, spleen, and gill of catfish challenged with two LAV and WT strains of *E. ictaluri.*

Materials and Methods

Bacterial Strains

E. ictaluri (WT strain 93–146, and EiΔevpB, and ESC-NDKL1) were cultured in brain heart infusion (BHI) agar or broth (Difco, Sparks, MD) at 30°C. When

required, media were supplemented with colistin (Col: 12.5 mg/ml, Sigma-Aldrich, Saint Louis, MN).

Catfish vaccination and tissue collection

One hundred specific-pathogen-free (SPF) channel catfish fingerlings (6-month old) were obtained from the College of Veterinary Medicine's fish hatchery at Mississippi State University by using the protocol approved by the Institutional Animal Care and Use Committee.

Catfish were stocked into four 80-L tanks (25 fingerlings per tank) supplied with flow-through water and continuous aeration. Catfish were maintained at 25-28°C and fed twice daily with a floating catfish feed. After a week of acclimation, catfish were exposed to vaccine strains *Ei*Δ*evpB* and ESC-NDKL1, *E. ictaluri* WT (positive control), and BHI (sham-control) as previously described (Abdelhamed, Lu et al. 2013). Exposure dose was approximately 3.67×10^7 CFU/ml of water, which was calculated by serial dilutions of bacterial cultures.

Following challenge, five catfish from each group were randomly removed from each tank at 6 h, 1, 3, and 7 d and euthanatized by 300 mg/L tricaine methanesulfonate (Sigma, St. Louis, MO). Catfish were placed in 10% neutral buffered formalin for 24 h and formalin was replaced, and fish were kept in formalin for another 24 h. Anterior kidney (AK), spleen, gill, and skin of catfish were isolated, dehydrated with a graded series of ethanol, and embedded in paraffin wax. Finally, tissue sections were cut as described (Lovy, Wright et al. 2008).

Immunohistochemistry (IHC)

To evaluate the number of L/CD207$^+$ cells during *E. ictaluri* infection, immunohistochemical staining of catfish AK, spleen and gill was performed with purified human antibody CD207-specific pAbs (R&D Systems, Inc.) as described previously (Kordon, Scott et al. 2016). Briefly, for antigen retrieval, sections were incubated in target retrieval solution (DAKO) for 40 min at 100°C. Then, sections were allowed to cool room temperatures and washed in 1X Phosphate-buffered saline (PBS) for 10 min. Following washing, sections were incubated in protein block (DAKO) for 1 h. After that, they were incubated with primary antibodies (0.2 mg/ml, 1:500 dilution). Normal goat IgG was used as negative controls at the same concentrations as primary antibodies. Primary and control antibody incubation was performed in a humid chamber for overnight followed by the addition of streptavidin/biotin for 15 min and then, incubated in Streptavidin-HRP (Vector Laboratories) for 1 h. Then, sections were stained in a solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB) for 10 min. Finally, samples were washed twice with water for 10 min and dehydrated through a graded series of ethanol to xylene. Slides were analyzed at 40x magnification with Olympus BX60 microscope (Olympus U-TV1 X) and photographed with Infinity analyze software (Lumenera corporation). The number of L/CD207$^+$ cells in AK, spleen, and gill of five catfish from each group was determined per each time point by using a "numbered indexed square grid" eyepiece graticule (1.00 mm^2 IN DEX SQU, PYSER-SGILTD) (Allen and Henshaw 2001). The L/CD207$^+$ cells were counted in 10 mm^2 per field, and total 10 fields and 100 mm^2 were counted for each organ. The mean value of L/CD207$^+$ cells was calculated for further statistical analysis.

Statistical Analyses

One-way and two-way ANOVA procedures of SAS (v 9.4, SAS Institute, Inc., Cary, NC) were used to evaluate differences in means of L/CD207$^+$ cells. The level of significance for all tests was set at $P < 0.05$.

Results

Monitoring of L/CD207$^+$cells in catfish challenged with *E. ictaluri* LAV and WT strains

In this study, we evaluated the presence of L/CD207$^+$ cells in immune-related tissues of catfish challenged with both *E. ictaluri* LAVs and WT strains by immunohistochemistry (IHC) as described previously [39]. The presence of the LC-like cells in the lymphoid organs and gills of catfish at the different time points of post-challenge is shown in Figure 7 and supplemental data (Fig. 1, Supplemental data). We provided the detailed quantitative assessment of the numbers of L/CD207$^+$ cells at 6 h, 1, 3 and 7 d post challenge that correlated with kinetics of initiation of innate and adaptive immune responses in the immune-competent organs of catfish fingerlings in the following chapters.

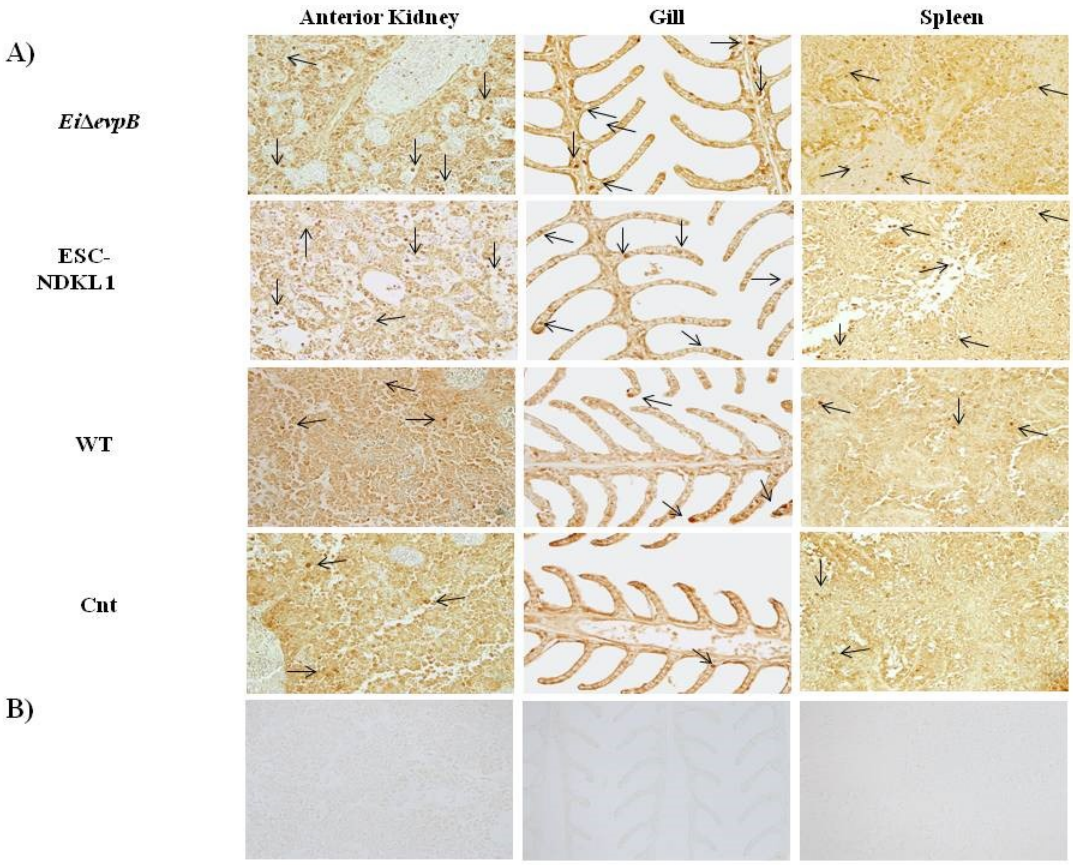

Figure 7 Identification of L/CD207$^+$ cells in the lymphoid organs (AK, spleen) and gill of channel catfish challenged with *E. ictaluri* LAV and WT strains by IHC.

The left columns show the L/CD207$^+$ specific (A) and background staining (B) in AK of catfish in all groups at 3 d post-challenge. Middle columns indicate the L/CD207$^+$ specific (A) and background staining (B) in the gill at 3 d post-challenge. The right column shows the L/CD207$^+$ specific (A) and background (B) staining in the spleen at 7 d post-challenge. The arrows indicate L/CD207$^+$ cells in the immune-related organs.

Assessment of the L/CD207$^+$ cell numbers in AK of catfish challenged with E. ictaluri LAV and WT strains

The numbers of the L/CD207$^+$ cells in the AK of catfish treated with two LAV and WT strains significantly increased at 6 h post-challenge compared to non-treated catfish (Figure 8). Interestingly, the L/CD207$^+$ cell numbers were significantly higher in

the AKs of catfish vaccinated with the LAVs compared to their counterparts challenged with the WT strain at this time point. However, there was no significant difference in the numbers of L/CD207$^+$ cells between the LAVs (Figure 8).

Similarly, at 6 h post-challenge, the numbers of L/CD207$^+$ cells were significantly higher in the challenged catfish AKs compared to non-treated controls at 1 d post-challenge (Figure 8). Furthermore, the numbers of L/CD207$^+$ cells were significantly higher in the AKs of catfish challenged with ESC-NDKL1 compared to the AKs of catfish challenged with WT strain, whereas there was no significant difference between the two LAV strains (Figure 8). Interestingly, the numbers of L/CD207$^+$ cells at 3 d challenge were significantly decreased in the AKs of catfish challenged with WT strain and did not differ from the uninfected control group (Figure 8). In contrast, the numbers of L/CD207$^+$ cells in the AK declined compared to the 1 d challenge but were still significantly elevated in the groups challenged with both LAVs compared to their WT- treated and control counterparts (Figure 8). After 7 d challenge, the treatment groups showed significant decreases compared to their 3 d challenge counterparts and did not differ from the controls in the numbers of L/CD207$^+$ cells (Figure 8). Overall, we documented the significant declines in the numbers of LC-like cells in the AK of catfish at 3 and 7 d post-challenge with both LAVs following similar patterns (Figure 8).

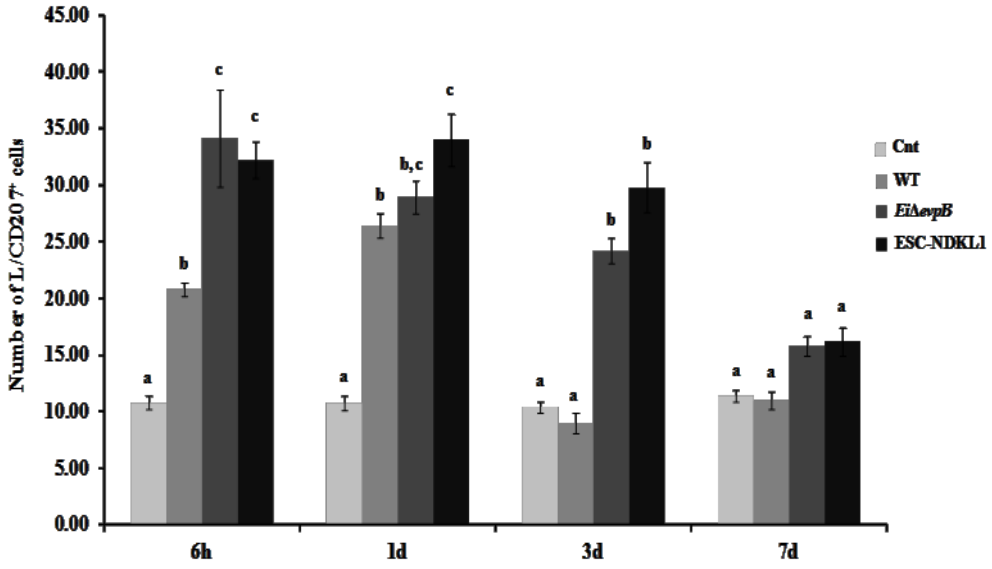

Figure 8 The kinetics of L/CD207$^+$ cells numbers in the anterior kidney of catfish challenged with *E. ictaluri* LAV and WT strains.

The (a, b, c) letters on top of bars indicate statistical group differences in L/CD207$^+$ cell numbers ($P < 0.05$). The data represented the mean of five fish ±SD.

Assessment of L/CD207+ cell numbers in spleen of catfish challenged with E. ictalurid LAV and WT strains

We evaluated the L/CD207$^+$ cell numbers in the spleen of catfish challenged with *E. ictaluri* LAV and WT strains (Figure 9). The L/CD207$^+$ cell numbers in the spleen at 6 h showed significant treatment-related differences but were comparable to the LC-like cell numbers at 1 d post-challenge (Figure 9). Namely, the L/CD207$^+$ cell numbers significantly increased in the spleen of catfish challenged with two LAVs and WT strains compared to non-vaccinated catfish whereas there was no significant difference between *Ei*Δ*evpB* and ESC-NDKL1 strains (Figure 9). As expected, the numbers of L/CD207$^+$ cells in the spleen dramatically decreased in all groups at 3 d post-challenge with no significant differences between treated and control fish (Figure 9). However, both LAV strains followed a similar pattern in the L/CD207$^+$ cell numbers different from the WT-

treated and control groups showing a significant increase in the L/CD207$^+$ cell numbers at 7 d post-challenge (Figure 9). Overall, we showed that the kinetics of the L/CD207$^+$ cell numbers in the *Ei*Δ*evpB*-treated groups were similar to the kinetics of their ESC-NDKL1-treated counterparts. Furthermore, significant treatment-related drops in the LC-like numbers occurred at the 3 d post-challenge followed by the dramatic increases in LAVs-treated groups at 7 d.

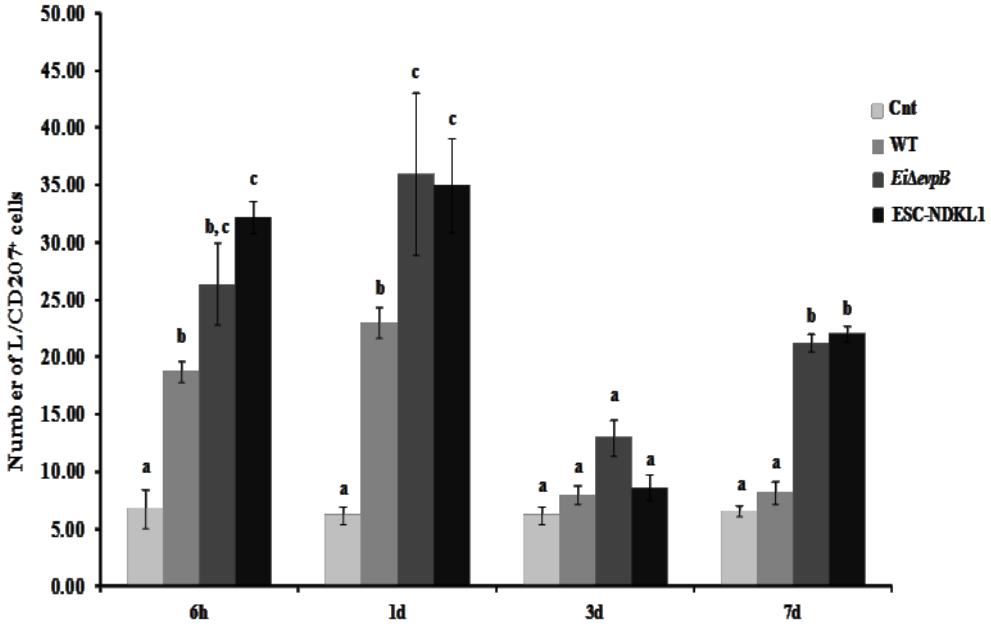

Figure 9 The kinetics of L/CD207$^+$ cells numbers in the spleen of catfish challenged with *E. ictaluri* LAV and WT strains.

The letters (a, b, c) show the significant differences between groups ($P < 0.05$). The data represented the mean of five fish ±SD.

Assessment of L/CD207+ cell numbers in gill of catfish challenged with E. ictalurid LAV and WT strains

In this study, we evaluated the L/CD207$^+$ cell numbers in the gill of catfish at different time points of in *E. ictaluri* LAVs and WT strain challenge (Figure 10). After 6 h infection, L/CD207$^+$ cell numbers significantly increased in the gill of catfish vaccinated with both LAVs and WT strain compared to non-vaccinated fish, and there was no significant difference in the L/CD207$^+$ cell number between LAVs and WT strains (Figure 10). The numbers of L/CD207$^+$ cells in the gill of all treatment groups continued to increase at 1 d of post-challenge, and there was a significant difference between *Ei*Δ*evpB* and ESC-NDKL1 strains but not compared to WT strain (Figure 10). No significant shifts have been documented in the L/CD207$^+$ cell numbers in the gill of catfish vaccinated with both LAVs at 3 d post-challenge. However, the L/CD207$^+$ cell numbers dramatically decreased in the gill of fish challenged with WT, but it was still significantly higher than non-challenged fish. On the other hand, after 7 d post-challenge, the L/CD207$^+$ cell numbers significantly decreased in the gill of fish vaccinated with both LAVs and were not different from non-vaccinated fish control group (Figure 10). On the other hand, the numbers of L/CD207$^+$ cells were significantly higher in catfish vaccinated with WT strain compared to LAVs-treated and control groups (Figure 10). In summary, the most dramatic changes in the numbers of LC-like cells in the gills were evident as decreases in the LAVs challenged gills compared to the WT strain treated and control groups (Figure 10).

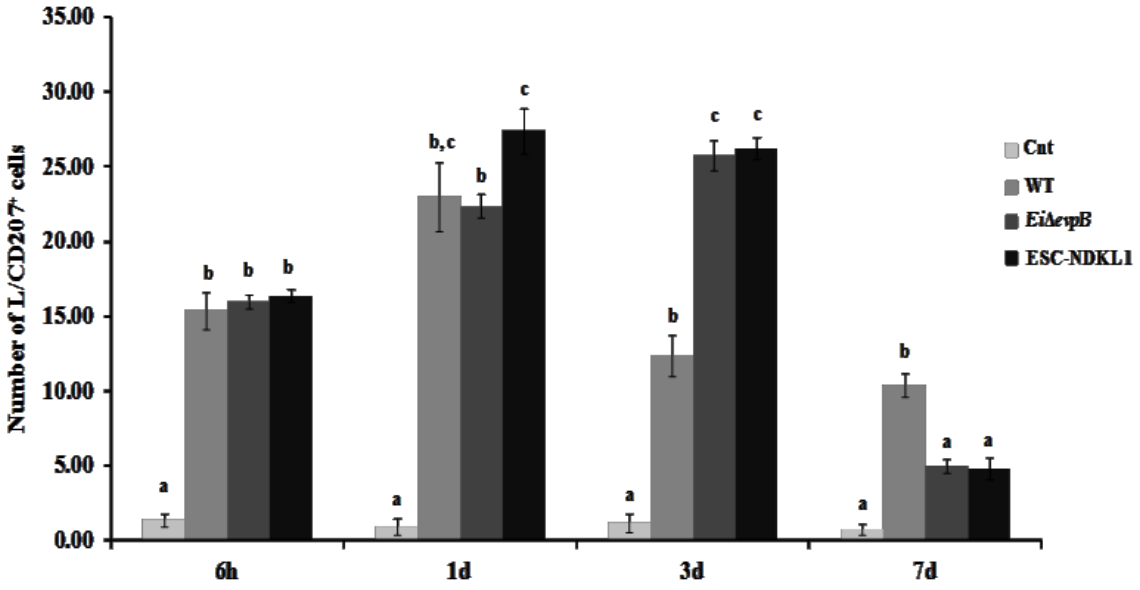

Figure 10 The kinetics of L/CD207$^+$ cells numbers in the gill of catfish challenged with *E. ictaluri* LAV and WT strains.

Different letters (a, b, and c) show the significant differences between groups ($P < 0.05$). The data represented the mean of five fish ±SD.

Comparative assessment of L/CD207$^+$ cell numbers in the immune-related organs of catfish challenged with E. ictaluri LAV and WT strains

We evaluated the L/CD207$^+$ cell expression kinetics in the lymphoid organs and gills of catfish in both *E. ictaluri* LAVs (*Ei*Δ*evpB* and ESC-NDKL10) and WT strain-challenged catfish (Figure 11). In the WT strain infected fish, the numbers of LC-like cells increased in all tissues, peaking at 1d and followed by a gradual decrease at 7 d post-challenge (Figure 11). The most dramatic differences in the numbers of L/CD207$^+$ cells were evident at 3 d and 7d in both LAVs challenged fish compared to the WT counterparts (Figure 11). The kinetics of L/CD207$^+$ cells numbers in the spleen was different from the kinetics in the AK and gill. Namely, the LC-like cell numbers picked at 3d followed by the rapid declines and finally showed increases at 7d while the

numbers of L/CD207^{+} cells continued to decline at 7d post challenge in the AK and gill (Figure 11).

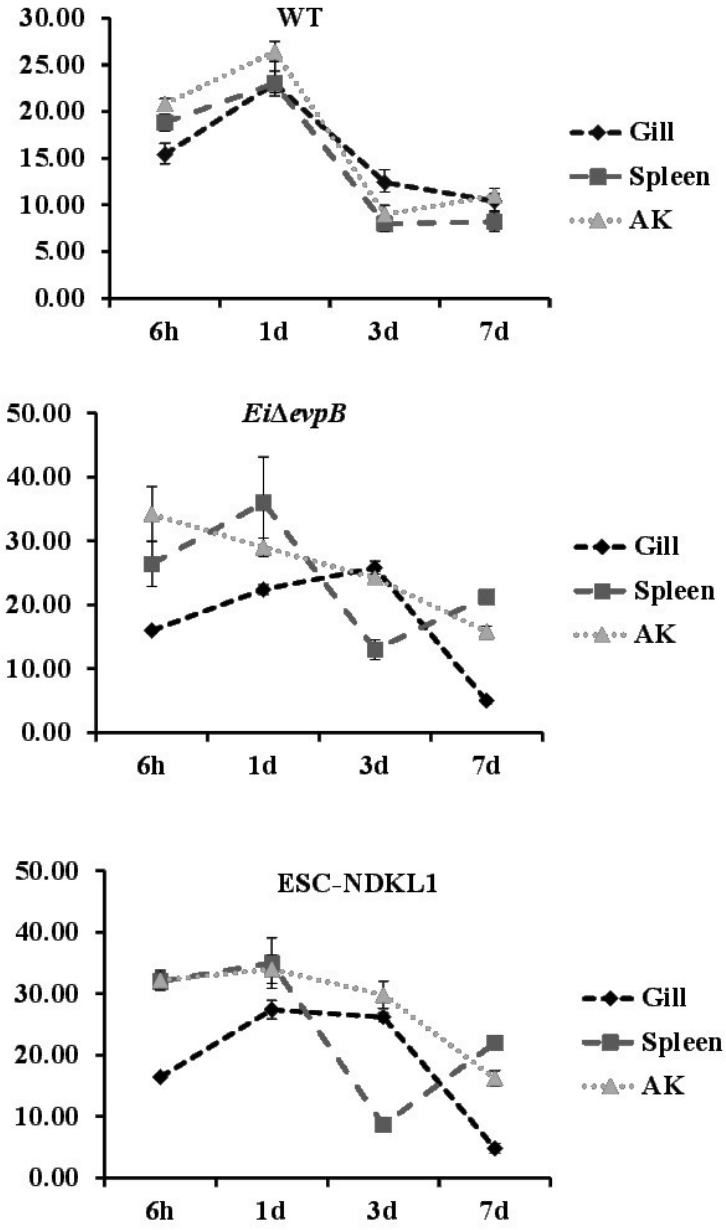

Figure 11 The kinetics of L/CD207^{+} cells numbers in the immune-competent organs of catfish exposed to the *E. ictaluri* LAV and WT strains.

