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Research article

Proteomic analysis of crocodile white blood cells reveals insights into the mechanism of the innate immune system

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ABSTRACT

Crocodiles have a particularly powerful innate immune system because their blood contains high levels of antimicrobial peptides. They can survive injuries that would be fatal to other animals, and they are rarely afflicted with diseases. To better understand the crocodile's innate immune response, proteomic analysis was performed on the white blood cells (WBC) of an *Aeromonas hydrophila*-infected crocodile. Levels of WBC and red blood cells (RBC) rapidly increased within 1 h. In WBC, there were 109 up-regulated differentially expressed proteins (DEP) that were up-regulated. Fifty-nine DEPs dramatically increased expression from 1 h after inoculation, whereas 50 up-regulated DEPs rose after 24 h. The most abundant DEPs mainly had two biological functions, 1) gene expression regulators, for example, zinc finger proteins and histone H1 family, and 2) cell mechanical forces such as actin cytoskeleton proteins and microtubule-binding proteins. This finding illustrates the characteristic effective innate immune response mechanism of crocodiles that might occur via boosted transcription machinery proteins to accelerate cytoskeletal protein production for induction of phagocytosis, along with the increment of trafficking proteins to transport essential molecules for combating pathogens. The findings of this study provide new insights into the mechanisms of the crocodile's innate immune system.

1. Introduction

Innate immunity is defined as the non-specific first line of host protection against pathogen infection, comprised of physical and chemical barriers, and humoral and cell-mediated innate immunity for eradicating or slowing down pathogen growth or breakthrough [1]. Among vertebrates, innate immunity has been widely investigated in mammals due to its significance for human health, whereas, in the case of reptiles, the investigation is still limited, although it is also important in comprehending the evolution of primitive

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creatures into humans. Reptiles have outstanding innate immunity, such as a prominent external protective structure for preventing pathogen invaders and a special skin structure that forms a thick epidermis [2]. Besides, reptiles have a wide innate immune response capable of encompassing various pathogens by using a variety of molecules and cells, including non-specific leukocytes, lysozymes and antimicrobial peptides [3]. In addition, the general components (i.e., antibodies, cytokines, complement system, and leukocytes) of the immune systems of reptiles tend to be similar to those of mammals [4]. Although our understanding of innate mechanisms in reptiles is still missing some pieces, at least what we know indicates evidence of coevolution between reptiles and mammals. Moreover, a major exploration of reptile innate immunity is crucial for understanding the complexity of human innate immune responses, and essential for finding new therapeutic agents to combat infectious pathogenic microorganisms.

Antimicrobial proteins and antimicrobial peptides (AMPs) are remarkable molecules in the innate immunity for host cell defense against intracellular pathogens. Likewise, cytoskeleton proteins (i.e., actin, microtubules, intermediate filaments and septins), are well known for their function in cytokinesis, cellular movement and cell shape. These molecules also play a vital role in innate immunity during phagocytosis for the cytoskeleton remodeling process [5]. Furthermore, these proteins are required for detecting bacteria and activating innate immune signaling pathways [6]. Moreover, during this immune cell sensing, the central metabolism of innate immune cells has to switch rapidly from the basal resting state to a hyper-fueled active cell. In this active state, the cells mainly use aerobic glycolysis to generate cellular ATP rather than oxidative phosphorylation [7].

The enormous tropical reptile known as the Siamese crocodile (*Crocodylus siamensis*) is sensitive to cold temperatures. There are many dangerous pathogens in their natural environments. It is possible that crocodiles have a strong immune system or can produce compounds that make them resistant to infection. It has also been proven that Siamese crocodile blood is a rich source of strong antibiotic substances. Many AMPs have been isolated from their blood, such as crocosin [8], leucrocin I and II [9], and cathelicidin [10]. *Aeromonas hydrophila* is a gram-negative bacterium that is frequently found in aquatic habitats and can infect a wide range of species, including crocodiles. This bacterium was isolated from the blood of a crocodile with septicemia [11]. Nevertheless, the mechanism underlying the immune response during *A. hydrophila* infection in crocodiles remains unclear.

It is well understood that insights into the processes behind immune function can be gained through the study of proteins and their roles in biological systems, termed proteomics. However, in comparison with conventional gel-based proteomics, the gel-free proteome approach is a relatively new advance in the field of proteomics that can offer a more comprehensive and sensitive analysis of proteins, as well as significant insights into the biology of a sample. By using this technique, it is possible to detect changes in protein expression that could be associated with immunological activation, such as the overexpression of certain cytokines or the activation of signaling pathways. Previously, this technique has been applied to studying reptiles. Darville et al. [12] used 2-Dimension gel electrophoresis accompanying mass spectrometry to analyze the proteome of white blood cells (WBC) from the American alligator (*Alligator mississippiensis*) and discovered 43 proteins that are mainly related to the cytoskeletal system, in which these proteins are known as regulator proteins in cell mobility and phagocytosis. In addition, the skin proteome of the Chinese giant salamander (*Andrius davidianus*) was established and 249, 155 and 97 proteins that are involved in various physiological processes were identified from skin, mucus and molting, respectively [13]. Besides, this method also was applied to the discovery of new antimicrobial peptides from Komodo Dragon (*Varanus komodoensis*) and at least seven peptides exhibit antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* [14]. In crocodiles, only eggshell proteome has been established [15]. There is currently little evidence of immunity in crocodiles. Therefore, in this work, we evaluate the innate immune response of *C. siamensis* infected with aquatic pathogenic *A. hydrophila* using a gel-free proteomics technique.

2. Materials and methods

2.1. Crocodile treatment

The *A. hydrophila* starter culture was prepared by culturing in Nutrient Broth (NB) at 37 °C with shaking for 18 h. Then it was inoculated in the same medium followed by incubation at 37 °C until OD_{600} reached 1.0, which corresponds to 1×10^9 CFU/ml. The bacterial culture was centrifuged and washed with phosphate-buffered saline (PBS 7.4). The washed bacterial cells were re-suspended with PBS again prior to use for immunization.

