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FAM46C inhibits lipopolysaccharides-induced myocardial dysfunction via downregulating cellular adhesion molecules and inhibiting apoptosis

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ABSTRACT

Aims: Sepsis is a syndrome of inflammatory response induced by infection. Cellular adhesion molecules may involve in sepsis-induced myocardial dysfunction (SIMD) which is a major predictor of morbidity and mortality of sepsis. Here we studied the role of FAM46C in AC16 cells and c57 mice with lipopolysaccharides (LPS) treatment.

Main methods: Real-time PCR and western blot were used to detect the expression level of relative genes and protein. Cell proliferation and apoptosis were evaluated.

Key findings: Interestingly, negative correlation between Toll-like receptor 4 (TLR4) and FAM46C in sepsis was observed. The overexpression of FAM46C reduced the apoptosis induced by LPS in AC16 cells. Inhibition of apoptosis contributed by FAM46C was mediated by adhesion molecule via blocking p38 and ERK/MAPK signaling pathway. Moreover, overexpression of Fam46c and inhibition of TLR4 by TAK-242 could attenuate apoptosis induced by LPS in vivo.

Significance: FAM46C played an important role in SIMD via inhibiting LPS-induced myocardial dysfunction by downregulating cellular adhesion molecules and inhibiting apoptosis. It was the first time to explore the role of FAM46C in SIMD in this study.

1. Introductions

Sepsis, a syndrome of inflammatory response induced by infection, is a major problem all around the world and based on the occurrence of serious complications of illness [1]. Severe sepsis and septic shock are parts of sepsis syndrome, which occurs with sepsis-related organ dysfunction [2]. Sepsis may induce multiple organ dysfunction, such as endothelial damage, acute lung injury, liver injury and myocardial dysfunction [3]. The cardiovascular systems are the most easily affected organ by sepsis and almost 40–50% of sepsis patients exhibit myocardial dysfunction [4]. Sepsis-induced myocardial dysfunction (SIMD) is a major predictor of morbidity and mortality of sepsis. Although there are many researches aim on SIMD, the underlying mechanism is still not understood and prognosis is still poor [5,6]. In recent years, early fluid resuscitation is the main treatment of sepsis, but how to prevent myocardial injury may be a novel view of sepsis treatment [7,8]. Currently, researchers find that mitochondrial dysfunction, nitric oxide, intracellular energetics, complements and cellular adhesion molecules maybe mediators of SIMD [5].

Cellular adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), are increased in the plasma of children with sepsis-induced multiple organ failure [9]. Further studies show that ICAM-1/VCAM-1 expression is increased in lipopolysaccharides (LPS) induced myocardial dysfunction [10]. TLR4 regulates ICAM-1 which contributes to endotoxic cardiac dysfunction [11]. TLR4, a key member of the TLRs, is involved in various diseases, including infectious disease [12], allergic diseases [13], neuronal degeneration [14] and cardiovascular disease [15]. Importantly, TLR4 mediates innate immunity and inflammatory response and plays a central role in LPS signaling and seems to be depended by the occurrence of SIMD [16]. It was well documented that TLR4 signaling pathways can be divided into MyD88-dependent and MyD88-independent respectively. The abnormal regulation of LPS/TRL4 signaling pathways has the potential to induce massive inflammation and cause acute sepsis [17].TLR4 existed on the surface of macrophages can cause myocardial dysfunction during endotoxemia [18]. Apoptosis is a major contributor to sepsis-induced multiple organ dysfunction and plays a critical role in SIMD [19,20].

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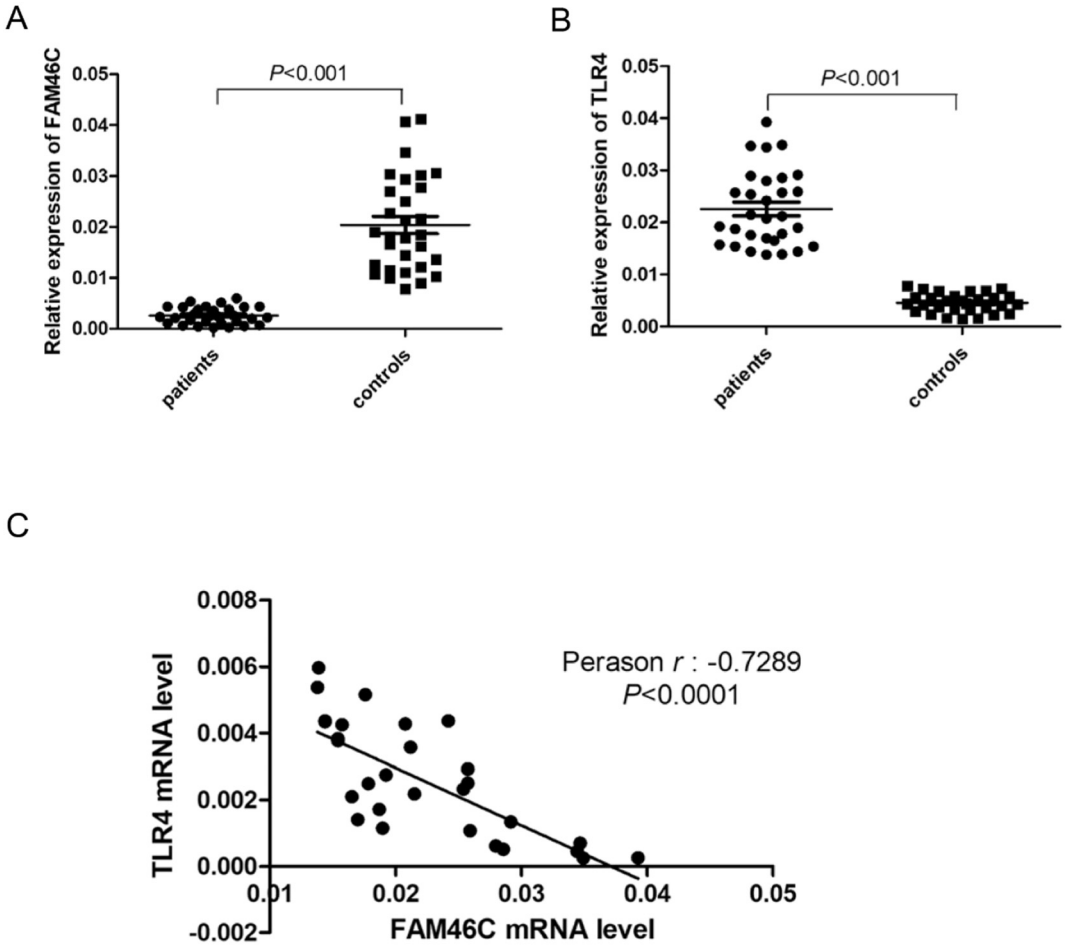

Fig. 1. Negative correlation between TLR4 and FAM46C in sepsis. A.B. Real-time PCR was performed to measure the relative expression level of FAM46C (A) and TLR4 (B) in blood cells of normal human and sepsis patients respectively (n = 30). Controls: blood cells of normal human; patients: blood cells of sepsis patients. (C): Pearson Correlation of TLR4 and FAM46C ($P < 0.0001$, $r = -0.7289$).

