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Involvement of JNK signaling pathway in lipopolysaccharide-induced complement C3 transcriptional activation from amphioxus Branchiostoma belcheri

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ABSTRACT

Complement C3 is a pivotal component of three cascades of complement activation. C3 in circulation is mainly provided by the hepatic cecum. The expression and secretion of C3 by hepatocytes is increased during acute inflammation. The detailed information on the regulationary mechanism underlying *C3* transcriptional activation is limited. Here, we characterized the 5′-flanking region of the amphioxus *C3* gene. To functionally analyze the upstream regulatory region of the *C3* gene, a series of luciferase reporter gene constructs containing deleted or mutant regulatory elements were prepared. Using luciferase assay, we revealed that a potential C-JUN-1 binding sites within the proximal promoter region were necessary for full activation of the C3 promoter, whereas NF-κB, AP-1, C-JUN-2 and NFAT transcription factor binding sites played roles in governing the promoter activity at a homeostatic level. Our data also indicated that sp600125, a c-Jun N-terminal kinase (JNK) inhibitor, decreased lipopolysaccharide (LPS)-stimulated C3 promoter activity, mRNA expression and protein secretion using western blotting and quantitative real-time PCR analysis. These findings demonstrated that JNK signaling pathway is involved in the regulation of *C3* gene transcription by targeting C-JUN transcription factor binding sites in the 5′-flanking promoter region, leading to LPS-induced *C3* activation and therefore providing a potential target for regulating *C3* expression.

1. Introduction

Amphioxus or lancelet, a cephalochordate, has recently been regarded as the basal chordate [1], and is becoming an emerging model organism for insights into the origin and evolution of vertebrates [2]. It has a vertebrate-like body plan including a circulation system with an organization similar to that of vertebrates. However, amphioxus is less complex than vertebrates for having a genome uncomplicated by extensive genomic duplication, and lacking lymphoid organs and free circulating blood cells. These findings together with the relative structural and genomic simplicity make amphioxus an ideal organism for gaining insights into the origin and evolution of the vertebrate immune system, especially adaptive immunity, and the composition and mechanisms of the vertebrate innate immunity [3]. Amphioxus has a hepatic cecum, the pouch that protrudes forward as an outpocketing of the digestive tube and extends along the right side of the posterior part of the pharynx, which has long been considered to be the precursor of vertebrate liver [4,5]. The liver is the major source of complement components including C3 family proteins in vertebrates, and participates in the immune response as an important line of host defense against invading pathogens. In addition, the acute phase response profile in amphioxus has been shown to be similar to that observed in vertebrates [6].

Complement C3 is a central protein that is indispensable for all complement activation cascades. In addition to its role in complement activation, C3 and its degradation products are able to promote phagocytosis, activate inflammatory responses against pathogens, and regulate differentiation and maturation of B- and T-lymphocytes and

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dendritic cells [7]. The complement system plays key roles in innate and adaptive immunity through mediating phagocytosis, chemotaxis and cell lysis, and belongs to the acute-phase proteins whose synthesis increase immediately upon inflammatory stimuli. The immunomodulators, such as lipopolysaccharide (LPS), can stimulate complement and antibody responses, thus enhance the resistance to bacterial pathogens and parasitic infections. LPS, a component of the outer membrane of Gram-negative bacteria, is a potent activator of a variety of cell types. LPS has been shown to initiate multiple intracellular signaling events, including both the activation of NF-κB, which ultimately leads to the synthesis and release of a number of proinflammatory mediators, and three mitogen-activated protein kinase MAPK pathways (extracellular regulated protein kinases ERK, c-Jun N-terminal kinase JNK/stress-activated protein kinase SAPK, and p38) which leads to a wide range of cellular responses, including cell differentiation, apoptosis, and inflammatory responses [8]. The c-Jun N-terminal kinases (JNKs) including JNK1, JNK2 and JNK3. The JNKs were initially isolated and characterized as stress-activated protein kinases on the basis of their activation in response to inhibition of protein synthesis [9]. In response to stimuli, the JNKs were then discovered to bind and phosphorylate the DNA binding protein c-Jun and increase its transcriptional activity. C-Jun is a component of the AP-1 transcription complex, which is an important regulator of gene expression [10]. AP-1 contributes to the control of many cytokine genes and is activated in response to environmental stress, radiation, and growth factors. JNKs are important in controlling programmed cell death or apoptosis [11]. The inhibition of JNKs enhances chemotherapy-induced inhibition of tumor cell growth, suggesting that JNKs may provide a molecular target for the treatment of cancer. JNK inhibitors have also shown promise in animal models for the treatment of rheumatoid arthritis [12]. In this study, from the genomic DNA library of adult amphioxus *Branchiostoma belcheri*, we isolated gene regulatory region fragment of *C3* gene. The purposes of this study were to examine the expression pattern of the *C3* gene in adult amphioxus, to explore its expression regulation in response to challenge with LPS and to test the involvement of JNK signaling pathway in LPS-induced *C3* transcriptional activation.

2. Materials and methods

2.1. C3 promoter constructs

Regulatory regions of the amphioxus C3 gene promoters were amplified from *Branchiostoma belcheri* genomic DNA by PCR. The resulting PCR products were cloned using TA cloning system (Invitrogen) to generate plasmids PT2AL-C3 and pGL3-C3. The C3 promoter was subcloned between the *Xho* I and *BamH* I sites of PT2AL vector to generate PT2AL-2160bp, PT2AL-963bp and PT2AL-466bp plasmids. Similarly, the C3 promoter was subcloned using *Hind* III and *Xho* I restriction sites into the polylinker upstream of luciferase in pGL3-Basic vector to generate pGL3-Luc-963bp and pGL3-Luc-466bp plasmids. Then a series of C3 promoter-luciferase-mutant constructs (covering 466 bp of *C3* proximal promoter) was constructed, driven by wild-type, five single point mutaion in NF-κB [13,14], AP-1 [13,15], C-JUN-1 [13,15], C-JUN-2 [13,15], NFAT [16] and triple point mutation C-JUN-1-2, AP-1-C-JUN-1-2, NF-κB-NFAT, respectively. All single point mutation plasmids are designed according to previous studies. C3 single point mutation promoters spanning nucleotides from −466 to −1 were generated by PCR using wild-type template and triple mutation promoters using single mutation templates.