A four-year-old male Siamese crocodile from Sriracha Moda Farm Co., Ltd. (Chon Buri, Thailand) was used in this experiment. Before treatment, a 15 ml blood sample was collected in a blood collection tube containing EDTA from the supra vertebral branch of the internal jugular vein as a pre-inoculation blood sample or 0 h. The crocodile was immunized with 1 ml of resuspended *A. hydrophila* cells (1×10^9 CFU/ml) through intraperitoneal injection into the blood circulation. After 1 h and 24 h of inoculation, blood samples were withdrawn, collected in a 15 ml sterile tube containing 0.89% EDTA and then utilized for determining the WBC and red blood cell (RBC) counts. Tubes of collected blood were kept cold by chilling on ice until the WBC and RBC layer appeared. Afterwards, the WBC and RBC fractions were carefully collected and washed immediately with PBS through centrifugation. The washed WBC fractions were used instantly for protein extraction. The animal ethics committee of Khon Kaen University approved the experimental procedures (ethical approval number 0514.175/66).

2.2. Analysis of protein pattern profile using SDS-PAGE

The WBC fractions were dissolved in a lysis buffer containing 1% SDS in 50 mM Tris-HCl pH 6.8. Before overnight storage at −20 °C, the dissolved solutions were homogenized with sonication and then precipitated in ice-cold acetone containing 10 % (w/v) trichloroacetic (TCA) and 0.07 % (v/v) β-mercaptoethanol. The frozen protein fractions were washed twice with 50 mM Dithiothreitol

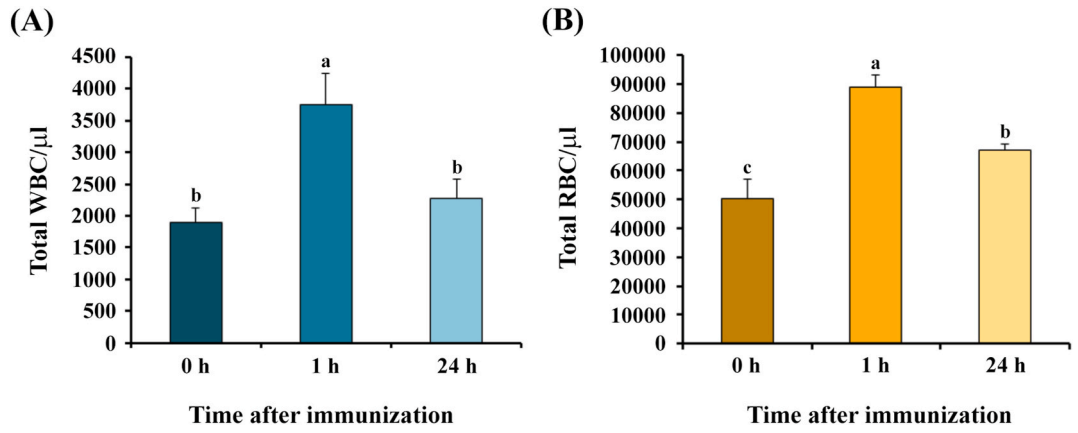

Fig. 1. Change in number of white blood cells (WBC) (A) and red blood cell (RBC) (B) among non-immunized (0 h) and after immunization of crocodile with *A. hydrophila* for 1 h and 24 h. Statistically significant differences among all groups are indicated by different letters (p-value <0.05).

(DTT) in ice-cold acetone, then were air-dried as crude protein pellets. A protein pellet was redissolved in 0.2 % RapiGest SF Surfactant (Water, USA) in 20 mM ammonium bicarbonate to enhance protein solubilization. Each solubilized crude protein sample was used for the protein pattern investigation by 4–20% gradient Tris-Tricine SDS-PAGE. An equal concentration (20 µg) of each crude protein condition was loaded onto the gel.

2.3. Protein identification by liquid chromatography with tandem mass spectrometry

The WBC protein was subjected to in-solution digestion following E-Kobin et al. [16] with minor modifications. Briefly, the solubilized protein fractions were mixed with 5 mM dithiothreitol (DTT) in 10 mM ammonium bicarbonate and incubated at 60 °C for 1 h for the reduction of disulfide bonds and heat denaturation. Next, in the alkylation step, the mixture was added with 15 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate, and then kept in the dark at room temperature for 1 h. Enzymatic digestion was performed by a trypsin-to-protein ratio of 1:50 (w/w) and incubated overnight at 37 °C. Before re-suspension in 0.1% formic acid, the tryptic peptide solution was dried under a vacuum. The suspended peptide was desalted by a C18-ZipTip (Metler, UK). Eluted peptides were further injected into the NanoAcquity UPLC system. An ACQUITY UPLC M-Class symmetry C18 Trap Column (5 µm, 180 µm × 20 mm) and an ACQUITY UPLC BEH C18 Column (1.7 µm, 100 µm × 100 mm) analytical reversed-phase column (Waters Corp., Milford, MA) were used. Linear gradient elution of 2 mobile phases (Mobile phase A: water + 0.1% formic acid and Mobile phase B: acetonitrile + 0.1% formic acid) was performed with a 400 nl/min flow rate. The positive mode of peptides was analyzed using an SYNAPT™ HDMS mass spectrometer (Waters Corp., Manchester, UK). A quadrupole mass analyzer was adjusted to Data-dependent acquisition (DDA) mode to scan the range of mass per charge ratio from 300 to 1800. For quantitative proteomics, DeCyder™ MS 2.0 was used to convert the multiple MS/MS dataset, including the observed mass per charge (m/z), intensity and charge state of the precursor peptide into mascot generic format (mgf) files. The mgf files were identified by searching the MS/MS data against the NCBI-nr database (August 07, 2015) using the MASCOT algorithm (Matrix Science version 2.2, London, UK) available in Bruker Daltonics BioTools 2.2 software (GE Healthcare Europe GmbH, Freiburg, Germany). The MASCOT search parameters were set following trypsin as a proteolytic enzyme, cysteine carbamidomethylation for fixed modification, three variable modifications (asparagine/glutamine deamidation, methionine oxidation and N-terminus acetylation) and allowing one missed cleavage. The level of each protein was represented as a log2 value.

