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IκBα phosphorylation and associated NF-κB activation are essential events in lymphocyte activation, proliferation, and anti-bacterial adaptive immune response of Nile tilapia

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ABSTRACT

Inhibitory protein IκBα plays a crucial role in the inflammatory process and immune response by regulating the activity of transcription factor NF-κB. In teleost, great progress has been achieved regarding NF-κB signaling for innate immunity, but whether this pathway modulates adaptive immunity, and how, remains largely unclear. In this study, after characterizing the sequence, structure, and phylogeny of Nile tilapia *Oreochromis niloticus* IκBα (defined as On-IκBα), we investigated the association between IκBα-regulated NF-κB activation and the lymphocyte-mediated adaptive immune response in Nile tilapia. We found that On-IκBα was evolutionarily conserved, and its mRNA was expressed widely in various tissues, with most abundance in the trunk kidney. mRNA expression of On-IκBα was significantly upregulated in spleen at both innate and adaptive immune stages after *Aeromonas hydrophila* infection. Moreover, phosphorylation of On-IκBα and the downstream On–NF–κB p65 was obviously elevated in spleen leukocytes at 3, 5, or 8 days after *A. hydrophila* infection, indicating the activation of NF-κB signaling. Correlating with the augmented protein phosphorylation, leukocyte proliferation was enhanced during the same immune stage, suggesting the potential association of IκBα and IκBα-regulated NF-κB signaling in the primary adaptive immune response. Although lymphocyte activation by the T cell-specific mitogen PHA did not alter On-IκBα mRNA expression significantly, lymphocyte activation by the agonist PMA obviously elevated On-IκBα and OnNF–κB p65 phosphorylation in spleen leukocytes. Together, the results suggest that IκBα phosphorylation and its regulated NF-κB activation are essential events associated with lymphocyte activation, proliferation, and anti-bacterial adaptive immune response in Nile tilapia. Our study aids to understand the regulatory mechanism of adaptive immunity in teleost.

1. Introduction

As master regulators of immune and inflammatory pathways, transcription factors of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) family are implicated in the response to injury and infection (Baldwin, 2001). More specifically, the eukaryotic NF-κB regulates the expression of various genes involved in acute-phase response, stress, inflammation, cell differentiation, and lymphocyte activation (Baeuerle and Henkel, 1994; Karin et al., 2002; Tak and Firestein, 2001). Mechanistically, NF-κB activation is the result of NF-κB inhibitor (IκB) phosphorylation and degradation, which releases NF-κB into the nucleus and activates target gene expression (Gilmore, 2006).

The IκB family acts as biological inhibitors of NF-κB activity (Baeuerle and Baltimore, 1988). The family consists of classical IκB proteins, including IκBα, IκBβ, and IκBε; the NF-κB precursor proteins p100 and p105; and the nuclear IκB proteins, such as IκBζ, Bcl-3, and IκBNS (Napetschnig and Wu, 2013). IκB proteins comprise an N-terminal signal-receiving domain (SRD); a central ankyrin repeat-containing domain (ARD) with a 33–amino acid motif that mediates protein–protein interactions; and a C-terminal proline-, glutamate-, serine-, and threonine-rich (PEST) sequence (Napetschnig and Wu, 2013). In unstimulated cells, IκBs are bound to NF-κB in the cytoplasm, masking the nuclear localization signal via the ARD of IκBα and sequestering NF-κB

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in an inactive state; thus, NF-κB cannot translocate into the nucleus to bind the DNA or exert its transcriptional regulation (Baeuerle and Baltimore, 1988; Gilmore, 2006; Huxford et al., 1998; Jacobs and Harrison, 1998). Following exposure to extracellular stimuli, for example infection, cytokines, and stress, IκBs are rapidly phosphorylated by IκB kinases (IKKs), which leads to IκB ubiquitination and degradation, resulting in NF-κB nuclear translocation and transcriptional activation of the target genes (May and Ghosh, 1999).

Tightly regulated by IκBα, the NF-κB pathway plays pivotal roles in both innate and adaptive immune responses (Li and Verma, 2002). With regard to adaptive immunity, NF-κB signaling is an indispensable regulator of various biological processes in both T and B lymphocytes (Li and Verma, 2002). IκBα degradation and its regulated NF-κB activation are crucial for T lymphocyte proliferation induced by interleukin-2 and interleukin-4 (Mora et al., 2001). In IκBα-deficient mice, the lack of IκBα promotes constitutive NF-κB activation; while in mutant mouse whose IκBα cannot be degraded, T cell proliferation are markedly impaired in response to mitogenic stimulation with phorbol 12-myristate 13-acetate (PMA) plus ionomycin (Beg et al., 1995; Ferreira et al., 1999). Additionally, T helper 1 (T_H1) cell differentiation is highly related to IκBα, as the NF-κB signaling pathway is involved in the production of interferon-γ, an essential cytokine for T_H1 cell function (Kojima et al., 1999). Meanwhile, B lymphocytes in mice with mutation in the IκBα nuclear export sequence show obvious impairment in proliferation, survival, and antibody production, accompanied by defective formation of the secondary lymphoid organs and tissues (Wuerzberger-Davis et al., 2011). Therefore, IκBα and its regulated NF-κB signaling plays global roles in the lymphocyte-mediated adaptive immune response.

In the last decade, an increasing number of IκBα homologues have been identified from early vertebrates and invertebrates, and their potential functions in innate immunity have been well investigated (Lee et al., 2014; Sangrador-Vegas et al., 2005; Wang et al., 2015; Yazawa et al., 2007; Zhang et al., 2012a, 2012b). However, whether and how IκBα functions in the adaptive immune response of early vertebrates, especially teleost, remain largely unclear. Recently, multiple functional components of the adaptive immune system in teleost have been identified, such as T cell receptors (TCR), co-receptors (CD4 and CD8), cytokines and markers of naïve, effector, and memory T lymphocytes (Castro et al., 2011; Laing and Hansen, 2011; Secombes et al., 2011; Wang and Secombes, 2013; Zou and Secombes, 2011). Meanwhile, the increasing potential of $CD3^+$, $CD4^+$, and $CD8^+$ T lymphocyte subsets in teleost has been identified (Koppang et al., 2010; Maisey et al., 2016; Takizawa et al., 2011; Toda et al., 2011; Xing et al., 2017). These subsets of teleost lymphocytes might exert similar functions as their counterparts in mammals (Nakanishi et al., 2002; Toda et al., 2009; Wan et al., 2016; Yamasaki et al., 2014). In teleost, B cells serve as phagocytic and pivotal antigen-presenting cells (Li et al., 2006; Zhu et al., 2014), while immunoglobulin (Ig)M^+ B cells and IgT^+ B cells perform antibody-like functions in the serum and mucosal tissues, respectively (Castro et al., 2013; Tacchi et al., 2014; Xu et al., 2013, 2016; Yu et al., 2018; Zhang et al., 2010). Although functional studies of teleost adaptive immunity have achieved some progresses, the related regulatory mechanism remains largely unknown. In the present study, we characterized the sequence, structure, and phylogeny of IκBα from Nile tilapia (*Oreochromis niloticus*). We then investigated the potential association between IκBα-regulated NF-κB activation and the lymphocyte-mediated adaptive immune response in Nile tilapia, aiming to foster better understanding of the adaptive immune system in teleost.