In each graph, the L/CD207^{+} cell numbers from the AK, spleen, and gill show the dynamics of DC distribution in the *EiΔevpB* and ESC-NDKL1 challenges and WT *E. ictaluri* infection

Discussion

The critical function of DCs is to bridge innate antigen recognition or processing or presentation and adaptive immune responses including memory. Recent studies showed that DCs in teleost fish have similar morphology and function with mammalian DCs (Lugo-Villarino, Balla et al. 2010, Bassity and Clark 2012). Previously, our group showed the presence of DCs with remarkable similarities to human LCs in the spleen, anterior and posterior kidneys, and gill of channel catfish (Kordon, Scott et al. 2016). The purpose of the current research was to gain valuable information on the mechanisms of LC-like cell-dependent immune responses of two LAV strains developed in our laboratory. The hemopoietic tissue is the primary tissue responsible for the production of all blood elements in both higher vertebrates and fish, and the fish AK is considered possess a hemopoietic tissue with various cell types (Rombout, Huttenhuis et al. 2005, Zapata, Diez et al. 2006, Abdel-Aziz, Abdu et al. 2010, Abdelhamed, Ibrahim et al. 2017).

The transcriptional regulator of the Zinc finger family DC-SCRIPT is expressed in all subsets of DCs in humans and mice (Triantis, Moulin et al. 2006, Triantis, Trancikova et al. 2006). Due to the lack of DC-specific markers in fish DC-SCRIPT was considered as one of the markers for the barramundi fish DCs (Zoccola, Delamare-Debouteville et al. 2015). A recent study showed that DC-SCRIPT in combination with MHCII expression was significantly upregulated in the AK of barramundi at both, 6 h and 1 d post-injection with peptidoglycan (PTG) and lipopolysaccharide (LPS), but relative expression of DC-SCRIPT was low at 3 d and 7 d (Zoccola, Delamare-Debouteville et al. 2015). Also, another study reported that TLR ligands, such as LPS

and Pam3CSK4, activated hematopoietic progenitor cells and induced their differentiation into myeloid cells, such as macrophages and DCs, in the bone marrow and spleen of mammals (Nagai, Garrett et al. 2006). Due to the lack of information available on the morphological and functional markers of catfish DCs, we applied the IHC approach described previously (Kordon, Scott et al. 2016) to evaluate the LC-like expression patterns in the immune-related tissues of catfish challenged with two LAVs and WT *E. ictaluri* strains to underscore their immune effector mechanisms. Several studies demonstrated that AK is the target organ at the early time of *E. ictaluri* infection. Leukocytes containing *E. ictaluri* were detected in the AK of channel catfish at 48 h post- infection, and *E. ictaluri* was detected in the posterior kidney at 15 min post-infection (Baldwin and Newton 1993). In addition, the dispersion of bioluminescent *E. ictaluri* was observed in the AK at 15 min after intraperitoneal injection (Karsi, Menanteau-Ledouble et al. 2006). Our data suggest that the elevated numbers of L/CD207$^+$ cells in the AK of channel catfish challenged with *E. ictaluri* LAVs and WT at the early stage can be explained by the development of innate immune responses at the site of infection, and two LAVs were more efficient to induce the DC numbers compared to their WT counterpart. The significant drops in the numbers of L/CD207$^+$ cells in the AK suggest the possible migration of these cells from the AK to the peripheral sites of infection.

Spleen in mammals is one of the secondary lymphoid organs in which antigen presentation occurs, and adaptive immune responses are activated (Ruddle and Akirav 2009). Similar to mammals, spleen serves as a secondary lymphoid tissue in teleost fish (Koppang, Fischer et al. 2010). Moreover, the spleen is also a target organ of *E. ictaluri*

after AK and posterior kidney in catfish. In the immersion-exposed channel catfish, bioluminescent *E. ictaluri* was detected in the abdominal area at 60-72 h post-infection, and spleen showed intense bioluminescence signal by dissection of catfish (Karsi, Menanteau-Ledouble et al. 2006). Our data on dramatic increases in the numbers of L/CD207$^+$ cells in the spleen at 6 h post-challenge, possibly via cytokine signaling cascades involving DCs are due to the two LAVs being more efficient to induce innate immune responses compared to their WT counterpart. The significant drops in the numbers of L/CD207$^+$ cells in the spleen at 3 d post-challenge suggest possible migration of these cells from the spleen to the site of infection followed by the remarkable increases in the LC-like numbers in the spleens of the vaccinated catfish after 7 d of the treatment. The LAVs induced increases in the spleens but not in the AKs after 7 days of challenge suggest the migration of the LC-like cells back to the spleen to present the pathogen- derived antigen to specific T cells thus initiating adaptive immune responses. Similarly to our findings, the study with barramundi reported that the expression of DC-SCRIPT was higher in the spleen at 6 h post-injection of LPS and PTG, but its expression level decreased in spleen after 1 d, and DC-SCRIPT expression was again elevated at 7d (Zoccola, Delamare-Debouttevillle et al. 2015).

The gill is one of the potential routes of entry for *E. ictaluri* into the channel catfish host (Nusbaum and Morrison 1996). Our data on the significant spikes in the numbers of L/CD207$^+$ cells in the gill in all fish at 6 h, 1 d, and 3 d post-challenge followed by the decreases after 7 days could be interpreted by the possible antigen recognition and capture by immature DCs. Our data support the previous observation that DC-like cells have been described in the gill of Chinook salmon heavily infected

with *L. salmonae* (Lovy, Wright et al. 2006). We expanded this earlier report by showing the kinetics of L/CD207$^+$ cells expression patterns in the catfish gills at different time points of exposure to the LAVs and WT *E. ictaluri* strains. Our data suggest that L/CD207$^+$ cells in the gill may recognize and capture antigens at 6 h, 1 d, and 3 d post-challenge, and after 7 d, L/CD207$^+$ cells in the gill of catfish vaccinated with two LAV strains have migrated to the secondary lymphoid tissue for antigen presentation. However, the APC function of L/CD207$^+$ cells, in particular, their migration to the spleen in the gill of catfish infected with WT strain was impaired.

In conclusion, based on our observation the differential LC-like cells expression patterns in the immune-related tissues of catfish exposed to the LAVs versus WT *E. ictaluri* strains suggest the functional differences of DC-like cells in different treatments. In the WT *E. ictaluri*-infected catfish, the expression of the DC-like cell patterns at 6 h and 1 d post-challenge resembled the patterns in the vaccinated fish in all three immune- related organs. However, significant declines in the numbers of L/CD207$^+$ cells in the AK and spleen at 3 and 7 d post-challenge comparable to the uninfected controls suggest the functional inability of the WT-induced APCs of migration/maturation and initiation of adaptive immune responses. Importantly, in the LAVs vaccinated catfish, the increased numbers of L/CD207$^+$ cells in the AK and spleen that were present at the early stage of infection suggest their involvement in the initiation of innate immune responses and migration/maturation from AK and spleen to the site of infection. Finally, at 7 d post- challenge, L/CD207$^+$ cells possibly migrated back to the spleen for antigen presentation and initiation of adaptive immune responses. Therefore, our data suggest that both LAVs possibly targeted the most potent APCs,

DC-like cells for the presentation on MHC molecules on the cell surface bridging innate and adaptive immunity thus completely satisfying the stringent requirements for successful LAVs.

CHAPTER IV

PHAGOCYTIC AND BACTERICIDAL PROPERTIES OF CHANNEL CATFISH PERITONEAL MACROPHAGES EXPOSED TO *EDWARDSIELLA ICTALURI* LIVE ATTENUATED VACCINE AND WILD TYPE STRAINS

Abstract

Edwardsiella ictaluri, a Gram-negative, intracellular, facultative bacterium, is the causative agent of enteric septicemia of catfish (ESC), which is one of the most significant diseases of farmed channel catfish. Macrophages have a critical role in primary defense mechanisms against bacterial infections by migrating to the site of infection, engulfing and killing pathogens, and priming adaptive immune responses. Vaccination of catfish with E. ictaluri live, attenuated vaccine (LAV) strains increased the efficiency of phagocytosis and bacterial killing in catfish peritoneal macrophages compared in vitro with macrophages from non-vaccinated fish. Recently, our group developed several protective LAV strains from E. ictaluri. However, their effects on the antigen uptake and bacterial killing in catfish macrophages have not been evaluated. In this study, we assessed the phagocytic and bactericidal activity of peritoneal macrophages in the uptake of E. ictaluri wild-type (WT) and two LAV strains. We found that phagocytosis of LAV strains was significantly higher compared to their WT counterpart in peritoneal macrophages. Moreover, the uptake of E. ictaluri opsonized with sera from vaccinated catfish was

more efficient than when opsonized with sera from sham vaccinated fish. Notably, catfish macrophages did not lose their phagocytic properties at 4° C, as described previously in mammalian and zebrafish models. Also, opsonization of E. ictaluri with inactivated (IN) sera from vaccinated and sham vaccinated catfish decreased significantly phagocytic uptake of bacteria at 32° C, and virtually suppressed endocytosis at 4° C, suggesting the important role of complement-dependent mechanisms in catfish macrophage phagocytosis. In conclusion, our data on enhanced phagocytic capacity and effective killing ability in macrophages of vaccine strains suggested the LAVs' advantage if processed and presented in the form of peptides to specific lymphocytes of an adaptive immune system and emphasize the importance of macrophage-mediated immunity against ESC. Furthermore, we showed the role of complement-dependent mechanisms in the phagocytic uptakes of E. ictaluri in catfish peritoneal macrophages at 4ºC and 32ºC. Finally, LAV vaccine-induced bacterial phagocytosis and killing properties of peritoneal macrophages emphasized the importance of the innate immune responses in ESC.

Introduction

Edwardsiella ictaluri, a Gram-negative, intracellular, facultative bacterium, is the causative agent of enteric septicemia of catfish (ESC), which is one of the most significant diseases of farmed channel catfish (Hawke 1981, Miyazaki and Plumb 1985, Wagner, Wise et al. 2006, Zhang and Arias 2007). In 1999, Klesius and Shoemaker developed a modified live *E. ictaluri* vaccine against ESC

(commercialized later as Aquavac-ESC) that stimulated protective immunity delivered by bath immersion in juvenile catfish (Klesius and Shoemaker 1999). Subsequent immersion studies showed effective protection in catfish fry, fingerlings, and eyed catfish eggs (Shoemaker, Klesius et al. 1999, Wise, Klesius et al. 2000, Shoemaker, Klesius et al. 2002, Shoemaker, Klesius et al. 2007), which also demonstrated that *E. ictaluri* live attenuated vaccines (LAVs) induced cell-mediated immunity to protect catfish against ESC (Shoemaker and Klesius 1997, Ellis 1999) because *E. ictaluri* could survive and replicate in channel catfish macrophages (Booth, Elkamel et al. 2006). Furthermore, vaccination of catfish with LAVs resulted in the specific antibodies production that enhanced the bactericidal activity of macrophages (Shoemaker and Klesius 1997).

Macrophages are professional phagocytes that have multiple functions in different species including immunity, inflammation and tissue repair (Godwin, Pinto et al. 2013). New evidence has accumulated on the progenitors of adult tissue resident macrophages, embryonic macrophages (Schulz, Perdiguero et al. 2012, Hashimoto, Chow et al. 2013, Epelman, Lavine et al. 2014, Hoeffel, Chen et al. 2015, Sheng, Ruedl et al. 2015). Professional phagocytes, including macrophages, in fish have a significant role in major defense mechanisms against bacterial infections as these cells can migrate to the site of infection and engulf and kill pathogens (Secombes and Fletcher 1992, Esteban, Cuesta et al. 2015). Multiple studies documented strong phagocytic capability and bactericidal activity of anterior kidney macrophages against intracellular pathogens including parasites, yeast, and bacteria (Bennani, Schmid-Alliana et al. 1995, Dieter and Katharina

1997, Esteban, Mulero et al. 1998, Muñoz, Álvarez-Pellitero et al. 2000, Qiu, Liu et al. 2016). Importantly, macrophages are present normally in the peritoneal cavity of fish; therefore, the peritoneal macrophage approach has been documented well for *in vitro* immunologic studies in catfish (Jenkins and Klesius 1998). Collection of peritoneal macrophages is relatively easy and does not require special isolation and purification procedures (Jenkins and Klesius 1998). Moreover, phagocytes response to intraperitoneal inflammation is easily observed and measured both qualitatively and quantitatively (Silva, Silva et al. 1989).

Peritoneal macrophages from sea bass had significantly greater phagocytic activity against bacteria, such as *Escherichia coli and Salmonella enterica serovar Typhimurium* than did monocytes and macrophages from blood and anterior kidney, respectively (Esteban and Meseguer 1997). Furthermore, a higher number of phagocytosed bacteria was observed in macrophages than other phagocytic cells in the peritoneal cavity of sea bass (Do Vale, Afonso et al. 2002). Phagocytosis of *Yersinia ruckeri* in peritoneal macrophages was significantly greater compared to the phagocytic activity of neutrophils in rainbow trout (*Oncorhynchus mykiss*) (António, Susana et al. 1998). Winter flounder (*Pleuronectes americanus*) peritoneal macrophages were capable of engulfing formalin-fixed bacteria (*Yersinia ruckeri* and *Bacillus cereus*) after a short exposure (Bodammer and Robohm 1996). The phagocytic and microbicidal activity of peritoneal macrophages were described in rohu (*Labeo rohita*) and walking catfish (*Clarias batrachus*) (M.Mamnur Rashid 2002, Awasthi, Rathore et al. 2015). Shoemaker et al. evaluated the role of peritoneal macrophages in immunity to ESC after infection with live *E. ictaluri*

(Shoemaker, Klesius et al. 1997). Phagocytic and bactericidal activity was significantly greater in macrophages from fish immunized with AL-93-75 compared to their counterparts from pathogen-free susceptible fingerling catfish (Russo, Shoemaker et al. 2009). Interestingly, opsonization of *E. ictaluri* with immune serum significantly enhanced the killing ability of macrophages from susceptible fish (Shoemaker, Klesius et al. 1997, Russo, Shoemaker et al. 2009).

One of the major manifestations of immunological autophagy is the destruction and elimination of invading pathogens (Deretic, Saitoh et al. 2013, Mizumura, Choi et al. 2014). Recently, autophagy has been described in fish (van der Vaart, Spaink et al. 2012, Yabu, Imamura et al. 2012, García-Valtanen, Ortega-Villaizán et al. 2014). Engulfment of microbial prey as part of autophagy is initiated at the plasma membrane of the macrophage where a vast repertoire of phagocytic receptors, in particular, complement receptors (CRs) recognize the bacterial surface directly or indirectly through deposition of serum opsonins such as IgG or the complement protein C3b (Herre, Marshall et al. 2004, Patel and Harrison 2008). Complement activation is a tightly regulated process that may proceed through three distinct pathways: the alternative pathway, classical pathway, and the lectin-dependent pathway. However, each pathway converges on the complement protein C3b to generate the bioactive components C3a and C3b (Merle, Church et al. 2015). Similar to higher vertebrates, the complement system in teleost fish can be activated through all three pathways and shows many effector functions identified in the mammalian complement system, such as opsonization, anaphylatoxic leukocyte stimulation, and target cell killing (Holland and Lambris 2002), (Nakao, Tsujikura

et al. 2011). However, some complement components in teleost fish are present in multiple active isoforms, in particular, the critical complement component, C3 is present in several isoforms produced by different genes (Boshra, Li et al. 2006), (Sunyer, Zarkadis et al. 1996, Sunyer, Tort et al. 1997, Nakao, Mutsuro et al. 2000). Also, catalytic residues of proteins of complement are different in teleost fish (Nakao and Yano 1998). Furthermore, unlike in mammals, fish complement components are active at low temperatures and show higher magnitude providing broader recognition of foreign substances in fish (Oriol Sunyer and Tort 1995, Sunyer and Lambris 1998, Boshra, Li et al. 2006). It is well documented that opsonization of bacteria with serum proteins and fixation of complement are formidable barriers that must be overcome to establish infection (Flannagan, Heit et al. 2015). Whereas the specific signaling molecules can vary, the requirement for actin remodeling in the engulfment process is absolute (Greenberg, el Khoury et al. 1991, Tse, Furuya et al. 2003). The lumen of phagolysosome is an inhospitable environment for intracellular pathogens (Flannagan, Heit et al. 2015). Lysosomal proteases that display a range of functions that shape the cellular immune response in macrophages, antimicrobial effectors that promote killing of phagocytosed pathogens through direct proteolytic attacks (Muller, Faulhaber et al. 2014), and other active substances comprise part of the macrophages antimicrobial arsenal that exerts microbicidal effects by compromising directly bacterial membranes or promoting the production of immunomodulatory compounds (Weinrauch, Elsbach et al. 1996, Wu, Raymond et al. 2010).

Recently, our research group has determined that *evpB* gene in the Type VI secretion system (T6SS) operon is differentially regulated during *in vitro* iron-restricted conditions (Dumpala, Peterson et al. 2015). We constructed *Ei*Δ*evpB* strain by an in- frame deletion of *evpB* gene and found that *E. ictaluri* is completely attenuated in catfish fingerlings and fry. Vaccination with *Ei*Δ*evpB but* did not cause mortality in fingerlings (100% survival) and low 3-4% mortality in fry catfish after WT *E. ictaluri* challenge (Nho, Abdelhamed et al. 2017). Our finding corroborated an earlier study showing that *evpB* plays a key role in *E. tarda* pathogenesis (Zheng and Leung 2007). Furthermore, our laboratory reported that genes encoded tricarboxylic acid cycle (*sdhCfrdA*) and one-carbon metabolism (*gcvP*) were essential for *E. ictaluri* virulence (Dahal, Abdelhamed et al. 2013). Similarly, we introduced an in-frame deletion of Glycine dehydrogenase (gcvP), Succinate dehydrogenase (sdhC), and Fumarate reductase (frdA) genes in E. ictaluri 93-146 strain and named it as ESC-NDKL1 (EiΔgcvPΔsdhCΔfrdA) (Nho, Abdelhamed et al. 2017). Vaccination of catfish fingerlings with ESC-NDKL1 showed similar 100% survival rates as *Ei*Δ*evpB*, however challenge of fry with ESC-NDKL1 showed moderatly elevated 3-4% mortality rates (Nho, Abdelhamed et al. 2017). The purpose of our study was to compare the phagocytic and bacterial killing activity of channel catfish peritoneal macrophages against *E. ictaluri* WT and two LAV strains in the presence of sera obtained from vaccinated fish. Increased phagocytic capacity and killing ability of macrophages against opsonized LAV strains will support the importance of macrophage-mediated immunity against ESC in catfish.

Materials and Methods

Animals

The fish hatchery at the College of Veterinary Medicine, Mississippi State University, provided specific pathogen free (SPF) channel catfish, which were maintained at 25-28 °C. All fish experiments were performed based on a protocol approved by the Mississippi State University Institutional Animal Care and Use Committee (IACUC). Tricaine methanesulfonate (MS-222, Western, Chemical, Inc.) was used to sedate (100 mg/ml) and euthanize (400 mg/ml) the catfish. Samples were obtained as described below.

Cell Harvesting

Channel catfish (250-300 g) were used in this study. Peritoneal macrophages were harvested as described previously as a simple reliable method to obtain tissue-resident macrophages that displayed high oxygen production and phagocytic ability (Jenkins and Klesius 1998). Briefly, 1 ml squalene (Sigma-Aldrich, St. Louis, MO) was injected intraperitoneally (IP) in sedated catfish. Four days after injection, catfish were sedated, the peritoneal area wiped with 70% ethanol, and cold, sterile phosphate-buffered saline (PBS) injected IP via a three-way valve attached to a syringe, 18- gauge needle, and Tygon tubing. The valve was then closed, the fish's abdominal region was massaged gently, and a cell suspension collected into a centrifuge tube placed on ice. Additional cold PBS was injected into the peritoneal cavity until IP fluid was clear.

Bacterial Strains and Opsonization

Bacterial strains used in this work are listed in Table 1. *E. ictaluri* 93-146 wild- type (WT) strain and vaccine strains were cultured in brain heart infusion (BHI) agar or broth (Difco, Sparks, MD) and incubated throughout the study at 30 °C. Each vaccine strain was labeled with bioluminescence by transferring pAK*gfplux*1 from an *E. coli* donor strain (SM10λ*pir*) by conjugation as described previously (Karsi and Lawrence 2007). When required, media were supplemented with following antibiotics and reagents; ampicillin (Amp: 100 mg/ml), and colistin sulfate (Col: 12.5 mg/ml, Sigma-Aldrich, Saint Louis, MN).

Overnight cultures of *E. ictaluri* and vaccine strains were grown and prepared as described above, followed by two PBS washes before opsonization with channel catfish serum for 30 min at room temperature. To opsonize *E. ictaluri*, sera were obtained from catfish treated with WT and vaccine strains and non-treated fish. Opsonized bacteria were then used for antigen uptake and infection experiments.