Family-with-sequence-similarity-46 (FAM46) has four members in humans and is a group activator of human nucleotidyltransferase (NTase), which play crucial roles in various biological processes [21]. Until now, the exact biological function of Fam46 is still unclear. As reported, FAM46C, which is a non-canonical poly(A) polymerase, promotes cell apoptosis in hepatocellular carcinoma [22,23] and is a tumor suppressor for multiple myeloma [24,25]. FAM46C improves the replication of certain viruses as it is a type I interferon stimulated gene [26]. In addition, FAM46C is related in various regulations of translation [27]. However, the roles of FAM46C in SIMD are needed to be recognized.

As reported, LPS is an effective method to induce septic models in vivo and in vitro [28]. Here we aimed to probe the role of FAM46C after treating with LPS in vitro and in vivo. So, AC16 cells and c57 mouse were used in this study. We found negative correlation between TLR4 and FAM46C in sepsis. The overexpression of FAM46C could reduce apoptosis induced by LPS in AC16 cells. Moreover, we found the similar effects in mice treated with LPS in vivo. Overall, we may reveal a novel mechanism of FAM46C in sepsis.

2. Methods and materials

2.1. Patient samples

30 pairs of SIMD patients and normal PBMC (Peripheral Blood Mononuclear Cell) were obtained from Huashan Hospital, Fudan University. Informed consents were signed by the patients. This study was approved by the ethics committee of Fudan University.

2.2. Cell culture and treatment

Human Cardiomyocyte Cell Line AC16 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured with DMEM media containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and penicillin/streptomycin. 37 °C, 5% CO_2 were the conditions of culture.

For the different concentrations of LPS (Sigma, St. Louis, MO, USA), AC16 cells were treated with 0, 50 ng/ml, 100 ng/ml, 200 ng/ml LPS for 24 h. For time points of LPS, AC16 cells were treated with 100 ng/ml LPS for 0, 6, 12, 24, 48 h. For the experiment, cells were treated with TAK-242 (100 μg/l, an inhibitor of TLR4), PD98059 (20 μM, an inhibitor of ERK1/2) and/or SB203580 (20 μM, an inhibitor of p38) after pretreatment of 100 ng/ml LPS. TAK-242, PD98059 and SB203580 were purchased from Selleck (Houston, TX, USA).

2.3. Lentivirus construction and infection

The human FAM46C and mouse Fam46c overexpression and human FAM46C knock-down lentiviruses were purchased from Genechem company (Shanghai, China). The sequences were shown as blow:

ShFAM46C-1: 5′-CCAGGGATTGCATGTCCTT-3′;
ShFAM46C-2: 5′-GGACGAGGCAACTTTCCAA-3′;
ShFAM46C-3: 5′-GCAACTTCAGCAACTACTA-3′;

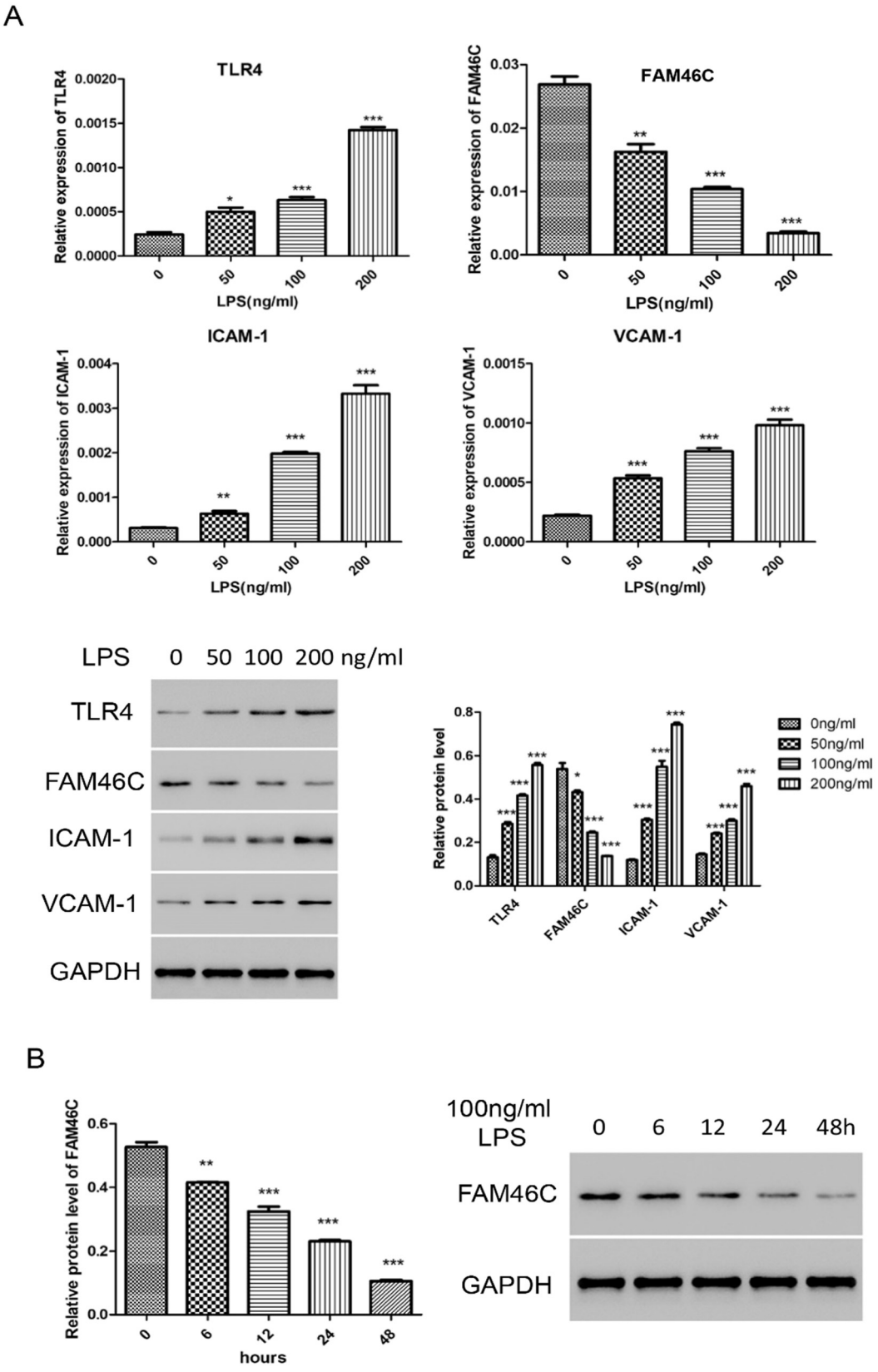

Fig. 2. Stimulation with LPS increased TLR4, ICAM-1 and VCAM-1 but suppressed FAM46C in AC16 cells. (A) Real-time PCR and western blot were used to detect the expression levels of TLR4, FAM46C, ICAM-1 and VCAM-1, when the cells were treated with gradient increased concentration of LPS. (B) Real-time PCR and western blot were used to measure the expression of FAM46C, when the cells were treated with 100 ng/ml LPS but different time course (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ vs 0 ng/ml LPS).

