Expressions of IL-6, TNF-α and NF-κB in the skin of Chinese brown frog (*Rana dybowskii*)

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Abstract

The cytokine interleukin-6 (IL-6) mediates a wide range of inflammatory and immune responses. Tumor Necrosis Factor α (TNF-α) has a myriad of pro-inflammatory effects on the skin. Nuclear factor κB (NF-κB) is a transcriptional factor that regulates a battery of genes that are critical to immune system. In this study, we investigated the localizations and expression levels of IL-6, TNF-α and NF-κB in the skin of *Rana dybowskii* during the breeding period and pre-hibernation. Histologically, the skin of *Rana dybowskii* consisted of epidermis and dermis. Four kinds of cells were identified in the epidermis, while the dermis was composed of homogenous gel, mucous glands and granular glands. IL-6, TNF-α and NF-κB were immunolocalized in the epithelial and glandular cells in both periods. Western blotting showed that IL-6, TNF-α and NF-κB were significantly higher in the pre-hibernation compared to the breeding period. Real-Time PCR revealed that the relative mRNA levels of IL-6 and NB-κB in the pre-hibernation increased significantly compared with the breeding period, while the TNF-α mRNA expression levels were not significantly different between these two periods. These results suggested that IL-6, TNF-α and NF-κB might collectively be involved in the skin immune system of *Rana dybowskii* during the breeding period and pre-hibernation.

Introduction

Amphibian skin is naked and directly exposed to harsh environments and damaged by varieties of external factors, such as predators, microorganisms, parasites, and some physical injuries.[1,2] As the first line of defensing against external infection, the skin provides the most important barrier against environmental influences. Wound healing, regeneration and the development of immune tolerance are main functions of the skin immune system.[3] Moreover, the skin, as a biochemically and physiologically complex organ, has functions of defensing against predators and microorganisms, which makes amphibians thrive in a wide range of habitats and ecological conditions.[1,4] The secretions of cytokines by epidermal keratinocytes, particularly tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β), play a key role in various immunological disorders and inflammation in the skin.[5,6] However, most relative reports were about the roles of IL-6, TNF-α and NF-κB in mammals skin, there were few reports about the physiological roles of IL-6 and TNF-α in amphibian skin, and until now there were no reports about the changes in the expressions of IL-6 and TNF-α in amphibian skin during different physiological states.

Cytokines are mediators with multiple functions, including the initiation or influence of numerous biological processes, such as, inflammation, sepsis and wound healing.[7,8] The pro-inflammatory cytokines IL-6 and TNF-α play key roles within the cytokine network.[9] As a multifunctional cytokine, IL-6 is involved in the regulation of growth of various malignant tumors and inflammation.[10] IL-6 is produced by various types of cells, such as leukocytes, keratinocytes, endothelial cells, fibroblasts, and some tumor cells. What's more, IL-6 is frequently associated with the early stages of host defense and mediates a wide range of inflammatory and immune responses.[11,12] IL-6 contributes to the growth and differentiation of numerous cell types, including those of dermal and epidermal origin[13] and is closely linked to skin wound healing.[14] IL-6 treatment also appears to modulate *stratum corneum* regeneration and skin barrier function[15] to maintain skin homeostasis. TNF-α acts as a mediator of both natural and acquired immunity, which could regulate many cellular and biological processes such as immune function, proliferation, cell differentiation, apoptosis and energy metabolism.[16] TNF-α plays an important role in host defense against viral, bacterial, fungal, and parasitic pathogens, in particular against intracellular bacterial infections, such as *Mycobacterium tuberculosis* and *Listeria monocytogenes*.[17] In addition, TNF-α participates in re-epithelialization and neovascularization and has a beneficial effect on tissue repair of the skin.[18,19]

Nuclear factor κB (NF-κB) is a transcriptional factor that regulates a battery of genes that are critical to innate and adaptive immunity, cell proliferation, inflammation, tumor development and inhibition of apoptosis.[20] NF-κB is involved in the activation of immune cells by upregulating the expressions of many cytokines (such as IL-1β, IL-6, IL-18 and TNF-α), which are essential for the immune response.[21] In the skin, NF-κB regulates the expressions of many genes that are involved in the initiation of the inflammatory response, including cytokines, adhesion molecules and chemokines, matrix metalloproteases, and nitric oxide synthase.[22]

