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Macrophage migration inhibitory factor is involved in inflammation response in pathogen challenged *Apostichopus japonicus*

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ABSTRACT

Macrophage migration inhibitory factor (MIF) is a cytokine and plays critical roles in inflammatory and immune responses in vertebrates. However, its functional role in inflammation has not been well studied in invertebrates. In the present study, we cloned and characterized MIF gene from *Apostichopus japonicus* by RNA-seq and RACE approaches (designated as *AjMIF*). A 1047 bp fragment representing the full-length cDNA of *AjMIF* was obtained, including a 5′ UTR of 100 bp, an open reading frame (ORF) of 366 bp encoding a polypeptide of 121 amino acids residues with the molecular weight of 13.43 kDa and theoretical isoelectric point of 5.63 and a 3′ UTR of 580 bp. SMART analysis showed that AjMIF has conserved MIF domain (2-117aa) similar to its mammalian counterparts. The amino terminal proline residue (P^2) and invariant lysine residue (K^{33}) which are critical active sites of tautomerase activity in mammalian MIF were also detected. Phylogenic analysis and multiple alignments have shown that AjMIF shared higher degree of structural conservation and sequence identities with other counterparts from invertebrates and vertebrates. For *Vibrio splendidus* challenged sea cucumber, the peak expression of AjMIF mRNAs in coelomocytes were detected at 6 h (23.5-fold) and remained at high levels until 24 h (4.01-fold), and returned to normal level at 48 h in comparison with that of the control group. Similarly, a significant increase in the relative mRNA levels of AjMIF was also found in 10 μg mL^{-1} LPS-exposed primary cultured coelomocytes. Functional analysis indicated that recombinant AjMIF incubation could promote inflammatory response related genes of *Ajp105*, *AjVEGF*, *AjMMP1* and *AjHMGB3* expression by 1.35-fold, 1.36-fold, 1.83-fold and 1.27-fold increase, respectively, which was consistent with the findings in vertebrate MIFs. All these results collectively suggested that AjMIF had a similar function to MIFs in higher animals and might serve as a candidate cytokine in inflammatory regulation in sea cucumber.

1. Introduction

The inflammatory process is a complex pathophysiological or natural biological response initiated by vascular tissues to defend against pathogens, cell damages or irritants [1,2]. The events in inflammation are well defined, regardless of the initiating agent, with an increase in blood flow, vasodilation, increased cellular metabolism and protein extravasation fluids, and the release of soluble mediators in the end. A key component of the process is the trafficking of inflammatory cells to the sites or infection. These so-called inflammatory cells include macrophages [3], T cells, B cells, natural killer cells, neutrophils, and granulocytes [4,5]. These cells excrete copious amounts of inflammatory cytokines into the microenvironment, including interleukin-6 (IL-6), IL-1α, IL-1β, tumor necrosis factor-α (TNF-α), and other cytokines [4–6]. However, activation of inflammation is a complex cascade process involving not only the execution of inflammatory cytokines but also the activation of proinflammatory cytokines.

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine found in 1966 which is initially shown to inhibit the random migration of macrophages upon its release from T lymphocytes during a delayed-type hypersensitivity response [7,8]. Today, MIF is widely recognized as a critical upstream player in the innate immune response, where it triggers and amplifies cytokine production by stimulating the production of inflammatory mediators, such as TNF-α, interferon-γ, interleukins (IL-1β, IL-2, IL-6, IL-8), nitric oxide, prostaglandin E2 and tissue-degrading matrix metalloproteinases (MMP) [9–11]. In ApoE knockout mice, blockage of MIF by anti-MIF antibody significantly reduced the production of regional and systemic inflammatory chemokines and cytokines [12] and suppression of inflammatory cell infiltration in the plaque [13]. MIF also promotes inflammation by orchestrating leukocyte trafficking [14], inhibiting p53-mediated apoptosis of inflammatory cells sustaining their survival span [12,15],

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and by counter-regulating the immunosuppressive action of glucocorticoids [16,17]. Furthermore, MIF exhibits tumor growth-promoting properties [18,19]. However, although the diversified roles of MIF have been extensively studied, and MIF can be involved in immune response through its unique expression, its role in immune responses remains unclear, especially in Echinodermata.