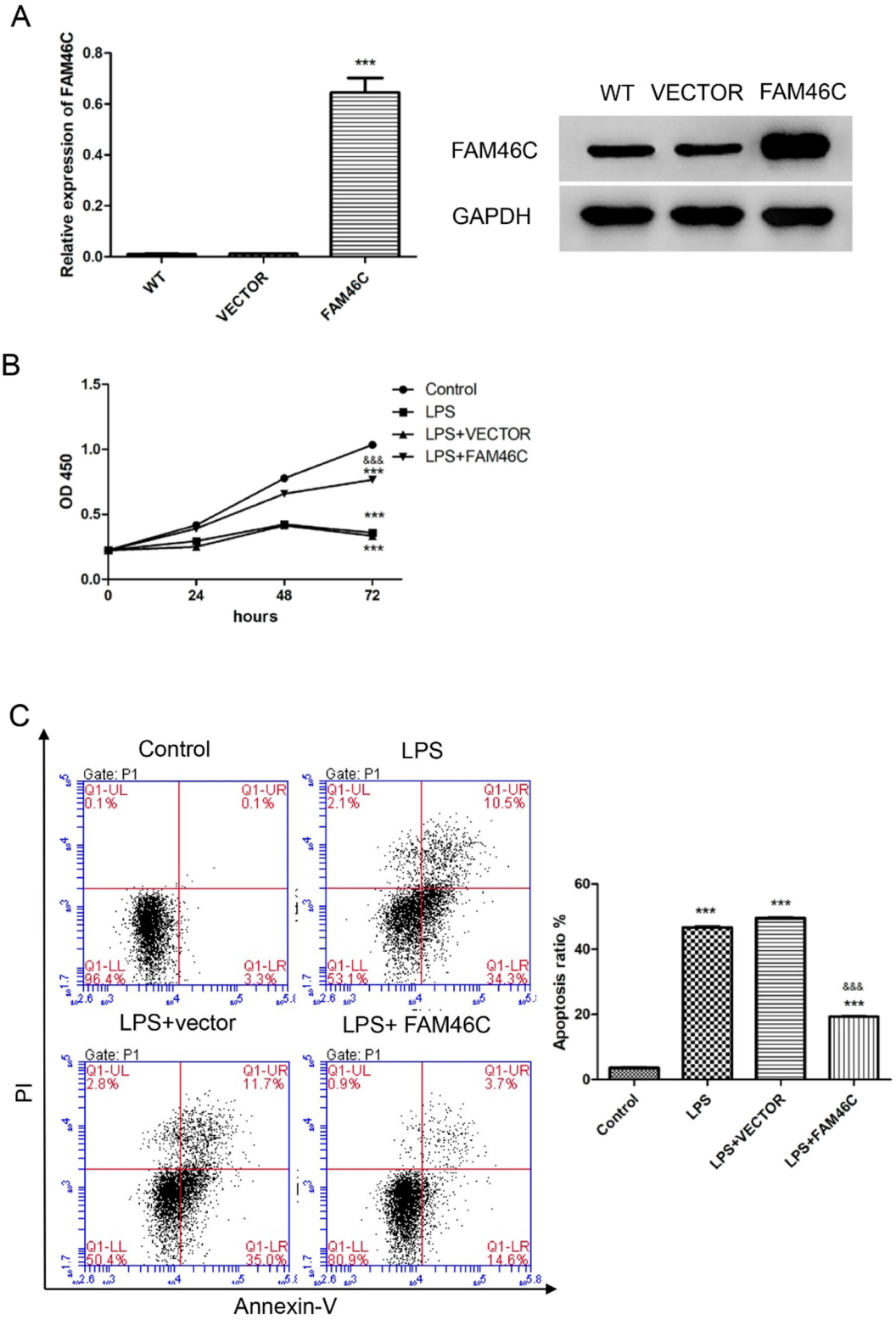

Fig. 3. Apoptosis induced by LPS treatment can be attenuated by FAM46C. (A) Real-time PCR and western blot were used to detect the expression of FAM46C. WT: wide type AC16 cells; VECTOR: transduced with empty vector lentivirus; FAM46C: transduced with FAM46C overexpression lentivirus (***: $P < 0.001$ vs VECTOR). (B) Cell proliferation was measured by CCK-8 (***: $P < 0.001$ vs Control; &&&: $P < 0.001$ vs LPS + VECTOR). (C) Cell apoptosis was measured by flow cytometry. Control: wide type AC16 cells; LPS: wide type AC16 cells treated with 100 ng/ml LPS; LPS + VECTOR: vector AC16 cells treated with 100 ng/ml LPS; LPS + FAM46C: FAM46C-AC16 cells treated with 100 ng/ml LPS (***: $P < 0.001$ vs Control; &&&: $P < 0.001$ vs LPS + VECTOR).