2.2. Cell culture

HEK 293T cells were maintained in DMEM supplemented with 10% FBS and antibiotics (100 units/mL penicillin G, 100 units/mL streptomycin sulfate; Gibco, Grand Island, NY, USA). Cells were grown in a 37 °C incubator with 5% CO_2, 95% humidity and subjected to a maximum of 20 cell passages.

2.3. Measurement of C3 promoter activity

The transcriptional regulation of *C3* was examined by the transient transfection of a C3 EGFP reporter construct (PT2AL-2160bp, PT2AL-963bp or PT2AL-466bp) and a promoter–luciferase reporter construct (pGL3-963bp and pGL3-466bp). 24 h post transfection, EGFP fluorescent signal was recorded under a Nikon fluorescence microscope equipped with digital microscope camera Te2000-S and the luciferase reporter assay was performed with a Dual Luciferase Reporter Assay System kit (Promega, Madison, WI, USA) according to the manufacturer's specification.

2.4. Transient transfection of C3 reporter constructs

For the reporter assay, HEK 293T cells were separately transfected with mutant *C3* promoter plasmids NF-κB, AP-1, C-JUN-1, C-JUN-2, NFAT, C-JUN-1-C-JUN-2, AP-1-C-JUN-1-C-JUN-2, NF-κB-NFAT and wild-type C3 promoter plasmid pGL3-Luc-466bp with or without LPS and SP600125 as indicated. The wild-type and mutant C3 promoter plasmids, as well as the pGL3-expressing plasmid, have been previously characterized.

For transfection, cells at approximately 80% confluence were transfected with 0.75 μg of total DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. All DNAs were prepared using endotoxin-free plasmid preparation kits. HEK 293T cells transfected with wild-type *C3* promoter plasmid pGL3-Luc-466bp in twenty four-well plates were treated with LPS (10 μg/mL) (Sigma, St. Louis, MO, USA) or JNK inhibitor SP600125 (20 μM) (Sigma, St. Louis, MO, USA) for 6 h. SP600125 was dissolved in 10% dimethyl sulfoxide (DMSO) and added directly to culture media before the addition of LPS. The final concentration of DMSO never exceeded 0.1%.

2.5. Dual luciferase reporter assays

The luciferase reporter assay was performed with a Dual Luciferase Reporter Assay System kit (Promega, Madison, WI, USA) according to the manufacturer's specification. Normalization was performed with renilla luciferase activity as the basis. Following 24 h culture, cells were washed with PBS and lysed with 100 mL of passive lysis buffer by freeze-thawing at −80 °C for 12 h. Then 20 μl of the cell lysate were added to 50 mL of Luciferase solution provided by the Luciferase Kit and determined with a spectrofluorimeter according to the manufacturer's protocol. .

2.6. In vivo infection

Amphioxus *Branchiostoma belcheri* with average body length of about 4 cm were collected from the professor Yi-quan Wang (Xiamen University, China). Adult *B. belcheri*, which were acclimatized in sterilized filtered seawater for two days, were divided into different groups and challenged with 0 (control), 20 μg/mL LPS or 40 μM SP600125 (a JNK inhibitor) in filtered seawater at room temperature. A total of 8 animals were sampled at intervals of 6 and 12 h, respectively. Amphioxus liver was separately collected for RNA extraction and western blotting anaylsis.

2.7. Western blotting analysis

Following the challenge of LPS and JNK inhibitor SP600125, amphioxus were homogenized in RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS), and protein concentration was measured using the BCA Protein Assay Kit (Sigma, USA). Tissue homogenate were lysed in SDS sample loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS,

10% glycerol, 50 mM DTT, 0.1% bromphenol blue) and the lysates were subjected to ultrasonication and 2 min boiling. All the samples were subject to SDS-PAGE gel at a 40 μg concentration per sample for 2 h at 120 V to isolate different proteins. The gels were transferred to the PVDF membranes followed by washing with TBST for three times. Following blocking with 5% BSA, the membranes were incubated with the indicated primary antibodies at 4 °C for 12 h. Membranes were washed three times and incubated with peroxidase-conjugated rabbit secondary antibodies for 2 h at room temperature and then washed in TBST. Antigen-antibody complexes were detected by enhanced chemiluminescence (Beyotime, Nantong, China) and analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

2.8. RNA extraction and cDNA synthesis

To evaluate mRNA expression levels of the *C3*, total RNA was extracted from several tissues collected from adult amphioxus, including gonad, notochord, intestine, gill, muscle and hepatic cecum. The post-stimulation expression patterns were analyzed using RNA isolated from hepatic cecum. Samples from 24 fish were pooled to yield 3 biological replicates of 8 individuals per petri dish. Total RNA was isolated from 3 biological replicates per sampling point, extracted with Trizol (Invitrogen, Carlsbad, CA, USA) and reversely transcribed to cDNA using an oligo (dT) primer and Superscript II (Invitrogen, Carlsbad, CA, USA). The concentration and quality of the RNAs were measured by spectrophotometry (Amersham Biosciences, Piscataway, NJ, USA), and the integrity were checked by electrophoresis in 1% agarose gel. The RNAs and cDNA were then stored at −80 °C for further use.