The sea cucumber *Apostichopus japonicus* (Echinodermata, Holothuroidea) as a deuterostome to the vertebrates in the evolution must play a special role in the evolution of immunology [20]. Similar to higher vertebrates, inflammatory responses were also reported in invertebrates through by histological examinations of wound repair and demonstration of early activation of infiltrating phase, such as: *Octopus vulgaris* [21], *Ruditapes decussatus* [22], *Crassostrea gigas* [23], and *Asterias rubens* [24]. In recent years, molecular patterns involved in inflammatory responses in invertebrates have been investigated. Although the discovery inflammasome member of NLR family pyrin domain containing 3 (NLRP3) [25] and inflammatory-related cytokine of vascular endothelial growth factor (VEGF) [26] provides molecular evidence for the occurrence of inflammation in invertebrates, no definitive conclusion was made on the mechanism of inflammation in invertebrates. MIF as an important pro-inflammatory cytokine, is implicated in tumors and inflammation and serves as an inflammatory mediator, inducing several immunological effects in vertebrates [27,28]. In invertebrates, more studies focus on the MIF's endocrine and enzymic properties [29–31]. Thus, the immune roles of MIF and the relationship between the AjMIF and inflammation in invertebrate remain poorly understood. In this study, we first cloned the full-length cDNA of MIF from *A. japonicus* (*AjMIF*) and then investigated its time course expression pattern. The functional characterization of AjMIF could regulated inflammatory-related protein expression, including: AjMMP1, AjVEGF, AjHMGB3 and Ajp105.

2. Materials and methods

2.1. Animals and challenge experiment

Sea cucumber *A. japonicus* (weight: 114 ± 13 g) were collected from Dalian Pacific Aquaculture Company and acclimatized in indoor aquaculture system with salinity of 30 and at 16 ± 1 °C [32], and acclimatized to laboratory conditions for one week prior to experimental use. For time-course expression analysis of AjMIF, one tank served as the control, and another five tanks contained fresh *V. splendidus* at a final concentration of 10^7 CFU mL^{-1}. Coelomic fluids from five individuals in the control and challenged groups were collected at 0, 6, 12, 24, 48, 72, and 96 h post-inoculation. Coelomic fluids were collected and then centrifuged at 800 × *g* for 5 min at 4 °C to collect coelomocytes. Five biological replicates were obtained from experimental and control groups, and all samples were stored at −80 °C before RNA extraction and cDNA synthesis.

2.2. Rapid application cDNA ends of AjMIF

Partial sequence of MIF was collected from our transcriptome data [33], which was further validated by PCR. Fullength cDNA of *AjMIF* was obtained by rapid amplification of cDNA ends (RACE) with 3′, 5′-Full RACE kit (TaKaRa) following manufacturer's instructions. Gene-specific primers (Table 1) were designed based on the candidate MIF fragment. Desired polymerase chain reaction (PCR) products were cloned into pMD19-T simple vector (TaKaRa), and three positive clones were produced for each product sequenced at Sangon Biotechnology (Shanghai). We obtained the full-length cDNA of *AjMIF* gene by overlapping the EST and the fragment from RACE. The completed sequences including the opening reading frame was further amplified and sequenced to ensure its accuracy.

Table 1
Primers used for cloning and quantitative real-time PCR.

Gene name	Primer sequence (5′ -3′)	Used for
AjMIF 3-1	CAAGCAGTGGAATCATCAACTT	3 ′ RACE
AjMIF 3-2	CTTGGTGACGGGAGCCTTTTGTC	3 ′ RACE
AjMIF ORF	ATGCCTTTAATATTTATAACGACGAA	Recombinant expression
AjMIF ORR	TCAATGCTGCTTTTCTGACAAAAG	
AjMIF BamHI-oF	GGATCCATGCCTTTAATATTTATAACGACGAA	Recombinant expression
AjMIF NotI-oR	GCGGCCGCTCAATGCTGCTTTTCTGACAAAAG	
AjMIF qF	ATTTCAGAGTTTGCGGAGGAGG	Real-time PCR
AjMIF qR	TATTCGGGCTCAGCTTGAGACC	
AjMMP1 qF	ATGGCAGATGACCAGCAACA	Real-time PCR
AjMMP1 qR	CGTGAGAGCCTCCTCGAATT	
Ajp105 qF	TCTTCGCATTCCATTGAGCTG	Real-time PCR
Ajp105 qR	ATGGTCCTTCACAGCCGTATCT	
AjVEGF qF	AGCCTAGGCCTGTGCTCCTA	Real-time PCR
AjVEGF qR	CCACTTGCGCTGTATATACTTTCA	
AjHMGB qF	CCCTCCAGCCCTACAGACTTTA	Real-time PCR
AjHMGB qR	TGATCGCCCTCCTTCACGT	
Ajβ-Actin qF	CCATTCAACCCTAAAGCCAACA	Real-time PCR
Ajβ-Actin qR	ACACACCGTCTCCTGAGTCCAT	