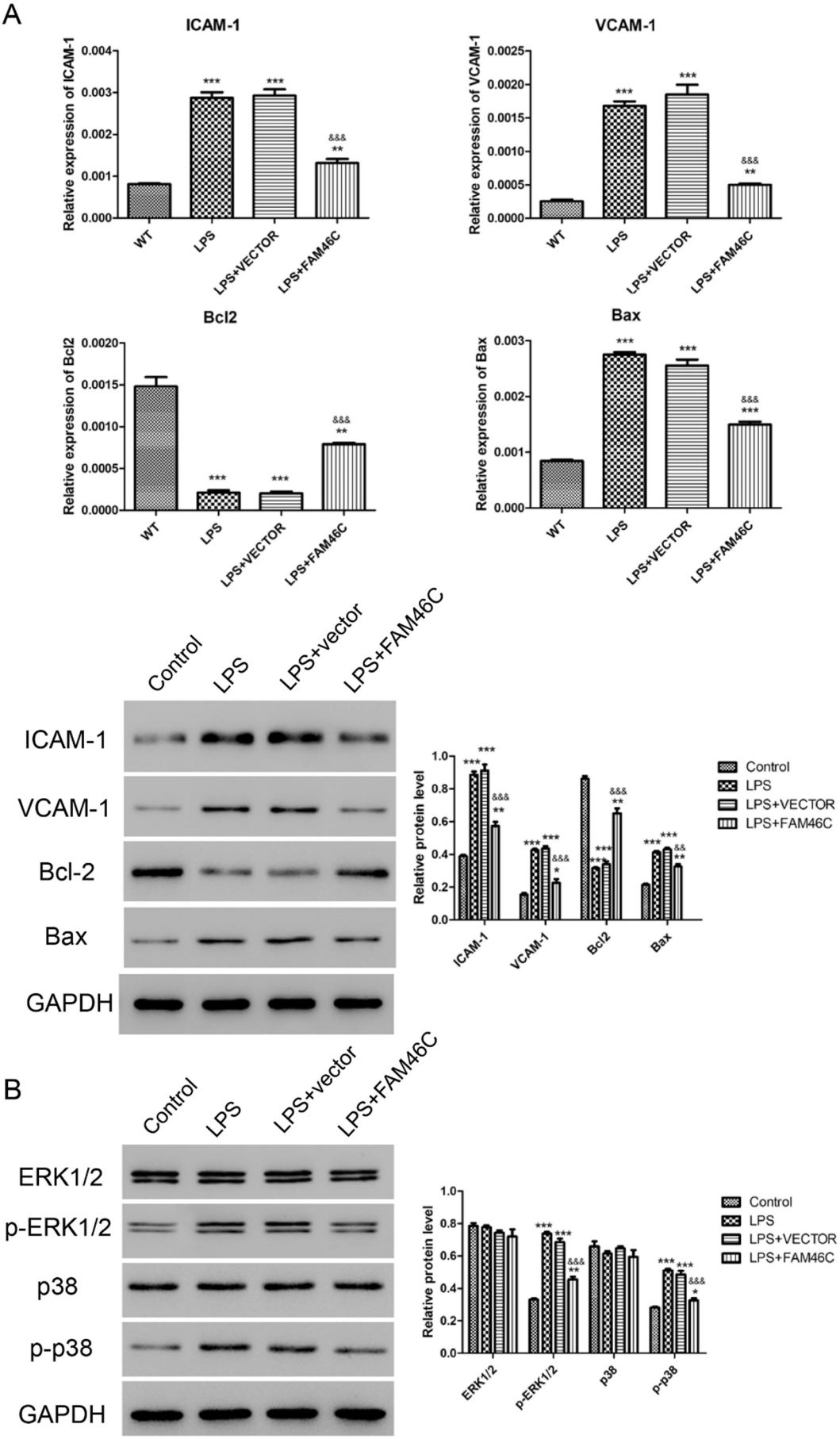

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Fig. 4. FAM46C contributed to resistance to LPS induced apoptosis by downregulating cell adhesion molecules. (A) Real-time PCR and western blot were used to measure the expression levels of ICAM-1, VACM-1, Bcl-2 and Bax. (B) Western blot was used to measure the expression of ERK1/2, p-ERK1/2, p38 and p-p38. Control: wide type AC16 cells; LPS: wide type AC16 cells treated with 100 ng/ml LPS; LPS + VECTOR: vector AC16 cells treated with 100 ng/ml LPS; LPS + FAM46C: FAM46C-AC16 cells treated with 100 ng/ml LPS (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ vs Control; &&: $P < 0.01$; &&&: $P < 0.001$ vs LPS + VECTOR).

Cells were planted into 6-well plates at a density of 5×10^5 cells/well. Lentivirus was added into each well respectively, according to lentivirus colony forming unit (CFU) when the cells had completely adhered to dish. After 48 h of transfection, cells were harvested to detect infection efficiency or cultured for other treatments. For FAM46C overexpression stable transfection AC16 cells, 0.8 mg/ml G418 (Sigma, St. Louis, MO, USA) was added to culture medium after 48 h of transfection. The culture medium was replaced every 2 days. Following 2 weeks the medium was replaced with normal medium.

2.4. RNA extraction and Real-time PCR

Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) was used to extract cellular total RNA. Using a cDNA synthesis kit (Promega, Madison, WI, USA) to obtain cDNA according the instruction. Real-time PCR was performed with SYBR Green qPCR Mixes (Thermo Fisher Scientifc Inc., Grand Island, NY, USA) and an ABI 7300 system (Applied Biosystems, FosterCity, CA, USA) following the user's manual. The primers were shown as followings.

Human FAM46C forward primer, 5′ CCGCCGTATAAGAACGGAG 3′,
reverse primer, 5′ AGAAGAGGAGGCGACAGAG 3′;
mouse Fam46c forward primer, 5′ TGGTTCTGTGCTCCCTTCTG 3′
reverse primer, 5′ CGTTCCTCCCGTTCTTGTTG 3′
human TLR4 forward primer, 5′ CCGCTTTCACTTCCTCTCAC 3′,
reverse primer, 5′ CATCCTGGCATCATCCTCAC 3′;
human ICAM-1 forward primer, 5′ GTTGTTGGGCATAGAGAC 3′,
reverse primer, 5′ CAGGGCAGTTTGAATAGC 3′;
mouse Icam-1 forward primer, 5′ CGTGATGGCAGCCTCTTATG 3′,
reverse primer, 5′ ATCCACCGAGTCCTCTTAGC 3′
human VCAM-1 forward primer, 5′ TGGGAACGAACACTCTTAC 3′,
reverse primer, 5′ CAGCAACTGAACACTTGAC 3′;
mouse Vcam-1 forward primer, 5′ GAAGATGGTCGCGGTCTTGG 3′,
reverse primer, 5′ CCGTAGTGCTGCAAGTGAGG 3′;
human Bcl2 forward primer, 5′ GCAGTGTGGTCTCCGAATGTC 3′,
reverse primer, 5′ CATTGCCTCTCCTCACGTTCC 3′;
mouse Bcl2 forward primer, 5′ ACATTGTGAGACTGAGTTAG 3′,
reverse primer, 5′ TTCCCAGGATTTCTGATTAG 3′;
human Bax forward primer, 5′ CTGAGCGAGTGTCTCAAG 3′,
reverse primer, 5′ CAGCCCATGATGGTTCTG 3′;
mouse Bax forward primer, 5′ GTGGTTGCCCTCTTCTAC 3′,
reverse primer, 5′ CAGCCCATCTTCTTCCAG 3′;
human GAPDH forward primer, 5′ AATCCCATCACCATCTTC 3′,
reverse primer, 5′ AGGCTGTTGTCATACTTC 3′;
mouse GAPDH forward primer, 5′ CTGCCCAGAACATCATCC 3′,
reverse primer, 5′ CTCAGATGCCTGCTTCAC 3′;

2.5. Western blot analysis

RIPA lysis buffer (Solarbio, Beijing, China) was used to extract total protein and a Bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientifc Inc., Grand Island, NY, USA) was used to measure concentration of protein. Equal amounts of protein (25 μg) were separated by 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Millipore Corp., Bedford, MA, USA). The membranes were blocked with TBST containing 5% BSA and incubated with primary anti-body at 4 °C overnight. After 3 times washing for 15 min, the membranes were incubated with horseradish peroxidase (HRP) linked secondary antibody for 1 h at room temperature. Then the blots were washed twice with TBST and developed using an enhanced chemiluminescence (ECL) kit (Millipore, Burlington, MA, USA). Protein expression was analyzed by Image J and normalized to GAPDH. The primary antibodies used were as follows:

TLR4 abcam 1:800 dilution; FAM46C abcam 1:800 dilution; ICAM-1 abcam 1:1000 dilution; VCAM-1 abcam 1:1000 dilution; Bcl2 abcam 1:1000 dilution; Bax abcam 1:1000 dilution; ERK1/2 CST 1:1000 dilution; p-ERK1/2 CST 1:800 dilution; p38 CST 1:1000 dilution; p-p38 CST 1:800 dilution; GAPDH abcam 1:2000 dilution.