2. Materials and methods

2.1. Experimental animals

Nile tilapia were obtained from an aquafarm in Guangzhou, Guangdong province, China. Prior to experimentation, healthy fish with body length of 8–10 cm were acclimated in glass jars at 28 °C for 1 week, and fish were fed with commercial pellets daily.

2.2. Sequence, structural, and phylogenetic analysis

cDNA and deduced amino acid sequences of *O. niloticus* IκBα (On-IκBα; XM_003445438 and XP_003445486) were obtained from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). The sequence data were analyzed using BLAST to derive a homologous comparison. The ARD homologues were predicted using SMART (Simple Modular Architecture Research Tool). Potential PEST sequences were found using the epestfind. Multiple sequence alignment was performed using ClustalW version 1.83. The presumed tertiary structures of ARD were established by combining SWISS-MODEL (http://swissmodel.expasy.org/) and PyMOL version 0.97. The phylogenetic tree of IκBα proteins was constructed via the neighbor-joining method with a bootstrap test (1000 replicates) using MEGA version 4.1.

2.3. Tissue distribution analysis of On-IκBα at the transcriptional level

Total RNA was extracted from the whole spleen, liver, head kidney, trunk kidney, and blood of Nile tilapia using TRIzol (Invitrogen) as per the manufacturer's instructions. First-strand cDNA was synthesized from the RNA using Reverse Transcriptase M-MLV (Promega). Then, the total cDNA products of different tissues were diluted for quantitative real-time RT-PCR by a SYBR Green fluorescence assay. The real-time RT-PCR results of On-IκBα were normalized to *O. niloticus* β-actin (XM_003443127). Quantitative primers specific for On-IκBα (forward: AAGGAGCAGCATAATGGTCGCA and reverse: CAGGTCAGGATGGGTC ACAGAG) and β-actin (forward: CGGAATCCACGAAACCACCTA and reverse: CCAGACGGAGTATTTACGCTCA) were designed based on the cDNA sequence of *O. niloticus* from GenBank. The real-time RT-PCR was performed on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad) using the SYBR Premix Ex Taq (Perfect Real Time) kit according to the manufacturer's protocol. On-IκBα expression levels were analyzed using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001).

2.4. Transcriptional expression analysis of On-IκBα during bacterial infection

Healthy Nile tilapia was infected with the pathogenic bacterium *Aeromonas hydrophila* by intraperitoneal injection. Each fish was injected with 50 μL of sterile phosphate-buffered saline (PBS) containing 4×10^6 CFU/mL *A. hydrophila*. Spleen from each group of fish was sampled at 6 h, 12 h, 24 h, and 3, 5, 8, 16 days after infection. A SYBR Green-based real-time RT-PCR assay was performed as described above to detect the dynamic changes in On-IκBα expression at the transcriptional level.

2.5. Isolation of spleen leukocytes

Spleen isolated from healthy or infected fish was mashed in a suitable amount of pre-cooled L-15 medium (Gibco) containing 2% fetal bovine serum. The tissue debris was filtered through a 300-mesh sieve. Following centrifugation of 800 × *g*, 4 °C for 5 min, the pellet was resuspended in 3 mL of L-15 medium. Then, leukocytes were separated using Percoll density gradient centrifugation. Briefly, Percoll (GE Healthcare) was diluted 9:1 (v/v) with 10 × PBS to obtain a 100% solution, which was further diluted with FBS-free L-15 medium to the final concentration of 34% or 52% (v/v). 4 mL of 52% Percoll was added to the bottom of 15-mL centrifuge tubes, followed by 4 mL of 34% Percoll, and the cell suspension was layered on top. Subsequently, the tubes were centrifuged at room temperature, 800 × *g* for 35 min, with the slowest acceleration and deceleration. The target phase containing leukocytes between the Percoll layers were collected and