The Chinese brown frog (*Rana dybowskii*) is distributed throughout China, Korea, Japan, and eastern Siberia.[23] Depending on the latitude and altitude, the hibernation of *Rana dybowskii* is from October to February next year, which is followed by the breeding period from February to June.[24] The skin of *Rana dybowskii* has been used extensively in traditional Chinese medicine to heal burnt wounds because of the antimicrobial components, which may contribute to efficacy in wound healing.[25] Our previous study demonstrated the presence and seasonal expressions of IL-1β and IL-1R in the *Rana dybowskii* skin,[26] which suggested that

IL-1β might play important role in the skin immune system of *Rana dybowskii* during the breeding period and pre-hibernation. In this study, we investigated the expressions of NF-κB, IL-6 and TNF-α in the skin of *Rana dybowskii* during the breeding period and pre-hibernation, in order to elucidate whether IL-6, TNF-α and NF-κB were collectively involved in the skin immune system of *Rana dybowskii.*

Materials and Methods

Animals

Fifty adult female Chinese brown frogs were obtained in April (breeding period, n=25) and October (pre-hibernation, n=25) from Jilin Baektu Mountain Chinese Brown Frog Breeding Farm, Jilin Province (125°40E-127°56E, 42°31N-44°40N), China. All animals were treated in accordance with the National Animal Welfare Legislation. All experimental procedures were conducted by the guidelines established and approved by the Beijing Forestry University. Skin samples from the back of *Rana dybowskii* were obtained after euthanized by 4% isoflurane. Part of the skin samples were fixed for 24 h in 4% paraformaldehyde in 0.05 M PBS, PH 7.4, for histological and immunohistochemical analysis. The remaining samples were stored at -80°C for protein and RNA extraction.

Histology

The skin samples were dehydrated by a certain concentration of ethanol (80% for 30 min, 90% for 30 min, 95% for 30 min, 100% for 45 min for twice) and xylene (10 min each, three times), and immersed in paraffin (1 h each, three times) before embedding. Serial sections (6 μm) were mounted on slides coated with poly-l-lysine (Sigma-Aldrich, St. Louis, MO, USA). Sections were stained with hematoxylin and eosin (HE) for general histological observations.

Immunohistochemistry

The skin sections were deparaffinized, rehydrated and immunostained using conventional methods as below. The sections were incubated with 10% normal goat serum to reduce background staining caused by the secondary antibody. And then the sections were incubated with primary polyclonal antibody against IL-6 (1:200) (bs-0379R, Beijing Biosynthesis Biotechnology Co., Beijing, China), TNF-α (1:200)

(bs-2150R, Beijing Biosynthesis Biotechnology Co.), NF-κB (1:200) (bs-0465R, Beijing Biosynthesis Biotechnology Co.) for 12 h at 4°C. The control sections were treated with normal rabbit IgG rather than the primary antibody. The sections were then incubated with a secondary antibody, goat anti-rabbit lgG conjugated with biotin and peroxidase with avidin, using a rabbit ExtrAvidin staining kit (Sigma-Aldrich), followed by visualizing with 30 mg 3,3-diaminobenzidine (Wako, Tokyo, Japan) solution in 150 mL of 0.05 M Tris-HCl buffer, plus 30 μL H_2O_2. Finally, the reacted sections for IL-6, TNF-α and NF-κB were counterstained with hematoxylin solution (Merck, Tokyo, Japan). The specificity of the NF-κB antibodies in this amphibian was described by previous study.[26]

Western blotting

Dorsal skin was weighed and diced into small pieces. The tissues were homogenized in a homogenizer containing 300 μL of 10 mg/mL phenylmethanesulfonyl fluoride (PMSF) stock and incubated on ice for 30 min throughout all the procedures. Homogenates were centrifuged at 12,000 × g for 10 min at 4°C. Protein extracts (25 μg) were mixed with an equal volume of 2 × Laemmli sample buffer. Equal amount of each sample was loaded and ran on a 12% (for IL-6, TNF-α) or 10% (for NF-κB) SDS-PAGE gel at 18 V/cm, and then transferred to nitrocellulose membranes using a wet transblotting apparatus for 20 min (Bio-Rad, Richmond, CA, USA). The membranes were blocked in 2% bovine serum albumin for 1 h at room temperature. Primary incubation of the membranes was carried out using a 1:200 dilution of rabbit anti-rat IL-6, TNF-α and NF-κB, which were the same as used in immunohistochemistry, for overnight. Secondary incubation of the membranes was then carried out using a 1:1000 dilution of goat anti-rabbit or anti-mouse IgG tagged with horseradish peroxidase for 1 h. The membrane was then stained with 10 mg 3,3-diaminobenzidine (Wako) solution in 50 mL phosphate buffer (0.03 M) plus 3 μL H_2O_2. β-actin was used

for the endogenous control. Preabsorptions of the antibodies were performed with an excess of relative antigens (Sigma Chemical Co.) for the negative control. The intensities of the bands were quantified using Quantity One software (ver. 4.5, Bio-Rad Laboratories, Shanghai, China).