2.9. Quantitative real-time PCR analysis

After qualification of the cDNA templates and primers, mRNA levels of target genes and the internal standard β-actin were measured by real-time quantitative PCR in triplicate on ABI StepOnePlus™ real-time PCR system (Stratagene, La Jolla, CA)). SYBR Premix Ex Taq (Takara, Dalian, China) was used according to the manufacturer's protocol with a primer concentration of 200 nM. The reactions (20 μL final volume) contained 2 μL of diluted template, 0.4 μM of each forward and reverse primer, 0.4 μL 50 × ROX Reference Dye and 10 μL 2 × SYBR Premix Ex Taq™II(Stratagene, La Jolla, CA, USA). The cycling parameters were as follows: 95 °C for 30s, followed by 40 cycles of 95 °C for 5s, 60 °C for 30s. The dissociation stage was 95 °C for 15s, 60 °C for 1 min, 95 °C for 15s. Dissociation analysis was performed at the end of each PCR reaction to confirm the amplification specificity. Fluorescence readings at the end of the extension phase of each cycle were used to estimate the values for the threshold cycles (Ct).

After the PCR, data were analyzed with StepOnePlus™ SDS software (Applied Biosystems, USA), and quantified with the comparative Ct method $2^{-\triangle\triangle Ct}$ based on Ct values for complement genes and β-actin in order to calculate the relative mRNA expression level. The primers for the transcripts were listed in Table 1.

2.10. Statistics

Results are presented as the means ± SD from at least 3 replicates at each time point and three independent experiments. Differences between the means were tested by one-way ANOVA and Dunn's multiple range test. Statistical analysis was performed using SPSS 20.0 for Windows. Data were considered significant if the two-sided $P < 0.05$. Image J was used to quantify the density of all visible spots.

3. Results

3.1. Characterization of the 5′-flanking region of the amphioxus C3 gene promoter

To examine the molecular mechanism underlying the induction of *C3* gene expression by LPS, we characterized the promoter of amphioxus *C3* gene. Based on *in silico* analysis, we predicted that the proximal promoter region of the amphioxus gene (NCBI Gene ID AB050668) is present in the 5′-upstream region extending to a nucleotide position about −2000 nt from the transcription initiation site. To map the promoter transcriptional activity, we cloned the *C3* 5′-upstream sequence (−2160/+1 nt, −963/+1, and −466/+1) into PT2AL plasmid as shown in Fig. 1A. Transfection of PT2AL-2160, PT2AL-963 or PT2AL-466 construct into HEK 293T cells resulted in the expression of EGFP fluorescent protein in all cells, suggesting that the three regulatory fragments all have promoter activity and the major regulatory region of the C3 promoter lies between –466 and + 1 (Fig. 1B and C). The −963/+1 and −466/+1 nt fragments were also subcloned into pGL3-basic vector and the two constrcuts were transiently transfected into HEK 293T cells for luciferase assay. Consistently, the cells transfected with these two plasmids showed similar luciferase activity (Fig. 1D), indicating that removal of −963/-466 region did not affect *C3* promoter activity. Together, these results suggest that the regulatory elements necessary for transcriptional activation of *C3* gene are present between the proximal −466 nt and the transcription start site of the promoter region.

3.2. LPS markedly induces C3 gene expression both in HEK 293T cells and in amphioxus

First, we examined the effect of LPS on *C3* gene expression in HEK 293T cells. HEK 293T cells were incubated with LPS (10 μg/mL) or PBS alone. After 24 h, cells were harvested, and the C3 promoter activity was measured using luciferase reporter assay. Luciferase analysis showed about 10-fold increase of *C3* expression in LPS-treated cells compared to the control, indicating that LPS dramatically activated *C3* gene expression in HEK 293T cells (Fig. 3A).

Furthermore, we examined *C3* gene expression in response to LPS *in vivo*. First, we investigated the tissue expression pattern of *C3* in adult amphioxus *Branchiostoma belcheri* (Fig. 2). The *C3* transcript was abundant in notochord, muscle, gill and hepatic cecum, while weakly

Table 1
Primer sequences for real-time PCR analysis.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
C3 promoter-1	CCTAGCTGTTCTTGGTTACTCTCG	CAGAGGTTTGGGATTTGGCTTAT (−466～-1, 466bp)
C3 promoter-2	CCTAGCTGTTCTTGGTTACTCTCG	GCAGTGATTGTTTTGACCGCTAT (−963～-1, 963bp),
C3 promoter-3	CCTAGCTGTTCTTGGTTACTCTCG	GTCTCTCCTAAGGTCCCCAAGTC (−2160～-1, 2160bp)
NF-κB mutant	CTTGGGTCCGCTCAGTTTCCTGGTACTG	CCAGGAAACTGAGCGGACCCAAGATTCA
AP-1 mutant	ACTTACTGCTACCTGTGTGCTGAGCTG	CAGCACACAGGTAGCAGTAAGTTAATG
NFAT mutant	AACACACGGGCCCGAGTTCCGCCTGAAC	GGCGGAACTCGGGCCCGTGTGTTTTACC
C-JUN-1 mutant	CGTATTCCGATGGAGTCAGCACAAATAT	TGTGCTGACTCCATCGGAATACGCCCGG
C-JUN-2 mutant	TATCAGACCACCAGTGACGCCCGCCGGCTTC	CGGCGGGCGTCACTGGTGGTCTGATATTTGT
β-actin	TCTGGCATCATACCTTCTACAA	TCTGTGTCATCTTTTCCCTGTT
C3	ACACTGATGAGAATGGCTTGGAC	GTCCACCACTCGCTTGTACTTCT