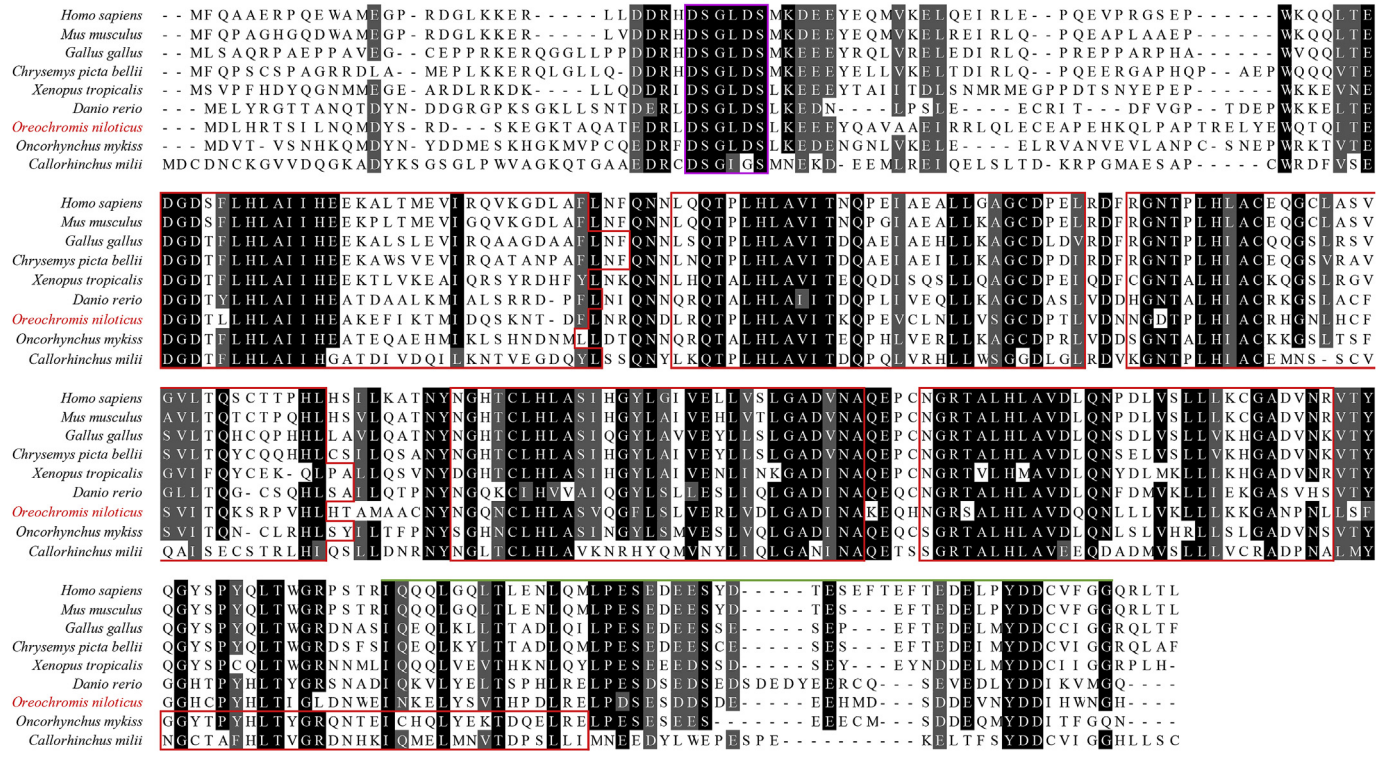

Fig. 1. Multiple sequence alignment analysis of On-IκBα amino acid with IκBα in other species. The same amino acid residues are in black background, and the 80% similar are in gray. The selected IκBα sequences include *Homo sapiens* (NP_065390), *Mus musculus* (NP_035037), *Gallus gallus* (NP_001001472), *Chrysemys picta bellii* (XP_005307579), *Xenopus tropicalis* (NP_001004851), *Danio rerio* (NP_955923), *Oreochromis niloticus* (XP_003445486), *Oncorhynchus mykiss* (ACO08648), *Callorhinchus milii* (AFP01506). In protein domain architecture, SRD, ARD and CTD are labeled with purple box, red box and green line, respectively. Two conserved and vital serine residues in IκBα are marked with a red asterisk.

washed with L-15 medium.

2.6. Phosphorylation analysis of On-IκBα and On–NFκB during primary immune response

Spleen leukocytes isolated from healthy or infected fish were centrifuged and resuspended in NP40 lysis buffer (Sigma) with freshly added protease inhibitor and phosphorylase inhibitor. The cells were lysed for 30 min on ice and then centrifuged at 4 °C, 13000 × *g* for 10 min. The protein supernatant was subjected to SDS-PAGE, and then transferred to nitrocellulose membrane. The membranes were blocked with 4% skim powdered milk in PBST (PBS containing 0.05% Tween-20) for 1 h. Then, the membranes were incubated with 1:1000-diluted rabbit anti-phosphorylated (p)-IκBα (CST), rabbit anti-p–NF–κB p65 (Affinity Biosciences), or mouse anti-β-actin (CST) antibodies at 4 °C overnight. Following three rinses in PBST, the membranes were then incubated with goat anti-rabbit/mouse IgG Alexa Fluor 790 (1:10000; Abcam) at room temperature for 1 h. After washing, the target proteins on the membrane were observed using the Odyssey CLx Image Studio system (LI-COR Biosciences).

2.7. Lymphocyte proliferation analysis during bacterial infection

To determine lymphocyte proliferation during *A. hydrophila* infection, healthy or infected fish were injected intraperitoneally with 500 μg of 5-bromo-2′-deoxyuridine (BrdU) in PBS one day before they were sacrificed. Spleen leukocytes were isolated at 0, 1, 3, 5, and 8 days after infection, and 2×10^6 cells were centrifuged at 2500 × *g*, 4 °C for 3 min into a 96-well V-bottom plate. BrdU staining was performed using the Cytofix/Cytoperm kit (BD Biosciences) as previously reported (Lin et al., 2016). Briefly, cells were fixed in BD Cytofix/Cytoperm for 30 min, permeabilized in BD Cytoperm Plus for 10 min, and placed in BD Cytofix/Cytoperm for another 5 min on ice. Between each permeabilization step, the cells were washed with BD Perm/Wash Buffer twice. Then, the cells were treated with 300 μg/mL DNase at 37 °C for 1 h, and stained with FITC-conjugated anti-BrdU antibody (BD; 1:100 diluted in BD Perm/Wash Buffer) for 20 min. The frequency of $BrdU^+$ lymphocytes was detected using a CytoFLEX flow cytometer (Beckman Coulter), and data were analyzed using FlowJo. Meanwhile, relative mRNA expression level of T/B cells marker, CD3ε/IgM, was examine by real-time RT-PCR with the following primers. CD3ε: forward, CTGGAGGACCAAAGTGACGCTG and reverse, CTCACACGCATTTCCTTCAACAT. IgM: forward, TGGCTTGTGGATGACGAGGA and reverse, AGCACTTGGAGTCTTGGTTGATG.

2.8. On-IκBα mRNA expression analysis after phytohemagglutinin (PHA) stimulation

The separated spleen leukocytes were resuspended in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. 10^7 cells in 1 mL DMEM was dispensed in 24-well cell plates and were stimulated with 5 μg/mL PHA for 0 h, 5 h or 10 h. The cells were incubated in an CO_2 incubator at 28 °C. Subsequently, the cells were collected at the indicated timepoints for first-strand cDNA synthesis and real-time RT-PCR, to analyze the On-IκBα mRNA expression pattern.

2.9. On-IκBα and On–NF–κB phosphorylation analysis after PMA stimulation

The spleen leukocytes were resuspended in 500 μL of DPBS (BBI Life Sciences), and incubated at 28 °C for 30 min to remove the original phosphorylation. The cells were then stimulated with 50 ng/mL PMA (Sigma) for 0 min, 10 min or 30 min at 28 °C. 500 μL pre-cooled DPBS was added to each group, and the cells were quickly placed on ice to

stop the stimulation. Protein samples were prepared for western blotting as described above to detect the On-IκBα and On–NF–κB phosphorylation. Rabbit anti-β-tubulin antibody (1:1000 in PBS, CST) was used as the internal control.