2.3. Sequence analysis

Sequence homology was analyzed using BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid (aa) sequence was assessed using Expert Protein Analysis System (http://www.expasy.org/). Domains in AjMIF aa sequence were detected using Simple Modular Architecture Research Tool (SMART) program (http://www.smart.emblheidelbergde/). Three-dimensional (3D) protein structures were predicted by Protein Homology/analog Recognition Engine V 2.0 (http://www.sbg.bio.ic.ac.uk/phyre2/html/login.html). A neighbor-joining (NJ) tree was constructed with Mega 6.0 software package (http://www.megasoftware.net/) and Clustal W2 (http://www.ebi.ac.uk/clustalw/) with 1000 bootstraps.

2.4. Spatial expression analysis of mRNA by quantitative PCR (qPCR)

Time-course expression of *AjMIF* was performed using the ABI 7500 real-time PCR detection system. β-actin was served as internal control in verifying successful reverse transcription according to our previous work and other reports [25,34], and calibrating cDNA templates using Primescript™ II 1st cDNA Synthesis Kit (TaKaRa). Employed primers were listed in Table 1. Real-time PCR amplification was performed in a 20 μL reaction containing 10 μL 2 × SYBR Green Mix (TaKaRa), 8 μL diluted cDNA (1:100), and 1 μL of each primer (10 mM). The following qPCR parameters were used: (1) denaturing step at 95 °C for 10 min; (2) 40 cycles of 95 °C for 15 s and 60 °C for 1min. Amplified products were subjected to melting curve analysis to confirm generation of a single PCR product. $2^{-\triangle\triangle CT}$ method was used to analyze relative expression levels of candidate genes. Data were presented as relative mRNA expression levels (means ± SD, n = 5), and one-way ANOVA was applied to discern significant differences between control and experimental groups.

2.5. LPS-exposed primary coelomocytes

Primary coelomocytes were prepared according to our previous work [35]. In brief, coelomic fluids were filtered through a 300 Mesh Cell Cribble to remove large tissue debris. Then, cells were washed twice with isotonic buffer (0.001 M EGTA, 0.53 M NaCl, and 0.01 M Tris-HCl, pH 7.6) and re-suspended in Leibovitz's L-15 cell culture medium (Invitrogen, USA) supplemented with penicillin (100 U mL^{-1})

and streptomycin sulfate (100 μg mL^{-1}) with a final concentration of 1×10^6 cells mL^{-1}; NaCl (0.39 M) was added to adjust osmotic pressure to 780 mmol L^{-1}. Cells were then dispensed in a 24-well culture microplate at 500 μL per well and pre-incubated in 18 °C for 12 h. For LPS (*Escherichia coli* 055:B5, Sigma, USA) challenge, cells were exposed to 10 μg mL^{-1} LPS for 0, 1, 3, 6, 12 and 24 h. Cells were collected and dissolved in Trizol for subsequently gene expression analysis.

2.6. Generation and purification of soluble recombinant AjMIF (rAjMIF)

For rAjMIF production, the positive transforms BL21 (DE3) with correct pET28a-AjMIF were inoculated into 5 mL LB broth containing Kn and then cultured at 37 °C and 180 rpm overnight. A grown culture (1 mL) was inoculated into flasks containing 100 mL of fresh LB media with Kn and cultured until OD_{600} = 0.6. The rAjMIF was generated by inducing with isopropyl-β-d-thiogalactopyranoside at a working concentration of 1 mM and continuously cultured at 37 °C for another 3 h. The bacteria were harvested through centrifugation at 6000 × *g* for 10 min at 4 °C, and the soluble His-tagged rAjMIF was purified with nickel-nitrilotriacetic acid agarose under native conditions according to the manufacturer's recommendations (Sangon Biotechnology, Shang Hai). Finally, the purified protein was subjected to 12% SDS-PAGE analysis.