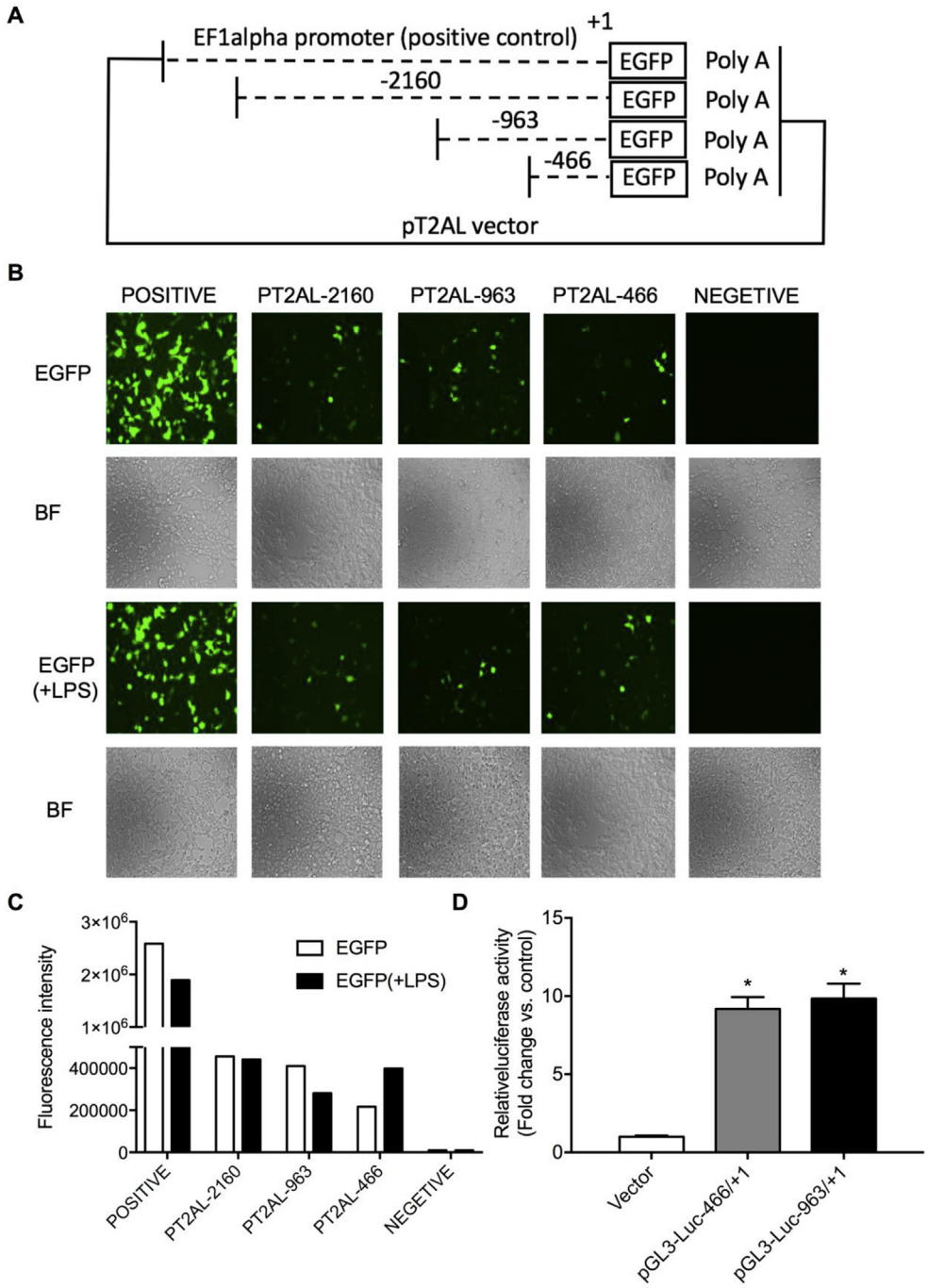

Fig. 1. The -466/+1 promoter region was important for transcriptional activity of the amphioxus *C3* gene. (A)The 293T cells were transfected with the *C3* promoter which subcloned between the *Xho I* and *Bam*H I sites of PT2AL vector to generate PT2AL-2160bp, PT2AL-963bp and PT2AL-466bp plasmids. (B) LPS induced *Branchiostoma belcheri C3* gene promoter activation in 293T cells by IFA (200 ×) as described under "Materials and Methods". Transfected with or without LPS (10 ng/μL). The 293T cells were transfected with PT2AL vector indicated the positive control and the 293T cells transfected nothing indicated the negative control. (C)The result represents EGFP fluorescence intensity measured by Image J software. (D) HEK 293T cells were transfected with pGL3 luciferase reporter constructs for 24 h as described under "Materials and Methods". The significant difference ($P < 0.05$) of *C3* promoter activities between different groups were indicated with asterisks*. Values are the means ± SD (standard deviation) for three independent experiments.

detected in gonad and intestine, indicating a tissue-specific expression pattern of *C3* gene in adult amphioxus. In the following study, the hepatic cecum was chosen for the analysis of *C3* expression after stimulation. The amphoixus was treated with LPS for 12 h. After stimulation, the amphioxus hepatocytes were harvested and the *C3* gene transcriptional level was measured using qRT-PCR. We found that *C3* mRNA expression was profoundly increased at about 3 fold after 12 h LPS treatment (Fig. 3B). Together, our data suggested that LPS strongly induced *C3* gene expression both *in vitro* and *in vivo* (Fig. 3A and B).