2.4. Statistical and data analysis

The average of triplicate protein intensities among 0 h, 1 h and 24 h samples were subjected to statistical analysis by the pair-wise t-test with a 95% significance level (p-value <0.05). Regarding altered protein intensity with statistically significant 1.5-fold change, both up-regulation and down-regulation compared with 0 h were considered as differentially expressed protein (DEP). All DEPs were further sorted according to their biological functions based on either the UniProtKB Protein knowledgebase (https://www.uniprot.org/uniprot/) or the NCBI database. The protein analysis through evolutionary relationships (PANTER) classification system was applied for candidate up-regulation of DEPs categorization according to their roles, which were related to molecular function, biological process and protein class using chicken (*Gallus gallus*) as a nearby evolutional organism reference [17].

3. Results and discussion

3.1. The level of WBC and RBC after infection

A crocodile was treated with 1×10^9 CFU/ml of *A. hydrophila*. Although this bacterial concentration is known to be a lethal dose

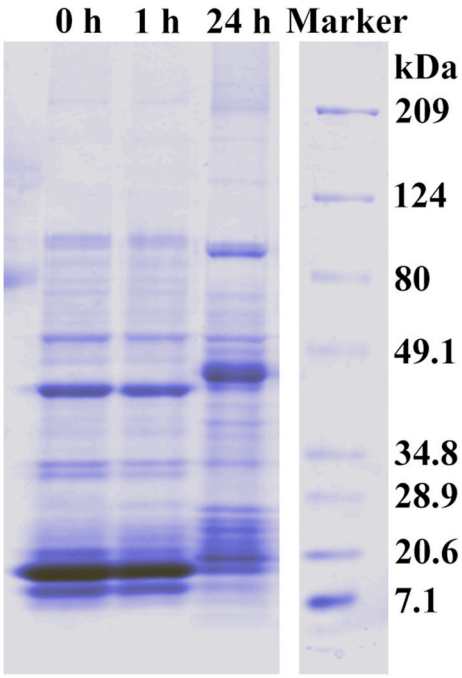

Fig. 2. Protein pattern of crocodile's white blood cells (WBC) by 4–20% gradient Tris-Tricine SDS-PAGE. An equal concentration (20 μg) of each crude protein condition was loaded onto the gel. Lane 1: pre-immunized WBC (0 h), lane 2: WBC after 1 h immunization with *A. hydrophila* (1 h), lane 3: WBC after 24 h immunization with *A. hydrophila* (24 h), lane 4: protein marker.

and induce pathological, hematological and immunological change within 24 h in various fish species [18–21], it is unlikely to be fatal in crocodiles due to their strong immunity. It is well known that RBC and WBC play a vital role in the immune response, which is the machinery of the organism for defending itself against harmful pathogens [22,23]. The RBC and WBC counts were investigated to understand how the infection with *A. hydrophila* affects the RBC and WBC levels in crocodiles. The results showed that RBC and WBC numbers are nearly two-fold increased at 1 h after inoculation (Fig. 1A and B). However, the amount of RBC and WBC apparently decreased at 24 h infection. It is not surprising that the WBC level increased, since during bacterial immunization, several types of WBCs are generally boosted because of migration of WBC from bone marrow to blood vessels for the killing of invaders [24]. However, the result indicated that the Siamese crocodile has a rapid immune reaction within only 1 h. In humans, WBC typically accumulate in the bloodstream 4–8 h after infection and are a maximum at 48 h [25]. This might be a piece of evidence that crocodiles have a robust innate immune system that functions through their WBC to prevent pathogens from spreading throughout their bodies. Additionally, rising RBC levels after infection implies that crocodile RBC might serve as an innate immune mediator for stimulating or maintaining the immune system. Moreover, RBC also performed essential immunological roles such as chemokine control, nucleic acid binding and pathogen eradication [22]. As a result, it raised an intriguing question on how the immune cell protein responds to infection.

3.2. The WBC protein profile on SDS-PAGE

WBC, also known as leukocytes, are the main immune system cells that directly participate in the phagocytosis or antibody production processes that are used to combat infections [26,27]. We investigated changes in the protein pattern on SDS-PAGE comparing before and after inoculation. The SDS-PAGE analysis of the WBC protein extract from an after-immunized 1 h sample presented a mostly similar pattern of protein bands compared to the protein profile bands of a before-immunized sample. Where the size of protein bands from both samples were similar in the range of 7–20 kDa, protein bands in the range of 20–29 kDa of the 1 h-infected sample appeared to have thicker protein bands (Fig. 2). However, the protein pattern of WBC after 24 h infection was a clearly distinct profile when compared to both pre-immunized and after-1 h immunized results. These results indicate that the WBC protein patterns were changed during infection, which might be responsible for the crocodile immune response. Furthermore, these changed proteins are probably an essential molecular machinery that functions via the aquatic pathogen *A. hydrophila* infection in crocodiles.