2.6. Cell proliferation and apoptosis

Cell proliferation was measured by a Cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China). AC16 cells were seeded in 96-well plates and treated as descripted above. At each time points, culture DMEM media was replaced with detection buffer (DMEM: CCK-8 = 9:1). After 1 h of incubation, cell proliferation was detected by an auto-microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 450 nm. Cell apoptosis was measured by an Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). AC16 cells were treated as descripted above and washed twice with PBS. Then, the cells were stained with Annexin V and PI for 15 min in the dark. The apoptosis was evaluated by flow cytometry (BD Biosciences, San Jose, CA, USA). Experiments were performed in triplicate.

2.7. Animal model and treatment

Wild C57BL/6J pathogen-free male mice were 18 to 22 g (Shanghai SLAC Laboratory Animal CO. LTD). Mice were fed with free access to food and water and maintained under a 12 h light/dark cycle at 22–24 °C and 40–60% humidity.

The mice were randomly divided into 4 groups and each group had 6 mice. (a) Control group (wild type), (b) LPS group, (c) LPS + FAM46C group, (d) LPS + TAK-242 group. To establish the LPS model, the mice were intraperitoneally injected with 15 mg/kg LPS (*Escherichia coli* 055: B5) expect control group. Each mouse of LPS + FAM46C group and LPS + TAK-242 group was intraperitoneally injected with FAM46C overexpressed lentivirus (1×10^7 PFU/ml) or TAK-242 (2 mg/kg) respectively before LPS administration. According to preliminary experiment, mice were sacrificed 12 h after LPS injection. Some of the hearts were fixed in a 10% formalin solution and some were frozen into the −80 °C refrigerator.

2.7.1. Myocardial function: two-dimensional echocardiography

Left ventricular end-systolic dimension (LVDs) and left ventricular end-diastolic dimension (LVDd) were measured by two-dimensional echocardiography using the Vevo 2100 Imaging System (Visual Sonics, Toronto, ON, Canada).

The mice were anesthetized and laid on the supine position on a temperature-controlled board, then the paws were taped to electrodes. Heart rate and temperature were monitored to ensure the data collected were physiologically relevant.

2.8. Histologic examination and TUNEL

After 48 h of fixing in a 10% formalin solution, the heart tissue was used for histologic examination. Sections (5 mm; Leica RM2125,

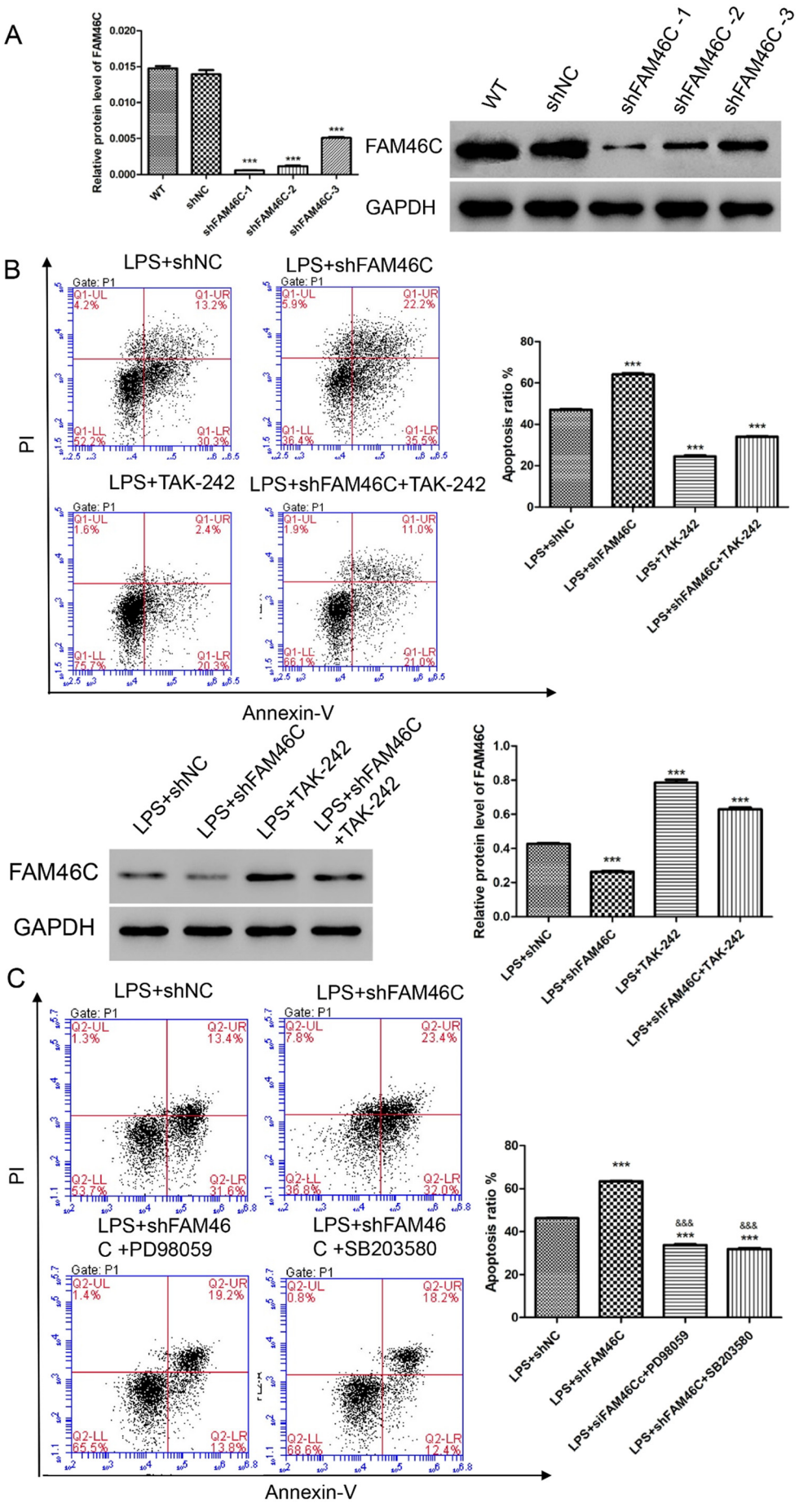

Fig. 5. TLR4 mediated apoptosis induced by LPS via inhibiting FAM46C. (A) Real-time PCR and western blot were used to measure the expression level of FAM46C. WT: wide type cell; NC: transduced with negative control; shFAM46C: transduced with FAM46C knockdown lentivirus; (B) Cell apoptosis was measured by flow cytometry after treated with LPS or TLR4 inhibitor in shFAM46C-AC16 or NC cells. LPS + shNC: NC cells treated with 100 ng/ml LPS; LPS + shFAM46C: shFAM46C-AC16 cells treated with 100 ng/ml LPS; LPS + TAK-242: NC cells treated with 100 ng/ml LPS and 100 μg/l TAK-242; LPS + shFAM46C + TAK-242: shFAM46C-AC16 cells treated with 100 ng/ml LPS and 100 μg/l TAK-242 (***: $P < 0.001$ vs LPS + shNC). (C) Cell apoptosis was measured by flow cytometry after treated with LPS or inhibitor of p38/ERK1/2 in shFAM46C-AC16 or NC cells. LPS + shNC: NC cells treated with 100 ng/ml LPS; LPS + shFAM46C: shFAM46C-AC16 cells treated with 100 ng/ml LPS; LPS + shFAM46C + PD98059: NC cells treated with 100 ng/ml LPS and 20 μM PD98059; LPS + shFAM46C + SB203580: shFAM46C-AC16 cells treated with 100 ng/ml LPS and 20 μM SB203580 (***: $P < 0.001$ vs LPS + shNC; &&&: $P < 0.001$ vs LPS + shFAM46C).