2.7. Identification of regulatory relationship between AjMIF and inflammatory

The relationship between AjMIF and inflammation was evaluated inflammatory-related gene expression pattern after incubated with purification AjMIF protein. Briefly, the cells were counted with a blood count plate and diluted to a concentration of 1.0×10^6 cells mL^{-1}, and a 500 μL cell suspension containing 5.0×10^5 cells was transferred to a 24-well microplate well and incubated at 18 °C for 12 h before rAjMIF was added. Then, coelomocytes were supplemented with 0, and 50 ng of rAjMIF and BSA as control. After incubating rAjMIF for 24 h, the expression pattern of *Ajp105*, *AjMMP1*, *AjVEGF* and *AjHMGB3* were detected by real-time PCR detection system.

3. Result and discussions

3.1. Molecular characterization of A. japonicus MIF

The full-length cDNA of *AjMIF* was obtained by overlapping each EST and RACE fragments (Fig. 1). This sequence was deposited in GenBank under accession number: MK426729. A 1047 bp fragment representing the full-length cDNA of *AjMIF* included a 5′ UTR of 100 bp, an ORF of 366 bp encoding a polypeptide of 121 amino acids residues with the molecular weight of 13.43 kDa and theoretical isoelectric point of 5.63, and a 3′ UTR of 580 bp. Sequence analysis revealed that AjMIF lacks a signal peptide. SMART analysis showed that AjMIF has conserved MIF domain (2-117aa) (Fig. 1) similar to its mammalian counterparts. Additionally, alignment analysis showed that AjMIF is highly similar to its fish and mouse counterparts and that it has the characteristic features of MIF proteins [36,37]. The amino-terminal proline residue (P^2) and invariant lysine residue (K^{33}) which were critical active sites of tautomerase activity in mammalian MIF are conserved in all selected species except sea urchins [38]. However, the conserved sequence motif Cys^{57}-XX-Cys^{60} that mediated oxidoreductase activity in mammalian [39] is absent in AjMIF (shown in Fig. 2). The similar phenomena were also found in other invertebrates, such as MIF from *Penaeus monodon*, *Ruditapes philippinarum* and *Eriocheir sinensis* [29–31]. Chauhan et al. suggested that oxidoreductase activity may not the signature enzymatic activity of MIF homologues, suggesting that the existence of common CXXC motif in MIFs was not positive correlation with the oxido-reductase activity [40,41]. Mounting CXXC in MIF from *Prochlorococcus marinus* also did not result in oxidoreductase activity [41]. In contrast, although this motif was found absent in *Wuchereria bancrofti*-MIF-2, significant oxidoreductase activity was also detected. Structural analysis revealed that Wba-MIF-2 showed the presence of two cysteine residues in close proximity in the tertiary structure of the protein [40]. Therefore, the similarity of spatial structure folding may lead to the similarity function, and we further performed tertiary structure prediction of AjMIF and human MIF. The results revealed that the predicted three-dimensional structure of AjMIF had eight-stranded β-sheet and three α-helices in a β1α1β2β3β4α2β5β6α3β7β8 arrangement (Fig. 2C), was very similar to human MIF (Fig. 2B), whose secondary structure was comprised of two alpha helices and six beta sheets in the following order: β1α1β2β3β4α2β5β6 [42]. The extreme similarity of the tertiary structure between the sea cucumber MIF and the human MIF proved that they may be very close in function.

3.2. Homology analysis of AjMIF

We assessed the homology relationship of AjMIF with some known MIFs, based on its derived amino acid sequence with that of other organisms MIF homologues available in the GenBank. The deduced amino acid sequence of AjMIF displayed 32% identity with MIF from *S. purpuratus* (XP_001177764.2), 37% with *Caenorhabditis elegans* MIF (NP_492069.1), 37% with *Azumapecten farreri* (ADF87941.1) and 29% with *Homo sapiens* (CAG46452.1), and Huang et al. reported that *Oncomelania hupensis* MIF was also homologous on 27% with *H. sapiens* (27%) [43], We selected 4-Oxalocrotonate_Tautomerase domain belonging to MIF domain for NCBI sequence analysis. The deduced amino acid sequence of 4-Oxalocrotonate_Tautomerase domain displayed 53% identity with MIF from *Apteryx australis mantelli* (XP_013800313.1), 50% with *Alligator mississippiensis* (XP_006277066.1) and 47% with *Azumapecten farreri* (ADF87941.1). We only compared the conserved key active regions, and found that homology was significantly improved compared with full-sequence alignment, further indicating that AjMIF may have the same activity as MIFs of other species. A phylogenetic tree was constructed to examine the AjMIF relationship with MIFs from other species. As shown in Fig. 3, AjMIF was closed to *S. purpuratus* MIF and formed one separate branch in MIF sub-clade.