3.3. Identification of WBC protein with LC-MS/MS

The fraction of trypsinized WBC peptides mixture was subjected to LC-MS/MS. The mass spectra of WBC obtained mostly corresponded with saltwater crocodiles (*Crocodylus porosus*). In comparison to 0 h, the 109 up-regulated DEPs were classified into two groups. The first group of 59 DEPs significantly increased expression after 1 h of inoculation (Fig. 3A–F and Table 1), while another group of 50 up-regulated DEPs increased at 24 h (Fig. 4A–F and Table 2) and 7 down-regulated DEPs (Supplemental Table S1). Heat

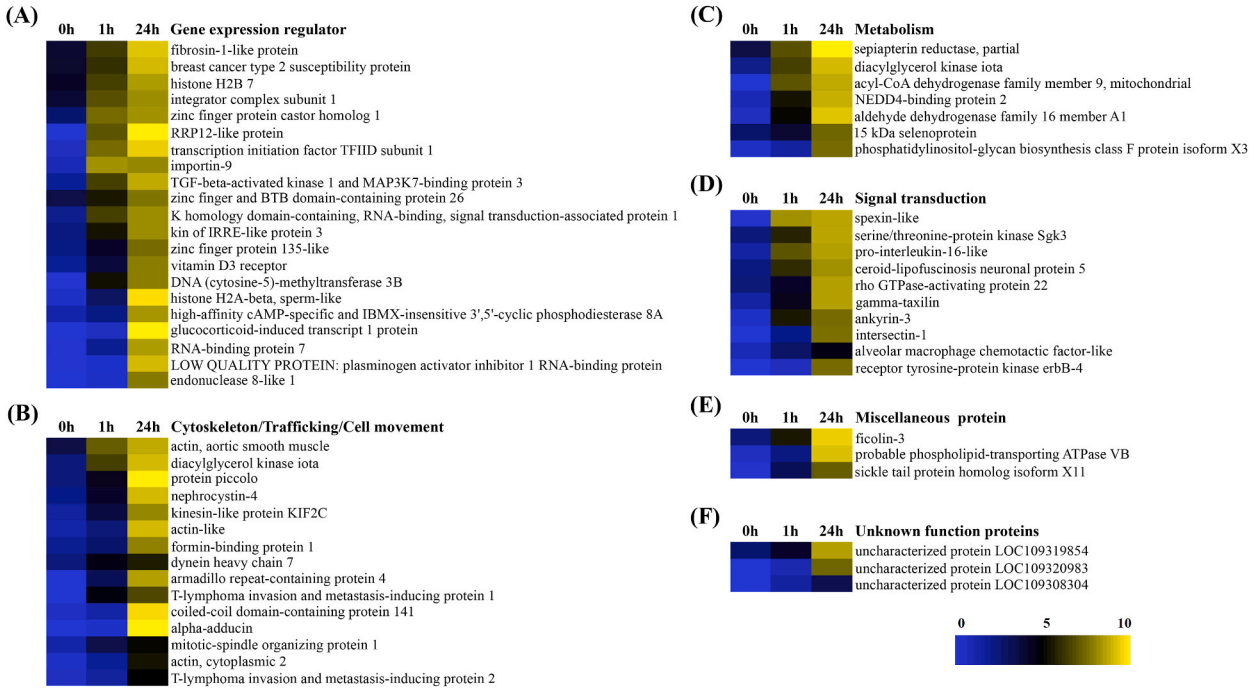

Fig. 3. Heat map analysis of up-regulated DEPs of crocodile's white blood cells (WBC) at 1 h. All DEPs were sorted according to their biological functions: Gene expression regulator (A), Cytoskeleton/Trafficking/Cell movement (B), Metabolism (C), Signal transduction (D), Miscellaneous protein (E), and Unknown function proteins (F). The relative protein expression values are color coded from the lowest (blue) to the highest (yellow).

map analysis of DEPs results illustrated that the heatmap intensities of 0 h and 1 h infection WBC have very similar protein profiles, which are clearly elucidated in Fig. 4A–F, whereas the heatmap intensity of the after 24 h infection WBC presented a distinctly diverse heat map profile (Fig. 3A–F and 4A-F). These results were consistent with SDS-PAGE analysis.

One hour after inoculation, the up-regulated DEPs were mainly involved in gene expression regulation and cytoskeleton/trafficking/cell movement for 21 and 15 DEPs, respectively. In addition, 10 DEPs played a role in cell signaling and 7 DEPs corresponded to cell metabolism. For miscellaneous protein and unknown function protein groups, at least 3 DEPs are classified in each biological function group.

3.4. An increment in gene expression regulators

The proteins involved in the regulation of gene expression (gene expression regulators) can be up-regulated during infection, depending on the specific pathogen and the host's immune response. In crocodiles, at least three zinc finger proteins such as zinc finger and BTB domain-containing protein 26 (ZBTB26), zinc finger protein 135-like, and zinc finger protein castor homolog 1 were up-regulated after *A. hydrophila* infection (Fig. 3A). These regulatory proteins bind DNA to make certain regions looser and to allow easier association with other molecules, thereby contributing consequently to transcription and translation [28]. Recently, many studies have revealed the potential functions of zinc finger proteins for regulation both at transcription and post-transcription in the immune system [29]. Besides, two important RNA processing machinery proteins, RRP12-like protein, and fibrosin-1-like protein, increased, suggesting that crocodile cells activated gene expression at the transcription level while infected. Moreover, two molecules of transcription factor and transcription activator presented a saturated increase in protein expression, indicated by 1 h comprise of transcription initiation factor TFIID subunit 1 (TAF1), zinc finger protein castor homolog 1 (CASZ1), importin-9 (IPO9), and K homology domain-containing, RNA-binding, signal transduction-associated protein 1 (KHDRBS1) (Table 1). In particular, importin-9 is implicated in importing nuclear proteins to the nucleus and as a histone chaperone [30] whereas KHDRBSI is an RNA-binding protein, that plays versatile roles in a variety of cellular processes such as regulation of RNA stability and alternative splicing [31]. It is probable that at 1 h after bacterial injection, these molecules might cooperate to accelerate the production of proteins in response to infection.