RNA isolation

Total RNA from skin tissue sample was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol. Approximately 0.1 g of skin tissues were pulverized in liquid nitrogen and immediately homogenized in 1 mL of TRIzol Reagent. The homogenate was placed at room temperature for 5 min so that the nucleoprotein complexes were separated completely. The mixture was vigorously shaken for 15 sec at room temperature after the addition of 0.2 mL of chloroform and then centrifuged at 12,000 × g for 15 min at 4°C. The aqueous phase was transferred to a fresh tube and 0.8 times volume of isopropanol (Beijing Hondar collet Technology Co., Beijing, China) was added. Samples were then kept at room temperature for 10 min. RNA was precipitated by centrifugation at 13,000 × g for 10 min at 4°C. Washing the RNA pellet with 70% ethanol for twice, allow it to air dry. The RNA was then dissolved in 60 μL of diethyl-procarbonate-treated water (Beijing Hondar collet Technology Co., Beijing, China).

Real-time PCR Analysis

The mRNA expressions of IL-6, TNF-α and NB-κB during the breeding period and pre-hibernation were analyzed by real-time PCR using one-step SYBR PrimeScript RT-PCR kit (TaKaRa Company, Dalian, China). Tissues dissected from 3 to 10 individuals were pooled from *Rana dybowskii* to analyze expression in the skin. The primers for real-time PCR analysis were designed using the Primer 3 program (Table 1). The PCR reactions were carried out in a 20 μL volume and performed with ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following conditions: reverse transcription at 42°C for 5 min and 95°C for 30 s,

Table 1. Oligonucleotide primers used for quantitative Real-Time PCR.

Gene	Primer sequence	Product size (bp)
IL-6	GCCAGTTGCCTTCTTGGG (forward) CGACTTGTGAAGTGGTATA (reverse)	107bp
TNF-α	TACACCTCACCCACACAGTC (forward) CGCACCTCACAGACTGTTTT (reverse)	91bp
NF-κB	GAAAAGTCACAGCCAGCCA (forward) CGCTGTCACACAAACTGTCA (reverse)	136bp

followed by PCR reaction of 40 cycles at 95°C for 5 s and 60°C for 34 s and dissociation protocol. Transcript levels of the target genes were normalized to the β-actin after correcting for differences in amplification efficiency. The expression level of each target mRNA relative to β-actin mRNA was determined using the $2^{-\Delta\Delta Ct}$ method.

Sequence analysis

DNA sequence was determined using the ABI-PRISM 3730 sequencer (Invitrogen). Searching of similar sequences was performed using BlastP in the nonredundant (nr) protein sequences database of the NCBI website.

Statistical analysis

Statistical comparisons were made with the Student's *t*-test using the SPSS computer package. A value of P<0.05 was considered as an indication of statistical significance. Mean values within the columns marked with asterisk were used to indicate significant difference.

Results

Histological structure of *Rana dybowskii* skin

Dorsal skin structural observations of the *Rana dybowskii* during the breeding period and pre-hibernation were shown in Figure 1 a,b. The skin of *Rana dybowskii* was composed of epidermis and dermis. The epidermis consisted of *stratum corneum, stratum granulosum, stratum spinosum and stratum germinativum.* The dermis was composed of *stratum spongiosum and stratum compactum*. The *stratum spongiosum* matrix was a homogenous gel, with mucous glands and granular glands embedded therein (Figure 1 a,b). The *stratum compactum* was mainly composed of collagen fibers, which were arranged in a tight, parallel wavy shape. Pigment cells were located between the epidermis and the dermis. They accumulated to the patch in the breeding period, while were dispersed in pre-hibernation period (Figure 1 c,d).