3.3. Identification of critical motifs maintaining basal activity of the amphioxus C3 gene promoter

To determine transcription factor binding sites critical for maintaining the promoter activity of the amphioxus *C3* gene, we searched for the known transcription-factor binding-consensus sequences in the C3 promoter region between −466 and + 1 nt using the Transfast program (Gesellschaft fur Biotechnologische, Braunschweig, Germany). The result revealed that the C3 -466/+1 promoter region contains multiple putative transcription factor binding sites (Fig. 4A). We next introduced piont mutation into each predicted transcription factor binding site, including NF-κB, AP-1, C-JUN-1, C-JUN-2, NFAT, C-JUN-1-C-JUN-2, AP-1-C-JUN-1-C-JUN-2 and NF-κB-NFAT, in the pGL3-466 as indicated in Fig. 4B. The luciferase reporter gene assay indicated that mutation of NF-κB, AP-1, C-JUN-2, NFAT and NF-κB-NFAT binding sites resulted in no significant increase in luciferase expression. In contrast, point mutations of either site C-JUN-1 or both C-JUN-1-2 and AP-1-C-JUN-1-2 which contain C-JUN-1 binding motifs markedly reduced the transcriptional activity of the C3 promoter. These data suggest that C-JUN-1 binding elements within the proximal sequence upstream of the

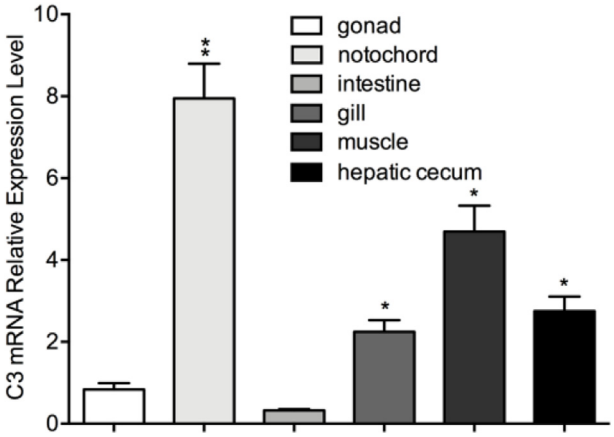

Fig. 2. Tissue distribution of *C3* gene of adult amphioxus by quantitative real-time RT-PCR. The statistical analysis was based on comparisons between the relative expression ratio of the *C3* gene among different groups by calculating $2^{-\Delta\Delta CT}$. The significant difference of *C3* expression between different groups were indicated with asterisk asterisks * ($P < 0.05$). Values are the means ± SD of six animals for three independent experiments.

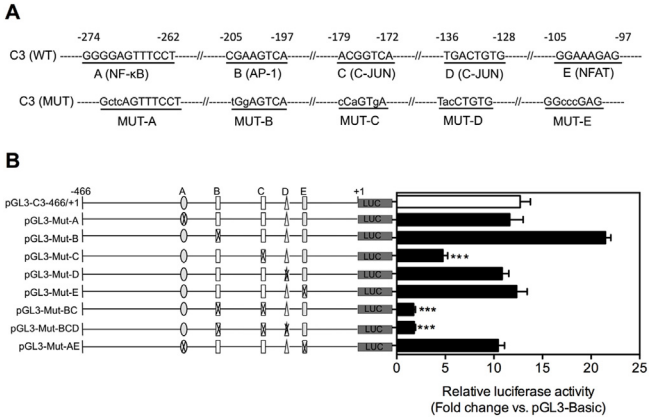

Fig. 4. Functional analysis of the *C3* gene promoter with site-directed mutagenesis. (A) Location of site-directed mutagenesis in putative transcription factor binding sites in the *C3* promoter. (B) Effect of site-directed mutagenesis of putative transcription factor binding sites on the *C3* promoter activities. The characteristics of *C3* promoter-luciferase reporter constructs used for the experiments are shown on the left panel, whereas the data of luciferase assay for the promoter activity are shown on the right panel. Circles named with A indicate the location of the NF-κB binding site. Rectangles named with B, C and E indicate the locations of the AP-1,C-JUN-1, and NFAT binding sites. Triangle named with D indicates the location of the C-JUN-2 binding site. The rectangles, triangle or circles are marked with an X when a site-replaced mutation in one or more positions are present. HEK 293T cells were transfected with pGL3 luciferase reporter constructs as described under "Materials and Methods". After 24 h culture, cells were harvested and cell lysates were assayed for luciferase activities. The significant difference ($P < 0.001$) of *C3* promoter activities between different groups were indicated with asterisks***. Values are the means ± SD for five independent experiments.

transcriptional initiation site are key motif for maintaing the basal activity of the C3 promoter function, whereas NF-κB, AP-1, C-JUN-2, NFAT binding sequence plays roles in governing the promoter activity at a homeostatic level.

3.4. Characterization of the LPS-induced functional motifs on the C3 promoter

In the experiment, we first transfected HEK 293T cells with PT2AL plasmid constructs containing −2160 to +1 nt, −963/+1, and −466/+1 regeions, respectively (as described in Fig. 1A) and pGL3 plasmid (−963/+1 nt, and −466/+1 nt). The pGL3 basic reporter was used as a negative control. 24 h after transfection, cells were treated with LPS (10 μg/mL) or PBS (control) for another 24 h and luciferase assay was performed with a standard protocol as previously described. We found that the relative luciferase activity was significant improved to about 10 fold in both pGL3-963/+1 and pGL3-466/+1 transfected cells 24 h after LPS treatment ($p < 0.05$) compared to control cells (Fig. 3A). These results indicated that the 5′-flanking region of the C3 promoter contains LPS-responsive sequences. To further evaluate the importance of these binding sites in response to LPS, HEK 293T cells were transfected with a set of pGL3-466/+1 constructs that have point mutations in the C-JUN elements as indicated in Fig. 3B. The wildtype promoter pGL3-466/+1 was used as the positive control. As shown in Fig. 5, after LPS stimulation, the relative luciferase activities were similar restrain in cells transfected with the construct containing the promoter region with single mutations at the C-JUN-1 motif (site C) and multiple mutations containing the C-JUN motif (C-JUN-1-2 and AP-1-C-JUN-1-2 sites). In contrast, LPS did not inhibited the relative luciferase activity with plasmids with single point mutation at the site NF-κB (A), AP-1 (B), C-JUN-2 (D), NFAT (E), or multiple mutations NF-κB-NFAT (AE).