At 24 h, the results presented that those three different histone isoforms (histone H1.01-like, histone H1-like and histone H1x) were up-regulated (Fig. 4A and Table 2). These molecules play a vital function in nucleolar chromatin condensation for the inactivation of transcription [32]. Recently a study indicated that histone H1s are an essential regulator of gene expression of late defense genes upon plant pathogens [33]. In addition, two histone lysine methyltransferases (histone-lysine N-methyltransferase ASH1L, and histone-lysine N-methyltransferase KMT5B) represented the increase of its expression. Both are responsible for the transfer of a methyl group to lysine and arginine residues of histone for chromatin compaction and regulating transcription. It is possible that, at 24 h, the

Table 1
Level expression of up-regulated DEPs of WBC since 1 h of bacterial inoculation.

GI number	Protein	0 h	1 h	24 h
Cytoskeleton/Trafficking/Cell movement				
gi|1121928835	actin, aortic smooth muscle	3.55	7.22	8.74
gi|1121868048	actin, cytoplasmic 2	0.28	1.87	5.44
gi|1121908360	actin-like	1.23	2.51	9.08
gi|1121914594	alpha-adducin	0.00	0.22	10.1
gi|1121798595	armadillo repeat-containing protein 4	0.00	3.21	8.55
gi|1121923033	coiled-coil domain-containing protein 141	0.44	1.33	9.67
gi|1121878367	diacylglycerol kinase iota	2.45	6.53	9.06
gi|1121919709	dynein heavy chain 7	2.40	4.66	5.61
gi|1121860261	formin-binding protein 1	1.90	2.81	7.98
gi|1121820145	kinesin-like protein KIF2C	1.58	3.74	8.07
gi|1121814631	mitotic-spindle organizing protein 1	1.23	3.48	5.10
gi|1121862190	nephrocystin-4	2.17	4.17	9.03
gi|1121875184	protein piccolo	2.43	4.52	10.2
gi|1121810931	T-lymphoma invasion and metastasis-inducing protein 1	0.00	4.73	6.76
gi|1121902185	T-lymphoma invasion and metastasis-inducing protein 2	0.54	1.59	4.98
Gene expression regulator				
gi|1121809861	breast cancer type 2 susceptibility protein	4.09	6.10	9.02
gi|1121854477[a]	DNA (cytosine-5)-methyltransferase 3B	0.07	5.31	7.90
gi|1121827581	endonuclease 8-like 1	0.01	0.28	7.84
gi|1121852039	fibrosin-1-like protein	3.96	6.38	9.30
gi|1121799480	glucocorticoid-induced transcript 1 protein	0.00	0.54	10.4
gi|1121828823	high-affinity cAMP-specific and IBMX-insensitive 3′,5′-cyclic phosphodiesterase 8A	1.18	2.25	8.36
gi|1121809713	histone H2A-beta, sperm-like	0.23	3.01	9.80
gi|1121880462	histone H2B 7	4.26	6.50	8.44
gi|1121884878[a]	importin-9	0.74	8.32	8.11
gi|1121866829	integrator complex subunit 1	3.93	6.95	8.23
gi|1121891341[a]	K homology domain-containing, RNA-binding, signal transduction-associated protein 1	2.01	6.51	8.14
gi|1121887933	kin of IRRE-like protein 3	2.30	5.47	8.14
gi|1121821053	LOW QUALITY PROTEIN: plasminogen activator inhibitor 1 RNA-binding protein	0.06	0.20	9.02
gi|1121807300	RNA-binding protein 7	0.10	1.93	8.52
gi|1121886350	RRP12-like protein	0.00	7.04	10.5
gi|1121812082	TGF-beta-activated kinase 1 and MAP3K7-binding protein 3	1.70	6.56	8.82
gi|1121838228[a]	transcription initiation factor TFIID subunit 1	0.57	7.47	9.45
gi|1121806779	vitamin D3 receptor	1.79	3.78	7.90
gi|1121861252	zinc finger and BTB domain-containing protein 26	3.53	5.57	7.74
gi|1121930072	zinc finger protein 135-like	2.25	4.18	7.50
gi|1121864870[a]	zinc finger protein castor homolog 1	2.73	7.47	8.32
Metabolism				
gi|1121819552	15 kDa selenoprotein	2.80	4.01	7.39
gi|1121841400	acyl-CoA dehydrogenase family member 9, mitochondrial	0.00	6.97	8.73
gi|1121906913	aldehyde dehydrogenase family 16 member A1	0.53	5.14	9.25
gi|1121878367	diacylglycerol kinase iota	2.12	6.53	9.06
gi|1121915605	NEDD4-binding protein 2	0.88	5.47	8.90
gi|1121898918	phosphatidylinositol-glycan biosynthesis class F protein isoform X3	0.36	1.56	7.48
gi|1121917615	sepiapterin reductase, partial	3.56	6.92	11.2
Signal transduction				
gi|1121834909	alveolar macrophage chemotactic factor-like	0.79	2.94	4.70
gi|1121850238	ankyrin-3	0.30	5.55	7.50
gi|1121814530	ceroid-lipofuscinosis neuronal protein 5	2.35	6.01	8.25
gi|1121812497	gamma-taxilin	1.43	4.50	8.61
gi|1121811187	intersectin-1	0.00	2.17	7.64
gi|1121808431[a]	pro-interleukin-16-like	1.40	7.04	8.54
gi|1121919755	receptor tyrosine-protein kinase erbB-4	0.00	0.67	7.53
gi|1121926835	rho GTPase-activating protein 22	2.54	4.14	8.56
gi|1121804795	serine/threonine-protein kinase Sgk3	2.38	5.88	8.72
gi|1121888712[a]	spexin-like	0.19	8.26	8.63
Miscellaneous protein				
gi|1121892031	ficolin-3	2.42	5.55	9.50
gi|1121833237	probable phospholipid-transporting ATPase VB	0.57	2.29	9.21
gi|1121798781	sickle tail protein homolog isoform X11	0.12	3.24	7.17
Unknown function proteins				
gi|1121824667	uncharacterized protein LOC109308304	0.00	1.51	3.39
gi|1121900401	uncharacterized protein LOC109319854	2.68	4.19	8.56
gi|1121907972	uncharacterized protein LOC109320983	0.00	1.05	7.37

[a] The saturated up-regulated protein expression level at 1 h.