Immunolocalizations of IL-6, TNF-α and NB-κB in *Rana dybowskii* skin

Immunoreactivities of IL-6, TNF-α and NF-κB were detected in the skin of *Rana dybowskii* during the breeding period and pre-hibernation (Figure 2). The expressions of IL-6 (Figure 2 a,b) and TNF-α (Figure 2 c,d) were localized in both the epidermal cells and gland cells of the breeding period and pre-

hibernation. In addition, NF-κB was also expressed in the epidermal cells and gland cells of dorsal skin (Figure 2 e,f). A substantial decrease in tissue immunostaning was observed after pre-absorption of the polyclonal antibody against IL-6, TNF-α and NF-κB with the specific recombinant proteins (Figure 2 g,h).
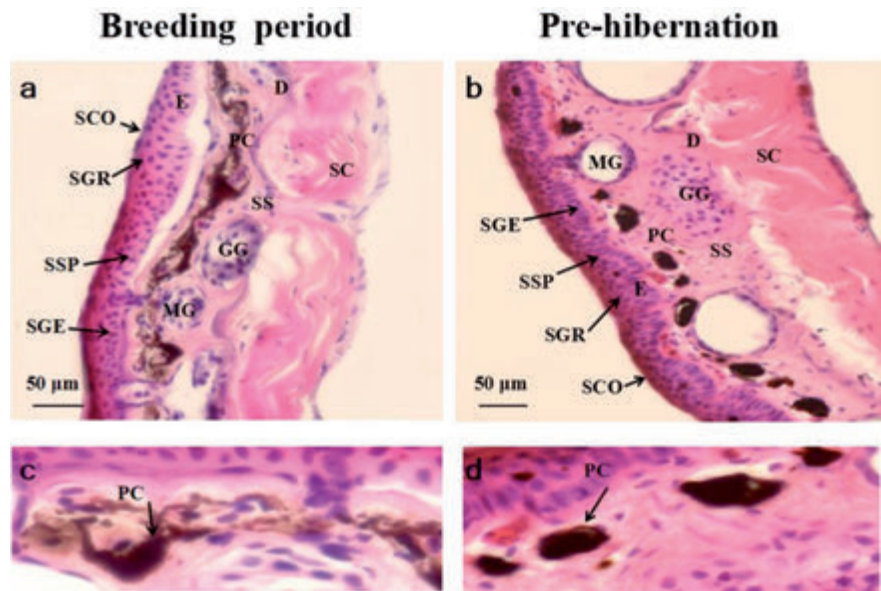

Figure 1. Structure observations of the skin from *Rana dybowskii* during the breeding period (a) and pre-hibernation (b). Pigment cells in the dermis during the breeding period (c) and pre-hibernation (d). E, epidermis; D, dermis; SCO, *stratum corneum*; SGR, *stratum granulosum*; SSP, *stratum spinosum*; SGE, *stratum germinativum*; SS, spongy layer; SC, dense layer; PC, pigment cells; MG, *mucus* glands; GG, granular glands.
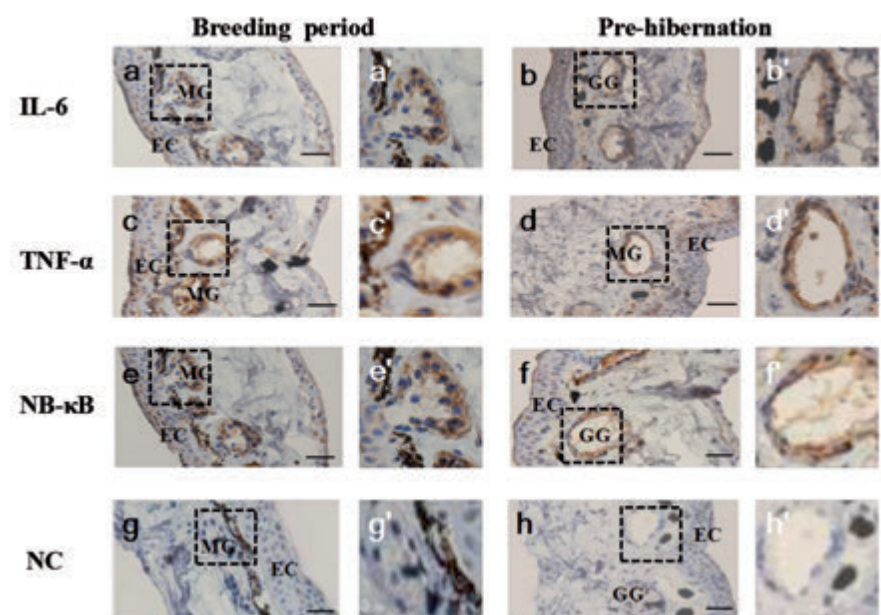