3.5. C-JUN modulates C3 promoter activity via JNK signaling pathway

C-JUN motif is a common downstream substrate of JNK in response to various stimuli. To furgher investigate whether JNK signaling pathway is involved in LPS-induced C3 promoter activation, lucifrease assay and western blotting experiments were performed using a JNK

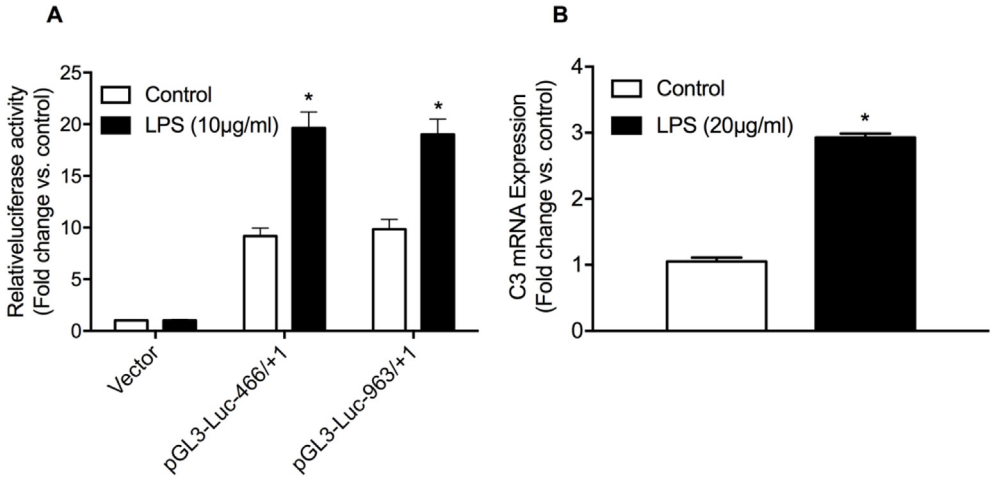

Fig. 3. LPS induced *C3* gene promoter activation and gene expression. (A) HEK 293T cells were transiently transfected with pGL3 luciferase reporter constructs as described under "Materials and Methods" and treated with LPS or PBS alone for 24 h as indicated in the figure. (B) Adult amphioxus were treated with LPS or PBS alone for 6 h as indicated in the figure. At the end of treatments, hepatic cecum were dissected for RNA extraction. Expression levels of *C3* transcripts were measured by qRT-PCR. The significant difference ($P < 0.05$) of *C3* promoter activities between different groups were indicated with asterisks*. Values are the means ± SD for five independent experiments.

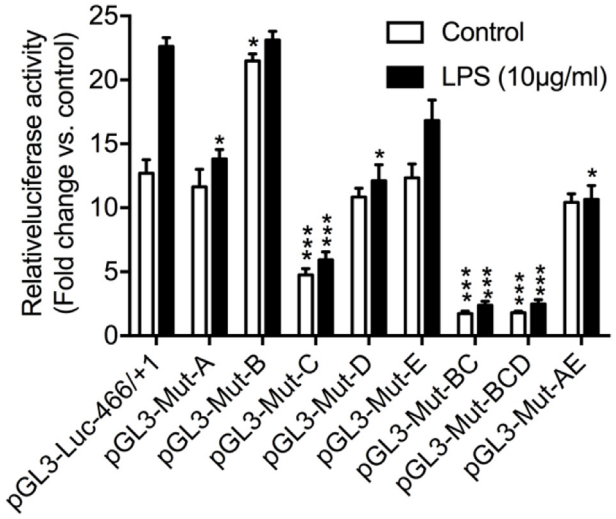

Fig. 5. LPS induced *C3* expression by targeting C-JUN motif in the *C3* gene promoter. HEK 293T cells were transfected with pGL3 luciferase reporter constructs as described under "Materials and Methods". The pGL3 plasmids containing a full length and 5′ truncated fragments of the *C3* promoter were used for the experiments in Fig. 4A, whereas pGL3 plasmid constructs harboring mutated *C3* promoter illustrated in Fig. 4B were used for experiments in Fig. 5. 24 h after transfection, cells were treated with LPS or PBS alone (control) for 24 h. Then, cells were harvested and lysed for luciferase acitivity dection. Values are shown as fold change of luciferase activity relative to controls. The significant difference of *C3* promoter activities between different groups were indicated with asterisks* ($P < 0.05$) and *** ($P<0.001$). Values are the means ± SD for five independent experiments.

inhibitor SP600125. As shown in Fig. 6A, incubation of cells transfected with −466/+1C3 plasmid with LPS resulted in a strongly enhanced luciferase activity in 293T cells, whereas SP600125 significantly reduced LPS-induced *C3* promoter activity to the basal level. This result showed that JNK regulated C3 promoter activity in LPS-stimulated HEK 293T cells. In addition, western blot analysis showed that phosphorylation level of JNK was strongly increased by LPS stimulation, while the treatment of SP600125 for 6 h partially inhibited *C3* expression with or without LPS stimulation. Moreover, the 12 h incubation of SP600125 resulted in a marked reduction in the level of phosphorylated JNK protein in amphioxus hepatic cecum tissues (Fig. 6C). Additionally, as shown in Fig. 6B, sp600125 also inhibited LPS-induced *C3* expression in amphioxus. These *in vitro* and *in vivo* findings showed the JNK was activated in response to LPS, which sebsequently induced C3 promoter activation through C-JUN motif.