Table 1 Create a short, concise table title and place all detailed caption, notes, reference, legend information, etc in the notes section below

Bacterial Strain	Description	Reference
***Edwardsiella ictaluri* Strain 93-146**	Wild type; pEI1^{+}; pEI2^{+}; Col^{r}; pAK*gfplux*1	(Lawrence, Cooper et al. 1997)
ESC- *Ei*Δ*evpB*	93–146 derivative; *ΔevpB*; pAK*gfplux*1	Dr. Attila Karsi Mississippi State Univ
ESC-NDKL1	93-146 derivative; Δ*gcv*PΔ*sdhC*Δ*mdh*; pAK*gfplux*1	Dr. Mark Lawrence Mississippi State Univ.

Fish Vaccination and Serum Collection

A total of 100 SPF channel catfish fingerlings (6-month old) with fully developed innate and adaptive immune systems (Patrie-Hanson and Jerald Ainsworth 1999, Petrie- Hanson and Ainsworth 2001, Rombout, Huttenhuis et al. 2005, Zapata, Diez et al. 2006) were stocked into four 40-L tanks (25 fish per tank) with a continuous water flow and aeration. Tanks were assigned randomly to *Ei*Δ*evpB*, ESC-NDKL1, *E. ictaluri* WT (positive control), and sham-vaccinated (negative control) groups. The fish were fed twice daily and acclimatized for one week. The water temperature was maintained at 24–26º C throughout the trial. After a week of acclimation, fish were exposed *Ei*Δ*evpB*, ESC- NDKL1, and wild-type *E. ictaluri* 93-146 by immersion challenge as described previously (Abdelhamed, Lu et al. 2013). Briefly, 100 ml of overnight cultures were added to 10 L water to yield infection dose of approximately 3.67×10^7CFU/ml of water.The negative control group was immersion challenged with BHI broth.

Blood samples were collected from caudal vein of ten fish at 14 and 21 days post- infection and were allowed to coagulate overnight at 4º C. Serum was obtained by centrifugation at 8000 rpm for 10 min.

Phagocytosis and Flow Cytometry

Harvested cells were washed three times in PBS at 2000 rpm for 10 min at 24° C and resuspended in Channel Catfish Macrophage Medium (CCMM), which included RPMI [(RPMI 1640 sans phenol red & L-glutamine, Lonza, Walkersville, MD, USA) containing 1x glutamine substitute (GlutaMAX –I CTS, Gibco, Invitrogen Corporation, Carlsbad, CA, USA)], 15 mM HEPES buffer (GIBCO), in 0.18% sodium bicarbonate

solution (GIBCO), 0.05 mM 2-beta-mercaptoethanol (all from Sigma Chemical Co., St.Louis, MO, USA), and 5% heat-inactivated (HI) pooled channel catfish serum. Cells were counted using a hemocytometer and trypan blue exclusion. Cell suspension was transferred into a 6 well plate (Fisher Scientific, Pittsburgh, PA), and the phagocytic capacities of peritoneal macrophages were determined by addition of GFP transformed bacterial strains in 1: 50 ratio and incubated at 32° C (active uptake) and 4° C (background levels of endocytosis, negative control) for 2 hrs in the dark. Following incubation, cells were harvested with cell scrapers (Fisher Scientific, Pittsburgh, PA), and washed three times by centrifugation in cold PBS and analyzed using FACSCalibur (Becton Dickinson), as follows. After setting a gate on large granular cells, the LAVs incorporation was measured and analyzed by using FlowJo 7.6.4 Software (Tree Star Inc.). To inhibit actin formation selectively, catfish macrophages were incubated for 10 min in the presence of cytochalasin D (CCD) [2.5 µg/ml], Sigma-Aldrich, St. Louis, MO) before the addition of LAVs bacterial strains (Watts and Marsh 1992). To determine differences between treatments, mean fluorescence intensity (MFI) of engulfed bacteria in catfish peritoneal macrophages was analyzed using single histogram and overlay histogram statistics.

Cytospin and Light Microscopy

Peritoneal macrophages were incubated in the dark with GFP-labeled wild-type *E. ictaluri* and LAVs at 32° C and 4° C for 2 hrs. Cells were then harvested, washed, and the cytospin preparations applied at 500 rpm for one min with a Cyto-Tek centrifuge machine. All samples were fixed and stained with Wright's stain (Haemacolor, Merck)

as described (Do Vale, Afonso et al. 2002), analyzed, and then photographed with Olympus BX60 microscope (Olympus U-TV1 X) and Infinity software.

Bacterial Killing Assay

The bacterial killing assay was performed as described previously with some modifications (Booth, Elkamel et al. 2006, Russo, Shoemaker et al. 2009). Briefly, harvested peritoneal macrophages were washed with PBS by centrifugation, resuspended in CCMM, and transferred to 96 well plates. Wild-type strain and LAVs were added to catfish macrophages in 1:1 ratio followed by centrifugation at 1500 rpm for 5 min at 24° C to compact cells and bacteria and then incubated at 32° C for 1 h. After incubation, plates were centrifuged at 2000 rpm for 7-10 min, and supernatants removed. Next, cell pellets were resuspended in CCMM containing 100 µg/ml gentamicin (Gibco, Life Technologies, Grand Island, NY) to kill extracellular bacteria, incubated at 32° C for 1 hr, and washed by centrifugation in PBS. After washing, plates were incubated at 32° C for 10 h and 24 hrs in CCMM containing 10 µg/ml gentamycin. For each time point, colony counting method was performed, as follows. Macrophages were lysed with 1x Triton X-100 (Sigma, St.Louis, MO, USA), as described (Russo, Shoemaker et al. 2009). Lysed macrophages were diluted in PBS and plated on a selective medium, and incubated at 32°C for 48 hrs.

Statistical Analyses

The significance of the differences between means was established by one-way ANOVA and two-way ANOVA procedures with Tukey's test in SAS for Windows 9.4

(SAS Institute, Inc., Cary, NC) to evaluate differences in mean of fluorescence intensity (MFIs). The level of significance for all tests was set at $P < 0.05$.

Results

Active Phagocytic Uptake of *E. ictaluri* LAVs in Peritoneal Macrophages

In this study, we evaluated endocytic uptake of *E. ictaluri* and the LAV strains in catfish peritoneal macrophages (Figure 12). To ensure active phagocytosis in macrophages, we measured the uptake of the GFP-labelled bacteria at 32° C and background endocytosis levels at 4° C. Also, we assessed the intensity of phagocytosis in peritoneal macrophages pre-incubated with the phagocytosis inhibitor, CCD (Figure 12A, B). The phagocytic intensity levels of both LAV strains at 32° C were significantly higher compared to the endocytosis of WT *E. ictaluri* in catfish peritoneal macrophages (Figure 1B). However, the uptake of *Ei*Δ*evpB* showed a significant increase compared to its ESC- NDKL counterpart (Figure 12B). Significant decreases but not the complete inhibition of uptake of LAVs and WT *E. ictaluri* were evident in the presence of actin formation inhibitor CCD (Figure 12B) and at 4° C (data not shown). In conclusion, active uptake of LAV strains was significantly higher compared to their WT counterpart in peritoneal macrophages at both temperatures.

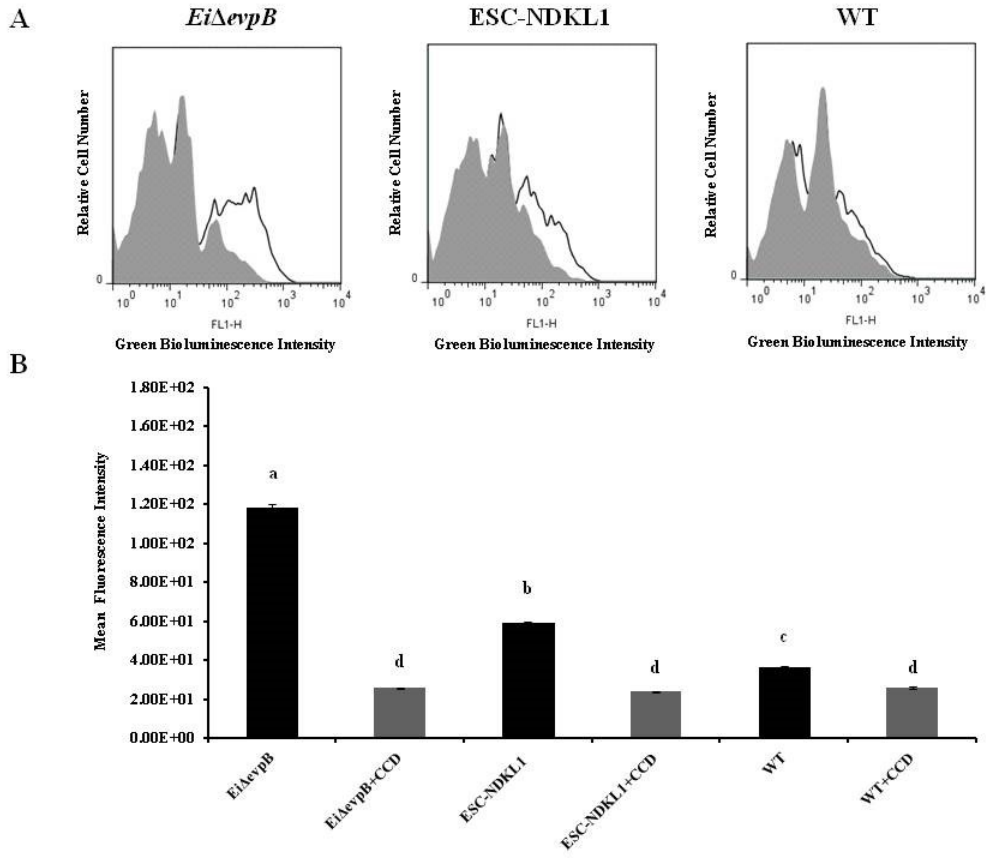

Figure 12 Active phagocytic uptake of - *EiΔevpB* and ESC-NDKL1strains in catfish peritoneal macrophages.

A) Original flow data by using overlay histogram statistics: Black line histograms indicate uptake of the LAVs and WT strains at 32° C. Grey histograms indicate antigen uptake in the presence of CCD at 32° C. B) Statistical analysis of the phagocytic uptake mean of fluorescent intensity (MFI). Black filled columns in the graph show MFI of LAVs and WT strains uptake at 32° C. Grey filled columns show MFI of antigen uptake in the presence of CCD at 32° C. The data represent the mean of MFI of macrophage phagocytic uptake from five fish ±SD. The letters (a,b,c,d) show the significant differences between treatments ($P < 0.05$).

The engulfed intracellular WT and the LAV strains were evident in catfish peritoneal macrophages at both 32° C and 4° C temperatures assessed, confirming our previous observation that catfish peritoneal macrophages did not lose their phagocytic properties at 4° C (Figure 13). In summary, our data showed the significant increases in

bacterial uptake at 32° C compared to 4° C; the inhibitory effects of CCD; and the presence of engulfed intracellular bacteria demonstrated an active uptake of WT and LAVs in catfish peritoneal macrophages. Furthermore, both LAVs were taken more vigorously by macrophages compared to their WT counterpart, suggesting the LAVs advantage for elimination and/or processing for antigen presentation.

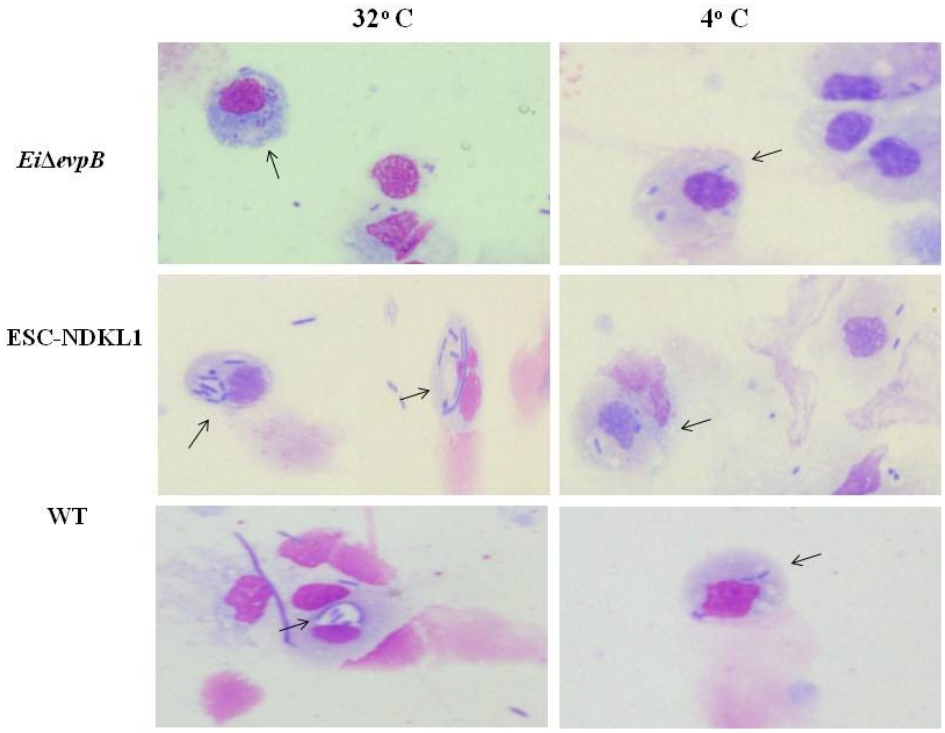

Figure 13 Active uptake of ESC vaccine strains in catfish peritoneal macrophages shown by light microscopy.

The column on the left shows phagocytosis of *E. ictaluri* strains at 32° C, and the right column shows phagocytosis *E. ictaluri* strains at 4° C. Arrows indicate intracellular, engulfed bacterial cells in the cytoplasm and phagosomes of peritoneal macrophages.

Active Uptake of *E. ictaluri* Opsonized with the LAVs-induced immune sera in Peritoneal Macrophages

Opsonization of WT *E. ictaluri* with sera from fingerlings vaccinated with both LAVs and non-vaccinated control was applied to determine the protective effect of antibodies and complement on *E. ictaluri* phagocytosis in peritoneal macrophages. The mean fluorescence intensity (MFI) of phagocytosis of WT *E. ictaluri* opsonized with intact (IN) and heat-inactivated (HI) sera from vaccinated and control catfish was assessed by flow cytometry (Figure 14). We found significant group- and treatment-related differences in the intensity of phagocytic uptake of WT *E.ictaluri* compared to LAV's induced phagocytosis in catfish peritoneal macrophages. Namely, peritoneal macrophages had increased significantly higher uptake of opsonized bacteria compared to the non-opsonized *E. ictaluri* (data not shown). Phagocytosis of WT *E. ictaluri* opsonized with ESC-NDKL1- derived serum was increased significantly compared to the *Ei*Δ*evpB*, and IN serum-opsonized bacteria and did not differ from the uptake of WT *E. ictaluri* opsonized the WT-derived serum (Figure 14). Additionally, we found significant increases in phagocytosis of opsonized bacteria at 4^{o} C showing similar patterns to the increases at 32^{o} C (Figure 15). These patterns of uptake at 4^{o} C, however, had significantly less phagocytic activity compared to phagocytosis occurring at 32^{o} C (Figure 14). As expected, our results indicated that *E. ictaluri* opsonized with sera from vaccinated fish were taken by peritoneal macrophages more efficiently compared to the bacterium opsonized with sera from control animals, suggesting an important role of humoral responses at the early step of *E. ictaluri* antigen presentation.

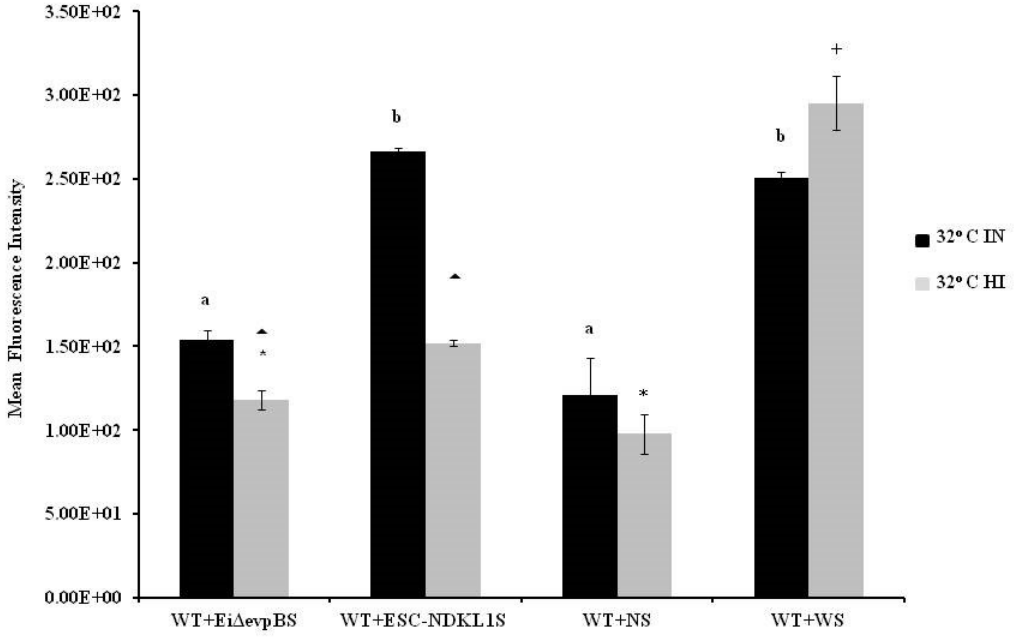

Figure 14 Active uptake of *E. ictaluri* LAVs opsonized with IN and HI sera from challenged fingerlings in catfish peritoneal macrophages at 32ºC.

*Ei*Δ*evpB* and ESC-NDKL1S indicate serum of vaccinated fish with *Ei*Δ*evpB* and ESC-NDKL1, respectfully; WS indicates serum from fish challenged with wild-type, and NS indicates serum from control fish. [a,b] Presence of letters on top of bars indicates group differences in uptakes with bars with different letters being different from each other and from bars without a letter designation ($P < 0.05$). *Indicates treatment differences in the uptakes ($P < 0.05$). [^+] Indicate group differences in the uptakes ($P < 0.05$). The data represent the mean of MFI of macrophage phagocytic uptake from five fish ±SD.

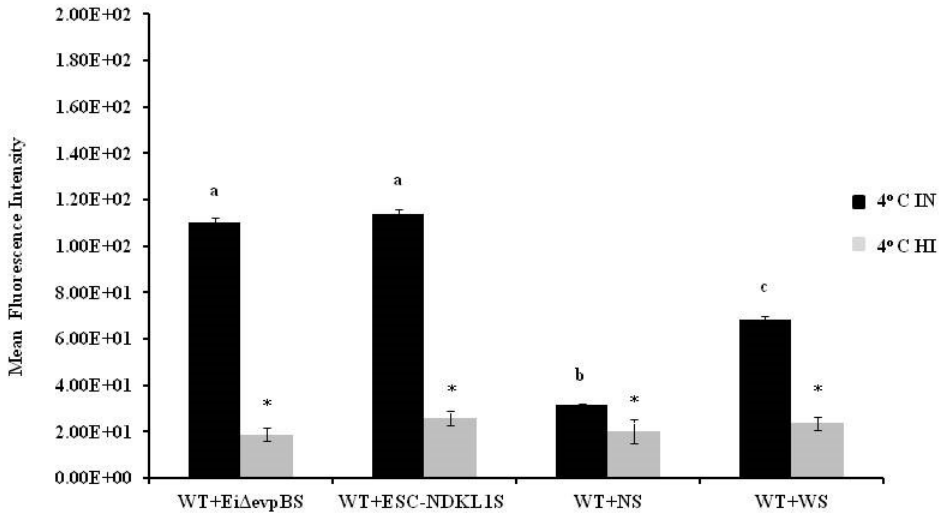

Figure 15 Active uptake of *E. ictaluri* LAVs opsonized with IN and HI sera from challenged fingerlings in catfish peritoneal macrophages at 4ºC.

[a,b,c] Presence of letters on top of bars indicates treatment differences in the uptakes with bars with different letters being different from each other and from bars without a letter designation ($P < 0.05$). *Indicates treatment differences in the uptakes ($P < 0.05$). The data represent the mean of MFI of macrophage phagocytic uptake from five fish ±SD.

Active Uptake of E. ictaluri Opsonized with the LAVs-induced Heat-Inactivated Immune Sera

Opsonization of WT *E. ictaluri* with HI sera from fingerlings vaccinated with both LAVs and non-vaccinated controls was utilized to determine the effect of complement on *E. ictaluri* phagocytosis in peritoneal macrophages. *E. ictaluri* opsonized with complement-inactivated sera derived from normal and ESC-NDKL -

challenged fingerlings was phagocytosed at significantly lower rates compared to the bacteria treated with *Ei*Δ*evpB* and WT treatment derived sera (Figure 14). There were no significant differences in the intensity of phagocytic uptake of bacteria treated with HI LAV1 and WT sera (Figure 14). Significant increases in the levels of phagocytosis were evident in all *E. ictaluri* treatment groups compared to the uptake of untreated bacterial cells (data not shown). However, inactivation of complement did not affect the uptake of WT serum opsonized *E. ictaluri* in peritoneal macrophages (Figure 14). Our results showed clearly that active uptakes of *E. ictaluri* and LAVs candidates in catfish peritoneal macrophages at 32^{o} C were mediated through the complement-dependent pathway.

We assessed phagocytic activity in catfish peritoneal macrophages exposed to WT *E.ictaluri* treated with IN and HI sera obtained from the challenged and control fish at 4^{o} C (Figure 15). Phagocytic uptake of bacteria opsonized with sera obtained from fish challenged with both LAVs did not show significant differences. However, they did express significant increases compared to *E. ictaluri* opsonized in the presence of HI sera from challenged and control fish (Figure 15). As expected, phagocytosis of *E. ictaluri* opsonized with sera from control animals was significantly lower compared to the uptake of bacteria opsonized with sera from all challenged fish groups (Figure 15). Notably, phagocytosis in peritoneal macrophages exposed to *E. ictaluri* opsonized with HI sera obtained from the challenged and control fish showed dramatically decreased background levels of phagocytic activity (Figure 15). These results indicate strongly that complement was a crucial role in phagocytosis of *E. ictaluri* and LAVs candidates by peritoneal macrophages.