Figure 2. Immunohistochemical staining of IL-6, TNF-α and NF-κB was performed in skin of *Rana dybowskii* during the breeding period and pre-hibernation. Positive signal of IL-6 was localized in both the epidermal cells and glandular cells of the breeding period and pre-hibernation (a, b). The expression of TNF-α was observed in the epidermal and glandular cells of both period (c, d). Nuclear factor κB (NF-κB) was also localized in the epidermal and glandular cells of both period (e, f). No immunostaining was detected in the negative control sections (g, h). EC, epidermal cells; MG, mucous gland; GG, granular glands. NC, negative control. Scale bars: 50 μm.

Expressions of IL-6, TNF-α and NF-κB in *Rana dybowskii* skin

The results of Western blotting analysis for IL-6, TNF-α and NF-κB in dorsal skin of *Rana dybowskii* of the breeding period and pre-hibernation were shown in Figure 3. Bands of approximately 28 kDa, 33 kDa and 61 kDa represented IL-6 (Figure 3a), TNF-α (Figure 3b), and NF-κB (Figure 3c), respectively. The quantification was normalized to the expressions of endogenous control β-actin. The protein concentrations of IL-6, TNF-α and NF-κB were significantly higher in pre-hibernation compared with the breeding period (Figure 3 a-c). The primary antibodies pre-absorbed with an excess amount of the antigens were used as the negative control (Figure 3 lane NC).

IL-6, TNF-α and NF-κB mRNA levels were detected in skin tissues of *Rana dybowskii* during the breeding period and pre-hibernation. The results showed that the relative mRNA levels of IL-6 and NF-κB in the pre-hibernation increased significantly compared with those in the breeding period (Figure 4 a,c), while the expression level of TNF-α mRNA in pre-hibernation was higher than that in the breeding period, but there was no significant difference between the breeding period and pre-hibernation (Figure 4b).

Discussion

The present study was the first attempt to investigate the seasonal immunolocalizations and expression patterns of IL-6 and TNF-α in the skin of *Rana dybowskii*. Our results demonstrated the presence of IL-6, TNF-α and NF-κB in the epithelial and gland cells in both the breeding period and pre-hibernation. Moreover, Western blotting data showed that IL-6, TNF-α and NF-κB protein levels were significantly higher in pre-hibernation compared to the breeding period. The mRNA expression levels of IL-6 and NF-κB were obviously increased during pre-hibernation, while there was no significant difference in the expression of TNF-α between these two periods. These findings suggested that IL-6 and TNF-α might participate in regulating *Rana dybowskii* skin function during the breeding period and pre-hibernation.

In amphibians, the mucus secreted by mucous glands helps to maintain a moist, slippery skin surface and prevents mechanical damage to the delicate skin. It also protects the skin from the harmful effects of prolonged contact with water, retards evaporative water loss and possesses a bacteriostatic effect.[2] The granular glands can synthesize a wide range of chemical compounds, which provide protection against bacterial and fungal infection as well as predators.[27] Salamanders (*Salamandra salamandra*) deprived of their skin gland secretions soon died from a variety of infections unless kept under sterile conditions,[28] indicating that the glands were important to
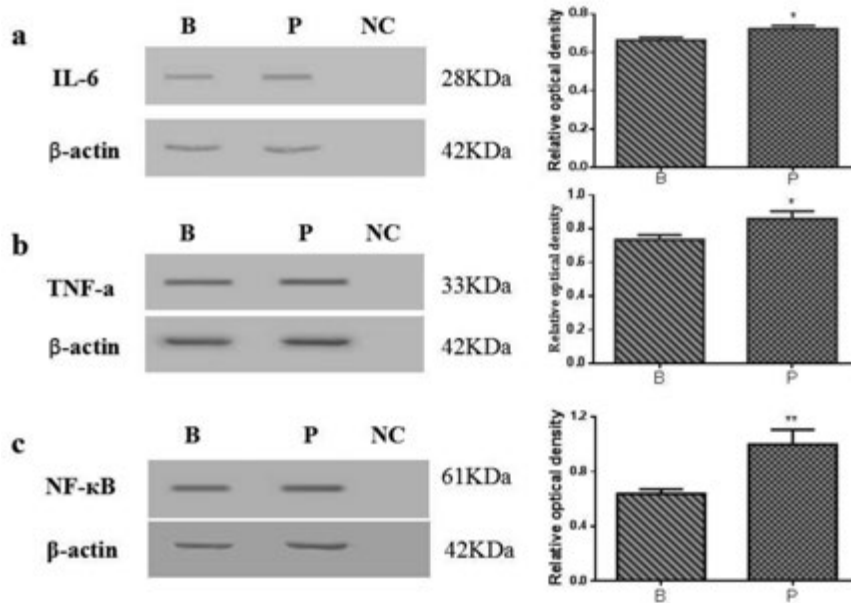