3.3. Time-course expression of AjMIF in vivo or in vitro

The hemocytes was the principal line of cellar immune defense involved in destruction of pathogen inside the invertebrate [44]. In echinoderms, cell-based immunity in the coelomocytes was important to recognize and neutralize pathogens like the hemocyte in other invertebrates [45]. Therefore, coelomocytes were selected to further investigate the possible immune function of AjMIF *in vitro* and *in vivo*. Current research showed that MIF can be released after stimulation with microbial products that include bacterial endotoxin (LPS), exotoxins, streptococcal pyrogenic exotoxin A, malaria pigment, gram-negative and gram-positive bacteria, mycobacteria, and proinflammatory cytokines [3,46,47]. The present study investigated potential roles and expression profiles of AjMIF after challenging *V. splendidus*, and results are shown in Fig. 4A. After pathogen challenge, levels of AjMIF transcripts reached their peak for 6 h (23.5-fold) and remained at high levels until 24 h (4.01-fold) post infection, and returned to normal level from 48 h in comparison with that of the control group. *AjMIF* in coelomocytes induced after *V. splendidus* infection and the up-regulation of *AjMIF* after bacterial challenge suggests that MIF played a critical role in inflammatory response to infection and tissue invasion. Similarly, up-regulated expression profiles were detected in LPS-challenged primary cultured cells. A total of 1.96-fold ($P < 0.05$), 2.13-fold, 2.48-fold and 3.04-fold ($P < 0.01$) increase were detected at 3, 6, 12 and 24 h, respectively (Fig. 4B), in comparison with that of the control group. LPS as an inflammatory inducer could stimulated cells showed higher expressions of pro-inflammatory genes as well as pro-inflammatory cytokines mediated by an elevated nuclear NF-κB level in human [48].

Fig. 1. Sequences analysis of MIF in *Apostichopus japonicus*. The cDNA and deduced amino acid of AjMIF. The start codon was blacked. The asterisk indicated the stop codon. Predicted MIF domain sequence was highlighted in gray shadow. The polyA tail-signal sequence (AATAAA) was shown in black and italics.

After stimulated with LPS, MIF was observed to significant up-regulated or with the cytokines TNFα and interferon-γ (IFNγ) in mice. MIF also was described to mediate certain pro-inflammatory effects, stimulating macrophages to produce TNFα and nitric oxide when given in combination with IFNγ [49,50]. Like TNFα and IL-1β, MIF played a central role in the host response to endotoxemia. Co-injection of recombinant MIF and LPS exacerbated LPS lethality, whereas neutralizing anti-MIF antibodies fully protect mice from endotoxic shock [46].

3.4. Characterization of regulatory relationship between AjMIF and inflammatory response

In mammals, some reports showed that MIF is closely associated with the innate immune response to a wide range of infectious and inflammatory diseases [51], and it can aggravate disease pathology [52]. However, no inflammation indicators gene like IL-1β, IL-6, and TNF-α were detected from the sea cucumber genomic [53]. In the sea cucumber, we only found that the alarmins (HMGB3) and inflammation-related transcription factor (NF-κB) that are critical for the regulation of inflammatory response in vertebrates, and VEGF protein and MMPs can be directly involved in the inflammatory response in vertebrates [26,54–56], so we chose them as detecting inflammation indicators to verify the relationship between MIF and inflammation. In our study, we found the incubated with rAjMIF 50 ng (Fig. 5) could up-regulate *Ajp105*, *AjVEGF*, *AjMMP1* and *AjHMGB3*, 1.35-fold, 1.36-fold, 1.83-fold and 1.27-fold, respectively (Fig. 6). Extracellular HMGB was essential to the activation of TLR4 signaling [57], and Lv et al. reported that MIF overexpression promoted HMGB release from the nucleus to the cytoplasm and activate NF-κB in human [58]. NF-κB, a ubiquitous transcription factor, was responsible for the transcription of a diverse range of genes involved in sepsis and is a critical regulator of genes that encode TNF-α, IL-6, chemokines, and other inducible enzymes. It was recently reported that MIF induced the expression of TNF-related apoptosis [59], inducing ligand and monocyte chemoattractant protein 1 in human diabetic podocytes, and that this MIF-induced expression was NF-κB dependent [60]. Furthermore, HMGB can induce expression of catabolic molecules, such as MMP-1, MMP-3, and MMP-9 responsible for the breakdown of the ECM. The continuous and sustained production of HMGB and its downstream pro-inflammatory and catabolic molecules can eventually lead to tissue damage [61]. In addition, elevated MIF activation had been shown to increase the expression of the MMP, uPA and VEGF genes [62,63]. Evidences pointed out that MMPs assemble in activation cascades and besides their classical extracellular matrix substrates, they cleaved several cytokines/chemokines from the ECM, and also considered important for regulating inflammatory responses, increasing their bioavailability and modifying their bioactivity and MMP activity decreased the secreted levels of IL-6, while at the same time it was responsible for increasing the secreted levels of CXC motif chemokine ligand 12 (CXCL12) [56] and VEGF-A [64]. At the same time, the up-regulated MMPs could degrade of endothelial vascular endothelial growth factor receptor (VEGFR) lead to a subsequent elevation of cellular/serum VEGF level, with the further leading to apoptosis and inflammation via the activation of caspase-1/3 and IL-1β release [65]. VEGF production can also push T cells toward a Th1 phenotype by increasing the production of IFN-γ and decreasing the production of IL-10 to promote inflammation [66]. Thus, as a pro-