Macrophages Killing of *E. ictaluri* Opsonized with the LAVs-induced Immune Sera

To examine how effective peritoneal macrophages are at destroying ingested bacteria, we performed the bacterial killing assay with *E. ictaluri* opsonized with IN sera from fish challenged with *E. ictaluri* LAVs, the WT strain, and control non-vaccinated fish (Figure 16). Initial numbers of colonies in all groups exposed to *E. ictaluri* did not show significant differences. However, the bacteria-killing capacity of peritoneal macrophages exposed to non-opsonized WT *E. ictaluri* was significantly lower compared to their counterparts treated with opsonized bacteria (Figure 16). No significant differences in killing capacity were evident in macrophages exposed to *E. ictaluri* treated with control, WT, or LAV challenged fish derived sera following 10 hrs exposure (Figure 16). Numbers of bacterial colonies did not differ significantly in all experimental groups exposed to opsonized *E. ictaluri* 24 hrs after infection. However, consistent numerical increases in numbers of colonies were evident in peritoneal macrophages exposed to the WT strain, *E. ictaluri* opsonized with sera derived from control, and the WT-infected fish compared to their counterparts treated with bacteria opsonized with both LAVs-induced immune sera (Figure 16). The virtually absent bacterial colonies in peritoneal macrophages exposed to the sera from fish challenged with *E. ictaluri* LAVs indicated that peritoneal macrophages efficiently killed LAVs strains after 24 hrs of *in vitro* infection. No bacterial colonies were evident in the negative control of uninfected macrophages (Figure 16). The use of HI sera in the bacterial killing assay, in general, showed the patterns that did not differ significantly from IN serum treated bacteria (data not shown).

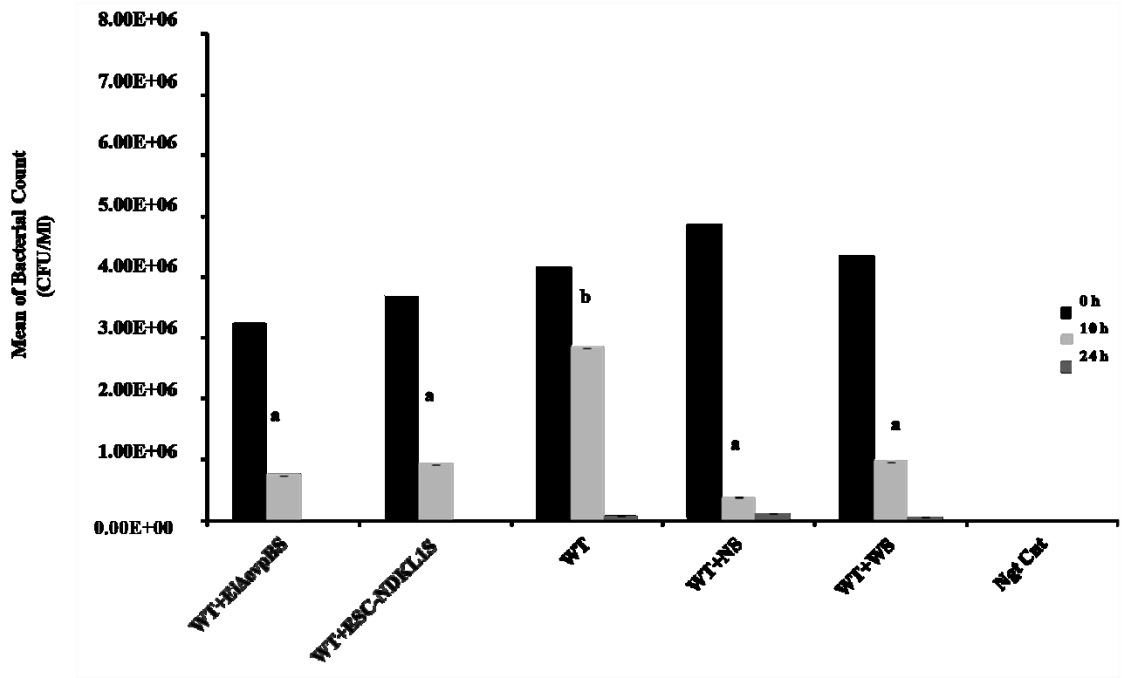

Figure 16 Bacterial killing of *E. ictaluri* LAVs opsonized with sera from challenged fingerlings in catfish peritoneal macrophages.

Black columns indicate the colony numbers at 0 h, and gray columns indicate the colony numbers at 10 h, dark gray color columns indicate the colony numbers after 24 h of *in vitro* infection. [a,b] Presence of letters on top of bars indicates group differences in the uptakes with bars with different letters being different from each other and from bars without a letter designation ($P < 0.05$). The data represent the mean of MFI of macrophage phagocytic uptake from five fish ±SD.

Discussion

Recent studies show that monocytes and macrophages are potent APCs that prime naïve T cells, and initiate adaptive cellular and humoral immune responses (Sakaguchi, Sakaguchi et al. 1995, Asano, Toda et al. 1996, Sakaguchi, Toda et al. 1996). Phagocytosis, which depends on membrane-cytoskeleton interactions is an important step that mediates innate immune recognition by professional antigen presenting cells (APCs) and triggers adaptive immune responses (Sakaguchi, Sakaguchi et al. 1995). The current research aimed to assess the phagocytic and bactericidal

activity of peritoneal macrophages in the uptake of WT *E. ictaluri* and two LAV candidate strains. Our conclusions agree with those of multiple studies that document strong phagocytic capability and bactericidal activity of peritoneal macrophages against intracellular pathogens (Esteban and Meseguer 1997, António, Susana et al. 1998, Do Vale, Afonso et al. 2002, Awasthi, Rathore et al. 2015). We documented enhanced vaccine strains phagocytic capacity and effective bacterial killing ability of catfish peritoneal macrophages *in vitro*. Interestingly, the intensity of *Ei*Δ*evp* LAV phagocytic uptake was significantly higher compared to the ESC-NDKL1 LAV uptake in catfish peritoneal macrophages suggesting its advantage to be destroyed and processed into peptides by the APCs compared to ESC-NDKL1 LAV strain. Our previous report showed that catfish fry vaccinated with ESC-NDKL1 had higher mortality rates compared to the fry vaccinated with *Ei*Δ*evp* LAV in E. *ictaluri* challenge (Nho, Abdelhamed et al. 2017). However, more research should be done to establish the necessary mechanistic framework for the LAV-dependent pathogenesis in catfish fry and fingerlings. Several earlier reports in humans and other mammals showed high-intensity antigen uptake at 37º C and low background levels of endocytosis at 4º C in professional APCs (Boyd, Lee et al. 2004, Ammari, Harris et al. 2014). Hohn et al. confirmed the data obtained in the mammalian studies on the low background levels of *E. ictaluri* phagocytosis at 4º C by using zebrafish anterior kidney/ monocyte/macrophage /granulocyte phagocytes (Hohn, Lee et al. 2009). However, contrary to the data derived from mammals, optimal conditions of antigen uptake in fish professional APCs are not well described yet. In contrast to previous observations, we report that active bacterial uptake in catfish peritoneal macrophages was detected at 32º

C and 4° C with significantly higher intensity at 32° C. Differences from earlier observations in our study regarding the intensity of active phagocytosis at 4° C could be due to several factors. First, there are phenotypic and functional differences between species in particular macrophages, and between APCs. Secondly, there are some fish species-specific differences in APC functions. Finally, monocytes, macrophages, and other professional phagocytes differ in the antigen uptake capacity due to their different locations and functions.

Importantly, our data agree with and contribute to the previous report that phagocytosis of LAV strains and WT *E. ictaluri* at 32° C was inhibited significantly in the presence of the actin formation inhibitor CCD, suggesting active uptake of *E. ictalurid* strains in catfish peritoneal macrophages (Hohn, Lee et al. 2009, Ammari, Harris et al. 2014). In particular, similar results were obtained with the uptake of WT *E. ictaluri* in the presence of CCD by zebrafish kidney phagocytes in the presence of CCD (Hohn, Lee et al. 2009). Also, a recent study showed that endocytosis of FITC-OVA in bovine monocytes was decreased significantly in the presence of CCD (Tomoda, Kishimoto et al. 1989). In addition to the significantly increased intensity of bacterial phagocytosis at 32° C and substantial inhibitory effect of CCD on the endocytic uptake, we demonstrated the presence of engulfed intracellular bacteria in the cytoplasm and phagosomes of peritoneal macrophages, thus confirming the active endocytic mechanisms of WT *E. ictaluri* and LAVs in catfish APCs. Furthermore, both LAVs were endocytosed more vigorously by peritoneal macrophages compared to their WT counterpart suggesting the LAVs advantage to be processed and presented in the form of peptides to specific lymphocytes, and subsequently destroyed.

Phagocytosis is a receptor-mediated process, and these receptors are classified into two groups: non-opsonic receptors (e.g., Dectin-1 and CD36) and opsonic receptors (e.g., FcγRIIA and Mac-1) (Araki, Johnson et al. 1996, Schlam, Bagshaw et al. 2015, Levin, Grinstein et al. 2016). Non-opsonic receptors can recognize directly and bind to chemical structures present on the surface of pathogens, whereas opsonic receptors can recognize indirectly phagocytic targets via binding to immunoglobulins (e.g., IgG) or complement C3b (Flannagan, Jaumouillé et al. 2012, Levin, Grinstein et al. 2016). Opsonin C3b molecules are generated by complement activation, bind covalently to the pathogen surface to create a destruction by phagocytes, which have receptors (e.g., CR1 (CD35) and CRIg) for complement C3b protein (Erdei, Sándor et al. 2016). In this study, we assessed the role of complement and antibodies in active uptake of *E. ictaluri* by peritoneal macrophages in catfish at 4° C and 32° C. As expected, our results indicated that *E. ictaluri* opsonized with sera from vaccinated fish was endocytosed by peritoneal macrophages more efficiently compared to the bacteria opsonized with sera from control animals, suggesting an important role of secondary humoral responses at an early stage of LAVs antigen presentation. Our results agree with previous reports on the enhanced phagocytosis of bacteria in the presence of immune sera from vaccinated fish. Namely, Russo et al. showed that opsonization of *E. ictaluri* with serum from vaccinated fish augmented the *in vitro* phagocytic ability of macrophages in catfish (Russo, Shoemaker et al. 2009). Also, another study (Esteban and Meseguer 1997) reported that macrophages from sea bass showed greater phagocytic activity against opsonized bacteria. Recently, human monocyte-derived macrophages infected with

Francisella tularensis showed 40 times more phagocytic activity in the presence of serum (Dai, Rajaram et al. 2013).

To confirm the significant contribution of complement-dependent mechanisms in the phagocytosis of *E. ictaluri*, we examined the uptake of the bacteria opsonized with HI sera from fish vaccinated with ESC-LAVs. We reported significant decreases in phagocytosis activity of peritoneal macrophages at both temperatures, which suggest the importance of CR ligation in *E. ictaluri* phagocytosis. Notably, the intensity of bacterial endocytosis was reduced dramatically to virtually background levels at 4° C, suggesting that bacterial uptake in catfish peritoneal macrophages at low temperatures, unlike that at 32° C, is predominantly CR-mediated. In contrast, the increased phagocytic activity of bacteria opsonized with HI WT serum in macrophages was evident at 32° C, suggesting a dominant role of the opsonic Fc receptors. Our findings are in agreement with earlier reports on the role of complement opsonic receptors in the endocytic activity of professional phagocytes. Namely, phagocytic activity of rainbow trout macrophages decreased significantly when *Mycobacterium marine* was treated with HI serum (Shih-Chu, Alexandra et al. 1998). Heat treated serum also suppressed phagocytosis of *Staphylococcus aureus* and zymosan particles in human macrophages significantly (Peterson, Verhoef et al. 1977, Shih-Chu, Alexandra et al. 1998, Mankovich, Lee et al. 2013). Heat inactivation disturbs the complement cascade and removes C3 opsonins thereby decreasing phagocytic activity in monocyte-derived human macrophages (Chan, Kedzierska et al. 2001). Taken together, various data indicate the importance of complement molecules engagement for efficient phagocytosis of bacterial cells. Our results also demonstrate that complement-mediated phagocytosis is temperature-

dependent in catfish peritoneal macrophages. Our data are in agreement with several previous observations that complement system in teleost fish is functionally active at low temperatures suggesting the enhanced complement-dependent phagocytosis compared to the mammalian counterpart (Sunyer and Lambris 1998), (Oriol Sunyer and Tort 1995), (Boshra, Li et al. 2006).

Finally, we applied a bacterial killing assay to assess the efficacy of the opsonized *E. ictaluri* destruction compared to the killing ability of the non-opsonized WT bacterial strain. We confirmed the previously reported observation by Shoemaker et al., on the effective killing of opsonized *E. ictaluri* by catfish peritoneal macrophages compared to the phagocytes exposed to the non-opsonized WT strain [28]. Our bacterial killing data in peritoneal macrophages showed that the numbers of bacterial colonies in peritoneal macrophages exposed to the opsonized WT *E. ictaluri* were significantly reduced compared to the phagocytes exposed to the non-opsonized WT strain showing significant differences at time 10 h post exposure. In our study, the intensity of the immune sera opsonized WT *E. ictaluri* uptake did not correlate with the bacterial uptake in peritoneal macrophages, suggesting the different sensitivity of the experimental approaches with active uptake by flow cytometry being more sensitive. We supported the data on active bacterial uptake in peritoneal macrophages by flow cytometry with significant inhibition of phagocytosis in the presence of actin formation inhibitor, CCD (a); significantly decreased uptake at 4^{o} C compared to the uptake at 32^{o} C(b), and finally, with the evidence of the internalized bacterial strains by light microscopy. However, in order to assess the bactericidal properties in professional phagocytes, we performed bacterial killing assay. The absence of bacterial colonies in peritoneal

macrophages exposed to the sera from fish challenged with *E. ictaluri* LAVs indicated that peritoneal macrophages efficiently killed WT *E. ictaluri* strain after 24 h of *in vitro* infection. Taken together, both approaches can provide valuable data on phagocytic properties and effective killing properties in professional phagocytes.

In conclusion, efficacious *E. ictaluri* LAVs are expected to induce active antigen uptake by phagocytosis and antigen presentation in catfish APCs such that infected monocytes/macrophages can initiate early activation of the innate immune system and acquired immunity mediated by T and B cells. Our study demonstrated that both vaccine candidates were endocytosed efficiently by catfish peritoneal macrophages showing significant increases in the intensity of uptake compared to their WT *E. ictaluri* counterpart. Importantly, both LAVs induced humoral immunity in the challenged fish resulting in significant increases in WT *E. ictaluri* uptake and bacterial destruction in catfish peritoneal macrophages *in vitro.* Our data on enhanced phagocytic capacity and effective killing ability of macrophages against ESC vaccine strains suggest the LAVs have advantage to be processed and presented in the form of peptides to the specific lymphocytes of the adaptive immune system and support the importance of macrophage- mediated immunity against ESC in catfish.

CHAPTER V

EFFECTS OF LIVE ATTENUATED VACCINE AND WILD TYPE STRAINS OF *EDWARDSIELLA ICTALURI* ON PHAGOCYTOSIS, BACTERIAL KILLING, AND SURVIVAL OF CATFISH B CELLS

Abstract

Edwardsiella ictaluri, a Gram-negative facultative and intracellular pathogen, is a causative agent of enteric septicemia of catfish (ESC) that is a devastating disease of farmed channel catfish. While the main function of B cells is to produce antibodies, two subsets (B-1 and marginal zone B cells) of B cells in mammals have phagocytic abilities and contribute to innate immunity. The innate roles of B cells have been demonstrated in several teleost fish, such as zebrafish, rainbow trout, and channel catfish. Recently, our group developed several protective *E. ictaluri* live attenuated vaccines (LAVs). However, the phagocytosis of *E. ictaluri* wild-type (WT) and LAVs strains in catfish B cells has not been evaluated. In this study, we assessed the efficacy of *E. ictaluri* WT and two LAVs on the innate functions (phagocytosis and microbial killing) and the survival of catfish B cells. First, we showed the active uptake of *E. ictaluri* WT and two LAVs in B cells by flow cytometry. Also, we determined that *E. ictaluri* opsonized with sera from vaccinated and non-vaccinated fish were engulfed by catfish B cells. However, *E. ictaluri* WT opsonized with serum from fish exposed to the WT strain significantly decreased the number of B cells compared to other groups.

Furthermore, catfish B cells were able to destroy *E. ictaluri* WT and LAV strains. However, catfish B cells more efficiently killed *E. ictaluri* opsonized with sera from vaccinated fish. Finally, we demonstrated that *E. ictaluri* WT and two LAVs induce early and late apoptotic changes in catfish B cells. These results indicate that efficacious *E. ictaluri* LAVs enhance the phagocytosis and killing of internalized bacteria in channel catfish B cells.

Introduction

Main function of B cells in immune system is to secrete antibodies, and B cells also contribute to innate immune responses. Recent discovery that live *Salmonella typhimurium* was engulfed by human primary B cells in B cell receptor (BCR)-mediated manner broke the long-held paradigm, which B cells are unable to uptake large particles (Souwer, Griekspoor et al. 2009). Two subsets of mammalian B cells, marginal zone (MZ) and B-1 B cells, were classified as "innate B lymphocytes" because of their developmental, phenotypic, and functional characteristics, thus contribute to innate immune responses, such as phagocytosis (Won and Kearney 2002, Zouali and Richard 2011). Different study showed that B cells from bone marrow, spleen, blood, and the peritoneal cavity are capable of phagocytosis in murine. The peritoneal cavity contains the largest percentage of phagocytic B cells (~10-17%); however, B cells showed phagocytic capacity less than 1.6% in other tissues (Parra, Rieger et al. 2012). In addition, phagocytic B cells from the peritoneal cavity can ingest latex beads and bacteria, and mature phagolysosomes were observed in these B cells, then B cells killed the ingested bacteria and presented the antigen to CD4+ T cells (Parra, Rieger et al. 2012).

Like mammalian B-1 cells, B cells in teleost fish were able to engulf particles and killing of internalized pathogens (Li J, DR. et al. 2006, Sunyer 2013). However, teleost B cells were present in all systemic compartments including blood, spleen, and anterior kidney (AK) and represented 60% of all B cells while phagocytic B cells in mammals were mainly found in the peritoneal cavity and represented a 5-15% of total B cell numbers. (Li J, DR. et al. 2006, Zhang, Salinas et al. 2010, Nakashima, Kinoshita et al. 2012, Parra, Rieger et al. 2012, Parra, Reyes-Lopez et al. 2015). Zebrafish B cells can ingest both soluble and particulate antigens (Zhu, Nie et al. 2013, Zhu, Lin et al. 2014). Furthermore, B cells had phagocytic ability in rainbow trout, and these B cells possessed phagolysosome, thus B cells have an important role in bacterial killing (Li J, DR. et al. 2006, Sunyer 2012). Additionally, fluorescent beads were ingested by B cells in Atlantic salmon and Atlantic cod, and B cells in these fish had higher phagocytic capacity than neutrophils in cod (Øverland, Pettersen et al. 2010). In addition to other teleost fish, the large amount of phagocytic B cells has also been found in catfish blood (Li J, DR. et al. 2006, Esteban, Cuesta et al. 2015).

Edwardsiella ictaluri, a Gram-negative, facultative, and intracellular fish pathogen, causes enteric septicemia of catfish (ESC), which is one of the most devastating diseases of farmed channel catfish in the US (Hawke, McWhorter et al. 1981, Miyazaki and Plumb 1985, Wagner, Wise et al. 2002, Zhang and Arias 2007). Immersion studies showed that live attenuated *E. ictaluri* live attenuated vaccines (LAVs) provided effective protection in catfish fry, fingerlings, and eyed catfish eggs (Shoemaker, Klesius et al. 1999, Wise, Klesius et al. 2000, Shoemaker, Klesius et al. 2002, Shoemaker, Klesius et al. 2007). Recently, Wise et al., developed an *E. ictaluri*

LAV isolate (S97- 773) that stimulated protection in fingerlings by oral vaccination (Wise, Greenway et al. 2015). Edwardsiella ictaluri is capable of surviving and replicating in channel catfish macrophages, and E. ictaluri LAVs triggered cell-mediated immunity to protect catfish against ESC(Shoemaker and Klesius 1997, Booth, Elkamel et al. 2006, Kordon, Abdelhamed et al. 2018). Additionally, humoral immune responses were induced in catfish vaccinated with E. ictaluri LAVs and facilitated the microbial killing activity of macrophages (Shoemaker and Klesius 1997, Booth, Elkamel et al. 2006, Kordon, Abdelhamed et al. 2018).

Recently, two novel *E. ictaluri* LAV strains, (*Ei*Δ*evpB* and ESC-NDKL1), were developed by our research group, and these LAVs provided efficient protection against ESC in both catfish fry and fingerlings (Lawrence and Karsi 2016, Karsi, Lawrence et al. 2017, Nho, Abdelhamed et al. 2017). We reported that two LAVs enhanced the phagocytic and bacterial killing abilities of catfish peritoneal macrophages (Kordon, Abdelhamed et al. 2018). However, the roles of these LAVs on the phagocytosis and intracellular killing properties in catfish B cells are still unexplored. Therefore, the purpose of this study was to assess the phagocytic and bacterial killing activity of channel catfish B cells against *E. ictaluri* WT and two LAV strains. Increased phagocytic and killing ability of catfish B cells will indicate the role of B cells in innate immune responses in *E. ictaluri* infection.

Materials and Methods

Animals

Specific pathogen free (SPF) channel catfish were obtained from the fish hatchery at the College of Veterinary Medicine, Mississippi State University. These fish were maintained at 25-28ºC, and all fish experiments were carried out based on a protocol approved by the Mississippi State University Institutional Animal Care and Use Committee (IACUC). To sedate and euthanize the catfish, tricaine methanesulfonate (MS-222, Western, Chemical, Inc.) was used. Samples were obtained as described below.

Bacterial Strains and Opsonization

Bacterial strains for this study are listed in Table 1 (Kordon, Abdelhamed et al. 2018). *E. ictaluri* 93-146 wild-type (WT) and two LAVs strains were cultured in BHI agar or broth (Difco, Sparks, MD, United States), and incubated at 30ºC for overnight. Two LAVs and WT strains were labeled with bioluminescence by transferring pAK*gfplux*1 from an *E. coli* donor strain (SM10aaa*pir*) by conjugation as described previously (Karsi and Lawrence 2007). Ampicillin (Amp: 100 mg/ml), and colistin sulfate (Col: 12.5 mg/ml, Sigma–Aldrich, St. Louis, MN, United States) were added to media when they are required.