Figure 3. The results of Western blotting analysis for IL-6, TNF-α and NF-κB in dorsal skin of *Rana dybowskii* of the breeding period (n=10) and pre-hibernation (n=10). Bands of approximately 28 kDa for IL-6 (a), 33kDa for TNF-α (b), and 61 kDa for NF-κB (c). The pre-absorbed primary antibody was used instead of primary antibody for the negative control (lane NC). The expression levels were determined by densitometric analysis. Bars represent means±SD for three independent experiments. B, breeding period; P, pre-hibernation period; *P<0.05; **P<0.01.

Figure 4. Real-time PCR results of IL-6, TNF-α and NF-κB in dorsal skin of *Rana dybowskii* of the breeding period (n=10) and pre-hibernation (n=10). The relative mRNA levels of IL-6, TNF-α and NF-κB in the skin of *Rana dybowskii* during breeding period and pre-hibernation were shown in (a), (b), and (c), respectively. Bars represent mean ±SD for three independent experiments. B, breeding period; P, pre-hibernation period; *P<0.05.

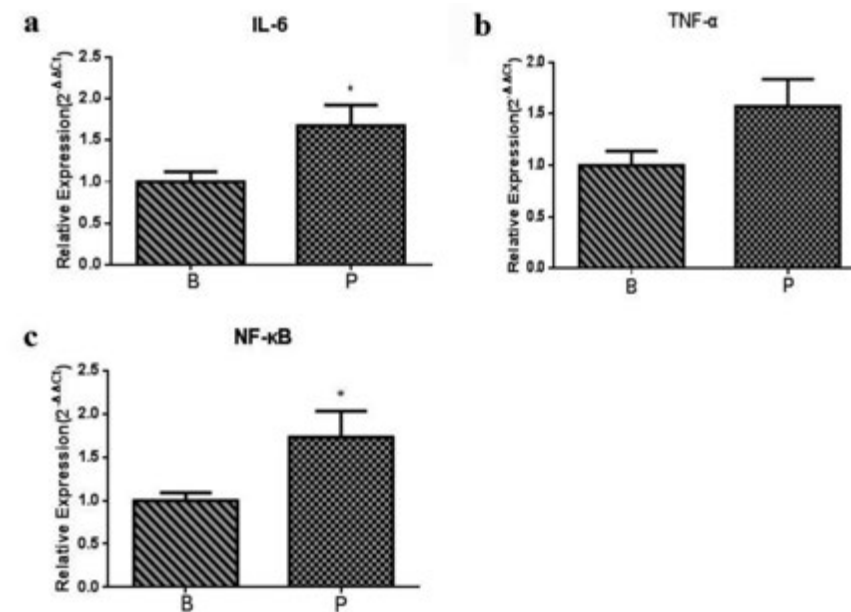maintain the skin immune system. Studies have also shown that there were 155 proteins in the skin mucus of Chinese giant salamander and these proteins participated in varieties of physiological activities, including defense, immune response, wound healing, respiration.[29] Our previous study demonstrated the expressions of IL-1β and IL-1R in the skin glandular cells of *Rana dybowskii*, which indicated that glandular cells had abilities to synthesize and secrete cytokines that were involved in the skin inflammation and immune response.[26] At present, we further proved that glandular cells could express pro-inflammation cytokines IL-6 and TNF-α, which suggested that the mucous glands and granular glands might play vital roles in the immune function of *Rana dybowskii* skin by secreting immune proteins and cytokines.