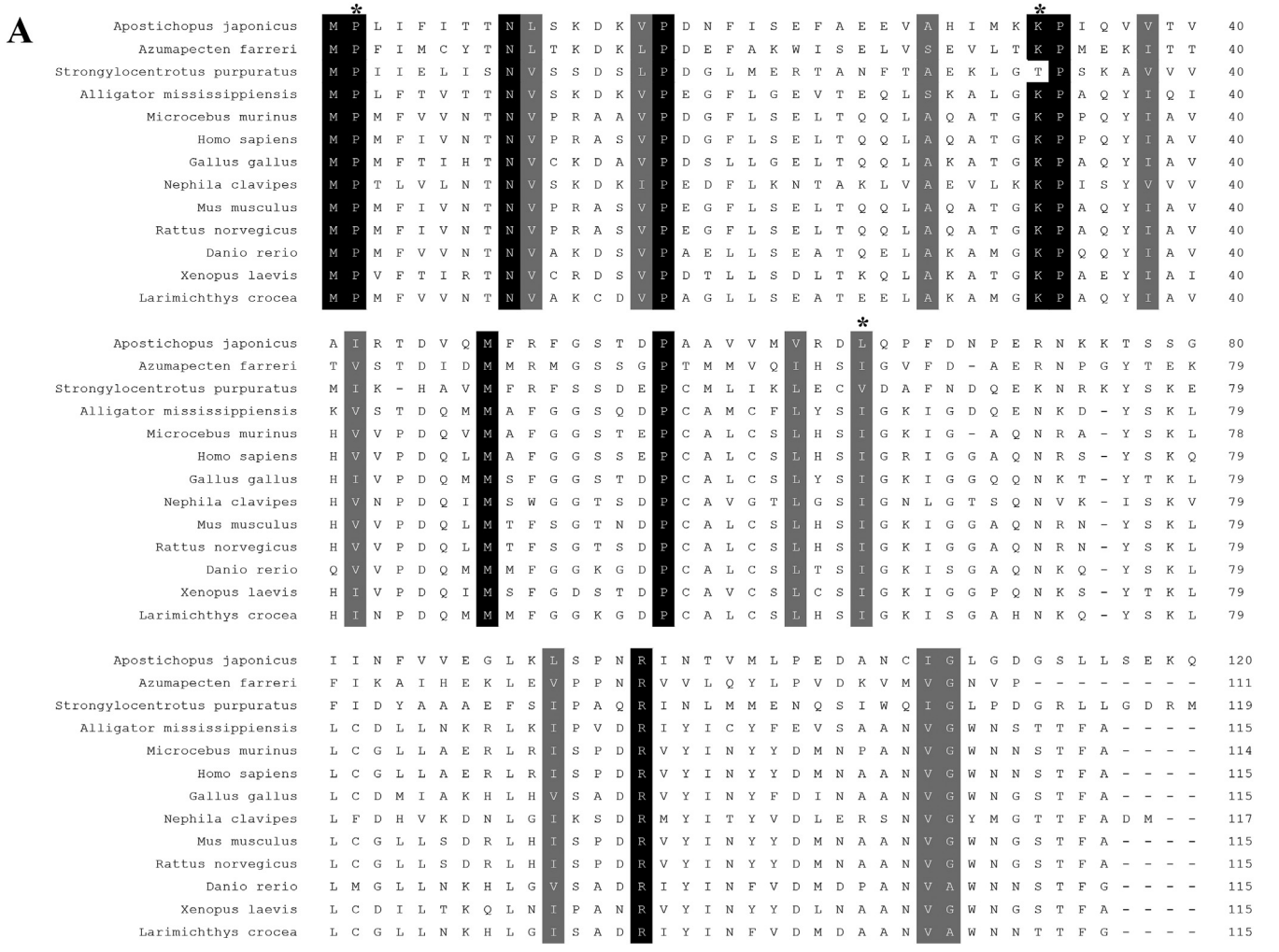

Fig. 2. Predictive analysis of amino acid sequences. A: alignment of predicted aa sequence of AjMIF by using Clustal W2 Multiple Alignment program. Consensus residues shaded with a threshold of more than 80% identity by using Multiple Align Show program. Conserved residues were marked with black, whereas analogous aa and non-identical aas were marked with dark gray and white colors, respectively. Conserved key enzyme sites were marked with black asterisks. Protein sequence accession numbers: *Azumapecten farreri* (ADF87941.1); *Strongylocentrotus purpuratus* (XP_001177764.2); *Alligator mississippiensis* (XP_006277066.1); *Microcebus murinus* (XP_012632205.1); *Homo sapiens* (CAG46452.1); *Gallus gallus* (NP_001292020.1); *Nephila clavipes* (PRD30574.1); *Mus musculus* (NP_034928.1); *Rattus norvegicus* (NP_112313.1); *Danio rerio* (NP_001036786.1); *Xenopus laevis* (NP_001083650.1); *Larimichthys crocea* (NP_001290329.1). B: 3D structure of AjMIF; C: 3D structure of human MIF. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