For opsonization, *E. ictaluri* strains were incubated with sera from channel catfish exposed to WT and two vaccine strains 30 min at room temperatures. Opsonized *E. ictaluri* strains were used for phagocytosis and bacterial killing assay assessments.

Fish Vaccination and Serum Collection

Channel catfish fingerlings (6-month-old) with fully developed innate and adaptive immune systems were used for fish vaccination (Patrie-Hanson and Jerald Ainsworth 1999, Petrie-Hanson and Ainsworth 2001, Rombout, Huttenhuis et al. 2005, Zapata, Diez et al. 2006). Four 40 L tanks with continuous water flow and aeration were assigned randomly to *Ei*Δ*evpB*, ESC-NDKL1, *E. ictaluri* WT (positive control), and sham-vaccinated (negative control) groups, and each tank contained 25 SPF catfish fingerlings. The fish were acclimated for one week and fed twice a day. The temperature of the water was maintained at 24-26ºC throughout the experiment. Following one week of acclimation, fish were exposed to two LAVs (*Ei*Δ*evpB*, ESC-NDKL1) and *E. ictaluri* WT by immersion challenge as described previously (Abdelhamed, Lu et al. 2013, Kordon, Abdelhamed et al. 2018). Briefly, 100 ml of overnight cultures were added to 10 L water to yield infection dose of approximately 3.67 × 107 CFU/ml of water. BHI broth was added to the negative control group for immersion challenge. After vaccination, blood samples were collected from caudal vein of 10 catfish at 14 and 21 days post- infection. Then, blood samples were incubated for overnight at 4ºC to coagulate, and serum was obtained after centrifugation at 8000 rpm for 10 min.

Cell Preparation

Channel catfish (150-200 g) were used in this study. Anterior kidney (AK) was dissected from catfish and placed in a sterile culture dish that contained Phosphate-buffered saline (PBS). Tissues were minced by using sterile forceps and passed through

cell dissociation sieves (Sigma, St. Louis, MO) to obtain a single-cell suspension of AK. After that, cell suspensions were resuspended and washed in PBS. Cell suspensions were then layered on Histopaque 1077 (Sigma) and centrifuged at 500 *g* for 30 min to obtain enriched white mononuclear cells. Following centrifugation, white mononuclear cells (WMCs) were collected from the interface and washed three times in PBS at 500 *g* for 10 min. Cell counts and viability were assessed by trypan blue exclusion. Finally, cells were used for phagocytosis assessment.

Phagocytosis and Flow Cytometry

Mononuclear white blood cells from AK were resuspended in L-15 medium (ThermoFisher Scientific) as described previously (Zhu, Lin et al. 2014). To detect catfish B cells, cells were incubated with primary antibody (anti-catfish IgM mAb 9E1) for catfish B cells on ice for 30 min (Sahoo, Edholm et al. 2008, Edholm, Bengtén et al. 2010). Following incubation, cells were washed three times with PBS and incubated with secondary antibody conjugated with fluorochrome (R-PE) (Mouse F (ab) 2 IgG (H+L), R&D Systems, Inc.) on ice in the dark for 30 min. After the second incubation, cells were washed three times within PBS.

Following the staining of catfish B cells, cells were resuspended in L-15 medium and transferred into a 6-well plate (FisherScientific, Pittsburgh, PA, United States). Green Fluorescence Protein (GFP) transformed bacterial strains were added in 1:50 ratio into cell suspension and incubated at 30oC in the dark to determine the phagocytic ability and capacity of catfish B cell. Different incubation times were performed for phagocytosis assessment: 15 min, 30 min, 60 min, and 90 min. After the incubation periods, cells were collected and washed three times by centrifugation in

cold PBS and analyzed using NovoCyte Flow Cytometry (ACEA Biosciences, Inc.). Catfish mononuclear white blood cells and lymphocytes were gated based on their relative size and granularity by using forward and side scatters (FSC and SSC, respectively). The B cells were identified based on their intensity of staining with the B cell-specific antibody. Phagocytic B cells were determined based on the intensity of GFP fluorescence. The percentage of phagocytic B cells was determined by NovoCyte Flow Cytometry using two-color analysis with Dot Plot Quadrant statistics. Samples were analyzed using FlowJo 7.6.4 Software (Tree Star Inc.). The live B cell and total lymphocyte numbers after exposure to *E. ictaluri* strains opsonized with sera from challenged fingerlings were assessed by NovoCyte Flow Cytometry using two-color analysis with Dot Plot Quadrant statistics.

Cell Sorting

B cells were positively selected by magnetic sorting from AK WBC populations as described previously with minor modifications (Zhu, Lin et al. 2014). Briefly, WMCs were obtained from AK by using Histopaque 1077 separation, resuspended in L-15 medium and passed through the pre-separation filters (Miltenyi Biotec) to remove cell clumps. Then, a monoclonal antibody specific to channel catfish IgM^+ B cells (anti-catfish IgM mAb 9E1) was used to positively identify catfish B cells (Sahoo, Edholm et al. 2008, Edholm, Bengtén et al. 2010). Followed by incubation on ice for 30 min, cells were washed and resuspended in MACS buffer (Miltenyi Biotec), and anti-mouse IgG (H+L)-magnetic microbeads (Miltenyi Biotec) were added to cell suspensions, incubated at 4^{o}C for 15 min in the dark, washed and transferred onto an LS separation

column (Miltenyi Biotec), according to manufacturer instructions. After magnetic separation, the positively selected cells were cultured in the L-15 medium at 28°C overnight to detach the magnetic microbeads. Finally, the purity of the sorted IgM^+ B cells was determined by a FACSCalibur Flow Cytometer (Becton Dickinson).

Apoptosis Assay

The apoptosis assay was performed as described previously with minor modifications (Pinchuk, Lee et al. 2007). Positively selected B cells were resuspended in L-15 medium and transferred to 24 well plates (Tissue Culture Plate, CELLTREAT). Then, WT and LAVs *E. ictaluri* strains were added to the plates in a 1:50 ratio and incubated at 30ºC in the dark for 30 min and 3 h to detect early and late apoptosis in catfish B cells. After incubation, cells were collected and washed with cold PBS by centrifugation at 4ºC. Apoptosis in catfish B cells was assessed with the Annexin V-FITC Apoptosis Kit according to manufacturer's instructions (BioVision, Inc., Mountain View, CA). Briefly, cells were resuspended in 1x Binding buffer and incubated with Annexin-V-FITC and propidium iodide (PI) for 5 min at room temperature in the dark. Samples were then analyzed by NovoCyte Flow Cytometry using two-color analyses with Dot Plot Quadrant Statistics. In addition, Staurosporine (10 µM, Sigma)-treated catfish B cells were used as positive control for apoptosis.

Bacterial Killing Assay

The bacterial killing assay was performed as described previously with some minor modifications (Booth, Elkamel et al. 2006, Russo, Shoemaker et al. 2009). Briefly,

sorted B cells were resuspended in L-15 medium supplemented with 10% FBS, 1% L-glutamine, and 1.5% HEPES buffer and transferred to 96-well plates (Evergreen Scientific).Then, WT and two LAVs strains were added to the cell suspension in a 1:20 ratio. Plates were centrifuged at 1500 rpm for 5 min at room temperature to compact cells and bacteria and incubated at 30ºC for 30 min. Plates were then centrifuged at 2000 rpm for 7-10 min to remove the supernatant. Next, the pellet was resuspended with L-15 medium supplemented with 10% FBS, 1% L-glutamine, 1.5% HEPES buffer, and 100 µg/ml gentamicin (Gibco, Life Technologies, Grand Island, NY, United States) to kill extracellular bacteria, and incubated at 30ºC for 1 hour. Following incubation, plates were washed in PBS and resuspended in L-15 medium containing 10 µg/ml gentamicin and transferred to the black 96 well plates (Fisher Scientific). Finally, plates were incubated for 48 h with 5% CO_2 at 30ºC to determine the number of survival *E. ictaluri* inside the catfish B cells. Statistical analysis was acquired with the results obtained from Cytation 5 Cell Imaging Multi-mode Reader (BioTek).

Statistical Analysis

One-way and two-way ANOVA procedures of SAS (v 9.4, SAS Institute, Inc., Cary, NC) were used to evaluate differences in means of treatments for this study. The level of significance for all tests was set at $P < 0.05$.

Results

Identification of Catfish B Cells by Flow Cytometry

To separate B cells of catfish white mononuclear cells (WMC)-derived from AK, the cells were analyzed for the intensity of staining with catfish B cell-specific mAbs. First, we determined lymphocyte population based on their size and granularity, and lymphocyte population was calculated around 41% of all WMCs (Figure 17A). Then, we identified B cells in the lymphocyte population based on the intensity of staining with B cell-specific antibodies by gating on positive cell population. The percentage of B cells was at the levels of ~ 48% of total lymphocytes (Figure 17B).

Followed by the magnetic sorting, the resulting cell populations (Figure 17C) were stained with catfish B cell-specific antibodies Based on the intensity of anti-IgM staining which indicated 94% pure catfish B cells in the resulting cell populations (Figure 17 D).

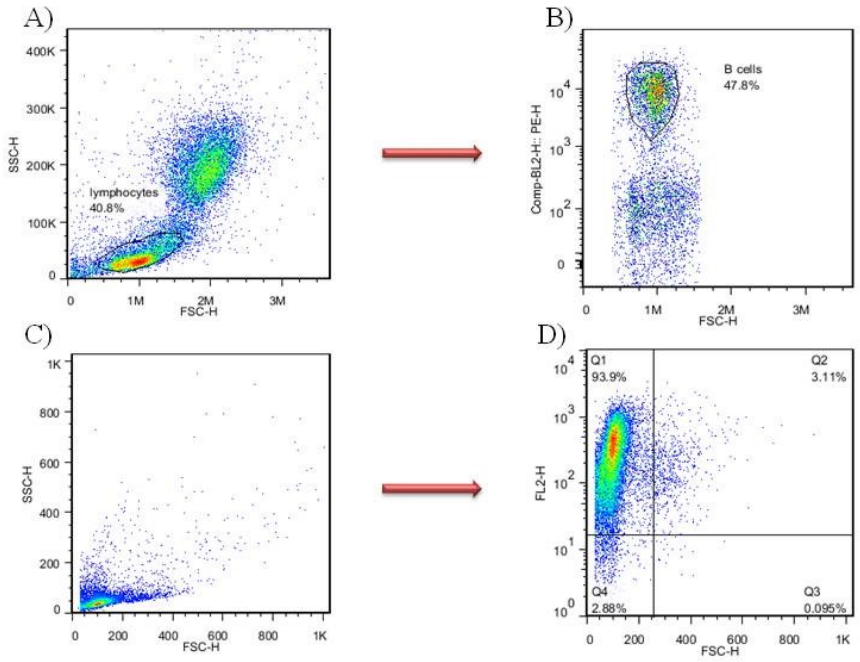

Figure 17 Separation of catfish B cells by flow cytometry.

(A) Assessment of lymphocytes based on their size and granularity; (B) Identification of B cells based on the intensity of B cell-specific staining; (C) Assessment of highly enriched cell population based on their size and granularity; (D) Identification of B cells based on the intensity of B cell-specific staining.

Active Phagocytic Uptake of *E. ictaluri* WT and LAVs strains in B cells

In this study, we determined the active uptake of *E. ictaluri* WT and two LAVs strains by sorted catfish B cells (Figure 18). We evaluated the uptake of GFP-labeled bacterial strains at 30ºC for 30 min. Then, we measured the percentage of phagocytic B cells in all groups treated with two LAVs and WT strains. Our data showed that there was no significant difference in the percentages of phagocytic B cells between LAVs

and WT treated groups, and both *E. ictaluri* WT and LAVs were engulfed by catfish B cells.

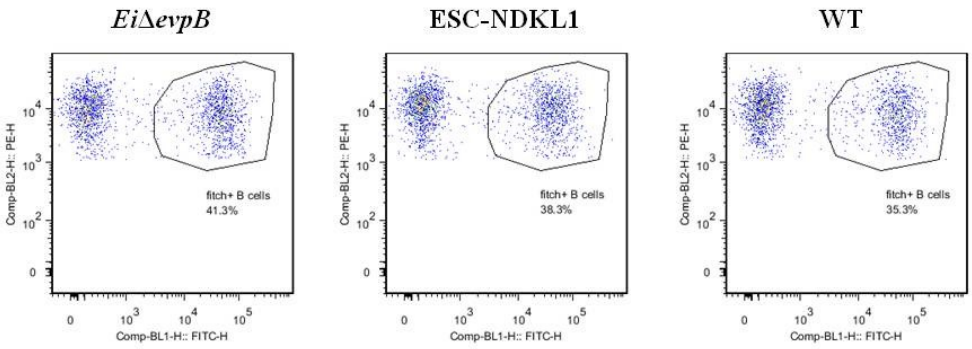

Figure 18 Active uptake of *E. ictaluri* WT, *EiΔevpB* and ESC-NDKL1 strains in catfish B cells.

B cells were observed by flow cytometry-two color with Dot Plot statistics. Dots in the circles indicate phagocytic B cells for each group.

Active Phagocytic Uptake of *E. ictaluri* Opsonized with the LAVs-Induced Sera by B cells

We evaluated the uptake of *E. ictaluri* WT opsonized with sera from catfish fingerlings vaccinated with both LAVs and non-vaccinated control groups. The phagocytosis of *E. ictaluri* WT strain after opsonization was monitored at multiple time

points: 15 min, 30 min, 60 min, and 90 min. The percentages of phagocytic B cells were assessed in groups treated with sera from vaccinated and non-vaccinated fish by flow cytometry (Figure 19). We determined that *E. ictaluri* WT opsonized with sera from vaccinated and non-vaccinated fish were taken up by catfish B cells. Interestingly, *E. ictaluri* WT opsonized with serum from fish exposed to WT strain significantly decreased the numbers of live B cells at all time points compared to their counterparts opsonized with sera from vaccinated fish and non-vaccinated fish (Figure 20A, B). These results suggested that *E. ictaluri* WT opsonized with serum from fish challenged with WT strain can have negative effects on the survival of B cells and lymphocytes.

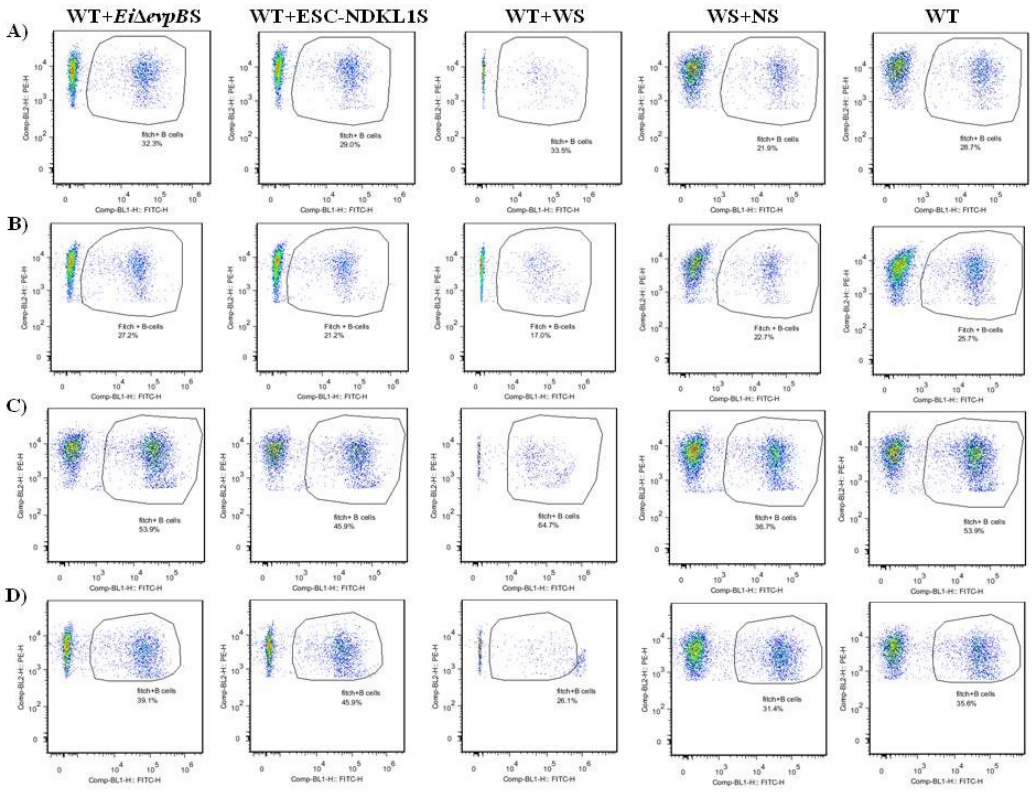

Figure 19 Active phagocytic uptake of GFP-labeled *E. ictaluri.*

WT strain opsonized with sera from challenged fingerlings with WT and two LAVs strains in catfish B cells for different incubation periods: 15 min (A), 30 min (B), 60 min (C), and 90 min (D). The treatments were: WT incubated with sera from *Ei*Δ*evpB*-exposed fish (WT + *Ei*Δ*evpB*S); WT incubated with sera from ESC-NDKL1-exposed fish (WT + ESC-NDKL1S); WT incubated with sera from WT-exposed fish (WT + WS); WT incubated with non-immune sera (WT + NS); WT in medium only (WT).

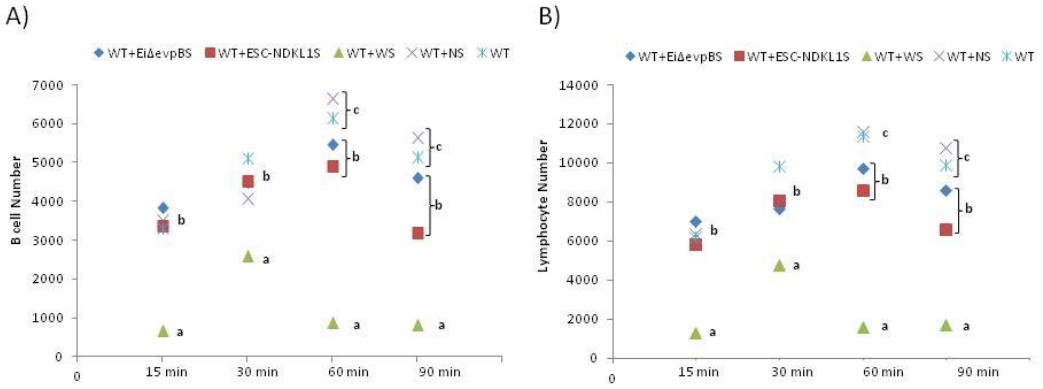

Figure 20 Survival rates of catfish anterior kidney B cells.

(A) and lymphocytes (B) exposed to B cells (A) and lymphocytes (B) exposed to *E. ictaluri* WT strain opsonized with sera from challenged fingerlings. Letters (a, b, and c) indicate significant differences ($P < 0.05$).

Killing of *E. ictaluri* and LAVs Strains by Catfish B Cells

After determining the levels of phagocytosis of *E. ictaluri* and LAVs strains in catfish B cells, we examined how effective catfish B cells were at killing ingested bacteria (Figure 21). The initial intensity of bacterial luminescence did not show significant differences in B cells treated with *EiΔevpB* and WT strains. However, the luminescence of ESC-NDKL1 in B cells was significantly lower compared to other groups. Surprisingly, the luminescence of bacteria in B cells increased in all treatments at 1 h, and there were significant differences between all treatments (Figure 21). For

example, the intensity of *Ei*Δ*evpB* luminescence in B cells was significantly higher than the luminescence of WT and ESC-NDKL1 strains. Moreover, the luminescence of WT strain in B cells was significantly higher than ESC-NDKL1 luminescence. Interestingly, the luminescence of both LAVs and WT in catfish B cells significantly decreased at 2 h; however, there were significant differences between the treatments and negative control in which B cells were not exposed to bacteria (Figure 21). The luminescence of *E. ictaluri* strains decreased in B cells at 36 h, and there were no significant differences in ESC-NDKL1 luminescence in B cells and negative control at 36 h. However, the intensity of WT and *Ei*Δ*evpB* luminescence at 36 h was still significantly higher compared to the group treated with ESC-NDKL1 and negative control group. Our data showed that catfish B cells are capable of killing *E. ictaluri* WT and both LAVs strains; however, they are more efficient at killing of ESC-NDKL1 than killing of WT and *Ei*Δ*evpB* strains.

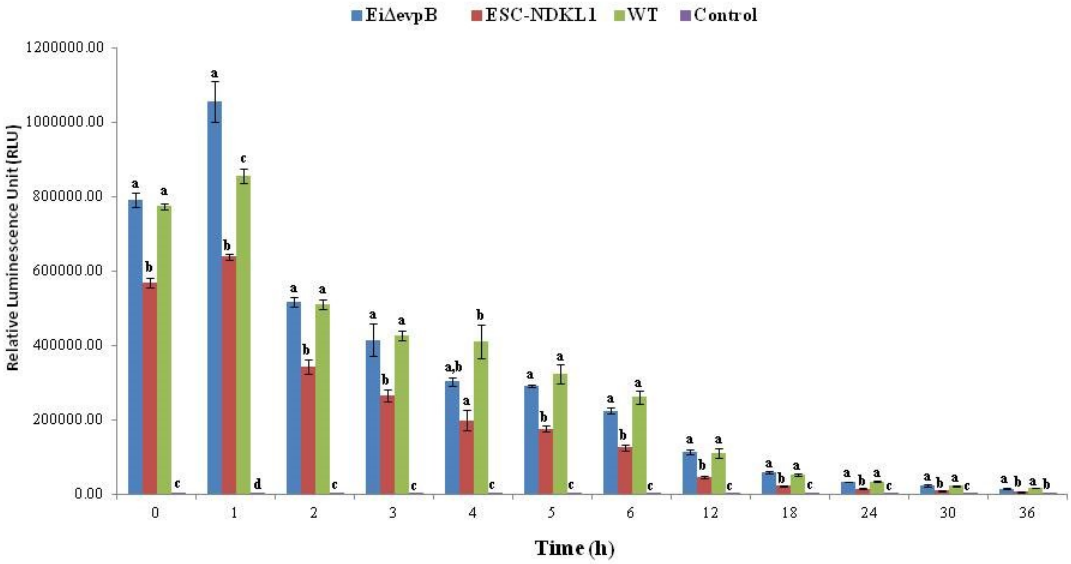

Figure 21 The bacterial killing of *E. ictaluri* WT and LAVs strains by catfish B cells. Letters show significant differences between the treatments ($P < 0.05$).