4. Discussion and conclusion

Amphioxus is widely held to be the closest invertebrate relative of the vertebrates and the best available stand-in for the proximate ancestor of the vertebrates and is shown by phylogenetic analysis to be derived directly from the common ancestor of vertebrate (supplemental Fig 1,2). The complement system has been discovered in invertebrates and vertebrates, and plays a crucial role in the innate defense against common pathogens. Bacterial structures such as polysaccharide capsule and those which mimic or bind host molecules function to prevent complement mediated lysis and phagocytosis. The complement system, which is composed of 35 fluid-phase and membrane-bound molecules, is initiated by the classical (CP) or the lectin (LP) pathways, and amplified by the alternative pathway (AP). Each pathway involves a series

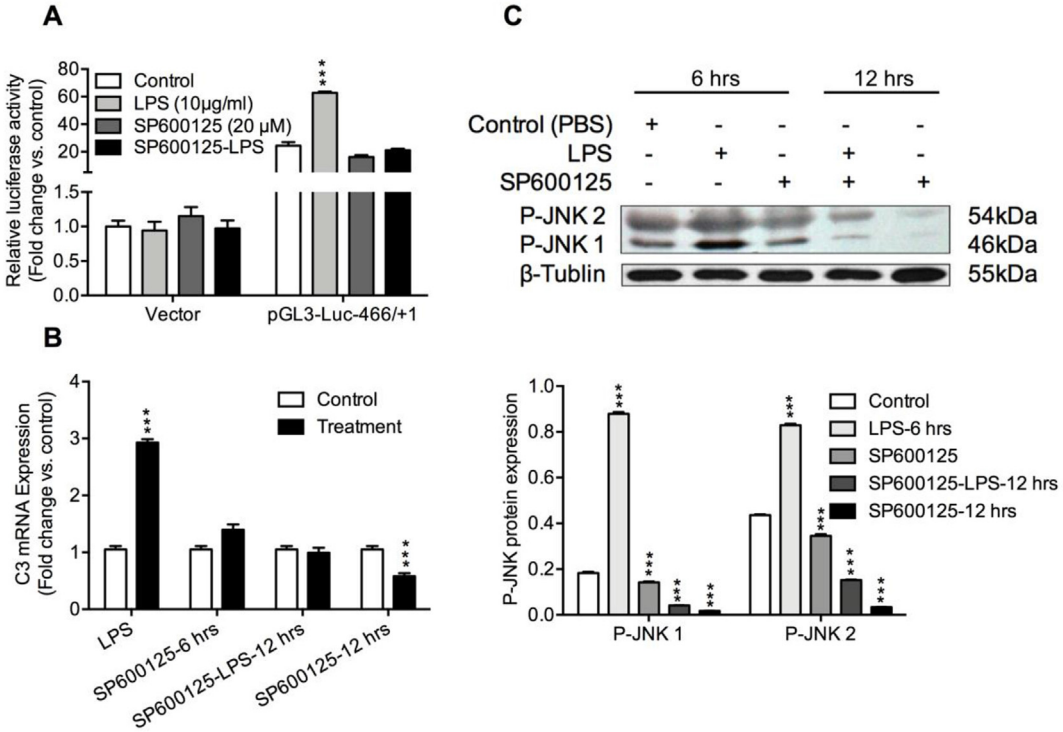

Fig. 6. Role of the JNK in the *C3* gene promoter activity. (A) HEK 293T cells were transfected with *C3* promoter and incubated overnight. Cells were pre-treated with LPS and/or JNK inhibitor SP600125 for 6 h. *C3* promoter activity was measured using the Luciferase assay. (B and C) Amphioxus were treated with LPS (20 ng/μL), JNK inhibitor SP600125 (40 μM) or the combination of SP600125 plus LPS. At the end of treatments, the hepatic cecum was separated under dissecting microscope at 4 °C. Levels of *C3* transcripts were measured by qRT-PCR (B), whereas p-JNK protein was assessed by western blotting analysis (C). The significant difference ($P < 0.001$) between the challenged and the control group were indicated with asterisks***. Values are the means ± SD for three independent experiments. The upper panel in C is a representative western blot result and the lower panel in C represents p-JNK protein expression as measured by densitometric analysis of immunoblot data. Bars represent mean signal intensity relative to that of control group. The levels of β-actin gene expression were used for normalizing equal protein loading in experiments.

of proteolytic steps that serve to activate the complement factors in a cascade. All pathways eventually lead to the generation of a complement C3 convertase, which cleaves C3 to liberate C3b. C3b becomes covalently bound to the surface of a pathogen resulting in its elimination through phagocytosis or lysis. Traditionally, C3 was thought to be present only on myeloid cells such as macrophages, eosinophils and mast cells, However, the demonstration that C3 was expressed throughout the body and in the central nervous system which is consistent with C3 having a much greater role in the pathogenesis of inflammatory and autoimmune diseases than previously suspected [17]. The C3 is a key molecule in the complement system, whose activation is essential for all the important functions performed by this system, but the molecular mechanism in regulating the initiation of *C3* transcription activity is less clear. Functional analysis of C3 promoter luciferase reporter plasmid will provide the theory basis for researching the function of transcription activity. The development of complement in Cephochordata is poorly understood, and little information is available about the acquisition of full complement capacities. In this study we demonstrated the differential expression of complement key component gene *C3* and its response to challenge with LPS during development of amphioxus Branchiostoma belcheri. Considering the significance of C3 in amphioxus, in this study we found a marked enhancing effect by LPS. Therefore, the effects on mutant *C3* expression in HEK 293T cells by LPS were further investigated. Furthermore, we showed that LPS induced *C3* expression through a JNK-mediated transcriptional regulation in HEK 293T cells. The possible underlying mechanisms were determined, which showed that LPS regulated *C3* activation through JNK dependent pathways.