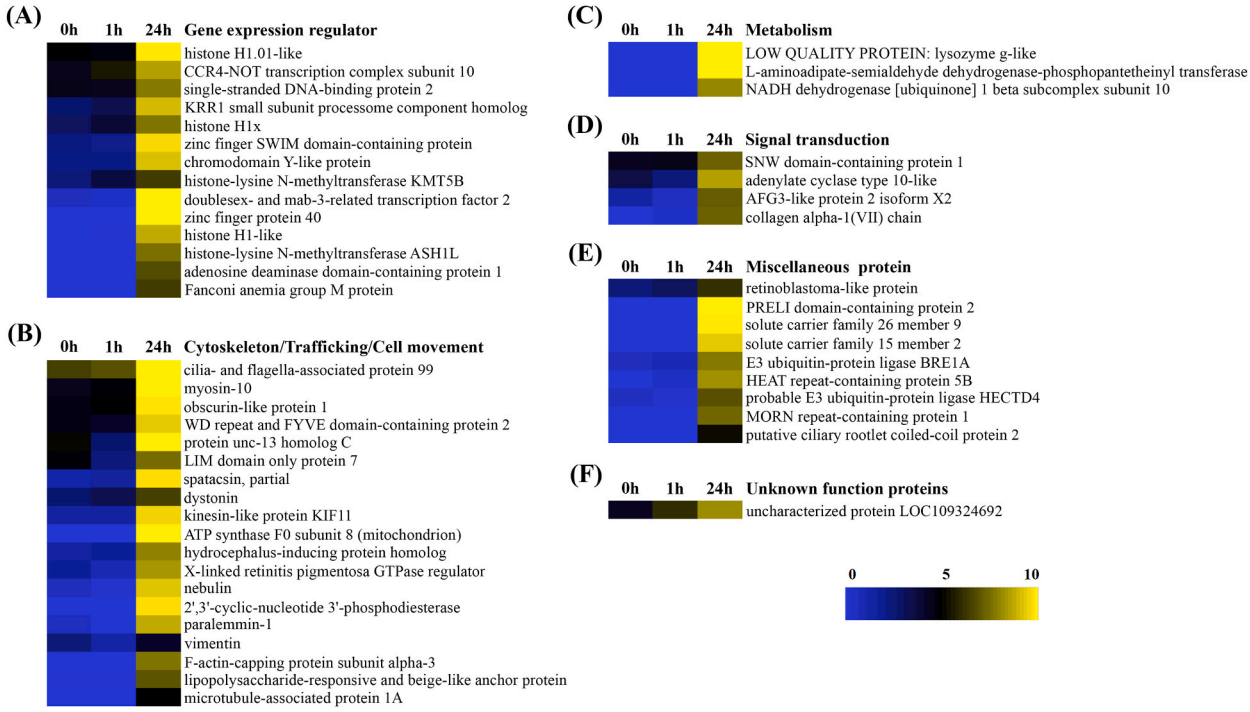

Fig. 4. Heat map analysis of up-regulated DEPs of crocodile's white blood cells (WBC) at 24 h. All DEPs were sorted according to their biological functions: Gene expression regulator (A), Cytoskeleton/Trafficking/Cell movement (B), Metabolism (C), Signal transduction (D), Miscellaneous protein (E), and Unknown function proteins (F). The relative protein expression values are color coded from the lowest (blue) to the highest (yellow).

crocodile's defense mechanism against the pathogen was complete and tended to suppress the expression of defensive molecules.

3.5. A rising number of mechanical forces proteins in WBCs

The response with proteins in Cytoskeleton/Trafficking/Cell movement tasks would be that WBC undergo phagocytosis or move towards pathogenic bacteria. First, three types of actins, a monomer of microfilament whose main responsibility is cell strength and movement, were up-regulated, including aortic smooth muscle actin (ACTA2), cytoplasmic 2 actin and actin-like (Fig. 3B and Table 1). The actin cytoskeleton is a key responsive protein for pathogen infection because actins are engaged in membrane remodeling activities such as phagocytosis, macropinocytosis, endocytosis, exocytosis and autophagy [34]. In addition, previous reports have demonstrated a key connection in the cellular defense against Salmonella infection between innate immunity and the actin cytoskeleton [35]. Moreover, alpha-adducin was produced during bacterial infection. Although there was a tiny level of expression at 1 h after infection, there was a massive expression level at 24 h (Fig. 4B and Table 2). Normally, adducin is mainly responsible for the formation of the spectrin-actin network, which brings physical support to the plasma membrane and facilitates signal transduction in various physiological functions of the cell [36]. Previous research has indicated that this molecule is highly expressed in T cells and plays a role in the actin cytoskeleton in regulating co-stimulation [37]. In addition, three actin-binding proteins were upregulated at 24 h including myosin-10, F-actin-capping protein subunit alpha-3 and nebulin. Myosin-10, an actin-based molecular motor, participates especially in intracellular functions such as phagocytosis and cell migration [38]. Actin filaments are stabilized by nebulin, which controls filament length [39], while the fast-growing ends of actin filaments are controlled by the F-actin-capping protein subunit alpha-3 [40]. This evidence might imply that crocodile WBCs respond to pathogens by a rapid remodeling process of their actin cytoskeleton.