The Killing of *E. ictaluri* Opsonized with Sera from Challenged Fingerling Fish

We performed the bacterial killing assay to assess the killing of *E.ictaluri* opsonized with sera from fingerling fish exposed to two LAVs and WT strains, and serum from non-vaccinated fish (Figure 22). The luminescence of GFP-labeled bacteria in B cells was different at the initial time point. However, the intensity of luminescence from *E.ictaluri* opsonized with LAVs-induced immune sera was significantly higher than *E.ictaluri* opsonized with serum from non-vaccinated fish and non-opsonized WT strain (Figure 22). The luminescence of bacteria significantly increased in all treatments

at 1 h except the group treated with WT strain opsonized with serum from fish exposed to WT strain (Figure 22). Furthermore, there were no significant differences between the WT strain opsonized with serum from fish vaccinated with WT and the negative control, which was not exposed to bacteria (Figure 22). Bacterial luminescence significantly decreased in all treatments, and there were no significant differences between WT opsonized with sera from vaccinated fish with LAVs, WT strains and negative control at 2h. However, the luminescence of WT opsonized with serum from non-vaccinated fish and non-opsonized WT strain were significantly higher compared to their counterparts (Figure 22). These results indicate that catfish B cells were able to more efficiently kill *E. ictaluri* opsonized with sera from vaccinated fish than *E. ictaluri* opsonized with serum from non-vaccinated fish and non-opsonized *E. ictaluri* WT strain.

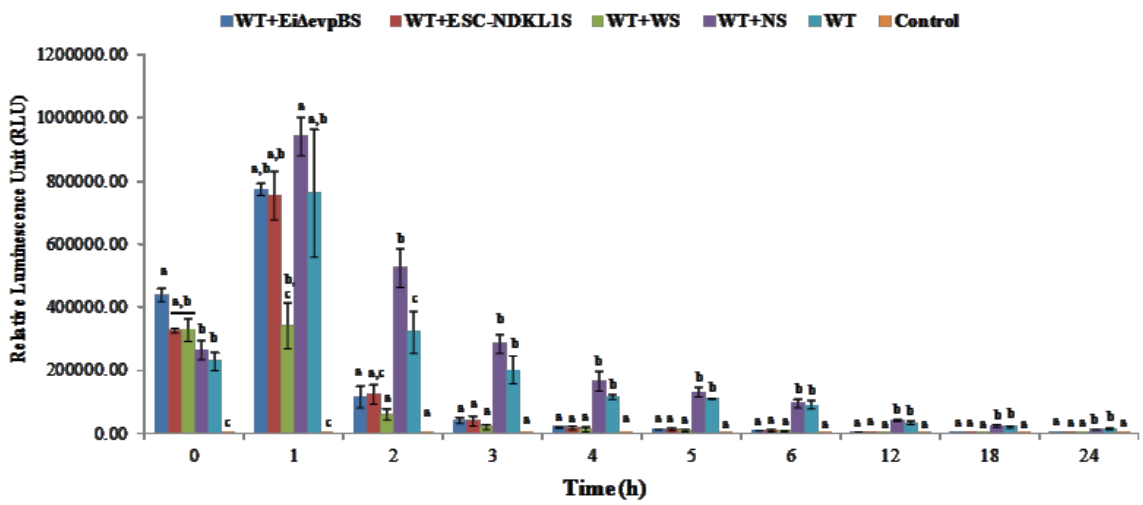

Figure 22 The bacterial killing of *E. ictaluri*opsonized with sera from challenged fingerlings in catfish B cells.

Letters indicate significant differences between treatments ($P < 0.05$).

Early and Late Apoptotic Changes in Catfish B Cells Exposed to WT *E. ictaluri* and LAVs

We showed previously that *E. ictaluri* WT opsonized with serum from fish exposed to WT strain significantly decreased the numbers of B cells and lymphocytes in the AK. Therefore, early and late apoptotic changes in B cells were assessed by flow cytometry at 30 min (Figure 23A) and 3h after exposure to WT *E. ictaluri* and LAV strains (Figure 23B). The flow cytomerty results are presented in graphic form in Figure 24. Interestingly, there were no significant differences in the percentages of live cells between B cells exposed to WT and LAV strains at both 30 min and 3 h incubations (Figure 24A). However, the percentages of live B cells treated with staurosporine were significantly decreased compared to other groups. Also, there were significant differences in the percentages of live B cells between 30 min and 3 h incubation times (Figure 24A). Live B cell numbers significantly decreased in all treatments at 3 h except the negative

control (Figure 24A). Furthermore, there were no significant differences in the percentages of early apoptotic cells between treatments exposed to LAVs and WT strains at 30 min; however, staurosporine-induced significantly higher levels of early apoptosis at 30 min compared to other groups (Figure 24B). In addition, there were no significant differences in the percentages of early apoptotic cells between the groups exposed to staurosporine, WT and ESC-NDKL1 strains at 3 h (Figure 24B). In contrast, *Ei*Δ*evpB* caused significantly less early apoptotic changes than staurosporine at 3 h (Figure 24B). Moreover, there were no significant differences in the early apoptotic cells between 30 min and 3 h incubation times. Surprisingly, the percentages of late apoptotic cells at 3 h significantly increased in all treatments except in the negative control (Figure 24C). There was no significant difference in the percentages of late apoptotic cells between treatments at 30 min. However, the percentages of late apoptotic cells in the groups exposed to WT and LAV strains were significantly higher than in the group treated with staurosporine at 3 h (Figure 24C, D). There were no significant differences in the percentages of necrotic cells between treatments at 30 min; however, the percentages of necrotic cells at 3 h significantly increased in the group exposed to staurosporine (Figure 24D).

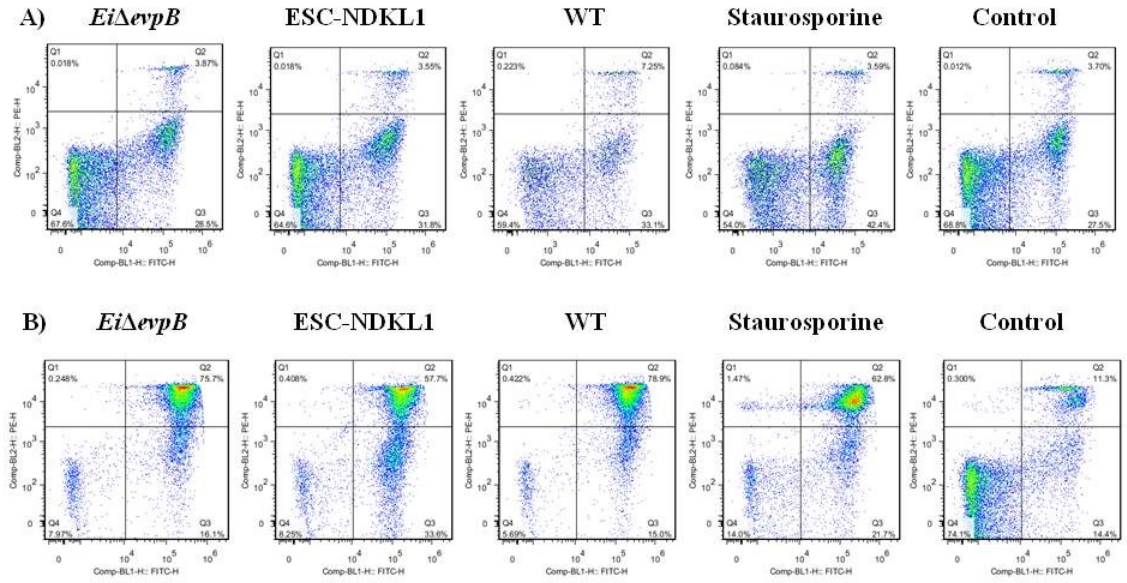

Figure 23 The effects of *E, ictaluri* WT and LAVs strains on B cell apoptosis.

Early and late apoptotic changes in catfish B cells exposed to EiΔevpB and ESC-NDKL1 and WT E. ictaluri strains at 30 min (A) and 3h (B). Q1 shows necrotic cells (PE+ cells); Q2 indicates late apoptotic cells (FITC and PE+ cells); Q3 shows early apoptotic cells (FITC+ cells); Q4 indicates live cells.

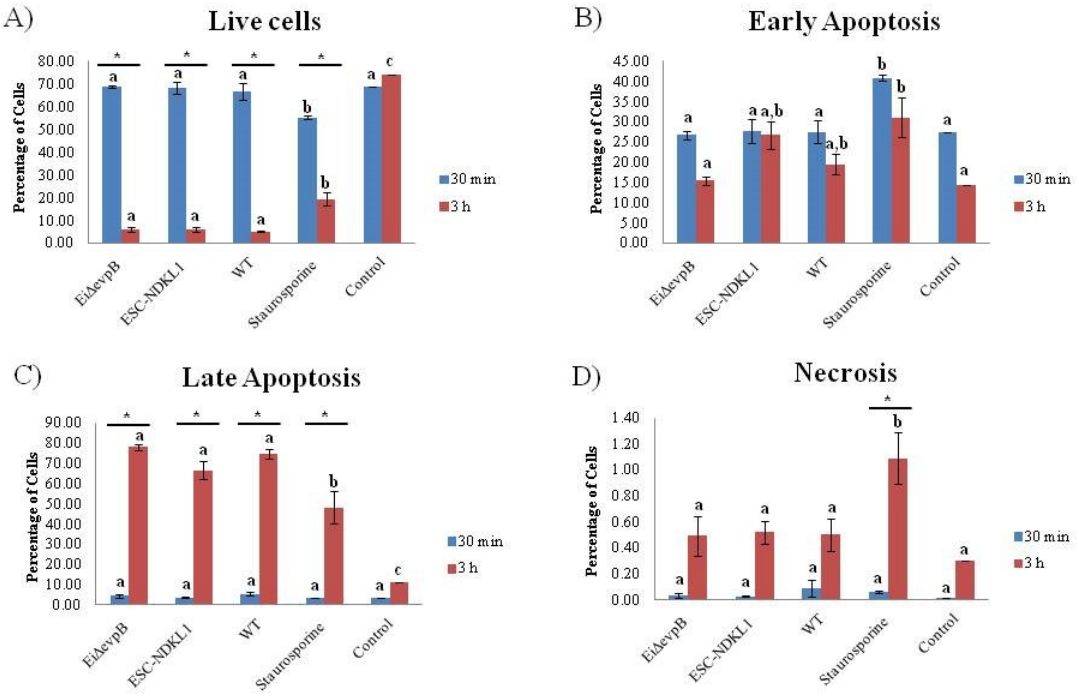

Figure 24 Statistical interpretation of early and late apoptotic changes in catfish B cells.

The percentage of live B cells (A), early apoptotic cells (B), late apoptotic cells (C), and Necrotic cells (D). Letters show significant differences between treatments at a given time point. * Symbol indicates A significant difference between 30 min and 3 h for each treatment ($P < 0.05$).

Discussion

B cells can capture antigens and process into peptides, then load the peptides onto MHC class II molecules for presentation to CD4 T cells (Avalos and Ploegh 2014). Recently, several studies reported that B cells in teleost fish served as a professional antigen presenting cells (APCs). For instance, zebrafish B cells were able to present

both soluble and particulate antigens to prime naïve $CD4^+$ T cells (Zhu, Nie et al. 2013). Also, this study showed that the expression of MHC class II molecules and co-stimulatory molecules (CD86 and CD83) was upregulated in B cells during presentation of antigens (Zhu, Nie et al. 2013). Other *in vivo* and *in vitro* studies have demonstrated that B cells in teleost fish were important APCs that activated T cells and initiated adaptive immunity (Lewis, Del Cid et al. 2014, Zhu, Lin et al. 2014, Abós, Castro et al. 2015). Phagocytosis and intracellular killing activities are crucial properties of all professional APCs. Therefore, the current research aimed to determine the phagocytic and bactericidal activity of catfish B cells in the uptake of *E. ictaluri* WT and two LAV strains. We documented phagocytic capability and bacterial killing activity of B cells in channel catfish against intracellular pathogens *in vitro* confirming the data obtained in the previous studies.

First, we showed that catfish B cells were able to engulf *E. ictaluri* WT and two LAV strains. Phagocytic ability of B cells has been shown in several teleost fish, such as zebrafish, rainbow trout, Atlantic salmon and Atlantic cod (Li, Barreda et al. 2006, Øverland, Pettersen et al. 2010, Zhu, Lin et al. 2014). In addition, B-1 and MZ subsets of B cells in mammals are capable of uptake of pathogens (Zouali and Richard 2011). Phagocytosis is mediated by two classes of receptors which are non-opsonic receptor (e.g., Dectin-1) and opsonic receptors (e.g., FcγRIIA) (Araki, Johnson et al. 1996, Schlam, Bagshaw et al. 2015, Levin, Grinstein et al. 2016). Although non-opsonic receptors can recognize and bind to the pathogen-associated molecules present on the surface of pathogens, opsonic receptors can recognize pathogen coated with immunoglobulins (e.g., IgG) or complement component C3b (Flannagan, Jaumouillé et

al. 2012). A recent study demonstrated that phagocytic B cells possessed both Fc and complement receptors (Sunyer 2012). Surprisingly, WT opsonized with sera from vaccinated fish significantly decreased the numbers of B cells and lymphocytes compared to WT opsonized with sera from non-vaccinated fish and the non-opsonized strain. Uptake of opsonized pathogens into phagocytic cells may have advantages because of the induction of antimicrobial mechanisms, such as reactive oxygen or nitric oxide (NO) production, or may show negative effects and harm the host (Eze, Yuan et al. 2000). Opsonization of pathogens with serum including IgM and complement can enhance the uptake of pathogens by fish granulocytes and monocyte/macrophages (Honda, Kodama et al. 1986, Jenkins and Ourth 1993, Leiro, Ortega et al. 1996, Kordon, Abdelhamed et al. 2018). In other studies, opsonized *Brucella abortus* in the presence of complement *in vitro* enhanced the uptake and killing of the pathogen in human neutrophils; however, opsonization increased the virulence of *Brucella melitensis* in humans (Young, Borchert et al. 1985). Our data suggest that *E. ictaluri* opsonized with serum from fish exposed to the WT strain may harm catfish B cells by enhancing the virulence activity of pathogen.

Professional phagocytic cells, such as macrophages, recognize and engulf pathogens into vesicles known as phagosomes that fuse with lysosomes to form phagolysosomes (Harrison, Bucci et al. 2003, Jeschke, Haas et al. 2018). Ingested pathogens are destroyed and killed in phagolysosomes by enzymes and antimicrobial substances, such as NO (Bronietzki, Kasmapour et al. 2014). In addition to professional phagocytes, B-1 cells in mice have been shown to mature their phagosomes into phagolysosomes (Parra, Rieger et al. 2012). In this study, we performed a bacterial

killing assay to determine the ability of catfish B cells to kill the WT and LAV strains. Our data showed that catfish B cells were capable of destroying *E. ictaluri* strains. We also assessed the efficacy in destruction of *E. ictaluri* opsonized with sera from vaccinated and non-vaccinated fish and non-opsonized strains. Our data demonstrated that *E. ictaluri* opsonized with sera from fish challenged with LAVs and WT strains were killed more efficiently compared to *E. ictaluri* opsonized with serum from non-vaccinated fish and the non-opsonized strains. Similar to mammals, the internalization of particles by phagocytic B cells in teleost fish induced the formation of phagolysosomes with the fusion of lysosomes with phagosomes and exhibited the capability to kill ingested particles (Li J, DR. et al. 2006, Sunyer 2012). Another study in rainbow trout showed that sorted IgM^+ and IgT^+ B cells were able to kill internalized bacteria, *Escherichia coli* (Zhang, Salinas et al. 2010). Also, phagolysosome formation in mammalian B cells supported the killing activity of phagocytic B cells. The B cells from the peritoneal cavity of mice engulfed *Staphylococcus aureus* (*S. aureus*), and uptake of bacteria led to the formation of phagolysosomes followed by activation the degradation pathways to kill the ingested bacteria (Gao, Ma et al. 2012).

Apoptosis, the process of programmed cell death, is identified by distinct morphological changes and biochemical modifications, such as protein cleavage and DNA fragmentation (Häcker 2000, Hengartner 2000, Elmore 2007). Apoptosis is a crucial component of numerous processes including development, normal cell turnover, and immune system, and this process occurs during normal development and aging (Elmore 2007). Also, apoptosis occurs in response to diverse physiological and pathophysiological stimuli and diseases (Ekert and Vaux 1997, Norbury and Hickson

2001). In this study, we applied an apoptosis assay to detect the early and late apoptosis in catfish B cells exposed to *E. ictaluri* WT and two LAV strains. Our data showed that *E. ictaluri* strains caused early and late apoptosis in catfish B cells. It was shown previously that mouse B cells from peritoneal cavity were incubated with *S. aureus,* and a very few numbers of B cells underwent apoptosis (Gao, Ma et al. 2012). Moreover, *Trypanosoma brucei* induced the loss of IgM^+ B cell population in mice by causing apoptosis (Radwanska, Guirnalda et al. 2008). Also, *Mycoplasma bovis* induces apoptosis of lymphocytes in the bovine model (Vanden Bush and Rosenbusch 2002).

In conclusion, channel catfish B cells can ingest *E. ictaluri* WT and two LAVs strains. Although WT opsonized with serum from fish exposed to WT strain significantly decreased the number of B cells, *E. ictaluri* obsonized with sera from vaccinated fish with LAVs strains did not induce the loss of B cells. Also, our data demonstrated that catfish B cells more efficiently killed *E. ictaluri* obsonized with sera from vaccinated fish. Our study showed that efficacious *E. ictaluri* LAVs facilitate the phagocytic activity and effective killing of internalized bacteria in channel catfish B cells. These results suggest that the advantage of LAVs is to be processed into peptides and presented to T cells to activate adaptive immune responses and support the important role of B cells against ESC in channel catfish.

CHAPTER VI

T CELL-MEDIATED IMMUNE RESPONSES IN IMMUNE ORGANS OF CHANNEL CATFISH, *ICTALURUS PUNCTATUS*, EXPOSED TO *EDWARDSIELLA ICTALURI* LIVE ATTENUATED VACCINES

Abstract

Edwardsiella ictaluri, a Gram-negative, facultative, and intracellular pathogen, is an etiological agent of enteric septicemia of catfish (ESC) which is a serious disease in channel catfish industry. T cells, specific lymphocytes, regulate immune responses by activating other immune cells or remove pathogen-infected host cells. Both subsets of T cells, helper T (Th) cells and cytotoxic T cells (CTLs) have been described in teleost fish with functions similar to their mammalian counterparts. Recently, our group developed two novel protective *E. ictaluri* live attenuated vaccines (LAVs). However, the effects of *E. ictaluri* wild-type (WT) and LAVs strains on T cell gene expression in catfish have not been determined. In this study, we assessed the expressions of the T cell-related genes, CD4-1, CD4-2, CD8-α, CD8-β, and the proinflammatory cytokine gene IL-1β, in the catfish spleen and anterior kidney (AK) in *E. ictaluri* infection. We showed that the expression levels of CD4-1 and CD4-2 were significantly elevated in the spleen of catfish treated with WT and LAVs strains compared to non- treated control group at 14 d post-treatment. However, the CD4-1 and CD4-2 expression were significantly increased in the catfish AK at 21 d. Similar to

CD4 genes, the expression levels of CD8-α and CD8-β in all treatment groups were significantly increased in the spleen at 14 d and in the AK at 21 d post-challenge. Also, the IL-1β gene expression was significantly increased in the spleen of catfish exposed to WT strain at 7d. However, the expression levels of IL-1β gene were significantly elevated in the AK at the beginning of treatment. These data indicate that two LAVs can trigger the expression of genes related to T cells without causing inflammation in catfish.

Introduction

T cells in mammals develop a diverse antigen repertoire of cell-surface receptors by the RAG proteins, and these receptors are called T-cell receptors (TCR) which recognize peptide of antigens presented by major histocompatibility complex (MHC) proteins (Langenau and Zon 2005). Also, T cells in teleost fish contain TCR genes, which may encode the TCR α, β, γ, and δ chains (Nam, Hirono et al. 2003). Like in mammals, T cells develop and mature in the thymus of teleost fish, and naïve T cells are activated and differentiate into cytotoxic Tcells (CTLs) or helper T (Th) cells (Nakanishi, Shibasaki et al. 2015). In mammals, CTLs express CD8 co-receptors on their surface which interact with MHC class I molecules while helper T cells express CD4 co- receptors that interact with MHC class II molecules (Andersen, Schrama et al. 2006). Furthermore, naïve Th cells are differentiated into several populations of effector T cells including Th1, Th2, Th17, Th9, follicular helper T cells (Tfhs), and regulatory T cells (Tregs) which have different function in immune responses (Dardalhon, Awasthi et al. 2008, Annunziato and Romagnani 2009, Wan and Flavell 2009). Both types of T cells, CTLs and Th cells, also differentiate into memory T cells which are involved in

long term protective immunity (Tanel, Fonseca et al. 2009, MacLeod, Kappler et al. 2010). T cell-related genes, such as CD3, CD4, CD8, and antigen presenting cells (APCs) related MHC class I and II genes, have been identified in teleost fish, and $CD4^+$ helper and $CD8^+$ CTL cell populations are similar to those of higher vertebrates (Toda, Shibasaki et al. 2009, Koppang, Fischer et al. 2010, Toda, Saito et al. 2011, Fischer, Koppang et al. 2013). CTLs in fish are similar to mammalian CTL and are primarily involved in antiviral immune responses by lysing of virus-infected host cells (Somamoto, Nakanishi et al. 2002). Additionally, teleost helper T cells function similar to their mammalian counterparts and are involved in the regulation of the immune responses by recruitment and activation of cell-mediated immunity, including CD8+ T cells, B cells, macrophages, and granulocytes (Castro, Bernard et al. 2011). For example, Th1 cells activate macrophages against intracellular bacterial and viral infections in fish (Díaz-Rosales, Bird et al. 2009, Mitra, Alnabulsi et al. 2010). In mammals, Th2 cells have a role in parasitic infections, and studies on teleost fish showed the expression of Th2 markers (IL- 4/13A and GATA-3) in the gills and skin with parasite infections (Takizawa, Koppang et al. 2011).