Table 1
The differences in LV dimensions and function at different groups.

	Control	LPS	LPS + Fam46c	LPS + TAK-242
EF (%)	75.82 ± 2.71	50.97 ± 5.14*	62.68 ± 3.89*,&	64.77 ± 3.18*,&

All values are means ± SE. EF, ejection fraction. Sample sizes are n = 5 animals for each group.
* P < 0.05 VS control.
& P < 0.05 VS LPS.

Germany) from the left ventricle were stained with hematoxylin and eosin (H&E) according to user's instructions. Then light microscopy (Olympus, Tokyo, Japan) was used to collect the images at ×200 magnification.

For apoptosis detection, the sections from left ventricle were stained with reaction buffer applied by a TUNEL Kit (Roche, Indianapolis, IN, USA) following the user's instructions.

2.9. Statistical analysis

All values are expressed as the mean ± SD. Data sets in groups were analyzed using a one-way analysis of variance (ANOVA). Significance was assigned where $P < 0.05$.

3. Results

3.1. Negative correlation between TLR4 and FAM46C in AC16 cells

In this study, considering the role of TLR4 in sepsis contributed by LPS-stimulation in vitro, we wanted to further test the correlation between TLR4 and FAM46C in sepsis. We firstly evaluated the transcription of TLR4 and FAM46C in blood cells of normal human and sepsis patients respectively. Real-time PCR was used to measure the relative expression levels of TLR4 and FAM46C respectively. As shown in Fig. 1A, we found dramatically reduction of FAM46C expression in sepsis patients compared with normal human ($P < 0.001$, $n = 30$). However, robust upregulation of TLR4 expression in sepsis patients compared with normal human was observed ($P < 0.001$, $n = 30$) (Fig. 1B). The data implied potentially negative correlation between TLR4 and FAM46C in sepsis. To test our hypothesis, we then performed correlative analysis and definitely found negative correlation between TLR4 and FAM46C in sepsis ($P < 0.0001$, $r = -0.7289$) (Fig. 1C).

3.2. LPS treatment increased TLR4, ICAM-1 and VCAM-1 but suppressed FAM46C in AC16 cells

Since cell adhesion molecules may be the mediators of SIMD, we then raised hypothesis as crosstalk among TLR4, ICAM-1, VCAM-1 and FAM46C might involve in the initiation and/or progression of sepsis. To test, we firstly evaluated transcription levels of TLR4, ICAM-1, VCAM-1 and FAM46C in AC16 cells after treatment with gradient increased concentration of LPS. As shown in Fig. 2A, we found dramatic reduction of FAM46C in the way of gradient dependence of LPS in AC16 cells. However, converse tendency was found for the expression levels of TLR4, ICAM-1 and VCAM-1. In detail, we found moderate increase of TLR4 after treatment with 50 and 100 ng/ml LPS in AC16 cells but much more significant increase when treated with 200 ng/ml LPS. For transcription of ICAM-1 and VCAM-1, 50 ng/ml of LPS slightly increase expression but more robust effects can be found when AC16 cells treated with 100 and 200 ng/ml LPS. Generally, 100 ng/ml of LPS seemed showing strong effects but not too high on suppressing or increasing related genes transcription in AC16 cells. We chose 100 ng/ml LPS for next studies. To further validate the findings, we then switched to use immune blotting to further evaluate the expression levels of TLR4, ICAM-1, VCAM-1 and FAM46C. We harvested cells and used whole cell lysates to perform immune blotting. We found similar tendency for the expression of the four proteins which indicated both reduction of FAM46C and upregulation of TLR4, ICAM-1 and VCAM-1 on the manner of LPS dosage dependence.

To further study the role of FAM46C in SIMD, we then treated AC16 cells with fixed concentration, 100 ng/ml but with time course from 0 to 48 h. As we found in Fig. 2B, reduction of FAM46C was observed when treated with LPS on the manner of time dependence. Most significant effects were observed at 48 h treatment. Similarly, protein level of FAM46C also decreases on time dependence when treated with 100 ng/ml LPS (Fig. 2B).

Collective data indicated LPS treatment inhibited expression of FAM46C on the manner of both dosage and time dependence.

3.3. Apoptosis induced by LPS treatment can be attenuated by FAM46C

Next, for deeper investigating the role of FAM46C in sepsis, we constructed stable cell line with overexpression of FAM46C in AC16 cells. Efficiency of overexpression was evaluated by real-time PCR and immunoblotting. As shown in Fig. 3A, FAM46C-AC16 cells can overexpress FAM46C ectopically. Moreover, efficiency of overexpression was also validated by immune blotting.

To test the effects of FAM46C on cell proliferation after treatment with LPS, FAM46C-AC16 stable cell line was chose to be treated with 100 ng/ml LPS for 24 h and then followed cell viability assay. As demonstrated in Fig. 3B, we found LPS alone induced robust reduction of cell proliferation. Of interest, FAM46C-AC16 can dramatically rescue cell proliferation. Meanwhile, no difference can be found in the group treated with LPS after transduction of empty vector comparation with LPS treatment alone (Fig. 3B).

To further test the function of FAM46C in cell apoptosis, especially under LPS treatment, we chose FAM46C-AC16 cells to be treated with 100 ng/ml LPS for 24 h and then followed by flow cytometry to detect apoptosis. We found LPS alone induced robust increase of apoptosis from 3.6 ± 0.1% to 46.73 ± 0.21%. Interestingly, FAM46C-AC16 can dramatically reduce apoptotic population from 46.73 ± 0.21% to 19.27 ± 0.29%. Meanwhile, no difference can be found in the group treated with LPS after transduction of empty vector comparation with LPS treatment alone (46.73 ± 0.21% vs 49.5 ± 0.26%) (Fig. 3C).

3.4. FAM46C contributed to resistance to LPS induced apoptosis by downregulating cell adhesion molecules

To test the hypothesis that resistance to LPS induced apoptosis was mediated by cell adhesion molecules in sepsis, we evaluated related genes expression in wide-type AC16 and FAM46C-AC16 cells under LPS treatment. LPS treatment alone dramatically increased cell adhesion molecules, including ICAM-1 and VCAM-1. However, upregulation of these two genes induced by LPS can be inhibited by overexpression of FAM46C. Conversely, LPS treatment suppressed Bcl2 but promoted Bax transcription respectively. Of note, these effects can be reversed by ectopic expression of FAM46C. Consistently, similar results were observed when we performed immune blotting to detect expression levels of ICAM-1, VCAM-1, Bcl2 and Bax (Fig. 4A).