First, we determined the regulatory region in the C3 promoter important for *C3* gene expression. EGFP fluorescence and Dual-luciferase gene reporter assays were used to assess different fragment effects on promoter activity. To test for putative promoter activity we cloned the -2610/+1 region of C3 promoter into PT2AL vector and luciferase gene reporter constructs, respectively. To evaluate the potential effect and further characterise this intronic promoter, we generated a series of progressive deletions from the 963 bp-long sense sequence. Analysis was conducted in the HEK 293T cell line which treated in the presence or absence of LPS, and the expression of enhanced green fluorescent protein was obviously induced both in all the experimental group which indicated three amphioxus C3 promoter fragments all have transcriptional activity. The Dual-luciferasereporter assay further revealed a core promoter located in the region up to −466 bp relative to the transcription start site of the *C3* gene in 293T cell lines. A consistent luciferase expression pattern was observed in the 293T cell line. Additionally, *C3* expression was analyzed from LPS-stimulated fish and PBS-dissolved controls to confirm the inflammatory state after stimulation. To understand how LPS regulates C3 transcription, we used a series of C3 promoter-luciferase mutant constructs (covering 466 bp of the proximal promoter) to determine the transcription factor binding sites critical for transcriptional activity of *C3* gene. We identified a 179-bp region upstream of the transcriptional start site that exhibited high promoter activity and was repressed by SP600125. Mutation of potential transcription factor binding sites within the promoter region revealed that C-JUN-1 (−179 bp from transcription start site) is required for C3 promoter activity.

Liver is the principal organ synthesizing acute phase proteins (APPs) (Steel, 1993). Amphioxus has a hepatic cecum, which has long been considered to be the precursor of vertebrate liver [18]. Furthermore, the acute phase action-relevant molecules including Bf and fibrinogenrelated protein genes that are primarily expressed in the vertebrate liver are also predominantly expressed in the hepatic cecum in *B. belcheri* [19]. All these not only support the hypothesis that the vertebrate liver evolved from the hepatic cecum of an amphioxus-like ancestor during early chordate evolution, but also suggest that like the liver, the hepatic cecum in amphioxus is the primary tissue possibly involved in acute phase response. *In vitro* studies demonstrated that the cells stimulated by LPS induced transcriptional increase of *C3* gene. In accordance with the *in vitro* findings, the amphioxus *C3* gene expression were upregulated after stimulation with LPS. Wang et al. has suggest that the complement operating via the AP is competent by responding to challenge with LPS in the hatched larvae of *Danio rerio* [13,15], which indicated that LPS could induce the complement siystem via AP pathway. In addition, our strudy provides several pieces of evidence indicationg that the c-jun binding site at −179 nt is a functional motif for transcriptional regulation of *C3* gene expression and JNK is involved in LPS-mediated C3 activation. First, we showed that mutation of this c-jun binding sequence resulted in a sharp decrease in the promoter activity of the *C3* gene. Secondly, the *in vivo* study indicated that JNK inhibitor downregulated the C3 mRNA expression. Collectively, our data suggest that JNK is involved in the transcriptional regulation of the *C3* gene via a complex mechanism.

NF-κB and AP-1 are the important transcription factors that regulate the expression of genes involved in inflammation, differentiation, and proliferation. Therefore, NF-κB and AP-1 are considered as the major targets and key players in inflammatory diseases that regulate the inflammatory process [4]. C-Jun is a component of the AP-1 transcription complex, which is an important regulator of gene expression and the AP-1 transcription factor is a major transcriptional parnter of NFAT [20–23]. Whereas, in our experiment, the mutation of NF-κB, AP-1, NFAT binding site resulted in a significant increase in luciferase expression and only the mutation in C-JUN binding sites markedly reduced the basal activity of the C3 promoter. This demenstrated that C-JUN binding sites strongly regulate C3 promoter activity. Next, Our results also illustrated that LPS-induced C3 promoter activity and C3 mRNA expression were significantly suppressed by JNK inhibitor SP600125. Moreover, Western blotting analysis indicated that JNK inhibitor SP600125 markedly inhibited LPS-induced JNK protein expression and JNK phosphorylation. Taken together, LPS stimulation phosphorylates and activates JNK, resulting in the increase of the C3 promoter activity and C3 up-regulation. The present study suggests that JNK may be a potential signaling pathway for mediating LPS-associated inflammatory diseases. The present study provided the first evidence that JNK may have an influence on the expression of C3 and revealed the mechanism of C3 transcriptional regulation.

Amphioxus C3 retains most of the functionally important residues of vertebrate C3 and only opsonic activity has been assigned to the invertebrate complement system until now. Therefore, this is the important molecular evidence for complement-mediated immunological in invertebrates. In conclusion, we characterized the important regulatory region of the amphioxus C3 gene promoter. Results from the present study suggest that the interaction among transcription factor c-jun and their binding motifs in the C3 promoter region plays a critical role in the transcriptional regulation of the *C3* gene. In this study, we clearly provide evidences for a pivotal role of JNK in LPS-induced C3 expression in amphioxus. However, further research is needed to understand the exact molecular mechanism by which inflammation target c-Jun and inflammation responsive motifs in the promoter of the *C3* gene to alter its expression.

CRediT authorship contribution statement

Mingliang Chen: Conceptualization, Funding acquisition, Methodology, Writing – original draft, Writing – review & editing. **Chenying Wang:** Data curation, Investigation, Formal analysis, Writing – original draft. **Zengpeng Li:** Formal analysis, Investigation, Resources, Validation. **Jianming Chen:** Conceptualization, Project administration, Writing – review & editing.

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Appendix A. Supplementary data

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