Edwardsiella ictaluri (*E. ictaluri*), a Gram-negative, facultative, and intracellular pathogen, causes a severe disease in the channel catfish (*Ictalurus punctatus*) industry known as enteric septicemia of catfish (ESC) (Hawke, McWhorter et al. 1981, Miyazaki and Plumb 1985, Wagner, Wise et al. 2002, Zhang and Arias 2007). Several studies demonstrated the several portal entries of *E. ictaluri* which includes mucosal epithelia on the gastrointestinal tract, skin, gills, and nares (Baldwin and Newton 1993, Morrison and Plumb 1994, Nusbaum and Morrison 1996, Karsi, Menanteau-Ledouble et al. 2006,

Griffin and Mitchell 2007). Following the entry, *E. ictaluri* colonizes in the lymphoid tissues of catfish upon oral or bath infection (Santander, Kilbourne et al. 2014) and induces acute and chronic infections in channel catfish (Shotts, Blazer et al. 1986, Newton, Wolfe et al. 1989).

In teleost fish, anterior kidney (AK) is considered the hemopoietic tissue, which is responsible for the production of all blood elements (Rombout, Huttenhuis et al. 2005, Zapata, Diez et al. 2006, Abdel-Aziz, Abdu et al. 2010, Abdelhamed, Ibrahim et al. 2017). The anterior kidney is one of the target organs in early *E. ictaluri* infection with leukocytes being observed in the channel catfish AK at 48 h post-infection, and *E. ictaluri* was detected in the posterior kidney of catfish after 15 min infection (Baldwin and Newton 1993). Moreover, the dispersion of *E. ictaluri* was found in the AK of catfish at 15 min post-intraperitoneal injection (Karsi, Menanteau-Ledouble et al. 2006). Recently, our research group showed that *E. ictaluri* causes necrosis in the hemopoietic tissue compartment of catfish kidney (Abdelhamed, Ibrahim et al. 2018).

The spleen in mammals is one of the secondary lymphoid organs in which antigen presentation takes place to activate the adaptive immune system (Ruddle and Akriav 2009). As in mammals, the spleen functions as a secondary lymphoid organ in teleost fish (Koppang, Fischer et al. 2010). Furthermore, the spleen is also one of the target organs of *E. ictaluri.* in channel catfish. After immersion infection, bioluminescent *E. ictaluri.* was observed in catfish abdominal area at 60-72 h post infection, and an intense bioluminescence signal of *E. ictaluri.* was obtained from the catfish spleen after dissection (Karsi, Menanteau-Ledouble et al. 2006). In spleen, the

maximum population of *E. ictaluri.* Reached at 96 h post-intraperitoneal injection, and necrosis is also observed in spleen (Areechon and Plumb 1983).

Edwardsiella ictaluri live attenuated vaccines (LAVs) provide effective protection in catfish fry fingerlings and eyed catfish eggs (Shoemaker, Klesius et al. 1999, Wise, Klesius et al. 2000, Shoemaker, Klesius et al. 2002, Shoemaker, Klesius et al. 2007). *Edwardsiella ictaluri* can survive and replicate in catfish macrophages, and *E. ictaluri* LAVs stimulated cell-mediated immune responses in catfish against ESC (Shoemaker and Klesius 1997, Booth, Elkamel et al. 2006, Kordon, Abdelhamed et al. 2018). Also, *E. ictaluri* LAVs induced humoral immunity and enhanced the bacterial killing activity of macrophages (Areechon and Plumb 1983, Booth, Elkamel et al. 2006, Kordon, Abdelhamed et al. 2018).

Our research group recently developed two novel *E. ictaluri* LAV strains, (*Ei*Δ*evpB* and ESC-NDKL1), which provided efficient protection in both catfish fry and fingerlings against ESC (Lawrence and Karsi 2016, Karsi, Lawrence et al. 2017, Nho, Abdelhamed et al. 2017). We demonstrated that two LAVs facilitated the phagocytic and bacterial killing abilities of catfish peritoneal macrophages (Kordon, Abdelhamed et al. 2018). However, the role of these LAVs on the expression of adaptive immune genes in channel catfish AK and spleen are still unexplored. Therefore, this study aimed to determine the gene expression of T cells ($CD4^+$ and $CD8^+$ T cells) after vaccination with *E. ictaluri* LAVs and WT strains. The elevated expression of adaptive immune genes will indicate the role of T cells against ESC and may also explain the role of LAVs in initiating adaptive immune responses successfully.

Materials and Methods

Animals

The fish hatchery at the College of Veterinary Medicine, Mississippi State University, provided specific pathogen free (SPF) channel catfish fingerlings (6-month old) that were maintained at 25-28ºC. All fish experiments were performed based on the protocol approved by the Mississippi State University Institutional Animal Care and Use Committee (IACUC). Tricaine methanesulfonate (MS-222, Western, Cehemical, Inc.) was used to euthanize the catfish. Samples were obtained as described below.

Bacterial Strains

E. ictaluri 93-146 wild-type (WT) and two LAVs (*Ei*Δ*evpB*, and ESC-NDKL1) listed in Table 1.1 were cultured in brain heart infusion (BHI) agar or (Difco, Sparks, MD, United States) incubated at 30ºC for overnight. Colistin sulfate (Col: 12.5 mg/ml, Sigma–Aldrich, St. Louis, MN) was added to media when required.

Fish Vaccination and Tissue Collection

One hundred catfish were stocked into four 40 L tanks (25 fingerling catfish per tank) which were supplied with flow-through water and continuous aeration. The fish were fed twice a day and acclimated for one week. After acclimation, fish were exposed to two LAVs (*Ei*Δ*evpB*, ESC-NDKL1) and *E. ictaluri* WT by immersion challenge as described previously (Abdelhamed, Lu et al. 2013, Kordon, Abdelhamed et al. 2018). Briefly, 100 ml of overnight cultures were added to 10 L water, and exposure dose was approximately 3.67 × 107 CFU/ml of water. BHI broth was added to the last tank as

negative control. Following vaccination, ten catfish from each group were randomly obtained from each tank at 6 h, 1 d, 3 d, 7 d, 14 d, and 21 d.

RNA Extraction and cDNA Synthesis

The spleen and AK were dissected from fish and immediately transferred into RNase-free tubes (ThermoFisher Scientific) containing ten volumes of RNA*later* (Ambion, Austin, TX). According to manufacturer's instructions, the FastRNA™ SPIN Kit for Microbes and the FastPrep-24™ Instrument (MP Biomedicals, Santa Ana, CA) were used to isolate total RNA from the collected tissues. To eliminate the catfish genomic DNA from the total RNA, we used on-column DNase treatment with RNase-Free DNase Set (QIAGEN, Hilden, Germany). The quantity and quality of total RNA were evaluated with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). The Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, USA) was used to convert tissue RNAs into cDNA according to the manufacturer's instructions. Briefly, the cDNA was synthesized using 2.5 µg of total RNA, 4 µl of 5X reaction mix, 2 µl of maximum enzyme mix, and nuclease-free water to complete a final volume 20 µl for a reaction and incubated at 25°C for 10 min followed by an incubation at 50°C for 15-30 min. Finally, reactions were incubated at 85°C for 5 min and stored at - 80 °C.

Quantitative real-time PCR and Data Analysis

Catfish-specific immune-related genes, their GenBank accession numbers, and primers in this study are listed in Table 2.2. Primer 3 software (http://www-genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) was used to design the primers

and synthesized commercially (MWG Eurofins Genomics). Gene expression was detected by using real-time PCR and FastStart Universal SYBR Green Master (ROX; Roche, Basal, Switzerland). Each qPCR reaction was composed of a final volume of 20 µl reactions which included 10 µl FastStart Universal SYBR Green Master (ROX), 0.6 µl primers, 6.8 µl nuclease free water, and 2 µl of cDNA. qPCR reactions were performed on the 7500 Real-Time PCR System (Applied Biosystems). Thermal cycling program was programmed with 45 cycles of 95°C for 10 s, 95°C for 15 s, 57°C for 30 s, and 72°C for 15 s, which was reduced to 5 s when required. Three technical replicates were used with each qPCR analysis to confirm the pattern of gene expression.

Table 2 Create a short, concise table title and place all detailed caption, notes, reference, legend information, etc in the notes section below

Genes	Accession NO.	Primers	References
18S ribosomal RNA	AF021880	F-GAGAAACGGCTACCACATCC R-GATACGCTCATTCCGATTACAG	(Karsi, Waldbieser et al. 2004)
CD4-1	DQ435305	F-GATGTCATCATTGTAGATCTCG R-GAGGTAGCTGGCATTTCACTCC	This study
CD4-2	DQ435304	F-CTGTATGTTGTATCAGCCTCTG R-CAGTCACCTCCTTACTTTGGCTA	This study
CD8-α	HQ446239	F-CTACGCGGAGAGACAGTCCCAA R-CTCACAACCCAAAAGCACATC	This study
CD8-β	HQ446240	F-CCATCAGGCCTGGAGAAAGCA R-TCACCACCAGGAGTAGGACA	This study
Interleukin 1 Beta (IL-1β)	DQ157743	F-TGATCCTTTGGCCATGAGCGGC R-AGACATTGAAAAGCTCCTGGTC	This study

The cycle threshold (Ct) of the reference gene (18S rRNA) was subtracted from the Ct value of the targets (gene of interest) for normalization with the formula: ΔCt = Ct (target gene) – Ct (reference gene). Then, the ΔΔCt value for each target was calculated by subtracting the ΔCt value of the target control from the ΔCt value of the target treated as described in the formula: ΔΔCt target= ΔCt target treated – ΔCt target control. Finally, fold changes were calculated for each gene using ΔΔCt value as described with the formula: Fold change=$2^{-\Delta\Delta Ct}$, and fold change values were used to compare the relative expression level of genes in response to *Ei*Δ*evpB*, ESC-NDKL1, and *E. ictaluri* WT strain.

Statistical Analysis

Statistical analysis was applied by using SAS 9.4 software (SAS Institute, Inc., Cary, NC). One-way and two-way ANOVA procedures of SAS 9.4 were used to evaluate differences in means between treatments for this study. The level of significance for all tests was set at $P < 0.05$.

Results

Relative CD4 Gene Expression in the Catfish Spleen and Anterior Kidney

To determine the effects of LAVs on initiating adaptive immune responses, we assessed the expression patterns of genes related to $CD4^+$ T cells, such as CD4-1 and CD4-2 in catfish spleen and AK after vaccination. Initially, we determined the expression levels of CD4-1 gene in the spleen and AK at different time points including 6 h, 1 d, 3 d, 7 d, 14 d, and 21 d post-vaccination (Figure 25). The expression of CD4-1 in catfish spleen was low in all treated groups at the beginning of the treatment,

and there were no significant differences between treated and non-treated control groups at the following time points, 6 h, 1 d, and 3 d (Figure 25A). However, CD4-1 gene expression significantly increased in the spleen of catfish exposed to WT strains at 7 d post-infection compared to other groups, while the expression of CD4-1 did not increase in the spleen of vaccinated fish, and there were no significant differences between vaccinated fish and non-vaccinated control fish groups (Figure 25A). Interestingly, the expression levels of CD4-1 in the spleen of fish treated with LAVs and WT strains significantly increased at 14 d post-exposure compared to the non-treated control group. The expression of CD4-1 in the spleen of catfish exposed to WT strain was significantly higher than that of vaccinated fish. Also, CD4-1 expression in the spleen of fish vaccinated with ESC-NDKL1 was significantly higher compared to fish vaccinated with *Ei*Δ*evpB* (Figure 25A). At 21 d post-treatment, the expression levels of CD4-1 significantly decreased in all groups, and there were no significant differences between the vaccinated groups and non-vaccinated control group (Figure 25A).

The expression levels of CD4-1in the AK of catfish was showed considerable differences compared to the expression levels in the spleen. Interestingly, the expression levels of CD4-1 in the AK of catfish exposed to WT strains was significantly higher compared to vaccinated fish and negative control groups at 6 h post-treatment (Figure 25 B). Also, CD4-1 expression was significantly decreased in the AK of catfish vaccinated with ESC-NDKL1 compared to the negative control group. However, the expression of CD4-1 was rather low in all groups at 1 d and 3 d, and there were no significant differences between vaccinated groups and the non-vaccinated control group (Figure 25B). Moreover, CD4-1 expression was significantly elevated in the AK of catfish treated

with WT strain after 7 d compared to other groups. At 14 d post-exposure, there were no significant differences in the expression of CD4-1 in the AK between vaccinated and non-vaccinated groups. Surprisingly, the expression levels of CD4-1 significantly increased in the AK of catfish treated with WT and *Ei*Δ*evpB*, and its expression in the AK exposed to WT strain was significantly higher compared to the AK of catfish vaccinated with *Ei*Δ*evpB*. However, there were no significant differences in CD4-1 expression in catfish AKs of vaccinated with ESC-NDKL1 and negative control groups.

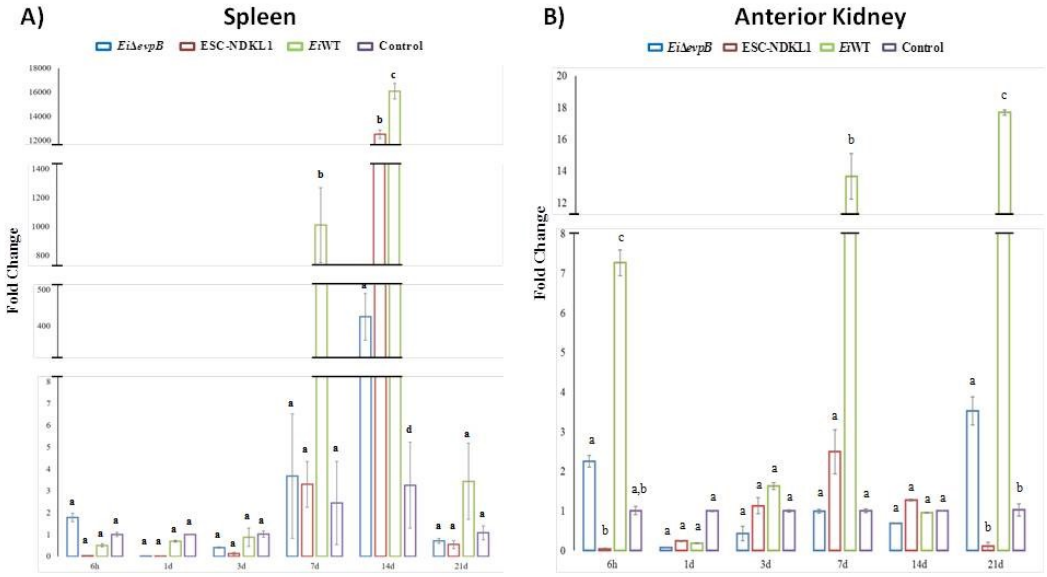

Figure 25 The expression pattern of CD4-1 in the spleen (A) and anterior kidney (B). Letters (a, b, c, d) indicate significant differences between the treatments at each time points ($P < 0.05$).

Another gene related to $CD4^+$ T cells is CD4-2, and the expression pattern of CD4-2 in the catfish spleen and AK was similar to CD4-1 expression in these organs (Figure 26). Like CD4-1 expression, the expression levels of CD4-2 in the spleen of catfish was low at the early time of infection, such as 6 h and 1 d, and there were no significant differences between vaccinated and non-vaccinated control groups (Figure 26A). However, the CD4-2 expression was significantly elevated in the spleen of fish exposed to WT strain at 3 d post-infection compared to catfish vaccinated with LAVs and non-vaccinated control group. After 7 d infection, the expression of CD4-2 significantly increased in the spleen of fish treated with WT and ESC-NDKL1 strains, and there was no significant difference between these two groups. The CD4-2 expression was low in the catfish spleen treated with *Ei*Δ*evpB* and was not different compared to non-treated control group at this time point. At 14 d post-infection, treatment groups showed significant increases compared to their 7 d post-challenge counterparts and were significantly different from the negative control group. Furthermore, the expression levels of CD4-2 in the spleen of fish challenged with WT strain was significantly higher than in fish challenged with two LAVs (Figure 26A). Also, CD4-2 expression in fish vaccinated with ESC-NDKL1 was significantly higher compared to fish vaccinated with *Ei*Δ*evpB*. In contrast, the expression levels of CD4-2 in the spleen significantly declined in all treatments at 21 d, but the levels in the spleen of fish vaccinated with ESC-NDKL1 strain was significantly higher than in the negative control group (Figure 26A).

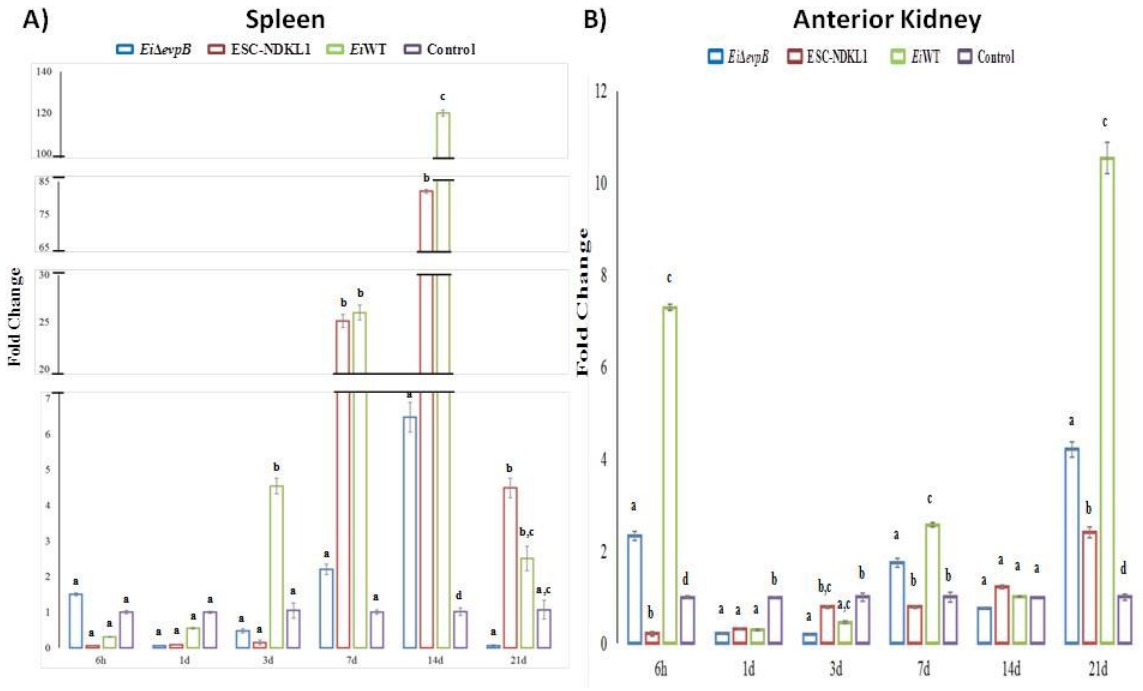

Figure 26 The expression pattern of CD4-2 in the spleen (A) and anterior kidney (B). Different letters show significant differences between the groups ($P < 0.05$).

Similar to the expression of CD4-1 in the AK, CD4-2 expression significantly increased in the AK of fish challenged with WT and *Ei*Δ*evpB* compared to the negative control group at 6 h whereas its expression in the AK of fish vaccinated with ESC-NDKL1 was significantly lower than the non-vaccinated control group (Figure 26B). Interestingly, the expression levels of CD4-2 in the catfish AK significantly declined in all treatment groups compared to the expression in non-treated control fish at 1 d and 3 d (Figure 26B). After 7 d challenge, catfish treated with *Ei*Δ*evpB* and WT strains showed significant increases compared to their counterparts treated with ESC-NDKL1 and

control group (Figure 26B). There were no significant differences between treatments and control group at 14 d post-infection. In contrast, CD4-2 expression was significantly elevated in the AK at 21 d post-exposure, and there were also significant differences between treatments (Figure 26B). The expression of CD4-2 in the AK challenged with WT strain was significantly higher than in fish vaccinated with LAVs. Also, CD4-2 expression in the AK of fish vaccinated with *Ei*Δ*evpB* was significantly higher compared to fish vaccinated with ESC-NDKL1 at this time point (Figure 26B). Overall, we documented the significant increases in the expression of genes related to $CD4^+$ T cells in the spleen at 14 days and in the AK at 21 d post-challenge.

Relative CD8 Gene Expression in the Catfish Spleen and Anterior Kidney

We also determined the effects of LAVs on the expression levels of genes related to $CD8^+$ T cells, such as CD8-α and CD8-β. The expression patterns of CD8-α in the catfish spleen and AK after challenge is shown in Figure 27. CD8-α expression in all treated groups did not differ from the non-treated group at the beginning of the treatment (Figure 27A). The expression levels of CD8-α in the spleen of catfish challenged with WT strain significantly increased at 7 d compared to its counterparts exposed to LAVs (Figure 27A). However, all treatment groups showed significant increases at 14 d, and the expression of CD8-α in the spleen of fish exposed to WT and ESC-NDKL1 strains were significantly higher than in fish exposed to *Ei*Δ*evpB* strain (Figure 27A). After 21 d infection, CD8-α expression in the spleen of vaccinated fish significantly declined compared to 14 d post-treatment, and there were no significant differences between the treated and the non-treated control group (Figure 27A).