3. Results

3.1. Homologous sequences of On-IκBα

The deduced sequence of On-IκBα contained 318 amino acids translated from a 1483-bp mRNA comprising an 84-bp 5′ untranslated region, a 957-bp open reading frame, and a long 442-bp 3′ untranslated region. BLAST analysis of On-IκBα revealed 98%, 48%, and 37% sequence similarities with the IκBα of *Maylandia zebra* (XP_004559368), *Homo sapiens* (NP_065390), and *Mizuhopecten yessoensis* (XP_021370618), respectively. Multiple sequence alignment among On-IκBα and IκBα from eight other species showed that amino acids, especially those in the SRD and the first five ARD, were moderately conserved in vertebrates (Fig. 1). Two serine phosphorylation sites associated with activation and degradation signal reception (Brown et al., 1995), namely Ser33 and Ser37 in *O. niloticus*, were well conserved across all species. The IκBα from each species had its own potential PEST sequence, which is highly associated with IκBα degradation (Rechsteiner and Rogers, 1996; Van Antwerp and Verma, 1996). On-IκBα had similar abundance of PEST in the C-terminal domain (33%) compared to *Callorhinchus milii* IκBα (35%), both of which were lower than other teleost (45–51%) and higher vertebrates (38–49%).

3.2. Structural characteristics of On-IκBα

To investigate the structure of On-IκBα and to better understand its potential function, the ARD tertiary structures were modeled using SWISS-MODEL. The On-IκBα ARD tertiary structure shared high similarity with the homologues from other five species. They were all characterized by ankyrin repeats that allow binding to NF-κB. *H. sapiens*, *Gallus gallus*, and *O. niloticus* had five ankyrin repeats, whereas *Oncorhynchus mykiss*, *C. milii*, and *M. yessoensis* had six (Fig. 2). These results indicate that On-IκBα is a structurally conserved molecule that might exert similar functions as its homologues in higher vertebrates.

3.3. Phylogenetic profile of On-IκBα

A phylogenic tree was built using the whole amino acid sequences of On-IκBα and its counterparts from other 20 species. The phylogenic tree was consistent with the evolutionary taxonomy. On-IκBα clustered with IκBα from selected bony fish and then formed a sister group with Tetrapoda, while invertebrate IκBα formed an isolated group from vertebrate IκBα (Fig. 3). These findings suggest a close phylogenetic relationship between On-IκBα and its homologues in teleost or even mammals.

3.4. Tissue expression pattern of On-IκBα mRNA

To study the possible function of On-IκBα in the immune response, On-IκBα mRNA distribution pattern in lymphoid tissues were examined using real-time RT-PCR. In healthy fish, On-IκBα mRNA was widely expressed, including the spleen, liver, head kidney, trunk kidney, and blood, and with the highest abundance in the trunk kidney (Fig. 4). Expression level of On-IκBα in head kidney or trunk kidney was significantly higher compared with that in blood (Fig. 4). The distribution in the immune-related organs suggests that On-IκBα is potentially involved in the immune response.

3.5. Expression of On-IκBα during anti-bacterial immune response

To examine the potential involvement of On-IκBα in anti-bacterial immunity, we infected Nile tilapia with *A. hydrophila* and then detected the expression of On-IκBα at different phases of the immune response via real-time RT-PCR. After *A. hydrophila* infection, transcriptional level of On-IκBα in spleen did not obviously change at 6 or 12 h, but was significantly up-regulated at 24 h after infection (Fig. 5a). While the mRNA expression of On-IκBα returned to normal levels compared with

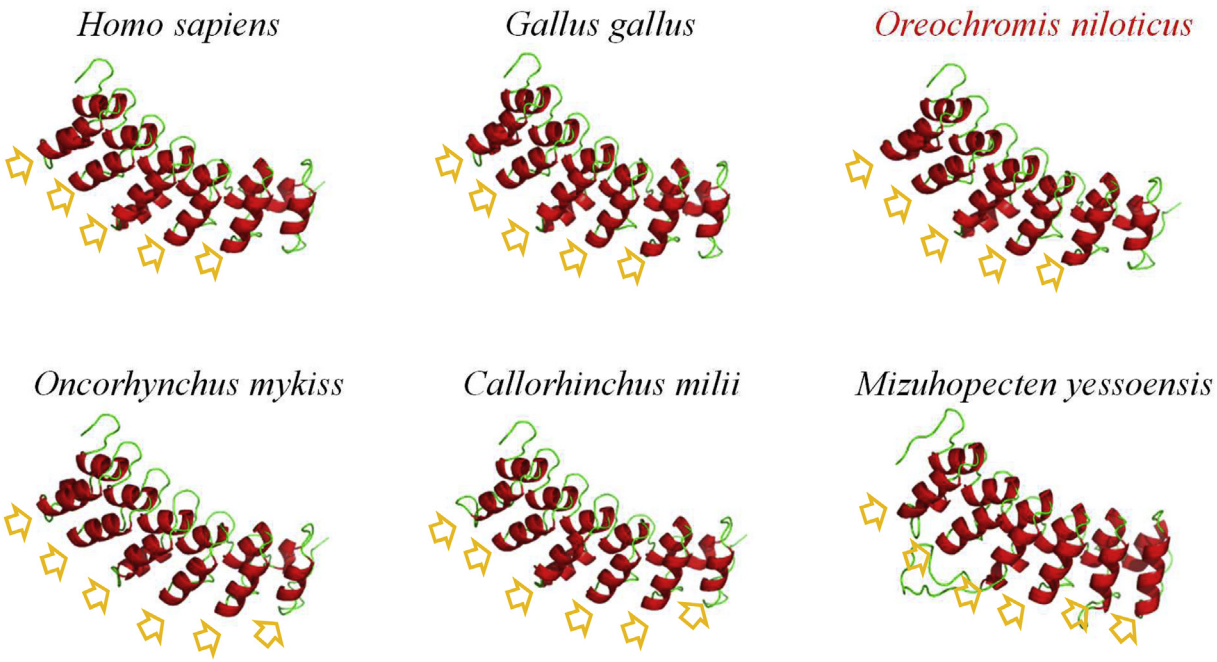

Fig. 2. Predicted tertiary structures of ARD of IκBα. IκBα from *H. sapiens* (NP_065390), *G. gallus* (NP_001001472), *O. niloticus* (XP_003445486), *O. mykiss* (ACO08648), and *M. yessoensis* (XP_021370618) were selected for homologous modeling. This diagram was generated by SWISS-MODEL online software. α-Helix are colored with red, loop and unassigned residues with green. Each predicted ARD is marked by an orange arrow. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