It has been documented that p38/MAPK pathway regulated Bcl2 transcription. To further explore the underlying mechanism contributing to upregulation of Bcl2, we then tested p38/MAPK pathway in our study. Interestingly, we found LPS treatment upregulated levels of p-p38 and p-ERK1/2 respectively. Of note, the effects can be reversed in FAM46C-AC16 cells. Collectively, FAM46C reversed apoptosis induced by LPS by downregulating ICAM-1, VCAM-1 and Bax via blocking p38/MAPK pathway in AC16 cells (Fig. 4B).

3.5. TLR4 mediated apoptosis induced by LPS via inhibiting FAM46C

As shown in Fig. 2, negative correlation between TLR4 and FAM46C

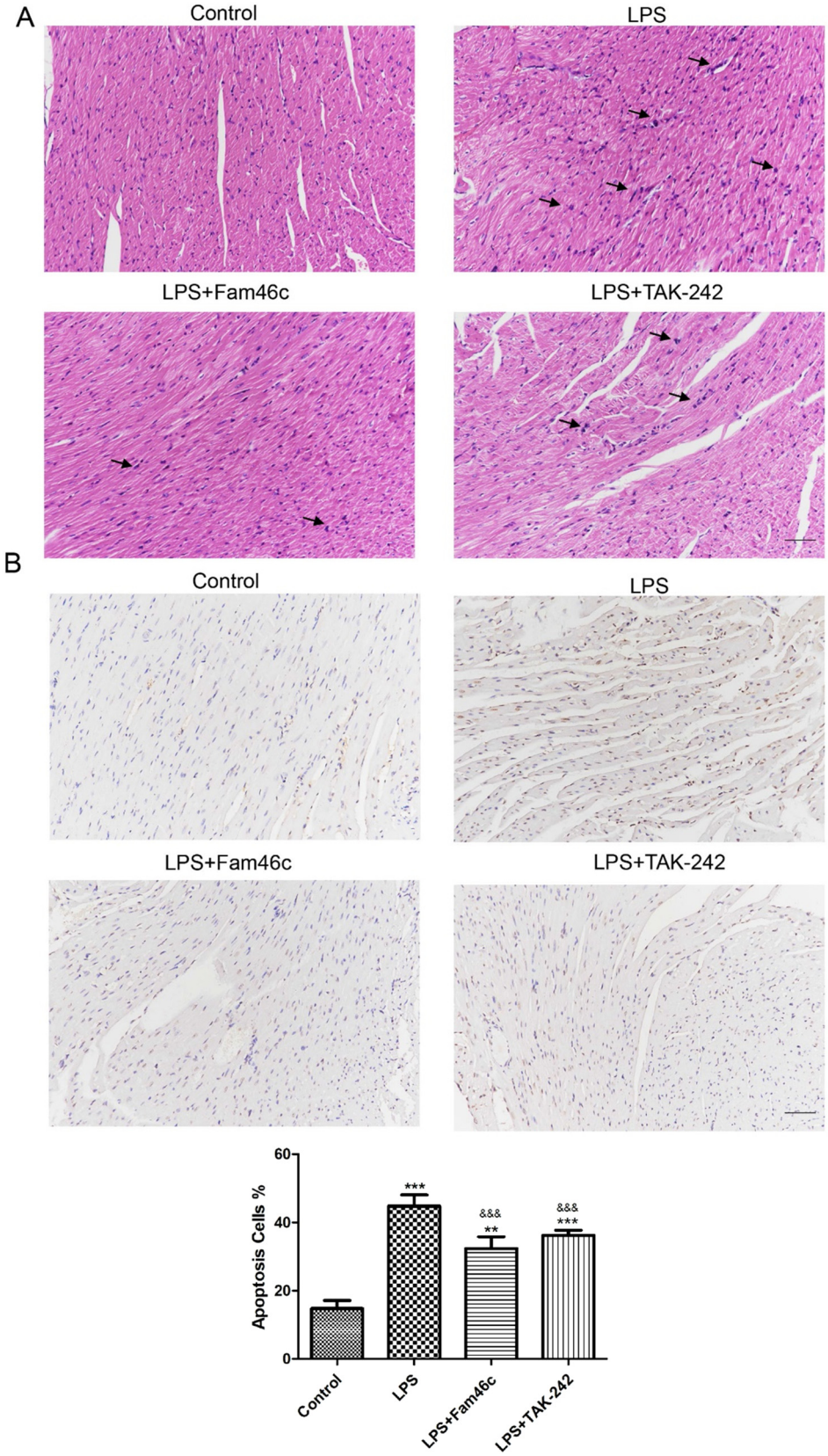

Fig. 6. Morphological characteristics and apoptosis of heart tissues. C57 mouse heart tissues were collected from different groups which were treated with LPS or LPS + Fam46c or LPS + TAK-242. HE staining (A) and TUNEL assay (B) were performed in heart tissues of animal models. Scale: 50 μm. (**: $P < 0.01$; ***: $P < 0.001$ vs Control group; &&&: $P < 0.001$ vs LPS group).