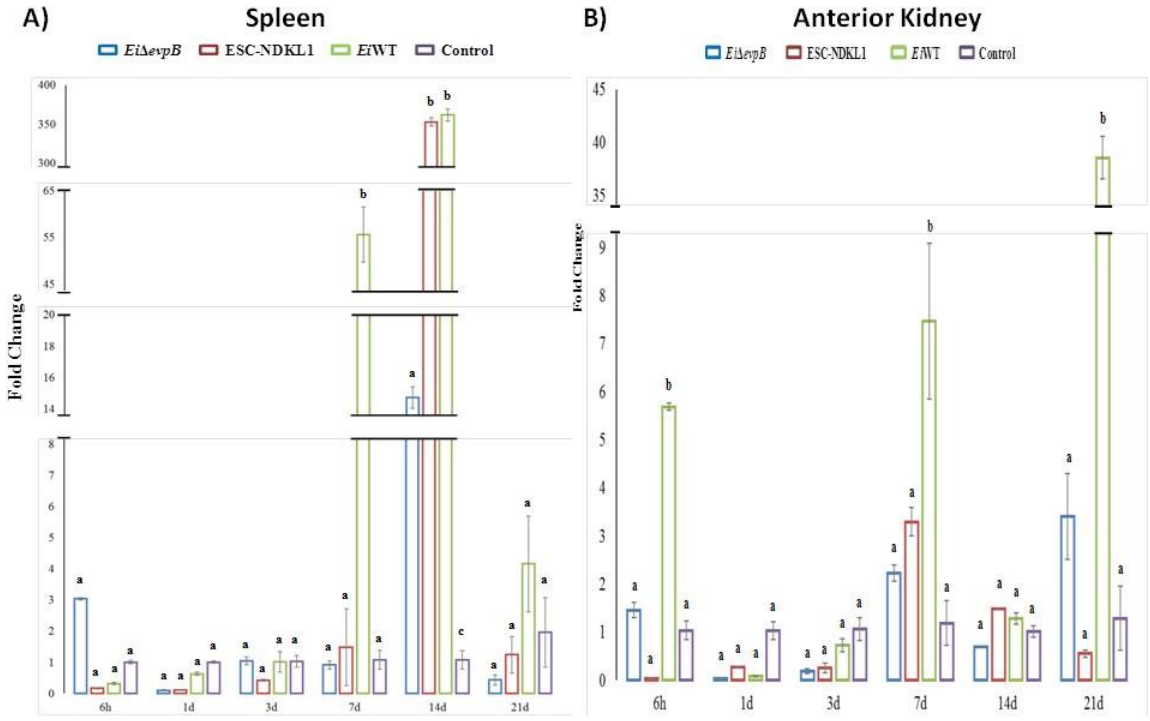

Figure 27 The expression pattern of CD8-α in the spleen (A) and anterior kidney (B). Letters (a, b, c, d) indicate significant differences between the treatments at each time point ($P < 0.05$).

On the other hand, CD8-α expression patterns in the AK of fish were different from the patterns in the spleen. The expression of CD8-α in the AK of fish treated with WT strain was significantly higher compared to its counterparts at 6 h post-infection (Figure 27B). The expression patterns of CD8-α in the AK of treated fish did not differ from the control group at 1 d and 3 d post-challenge (Figure 27B). After 7 d infection, its expression significantly increased in the AK of the fish exposed to WT strain compared to the fish vaccinated with two LAVs and non-vaccinated fish (Figure 27B). There were no significant differences between vaccinated and non-vaccinated fish at 14 d post-

exposure. After 21 d challenge, the expression of CD8-α significantly increased only in the AK of fish challenged with WT strain (Figure 27B). In addition to the CD8-α gene expression, we demonstrated the expression patterns of CD8-β gene in the catfish spleen and AK (Figure 28). Similar to the CD8-α gene, the expression of the CD8-β in the spleen of all treatment groups was low and did not differ from the non-treated control group at the early time of infection (Figure 28A). The CD8-β gene expression was elevated in the vaccinated fish at 7 d post-challenge, but was not significantly different from the control group (Figure 28A). However, the expression levels of CD8-β in the spleen of fish challenged with WT strain significantly increased at the same time point (Figure 28A). After 14 d infection, CD8-β expression significantly increased in the spleen of fish treated with ESC-NDKL1 and WT strains compared to the fish with exposed to the *Ei*Δ*evpB* strain and the control group (Figure 28A). Also, there was no significant difference between fish vaccinated with the *Ei*Δ*evpB* strain and the non-vaccinated control group at 14 d post-exposure (Figure 28A). On the other hand, the expression levels of CD8-β in the spleen significantly declined in all treatments and was not significantly different from the control group at 21 d post-infection (Figure 28A).

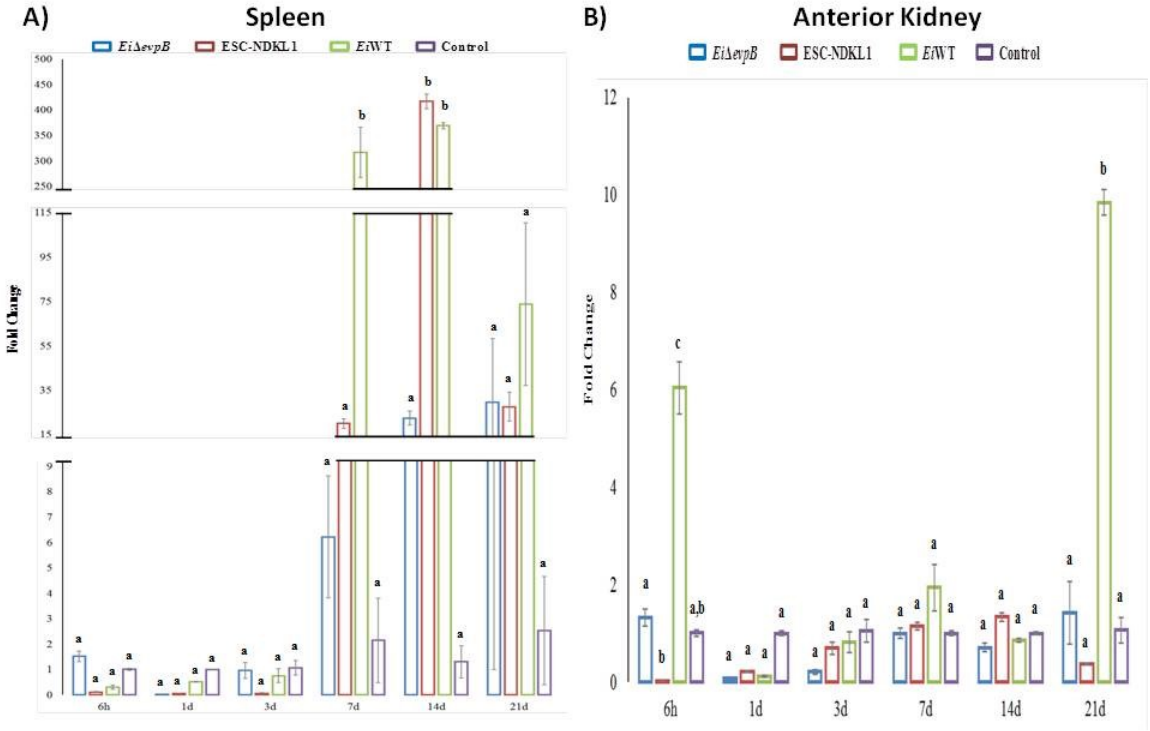

Figure 28 The expression pattern of CD8-β in the spleen (A) and anterior kidney (B). Letters (a, b, c, d) indicate significant differences between the treatments at each time point ($P < 0.05$).

The expression pattern of CD8-β gene in the AK of catfish differed from the expression levels of this gene in the spleen (Figure 28B). The expression levels of CD8-β gene were significantly elevated only in the AK of fish exposed to WT strain at 6 h post-infection. However, its expression significantly declined in fish treated with the WT strain at 1 d, and there were no significant differences between the treatments and control group at 1 d, 3 d, 7 d, and 14 d post-exposure. After 21 d infection, the CD8-β expression level significantly increased in fish challenged with WT strains, but its expression was low in the AK of vaccinated fish and did not differ from the control group. These results

indicated that the expression of genes related to $CD8^+$ T cells significantly increased in the spleen at 14 d and in the AK at 21 d.

Relative Gene Expression of IL-1B in the Spleen and Anterior Kidney of Catfish

We also performed qPCR to assess the expression level of IL-1β, which is a hallmark of the proinflammatory responses in the catfish spleen and AK (Figure 29). We detected expression of IL-1β gene in both tissues after exposure to WT *E. ictaluri* and LAVs. At the beginning of the exposure, its expression was low in the treatment groups and did not differ from the non-treated control group in the spleen (Figure 29A). After 7 d challenge, IL-1β gene expression in the spleen of fish challenged with WT strain significantly increased compared to its counterparts and significantly decreased at 14 d compared to 7 d post-infection (Figure 29A). However, the IL-1β gene expression was significantly higher after in the WT treated group than in vaccinated and non-vaccinated groups at 14 d (Figure 5A). The expression levels of IL-1β gene in the spleen of fish exposed to WT strain significantly declined at 21 d, and there were no significant differences between treated and non-treated groups (Figure 29A).

We detected the high levels of IL-1β in the AK of catfish after the challenge (Figure 29B). After 6 h, its expression was significantly elevated in the AK of catfish challenged with WT strain compared to catfish challenged with LAVs and control groups. Treatment groups showed significant increases at 1d post-infection compared to control group (Figure 29B). However, its expression level significantly declined in all treatments after 3 d, and there were no significant differences between treatments and control groups. After 7 d infection, IL-1β gene expression significantly increased again in the AK of catfish exposed to WT strain, but its expression significantly decreased at 14 d

post-infection (Figure 29B). Also, there were no significant differences between treatment and control groups at this time point. Furthermore, low levels of IL-1β gene expression were detected at 21 d in all groups (Figure 29B). In conclusion, WT and the two LAVs induced high expression levels of IL-1β gene in the AK and spleen at different time points.

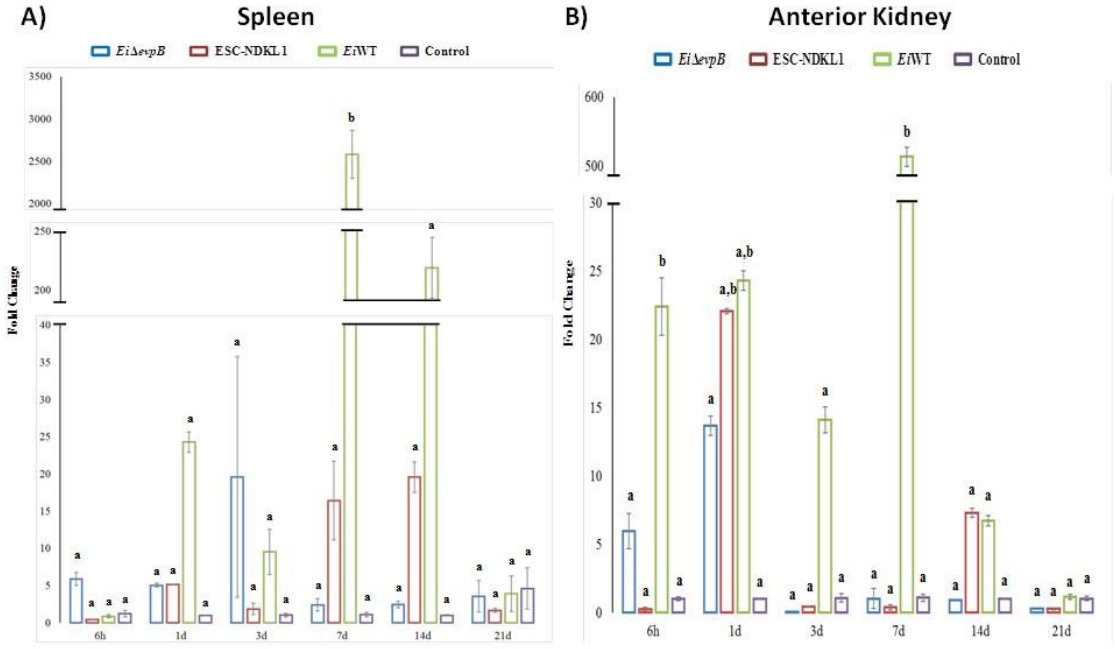

Figure 29 The expression pattern of IL-1β in the spleen (A) and anterior kidney (B). Letters (a, b, c, d) indicate significant differences between the treatments at each time point ($P < 0.05$).

Discussion

T cells are specific lymphocytes that play a crucial role in adaptive immune responses. Naïve T cells differentiate into helper T (Th) cells or cytotoxic T cells (CTLs) after activation in the secondary lymphoid organs, such as spleen and lymph nodes. Both types of T cells have different roles in the immune system. For example, Th cells produce robust chemokines which recruit new immune cells to the site of infections and also secrete cytokines to activate neighboring cells for their specific function. In contrast, CTLs generate diverse cytokines to remove the pathogen-infected host cells (Pennock, White et al. 2013). Several studies demonstrated that teleost fish possessed both subsets of T cells and their functions are similar to those in mammals (Laing and Hansen 2011, Secombes, Wang et al. 2011, Toda, Saito et al. 2011, Kono and Korenaga 2013). Therefore, the purpose of this study was to assess the expression of genes related to T cell co-receptors in the lymphoid organs of channel catfish in *E. ictaluri* infection. We determined the expression of the T cell-related genes, CD4-1, CD4-2, CD8-α, CD8-β, and proinflammatory cytokine gene IL-1β, in the catfish spleen and AK during *E. ictaluri* infection.

CD4 molecules of mammals contain four extracellular immunoglobulin (Ig) domains (D1, D2, D3, D4), a transmembrane (TM) region, and a cytoplasmic tail (CYT) (Turner, Brodsky et al. 1990). Two CD4-like molecules, which are CD4-1 and CD4L-2, have been characterized in several teleost fish (Dijkstra, Somamoto et al. 2006, Suetake, Saha et al. 2006, Edholm, Stafford et al. 2007, Buonocore, Randelli et al. 2008, Nonaka, Somamoto et al. 2008). Similar to mammalian CD4 molecules, teleost CD4 molecules are composed of four Ig-like domains; however, CD4L-2 in teleost consists of two or

three extracellular domains (Kono and Korenaga 2013). In this study, we assessed the expression levels of CD4 molecules in the spleen and AK of catfish challenged with two LAVs and WT strain. Our data showed that the high level of CD4-1 expression was detected only in the spleen of fish exposed to WT strain after 7 d infection. As expected, CD4-1 was highly expressed in the spleen of all vaccinated fish at 14 d post-infection. In contrast, it was expressed in the AK of fish challenged with EiΔevpB and WT strain at 21 d post-infection. In addition to the CD4-1 molecule, CD4-2 was expressed in the spleen of fish treated with ESC-NDKL1 and WT strains after 7 d challenge. Similar to CD4-1 expression, CD4-2 was expressed at the high levels in all treatment groups in the spleen at 14 d post challenge. Furthermore, the high levels of CD4-2 were detected in the AK of the treated groups after 21 d infection. In other studies using *Vibrio anguillarum* and *nodavirus* injection, CD4 expression was low in the spleen of AK of Atlantic halibut at the early time points, 24 h and 48 h post-injection (Patel, Øvergård et al. 2009). In studies with mammals, the percentages of CD4+ T cells significantly increased in the spleen of mouse infected with an attenuated strain of *Listeria monocytogenes* after 7 d (Pepper, Linehan et al. 2010). Also, CD4+ T cells increased in the spleen and lymph nodes of mice infected with *L. monocytogenes* at 10 d (Pepper, Pagán et al. 2011).

In mammals, CD8 molecules, CD8-α and CD8-β, are usually found in the form of heterodimers and are also described in teleost fish including channel catfish (Anne, Nadine et al. 1990, Quiniou, Sahoo et al. 2011). We assessed the expression of CD8-α and CD8-β in the spleen and AK of catfish after exposure to WT and LAVs of *E. ictaluri*. Our data demonstrated that the two LAVs and WT strains induced the CD8-α expression in the spleen of catfish at 14 d post-infection. However, only the WT strain triggered the

expression of this gene in the AK of fish at 21 d. Also, ESC-NDKL1 and WT strains stimulated the expression of CD8-β in the spleen at 14 d whereas its expression only increased in the AK of fish exposed to WT strain at 21 d. Recently, it was reported that CD8+ T cells responded to infection caused by *Viral Haemorrhagic Septicaemia* Virus (VHSV) and their number increased in the spleen of rainbow trout (Castro, Takizawa et al. 2013). Moreover, the expression of CD8-α increased in ginbuna crucian carp infected with crucian carp hematopoietic necrosis virus (CHNV) (Somamoto, Yoshiura et al. 2006). Additionally, the percentage of CD8+ T cells increased in dogs infected with *Leishmania infantum*, and also the percentage of CD8-α and CD8-β increased in the dogs (Moreno, Nieto et al. 1999).

IL-1β, member of 11 interleukin-1 family, is a pro-inflammatory cytokine which plays an important role in innate immune responses to infection and injury (Dinarello 1996). Pro-inflammatory caspase-1 cleaves pro- IL-1β to generate IL-1β that induces the formation of inflammasome (Thornberry, Bull et al. 1992, Schroder and Tschopp 2010). IL-1β has been identified in channel catfish in E. ictaluri infection (Wang, Wang et al. 2006). In this study, we assessed the expression of IL-1β in the spleen and AK of catfish challenged with two LAVs and WT strains. Our data showed that expression of this inflammatory cytokine gene only increased in the spleen of fish challenged with WT strains at 7 d and 14 d. Similar to spleen, IL-1β expression did not significantly increase in the AK of vaccinated fish and only increased in fish exposed to WT strain at the early time of infection, 6 h, 1 d, and 7 d. In studies with *V. anguillarum* infections, IL-1β expression increased in the AK and spleen of gilthead seabream (Angosto, Montero et al. 2014). Moreover, a crude preparation of lipopolysaccharide (LPS) induced the expression

of IL-1β in the AK macrophages in rainbow trout at 24 h (Hong et al., 2013). In LPS-induced rats, IL-1β expression increased at 6 h (Hwang, Lee et al. 2008).

In conclusion, our data showed that two LAVs can induce the expression of T cell-related genes, CD4-1, CD4-2, CD8-α, and CD8-β, in channel catfish lymphoid organs. These data suggest that naïve T cells are activated in the spleen of fish and differentiate into Th cells and CTLs. After activation, T cells migrate to the site of inflammation, such as AK. Activated Th cells will regulate the immune responses against *E. ictaluri* by stimulation of other immune cells, such as macrophages. In addition, CTLs will eliminate the infected cells of catfish. Also, our data demonstrated that our recently developed vaccines, *Ei*Δ*evpB* and ESC-NDKL1, did not cause inflammation in the catfish fingerlings as they did not induce the expression of pro-inflammatory cytokine, IL-1β, in catfish. Overall, two LAVs are protective and provide efficient immune responses against ESC.

CHAPTER VII

CONCLUSION

The overall goal of this project is to assess the role of three APCs; DCs, macrophages, and B cells in the LAV-induced innate and adaptive immune responses in channel catfish. Langerhans cells (LC), are a subset of DCs, that are located in the skin of mammals. In this project, we reported the identification of Langerhans-like cells in channel catfish. Results in chapter II showed that LC-like cells are found in the immunocompetent tissues of channel catfish. Moreover, Birbeck-like (BL) granules have been observed in channel catfish spleen. These results indicated that the LC-like cell populations in catfish lymphoid organs might represent an earlier lineage to Langerhans cells that are found in the epidermis in humans and other mammals. To our knowledge, this is the first demonstration of Langerhans-like cells in channel catfish. Moreover, results from chapter III showed that LC-like cell numbers increased in the spleen, AK, and gills of channel catfish in *E. ictaluri* infection. The expression patterns of LC-like cells are significantly different in the immune-related tissues of catfish exposed to WT and two LAVs. These results suggest that there are functional differences of DC-like cells in the different treatments. The numbers of L/CD207$^+$ cells in vaccinated fish was significantly elevated at the beginning of treatment, 6 h and 1 d, but their numbers decreased in the spleen at 3d. Furthermore, L/CD207$^+$ cell numbers increased in the spleen at 7 d post-challenge. On the other hand, the numbers of DC-like cells in the

immune-related organs of fish exposed to WT strains were similar to the numbers in the vaccinated fish at 6 h and 1 d; however, L/CD207^{+} cell numbers significantly decreased in the AK and spleen at 7 d post-treatment compared to non-treated control group. These results suggest that DC-like cells in vaccinated fish migrate to the site of infection for antigen uptake and migrate back to the spleen for antigen presentation to initiate the adaptive immune responses. However, WT strains reduced the ability of DC-like cell migration/maturation, which is critical for the initiation of adaptive immune responses.

The results in chapter IV showed that the efficacious *E. ictaluri* LAVs enhanced the phagocytosis activity and effective killing ability of ingested bacteria in catfish peritoneal macrophages. The uptake of both vaccines by catfish peritoneal macrophages was significantly higher compared to their WT counterpart, and the opsonization of *E. ictaluri* with sera from vaccinated fish increased the phagocytic activity of catfish peritoneal macrophages compared to non-vaccinated fish. These results indicate that monocytes/macrophages, professional APC, engulf two LAVs and can initiate early activation of the innate immune system. In addition, macrophages can process antigens and present in the form of peptides to the specific lymphocytes of the adaptive immune system, thus supporting the importance of macrophage-mediated immunity against ESC in catfish. In addition to catfish peritoneal macrophages, the results in chapter V showed that catfish B cells can engulf *E. ictaluri* WT and two LAVs strains. Furthermore, opsonized WT with serum from fish exposed to the WT strain caused a significant reduction in the number of B cells compared to the LAV strains opsonized with sera from LAV-vaccinated fish. Also, these results demonstrated that catfish B cells more efficiently killed *E. ictaluri* obsonized with sera from vaccinated fish. This study

indicates that the efficacious *E. ictaluri* LAVs facilitate the phagocytic activity and effective killing of internalized bacteria in channel catfish B cells. These results suggest that the advantage of LAVs is to be processed into peptides and presented to T cells for activation of adaptive immune responses and support the important role of B cells against ESC in channel catfish.

Finally, the results from chapter VI showed that the two LAVs can induce the expression of T cell related genes, CD4-1, CD4-2, CD8-α, and CD8-β, and the pro-inflammatory cytokine, IL-1β, in channel catfish lymphoid organs. The expression levels of T cell-related genes, CD4-1, CD4-2, CD8-α, and CD8-β, significantly increased in all treatment groups in the spleen at 14 d post-treatment and in the AK of fish at 21 d. In contrast, IL-1β gene expression was significantly increased in the spleen and AK of fish exposed to the WT strain compared to vaccinated fish in the early time of treatment. These results indicated that two LAVs can trigger the expression of T cell-related genes and did not cause inflammation. Therefore, two LAVs are protective and provide efficient immune responses against ESC.

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