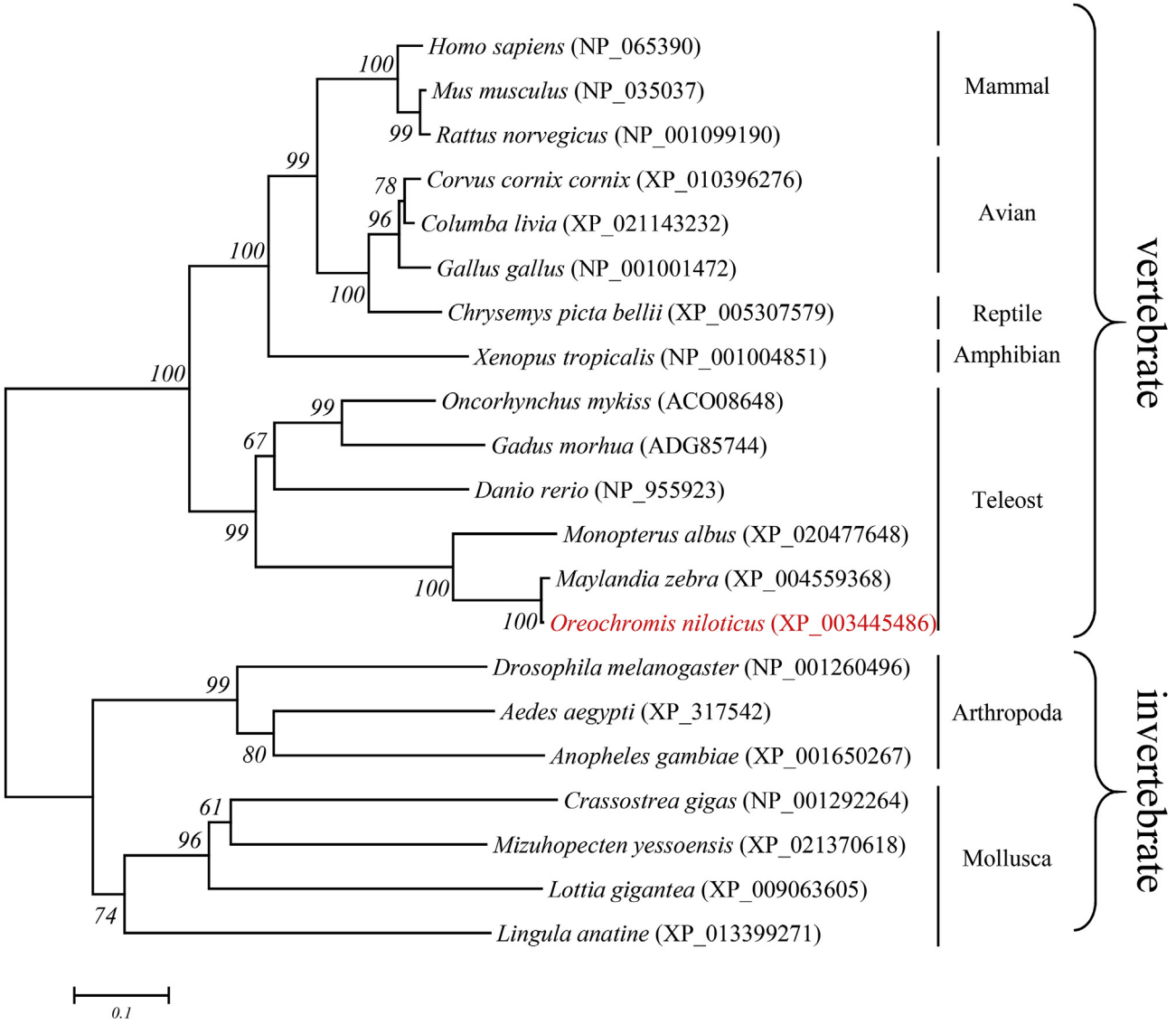

Fig. 3. A phylogenetic tree of IκBα family members. The tree was constructed by NJ method. Numbers for each branch indicated the percentage bootstrap values on 1000 replicates. The species names and GenBank accession numbers are included in the figure.

the control group at 3 or 5 days after infection (Fig. 5b). Subsequently, On-IκBα transcript was significantly upregulated for the second time at 8 days after infection, and then decreased to the original level again at 16 days after infection (Fig. 5b).

As almost all the leukocytes isolated from Nile tilapia spleen are lymphocytes using our isolation protocol (Wei et al., 2019), these cells are used for phosphorylation assay to exclude the affection from other cell lineages. The phosphorylation levels of On-IκBα or the downstream On–NF–κB p65 in spleen leukocytes were examined by western blotting after *A. hydrophila* infection. At the early stage of infection, phosphorylation level of On-IκBα in spleen leukocytes was comparable between infected and control group (Fig. 5c). While On-IκBα phosphorylation was slightly increased at 1 and 3 days, followed by an obvious augmentation at 5 days and a subsequent recovery to baseline level at 8 days after infection (Fig. 5d). The On–NF–κB p65 phosphorylation levels were not obviously increased at 1 and 3 days; however, enhanced phosphorylation was found at 5 and 8 days after infection (Fig. 5d), indicating the activation of NF-κB signaling during the adaptive immune response.

Together, these expression patterns of On-IκBα, as well as On-NF-κB p65, suggest that the activation of NF-κB signaling is an important event in the anti-bacterial adaptive immune response of Nile tilapia.

3.6. Lymphocyte proliferation during anti-bacterial adaptive immune response

To determine lymphocyte proliferation during the anti-bacterial immune response, we examined the frequency of BrdU-labeled newborn lymphocytes at the timepoints when On-IκBα or On–NF–κB p65 phosphorylation was elevated. Spleen leukocytes were used because the predominant population are lymphocytes (Wei et al., 2019). The frequency of lymphocyte population in spleen leukocytes did not obviously change during bacterial infection (Fig. 6a, upper panel). In healthy fish, the frequency of $BrdU^+$ lymphocytes, which represents the constitutive lymphocyte proliferation, was ~12% (Fig. 6a, bottom panel). At 1 day after infection, the frequency of $BrdU^+$ lymphocytes was almost the same as that in the control group. Lymphocytes began to proliferate at 3 days after infection, as revealed by the increased frequency of $BrdU^+$ lymphocytes, and lymphocyte expansion peaked at 5 days after infection, where ~50% of lymphocytes were $BrdU^+$ (Fig. 6a, bottom panel). Subsequently, the frequency of $BrdU^+$ lymphocytes began to decrease at 8 days after infection, although > 40% of the

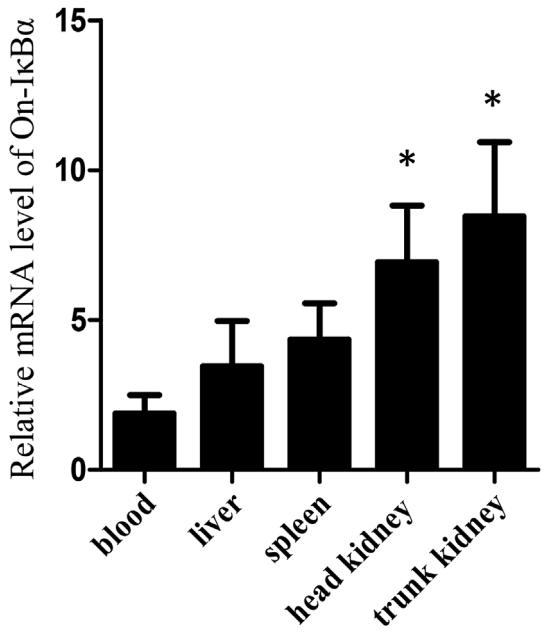

Fig. 4. The mRNA expression of On-IκBα in different tissues. On-IκBα expression levels in blood, liver, spleen, head kidney and trunk kidney were detected by real-time RT-PCR and normalized to β-actin. The vertical bars represent mean ± SE (n = 6). * represented $p < 0.05$, determined by 2-tailed Student's test.