IL-6 is a multifunctional cytokine involved in regulation of immune responses, acute-phase responses, hematopoiesis, and inflammation.[17] Evidence have shown that IL-6 deficiency exacerbated the skin inflammation in an irritant dermatitis murine model, which suggested that IL-6 acted in an anti-inflammatory manner during irritant dermatitis.[30] Lin *et al.* proved that IL-6 had a crucial role in the skin wound-healing process by regulating leukocyte infiltration, angiogenesis, and collagen deposition.[14] IL-6 was involved in the growth and differentiation of dermal and epidermal cells, and acted as a chemotactic factor for T cells.[31] In this study, we examined IL-6 expression levels during the breeding period and pre-hibernation, which indicated that IL-6 might potentially be secreted in the skin of *Rana dybowskii* and participate in the skin's biological function, such as host defense.

TNF-α is a proinflammatory cytokine that controls multiple cellular processes, such as, the production of inflammatory mediators, cell proliferation and survival and different modalities of cell death, which are intricately linked to the epithelial response to injury.[32] It exerts pleiotropic effects on various cell types and plays a critical role in the pathogenesis of chronic inflammatory diseases.[33,34] There were excellent evidence that TNF-α in the skin induced adhesion molecules and chemokines in the skin, leading to attachment of inflammatory cells to vessels, rolling, emigration, and eventually chemotaxis into the skin.[35,36] In this study, we demonstrated the expression of TNF-α at the protein and molecular level in the skin of *Rana dybowskii*, thus suggesting that the skin could synthesize and secrete TNF-α

which might participate in the immune defense function of the skin. Besides, TNF-α had been shown to promote the immune/inflammatory reactions via the activation and induction of cytokines IL-6 and IL-1β.[37] The expression of IL-1β in the skin of *Rana dybowskii* has been previously demonstrated.[26] Combining the expressions of IL-6 and TNF-α in the skin at present study, we speculated that IL-6, TNF-α and IL-1β might play a synergistic role in mediating the progression of immune/inflammation in the skin of *Rana dybowskii.*

NF-κB was involved in cellular responses to stimuli, and plays a key role in regulating the immune response to infection becoming activated by cytokines and bacterial and viral antigens.[38,39] NF-κB also played an important role in regulating the expressions of the IL-1 and IL-17 cytokine families as well as IL-6 and TNF-α.[40] Furthermore, some of these cytokines activate NF-κB themselves, thus initiating an autoregulatory feedback loop. TNF-α was believed to mediate immune responses and inflammation by activating the NF-κB signaling pathways. NF-κB activation subsequently leads to increased expressions of other inflammatory cytokines, such as IL-6 and IL-1β, and endothelial adhesion molecules.[41] Therefore, the presence of NF-κB, IL-6 and TNF-α in the skin of *Rana dybowskii* raised the possibility that NF-κB signaling pathway might be crucial for IL-6 and TNF-α activity in the skin of *Rana dybowskii.*

Interestingly, the protein expressions of NF-κB, IL-6 and TNF-α were significantly higher in pre-hibernation compared to those of the breeding period. Seasonal immune function had often been observed in vertebrates.[42] As an amphibian, *Rana dybowskii* goes into hibernation during the winter. Hibernation is a survival strategy enabling animals to cope with low temperatures and food restriction in winter.[43] The skin separates the external environment from the internal milieu of the body. Reduced temperature of the habitat during winter affect physiological processes that take place in the skin of frogs, including respiration and transport of water and ions.[44] One strategy to increase survival is to enhance immune function prior to the onset of the poor conditions that may compromise it.[45] Expressions of TNF-α, IL-1β, IL-1α and IL-6 mRNA in alpacas and llamas exposed to cold weather were increased quite significantly: these cytokines might participate in the immune and inflammatory responses against adverse weather conditions.[46] The expressions of NF-κB, IL-6 and TNF-α were significantly increased in pre-hibernation,

which suggested that IL-6 and TNF-α immune response pathway in the skin, mediated by NF-κB transcriptional regulation, might be enhanced in pre-hibernation, for the purpose of improving defense against bacterial infections during hibernation.

In conclusion, the present study demonstrated the presence and seasonal expressions of IL-6, TNF-α as well as NF-κB in the *Rana dybowskii* skin. These data shed light to the understanding on the roles of cytokines including IL-6, TNF-α as well as NF-κB in the skin of hibernating amphibians. Further studies are needed to assess the concentrations of IL-6 and TNF-α in skin tissues of frog and clarify whether upstream signaling molecules of NF-κB signal pathway are present in the skin and regulate skin functions of *Rana dybowskii* during pre-hibernation and the breeding period.

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