Microtubules are highly dynamic tubular polymers of tubulin distributed throughout the cytoplasm, and play a role in structural support, cell organization and intracellular transport. Furthermore, microtubules provide a function in innate and adaptive immune responses [41]. The results indicate that two microtubule-binding proteins (kinesin-like protein (KIF2C) and dynein heavy chain 7 (DNAH7)) displayed increased production starting at 1 h–24 h (Fig. 3B and Table 1). KIF2C had previously been known as a mitotic centromere-associated kinesin, which facilitates the separation of chromosomes during cell division [42], being involved in intracellular cargo transport. Recently, a new role of KIF2C has been established in DNA damage repair [43]. It is possible that an increase in white blood cells (Fig. 1A) might result from the upregulation of KIF2C. Furthermore, KIF2C might play a vital role in DNA repair during bacterial infection in crocodiles, although the function of DNAH7 has not been clarified. Nonetheless, dynein has been reported to have a molecular function in the antiviral process [44] and regulation of adaptive and innate B cell development [45]. Accordingly, an abundance of KIF2C and DNAH7 might imply that cytoplasmic transport machinery molecules are engaged in host-bacteria

Table 2
Level expression of up-regulated DEPs of WBC at 24 h of bacterial inoculation.

GI number	Protein	0 h	1 h	24 h
Cytoskeleton/Trafficking/Cell movement				
gi|1121903935	2′,3′-cyclic-nucleotide 3′-phosphodiesterase	0.00	0.00	9.75
gi|669017076	ATP synthase F0 subunit 8 (mitochondrion)	0.00	0.00	11.4
gi|1121917454	cilia- and flagella-associated protein 99	6.48	6.89	10.9
gi|1121844691	dystonin	2.67	3.43	6.55
gi|1121877613	F-actin-capping protein subunit alpha-3	0.00	0.00	7.73
gi|1121835463	hydrocephalus-inducing protein homolog	1.45	1.78	7.98
gi|1121929206	kinesin-like protein KIF11	1.53	1.52	9.54
gi|1121814560	LIM domain only protein 7	4.85	2.45	7.53
gi|1121831752	lipopolysaccharide-responsive and beige-like anchor protein	0.02	0.09	7.00
gi|1121829735	microtubule-associated protein 1A	0.00	0.00	5.02
gi|1121870527	myosin-10	4.45	4.88	10.6
gi|1121918650	nebulin	0.68	0.14	9.22
gi|1121918446	obscurin-like protein 1	4.70	5.02	9.84
gi|1121848333	paralemmin-1	0.60	0.00	8.82
gi|1121828404	protein unc-13 homolog C	5.12	2.69	10.0
gi|1121827139	spatacsin, partial	1.25	1.66	9.76
gi|1121796649	vimentin	2.53	1.39	4.14
gi|1121809837	WD repeat and FYVE domain-containing protein 2	4.69	4.14	9.35
gi|1121811985	X-linked retinitis pigmentosa GTPase regulator	1.72	0.83	8.38
Gene expression regulator				
gi|1121830033	adenosine deaminase domain-containing protein 1	0.00	0.00	6.79
gi|1121800402	CCR4-NOT transcription complex subunit 10	4.46	5.56	8.56
gi|1121802640	chromodomain Y-like protein	2.23	2.26	9.20
gi|1121923336	doublesex- and mab-3-related transcription factor 2	0.64	0.31	11.1
gi|1121807579	Fanconi anemia group M protein	0.00	0.00	6.41
gi|1121880353	histone H1.01-like	4.93	4.75	9.91
gi|1121880343	histone H1-like	0.00	0.02	8.82
gi|1121840796	histone H1x	3.11	3.93	7.73
gi|1121808693	histone-lysine N-methyltransferase ASH1L	0.00	0.00	7.58
gi|1121889184	histone-lysine N-methyltransferase KMT5B	2.50	3.72	6.43
gi|1121872008	KRR1 small subunit processome component homolog	2.68	3.36	9.06
gi|1121821484	single-stranded DNA-binding protein 2	4.54	4.50	7.75
gi|1121802807	zinc finger protein 40	0.00	0.00	10.5
gi|1121854609	zinc finger SWIM domain-containing protein	2.29	2.13	9.68
Metabolism				
gi|1121816170	L-aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase	0.00	0.00	10.3
gi|1121813419	LOW QUALITY PROTEIN: lysozyme g-like	0.00	0.00	10.4
gi|1121865952	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	0.00	0.00	8.04
Signal transduction				
gi|1121886705	adenylate cyclase type 10-like	3.60	2.38	8.61
gi|1121803654	AFG3-like protein 2 isoform X2	1.41	0.58	7.16
gi|1121840804	collagen alpha-1(VII) chain	0.00	0.26	7.28
gi|1121912072	SNW domain-containing protein 1	4.40	4.59	7.30
Miscellaneous protein				
gi|1121925049	E3 ubiquitin-protein ligase BRE1A	0.64	0.82	7.75
gi|1121900695	HEAT repeat-containing protein 5B	0.00	0.37	8.26
gi|1121862753	MORN repeat-containing protein 1	0.00	0.00	7.45
gi|1121833632	PRELI domain-containing protein 2	0.00	0.00	11.3
gi|1121851137	probable E3 ubiquitin-protein ligase HECTD4	0.54	0.13	6.93
gi|1121871440	putative ciliary rootlet coiled-coil protein 2	0.00	0.00	5.27
gi|1121905442	retinoblastoma-like protein	2.50	3.12	6.14
gi|1121921562	solute carrier family 15 member 2	0.00	0.00	9.39
gi|1121885866	solute carrier family 26 member 9	0.00	0.00	9.94
Unknown function proteins				
gi|1121808090	uncharacterized protein LOC109324692	4.36	6.06	8.18

interactions, assuming that intracellular transport is a key point in the host's weapon process to combat pathogens. Likewise, Kinesin-like protein (KIF11) and microtubule-associated protein 1A (MAP1A) showed increased expression after 24 h (Fig. 4B and Table 2). KIF11 is a molecular motor protein required for mitosis [46], whereas MAP1A has been established to play a role in regulating the neuronal cytoskeleton [47]. Moreover, other proteins also involved in cell movements such as T-lymphoma invasion and metastasis-inducing protein (Tiam) 1 and 2 contribute to cancer cell migration [48,49], while nephrocystin-4 is an actin cytoskeleton organization protein [50]. These proteins were increased at 24 h (Fig. 4 and Table 2).