lymphocytes remained BrdU$^+$ (Fig. 6a, bottom panel). Meanwhile, mRNA expression level of T or B lymphocyte marker, CD3ε or IgM, was also examined during bacterial infection. mRNA level of CD3ε was significantly up-regulated at 3 days after infection (Fig. 6b), while IgM transcription was obviously induced by bacterial infection at day 5 or 8 (Fig. 6c). These results suggest that lymphocyte proliferation is accompanied by the On-IκBα phosphorylation and On–NF–κB p65 activation, during the anti-bacterial immune response of Nile tilapia.

3.7. Regulation of On-IκBα expression during lymphocyte activation

Considering On-IκBα and On–NF–κB signaling may play roles in the adaptive immune response, we next investigated the potential participation of this signal in lymphocyte activation. On-IκBα mRNA expression was examined following PHA stimulation of spleen leukocytes in vitro. On-IκBα mRNA expression was slightly, but not significantly, increased after 5-h or 10-h stimulation as compared to the unstimulated control (Fig. 7a). This result suggests that mRNA regulation may not be the key means of On-IκBα involvement in lymphocyte activation.

To investigate whether lymphocyte activation correlates with the induced phosphorylation of On-IκBα or On–NF–κB, we detected On-IκBα and On–NF–κB p65 phosphorylation levels using western blotting after spleen leukocytes were stimulated with PMA (Castagna et al., 1982). PMA induced prompt IκBα and NF-κB p65 phosphorylation within 10 min as compared with the resting control (Fig. 7b). Then IκBα and NF-κB p65 phosphorylation both began to decline at 30 min after stimulation (Fig. 7b). These observations indicate that On-IκBα phosphorylation and the downstream On–NF–κB activation are crucial events that correlate with lymphocyte activation in Nile tilapia.

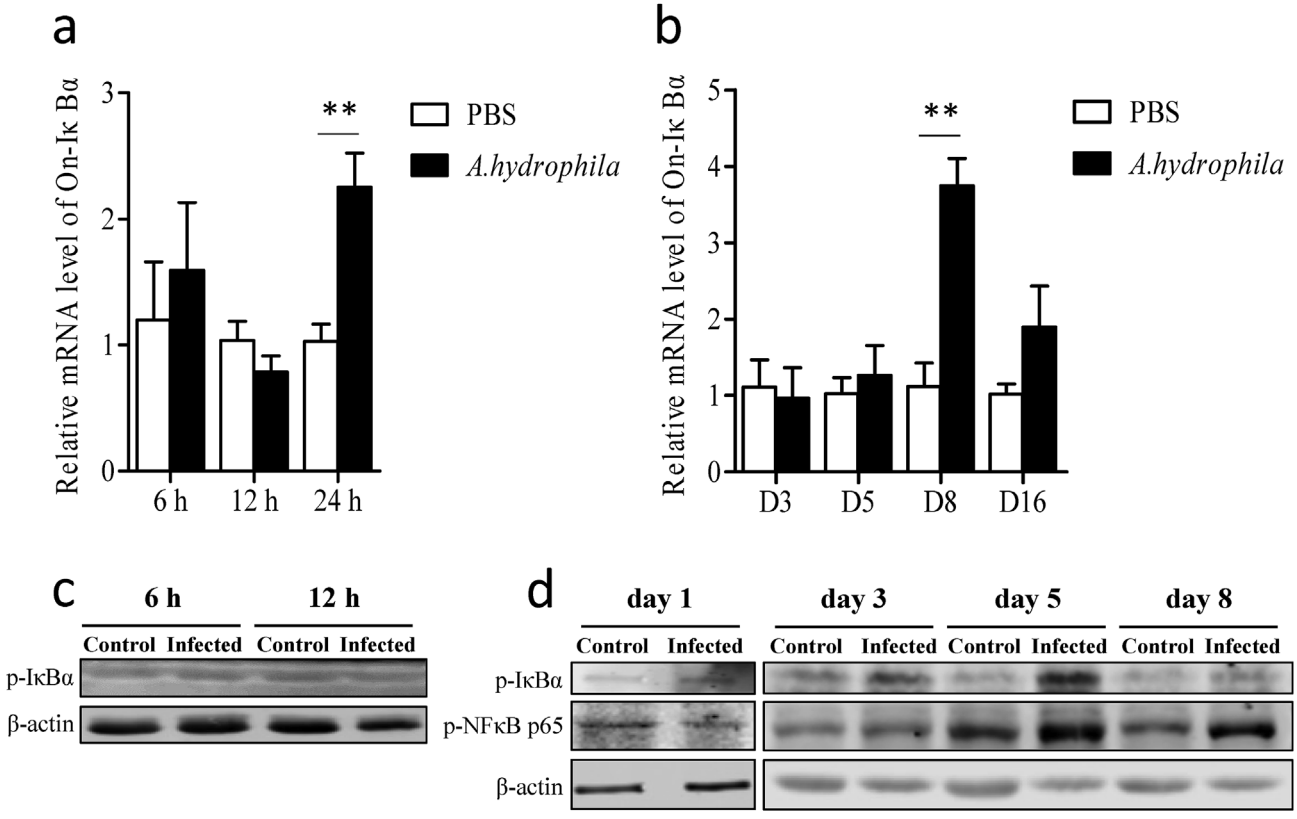

Fig. 5. Temporal expression of On-IκBα or On-NF-κB after *A. hydrophila* stimulation. The whole spleen or spleen leukocytes of infection group and PBS control group were collected at indicated timepoint after infection for real-time RT-PCR or western blotting assay. (a-b) The mRNA expression level of On-IκBα in spleen detected by real-time RT-PCR, vertical bars represent mean ± SE (n = 4). ** represented $p < 0.01$, determined by 2-tailed Student's test. (c–d) The phosphorylation of On-IκBα or On-NF-κB in spleen leukocytes after *A. hydrophila* infection.