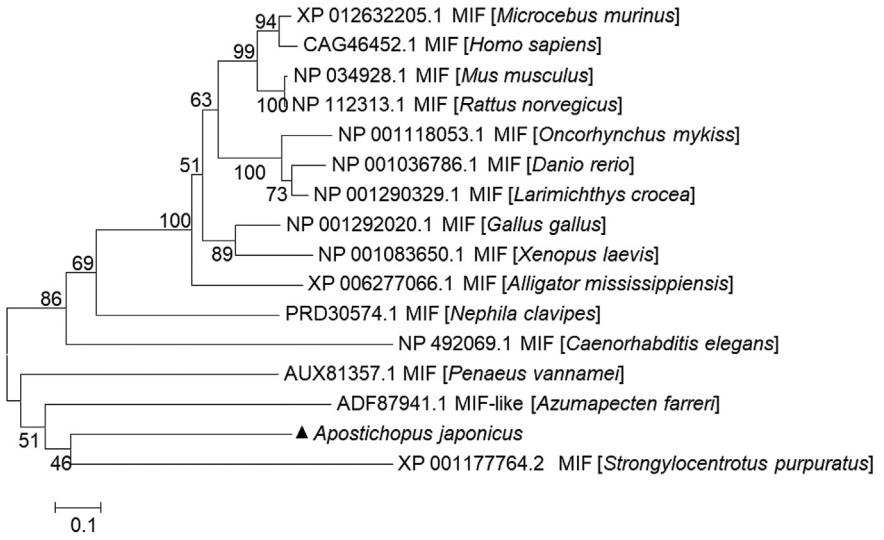

Fig. 3. Phylogenetic tree based on aa sequences of VEGF family from other original samples. The tree was obtained by bootstrap analysis with NJ method, and the numbers on branches represent bootstrap values for 1000 replications. Protein sequence accession numbers: *Azumapecten farreri* (ADF87941.1); *Strongylocentrotus purpuratus* (XP_001177764.2); *Alligator mississippiensis* (XP_006277066.1); *Microcebus murinus* (XP_012632205.1); *Homo sapiens* (CAG46452.1); *Gallus gallus* (NP_001292020.1); *Nephila clavipes* (PRD30574.1); *Caenorhabditis elegans* (NP_492069.1); *Mus musculus* (NP_034928.1); *Rattus norvegicus* (NP_112313.1); *Danio rerio* (NP_001036786.1); *Xenopus laevis* (NP_001083650.1); *Oncorhynchus mykiss* (NP_001118053.1); *Larimichthys crocea* (NP_001290329.1); *Penaeus vannamei* (AUX81357.1).