In addition, certain proteins are involved in metabolism including acyl-CoA dehydrogenase family member 9 (ACAD9) and aldehyde dehydrogenase family 16 member A1 (ALDH16A1) that clearly increased from 1 h to 24 h (Fig. 3C and Table 1). ACAD9 is a mitochondrial enzyme in fatty acid β-oxidation that contributes to the initial step of fatty acid metabolism [51]. ALDH16A1

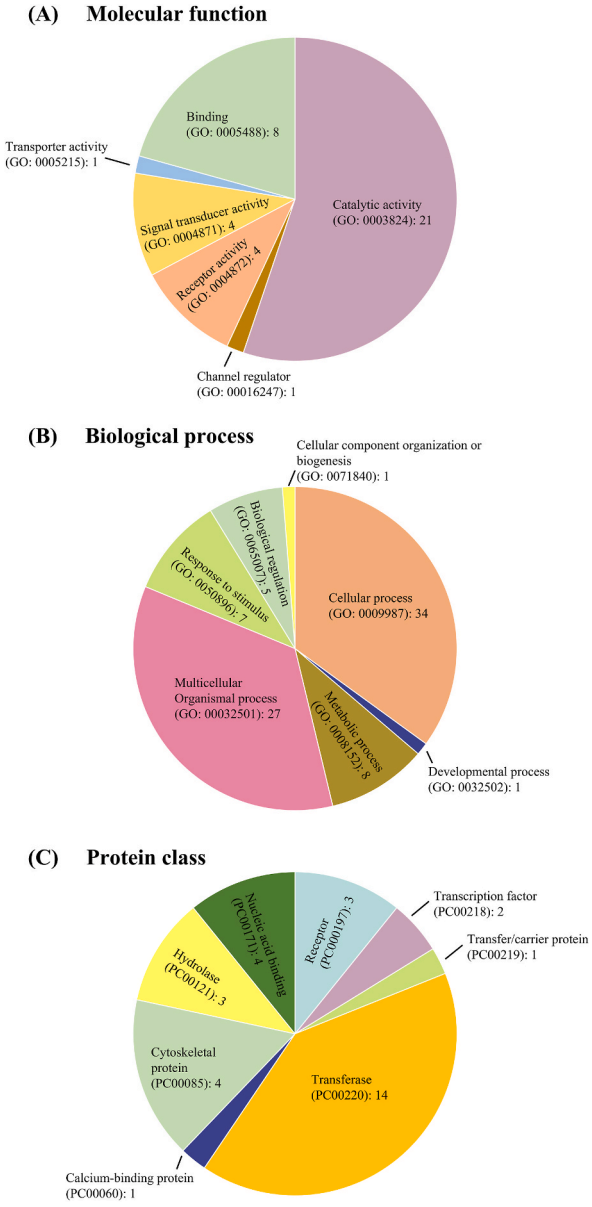

Fig. 5. Classification of Up-regulated crocodile's white blood cells (WBC) proteins by the Panther classification system. The letter and number in parentheses represent gene ontology (GO), and protein classifiers (PC). Numbers after parentheses represent the number of hits in each biological function. Proteins are classified into (A) molecular function, (B) biological process and (C) protein class.

participates in catalyzing the oxidation of aldehydes to carboxylic acids, and produces NADH to generate ATP. In general, vertebrate cells can generate endogenous aldehydes through the metabolism of amino acids, carbohydrates, lipids and biogenic amines [52]. It is feasible that crocodiles might use these enzymes for increasing ATP to supply the cytoskeletal protein function during movement or phagocytosis. Hence, this evidence suggests that cytoskeleton remodeling is a crucial process during infection, which involves the cellular trafficking and movement of crocodile immune cells.

3.6. Sorting up-regulated WBC proteins with the Panther classification system

The roles of the up-regulated WBC proteins were analyzed using the Panther classification system (http://pantherdb.org/). The chicken was used as a reference species for this analysis because it is phylogenetically grouped into Archosaur, which includes birds and crocodilians [53]. All 109 up-regulated DEPs could be hit to 39 proteins of *G. gallus* following the criteria of the Panther classification system. Although previous research on Siamese crocodile eggshell proteins used human proteins for searching in the Panther system [15], most of the proteins are involved in catalytic activity function and are associated with the cellular process (Fig. 5A and B).

There are also transferase, nucleic acid binding, transcription factors and cytoskeleton proteins (Fig. 5C). Although only 39 proteins were matched in the sorting system, it could confirm that the majority of up-regulated DEPs participate in the gene expression process and cell motility during crocodile infection.

4. Conclusion

The proteomic analysis of WBCs from an *A. hydrophila*-infected crocodile revealed that the crocodile's innate immune response is characterized by the rapid up-regulation of gene expression regulators and cell mechanical forces. These changes are likely responsible for the crocodile's ability to survive injuries and infections. The up-regulation of gene expression regulators suggests that the crocodile's immune system is able to quickly ramp up production of proteins that are essential for fighting infection. This includes proteins that are involved in phagocytosis. The up-regulation of cell mechanical forces suggests that the crocodile's immune system is able to rapidly reorganize the cytoskeleton. This reorganization is necessary for the crocodile's WBCs to move quickly to the site of infection and to engulf pathogens. The findings of this study provide new insights into the mechanisms of the crocodile's innate immune system. This information could be used to develop new treatments for infectious diseases, as well as to improve our understanding of the reptile immune system.

Data availability statement

Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Anupong Tankrathok: Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Conceptualization. **Bancha Mahong:** Writing – original draft, Visualization, Investigation, Formal analysis. **Sittiruk Roytrakul:** Resources, Methodology, Investigation, Formal analysis. **Sakda Daduang:** Writing – review & editing, Supervision, Resources, Conceptualization. **Yosapong Temsiripong:** Resources. **Sompong Klaynongsruang:** Writing – review & editing, Supervision, Resources, Conceptualization. **Nisachon Jangpromma:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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