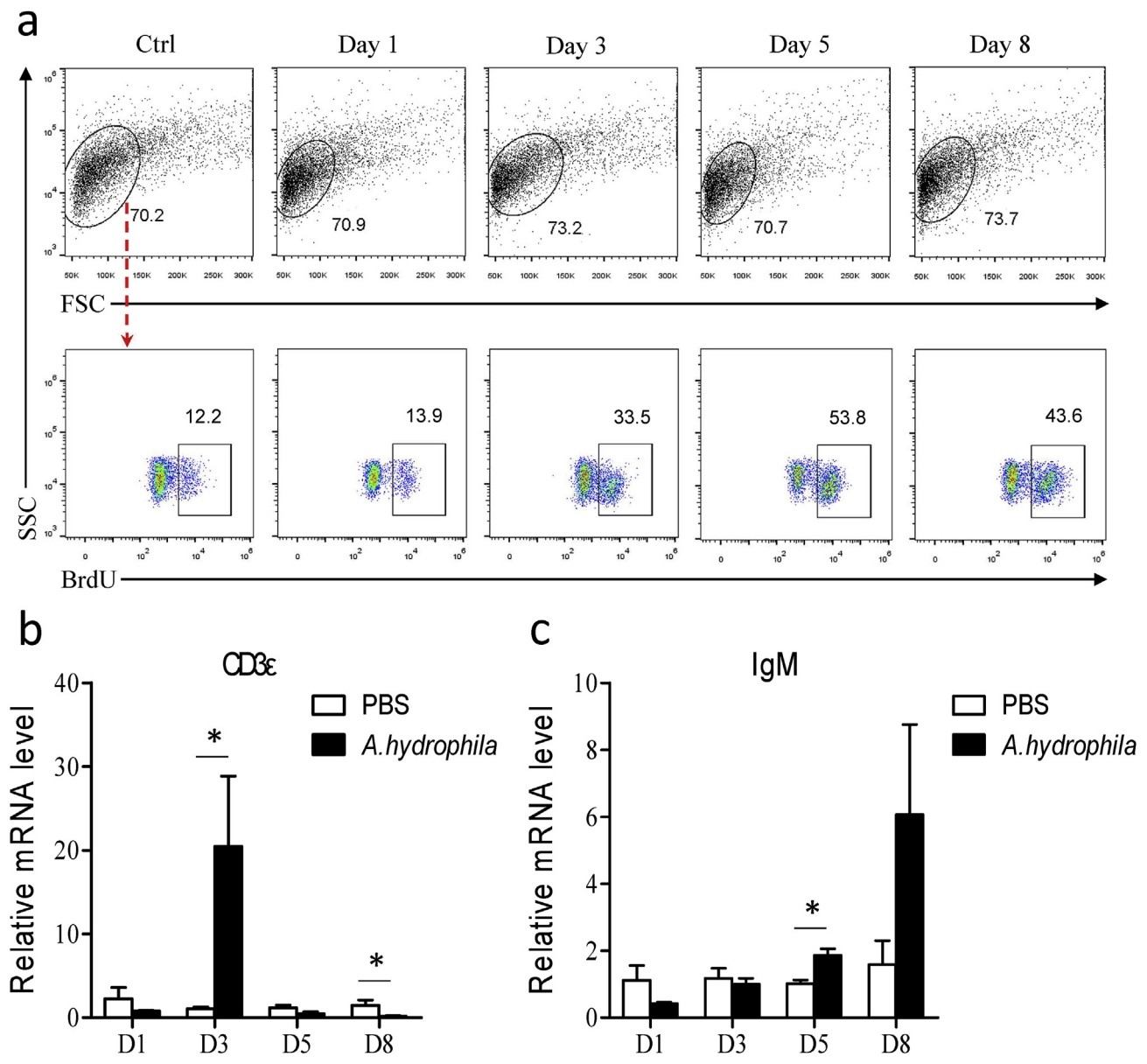

Fig. 6. Lymphocyte proliferation during *A. hydrophila* infection. The spleen leukocytes of healthy or infected fish, which were *i.p.* injected BrdU 24 h before sacrificed, were collected at day 1, 3, 5 or 8 after infection for BrdU staining assay. (a) The frequency of $BrdU^+$ cells (bottom panel) were analyzed by flow cytometer on gated lymphocyte population (upper panel). (b–c) The mRNA expression level of CD3ε (b) or IgM (c) in spleen leukocytes detected by real-time RT-PCR, vertical bars represent mean ± SE (n = 6). * represented $p < 0.05$, determined by 2-tailed Student's test.

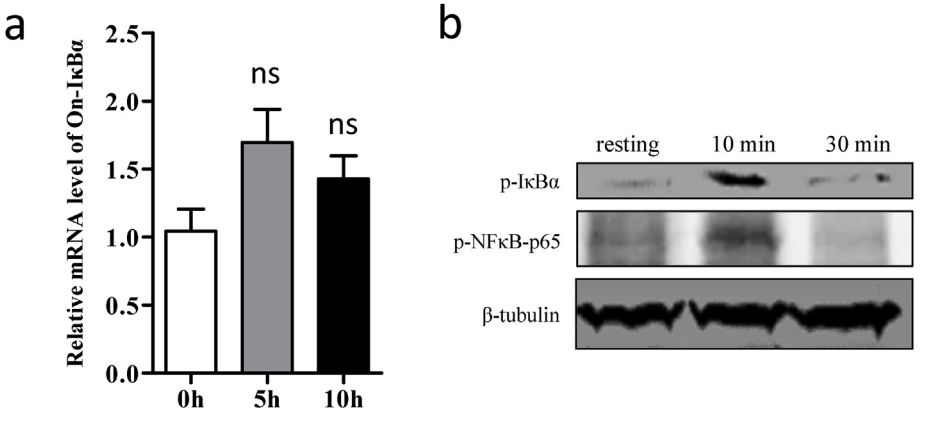

Fig. 7. Expression of On-IκBα or On-NF-κB during lymphocyte activation. Spleen leukocytes were stimulated with PHA or PMA in vitro for real-time RT-PCR or western blotting assay. (a) The mRNA expression level of On-IκBα detected by real-time RT-PCR at 5 h and 10 h post PHA stimulation. The vertical bars represent mean ± SE (n = 4).“ns”represented non-significant, determined by 2-tailed Student's test. (b) Phosphorylation of On-IκBα or On-NF-κB detected by western blotting assay at 10 and 30 min after PMA stimulation.

4. Discussion

IκBα, which plays an important role in the NF-κB pathway, is involved in various biological processes (Baldwin, 1996; Liu et al., 2017). Although IκBα has been well studied in higher vertebrates, the related research in bony fish remains preliminary. Here, we found IκBα phosphorylation and NF-κB activation in the teleost Nile tilapia were associated with anti-bacterial adaptive immune response, lymphocyte activation, and proliferation. Our results provide helpful evidence for understanding the adaptive immunity of teleost.