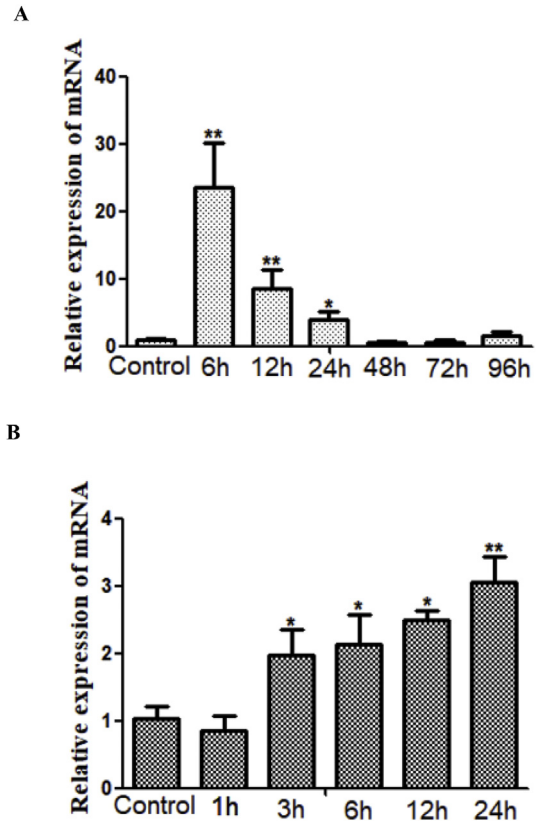

Fig. 4. Temporal expression analysis of AjMIF in *V. splendidus*-challenged sea cucumber (A) and LPS-exposed coelomocytes (B). Values were given as the mean ± SD (n = 5). Asterisks indicate significant differences: $^{*}P < 0.05$ and $^{**}P < 0.01$.

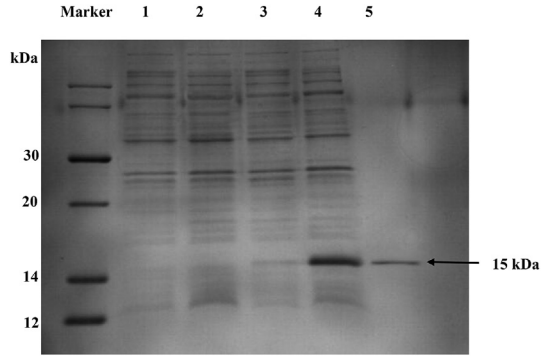

Fig. 5. SDS-PAGE analysis of rAjMIF. Marker: protein molecular standard; lane 1: the empty pET-28a plasmid without induction; lane 2: the whole bacteria lysate without induction; lane 3: the induced recombinant bacteria 1 h; lane 4: the induced recombinant bacteria 3 h; lane 5: purified rAjMIF.

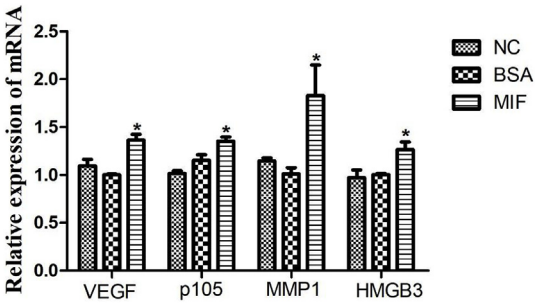

Fig. 6. Expression analysis of AjHMGB3, AjVEGF, Ajp105 and AjMMP1 after incubated with rAjMIF (50 ng) 24 h in primary coelomocytes. Values were presented as mean ± SD (n = 5). Asterisks indicate significant differences: $^{*}P < 0.05$ and $^{**}P < 0.01$.

inflammatory factor, AjMIF may promote AjHMGB release, further activates and regulates NF-κB, thereby regulating AjVEGF and AjMMP1 and further regulating inflammation in sea cucumber.

To conclude, we cloned a new MIF molecule in the sea cucumber. The pathogens *V. splendidus* and LPS can promote the production of MIF, and overexpressed MIF can regulate HMGB and activate NF-κB, as well as regulate the production of VEGF and MMP to participate in the inflammatory response.

Notes

The authors declare no competing financial interest.

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