On-IκBα shares moderate similarity with its homologues in mammals and even in some mollusks, as revealed by sequence analysis in this study. On-IκBα has three characteristic features of IκBα: the SRD, as the target of phosphorylation and ubiquitination (Brown et al., 1995); the ARD, for direct protein–protein interaction with NF-κB (Jacobs and Harrison, 1998); and the PEST sequence, related to IκBα degradation (Rechsteiner and Rogers, 1996; Van Antwerp and Verma, 1996). The main difference in IκBα among the species discussed in the present study is the number of ankyrin repeats. The IκBα of higher vertebrates tends to have five ankyrin repeats, fewer than the six in invertebrates. Teleost appear to be a transitional group, with IκBα including both five- and six-repeat isotypes (Lee et al., 2014; Sangrador-Vegas et al., 2005; Wang et al., 2009, 2015; Yazawa et al., 2007). The potential relevance between the number of ankyrin repeats and IκBα functions, for example the specificity and affinity to NF-κB, requires further exploration. The distinction between vertebrate and invertebrate IκBα is the gap in the third and fourth ankyrin domains: in invertebrates, it is longish compared to that of the other ankyrin domains; by contrast, this phenomenon disappears in teleost and higher vertebrates, which might be due to advanced evolution. Multiple sequence alignment revealed the existence of two important serine residues in On-IκBα, i.e., Ser33 and Ser37, which are well conserved from mollusks to mammals. This result suggests the close association of IκBα phosphorylation with its biological function. Meanwhile, the structural similarities between On-IκBα and its mammalian counterparts suggest that On-IκBα may exert a similar function in the immune response of teleost as its homologues in mammals.

On-IκBα was constitutively expressed in the immune-related tissues of healthy Nile tilapia, including the blood, liver, spleen, and kidney. The On-IκBα tissue distribution agrees highly with that of previous studies on other teleost, which display wide IκBα distribution in most tissues (Sangrador-Vegas et al., 2005; Wang et al., 2009; Yazawa et al., 2007). As the spleen and kidney serve as two major lymphoid organs in teleost (Zapata et al., 2006), their high On-IκBα expression might be closely associated with immune-related functions. Moreover, the highest expression of On-IκBα in the trunk kidney differed from that in other teleost, where *Oplegnathus fasciatus* IκBα was poorly expressed in the kidney (Lee et al., 2014; Wang et al., 2009), and this interesting difference warrants further elucidation.

Mammalian IκBα plays a vital role in regulating the immune response to bacterial infection (Johannessen et al., 2013; Yamamoto and Takeda, 2008). On the other hand, bacteria resist the innate immune system by impeding the NF-κB pathway (Le Negrate, 2012). The teleost NF-κB signaling pathway participates in innate immunity via NOD-like receptors and Toll-like receptors (Bi et al., 2017; Huo et al., 2018; Li et al., 2015; Swain et al., 2013; Tang et al., 2012). To determine whether On-IκBα also participates in the adaptive immune response, we analyzed On-IκBα mRNA expression and protein phosphorylation levels following *A. hydrophila* infection. The transcriptional expression of On-IκBα in the spleen was dramatically upregulated in both innate and adaptive immune stages, suggesting its potential involvement in these processes. As IκBα is degraded rapidly after its phosphorylation and NF-κB release, IκBα transcription and re-synthesis are crucial events for preventing excessive NF-κB activation (Verma et al., 1995). Meanwhile, NF-κB signaling is indispensable for lymphocyte expansion, and mammalian lymphocytes lacking IκBα or NF-κB p65 have abnormal proliferation and immune function (Chen et al., 2000; Doi et al., 1997). Here, our data suggest obvious elevation of On-IκBα and On–NF–κB p65 phosphorylation levels in spleen leukocytes during the adaptive immune response of Nile tilapia. More importantly, the NF-κB phosphorylation augmentation was accompanied by significantly enhanced lymphocyte proliferation. The strong synchronization between NF-κB phosphorylation and prompt leukocyte proliferation primarily suggests a potential role for IκBα-regulated NF-κB signaling in the adaptive immune response of Nile tilapia. IκBα degradation and NF-κB nuclear translocation are crucial events for NF-κB signaling activation, however, owing to the lack of effective antibodies for total IκBα and NF-κB protein of Nile tilapia, at this stage we cannot detect the expression pattern of IκBα and NF-κB on the protein level during bacterial infection. Further study using Nile tilapia specific IκBα and NF-κB antibodies will provide more convincing evidences for NF-κB signaling activation, and IκBα or NF-κB phosphorylation assay.

The canonical NF-κB pathway (IKK–IκB–NF-κB) also plays an essential role in mediating the TCR signaling pathway and in T cell differentiation to several $CD4^+$ subsets (Oh and Ghosh, 2013). NF-κB activation and translocation could also be a foundation for production of the transcription factors T-bet, GATA3, and RORγt, which in turn facilitates T_H1, T_H2, and T_H17 cell differentiation, respectively (Aronica et al., 1999; Das et al., 2001; Ruan et al., 2011; Szabo et al., 2000). Here, Nile tilapia spleen leukocytes were stimulated with the T cell-specific mitogen PHA, which triggers T cell activation and proliferation (Movafagh et al., 2011), or with PMA, a protein kinase C (PKC)-specific activator that acts as a diacylglycerol analogue to activate the PKCθ–NF-κB pathway (Castagna et al., 1982). On-IκBα protein phosphorylation, but not On-IκBα mRNA expression, was strongly induced upon lymphocyte activation, which was accompanied by obvious phosphorylation of NF-κB p65. These results suggest that NF-κB activation regulated by IκBα phosphorylation is also a pivotal event during the activation of teleost lymphocytes. Since previous research has demonstrated the involvement of teleost IκBα in innate immunity (Lee et al., 2014; Sangrador-Vegas et al., 2005; Wang et al., 2015; Yazawa et al., 2007; Zhang et al., 2012a, 2012b), here, we rule out the close association between IκBα-regulated NF-κB signaling activation and lymphocyte activation in Nile tilapia, and suggest that On-IκBα might play a conserved role in lymphocyte signal transduction as compared to that in mammals.

In summary, in this study, we demonstrate the evolutionary conservation of IκBα in Nile tilapia. We also reveal the involvement of IκBα in the adaptive immune response of Nile tilapia at both mRNA expression and protein phosphorylation levels during bacterial challenge. More importantly, we suggest that IκBα phosphorylation and the downstream NF-κB activation are essential events associated with lymphocyte activation and proliferation in Nile tilapia. Our study aids better understanding of the lymphocyte-mediated adaptive immunity of teleost species.

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