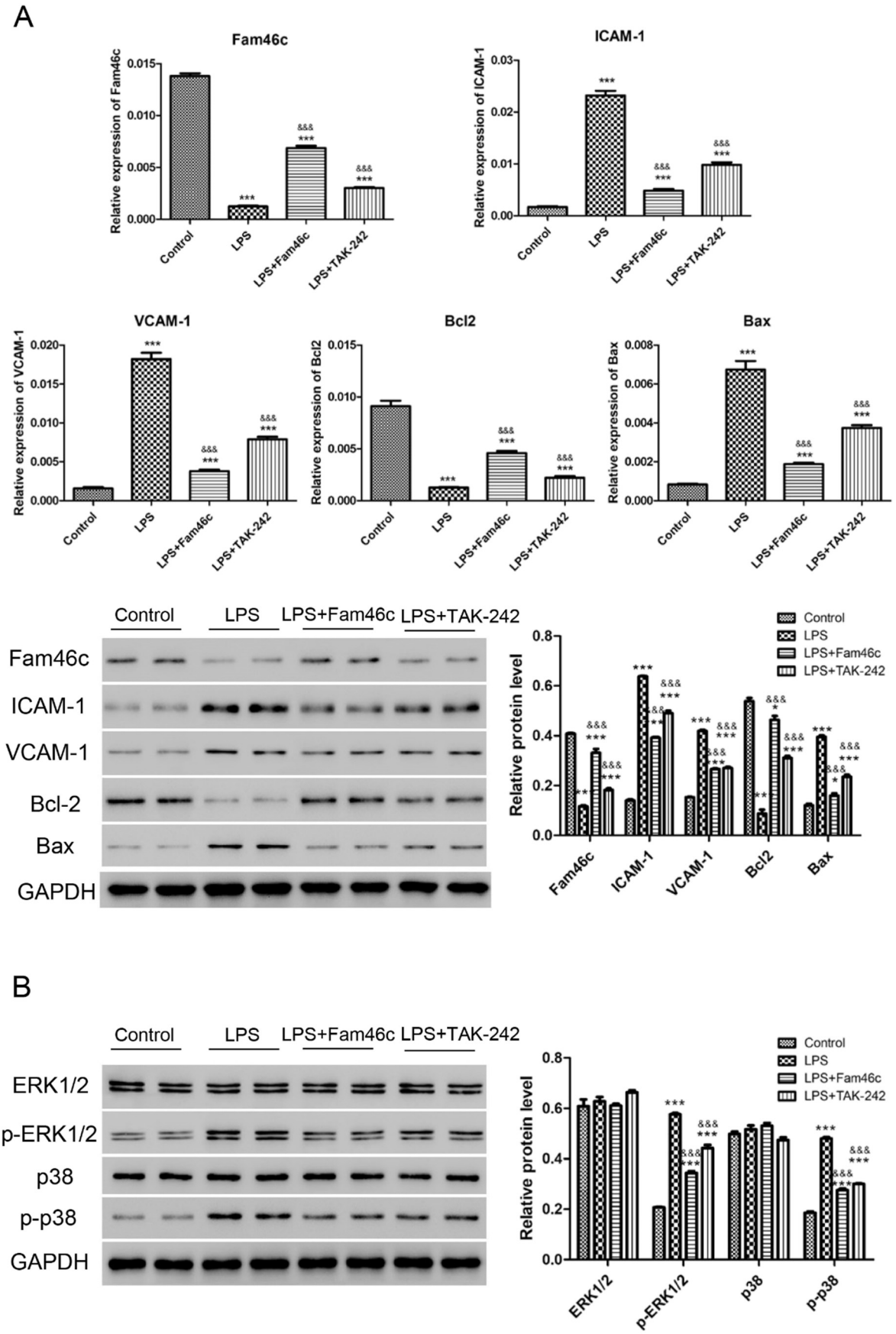

Fig. 7. Overexpression of Fam46c and TLR4 inhibition reversed apoptosis induced by LPS by upregulating ICAM-1, VCAM-1 and Bax via blocking p38/MAPK pathway. (A) Real-time PCR and western blot were used to measure the expression of ICAM-1, VACM-1, Bcl-2 and Bax in heart tissues of animal models. (B) Western blot was used to measure the expression of ERK1/2, p-ERK1/2, p38 and p-p38. (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ vs Control; &&: $P < 0.01$; &&&: $P < 0.001$ vs LPS).

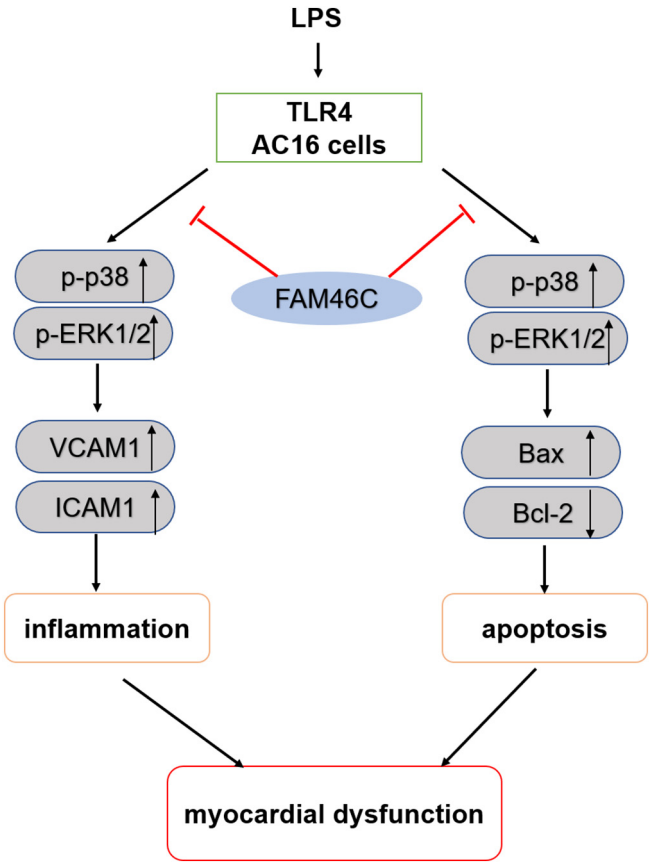

Fig. 8. Schematic representation of the signaling pathways involved in the SIMD. LPS induced inflammation and apoptosis in SIMD via upregulating VACM-1, ICAM-1 and Bax. FAM46C inhibited LPS-induced myocardial dysfunction via downregulating cellular adhesion molecules and inhibiting apoptosis.

was observed. To digest the role of TLR4 in resistance to apoptosis induced by LPS mediated by FAM46C, shFAM46C-AC16 or NC cells were treated with LPS as descripted previously. Then cells were treated with TAK-242, an inhibitor of TLR4, or not. Flow cytometry was used to detect apoptosis. As observed in Fig. 5A, FAM46C knockdown slightly increased apoptosis proportion compared with LPS treatment alone. Interestingly, inhibition of TLR4 attenuated apoptosis induced by LPS no matter with or without FAM46C knockdown. Moreover, inhibition of TLR4 can also restore FAM46C level in AC16 cells.

To further study the underlying mechanism, we introduced PD98059, an inhibitor of ERK1/2 and SB203580, an inhibitor of p38. As shown in Fig. 5B, FAM46C knockdown induced more apoptosis, which can be attenuated not only by ERK1/2 inhibition but also by p38 inhibition.

The collective data suggested inhibition of p38/MAPK signal pathway by PD98059 and SB203580 could attenuate apoptosis induced by FAM46C knockdown.

3.6. Morphological characteristics and apoptosis of heart tissues

According to the results in vitro, we then wanted to confirm our hypothesis in vivo. As shown in Table 1, EF was significantly lower in the LPS than the control mouse, indicating reduced cardiac function. But Fam46c and TAK-242 significantly increased EF comparing with LPS. Morphological characteristics of heart tissues, which collected from mice belonged to different groups, were demonstrated in Fig. 6. Comparing to control group, LPS + Fam46c and LPS + TAK-242 induced edema and rupture in the myocardial fibers. More severe edema and rupture were observed in mice treated with LPS (Fig. 6A). Furthermore, apoptosis cells were found in both LPS + Fam46c and LPS + TAK-242 groups. However, more apoptosis was observed in LPS group comparing with control group after staining with TUNEL assay (Fig. 6B). Of note, pretreatment of Fam46c and TAK-242 can't protect structural alterations. It seemed to only reduce the effect which induced by LPS.

3.7. Overexpression of Fam46c or TLR4 inhibition reversed LPS induced apoptosis by downregulating ICAM-1, VCAM-1 and Bax contributed by blocking p38 and ERK1/2/MAPK pathway

Based on the results that the mice treated with Fam46c and TAK-242 can resist apoptosis induced by LPS, we then evaluated related genes expression. LPS treatment alone dramatically increased ICAM-1 and VCAM-1. However, upregulation of these two genes induced by LPS can be inhibited by overexpression of Fam46c and TAK-242. Conversely, LPS treatment suppressed Bcl2 but promoted Bax transcription respectively. Of note, these effects could be reversed by ectopic expression of Fam46c or TAK-242 treatment. Consistently, similar results were observed when we performed immune blotting to detect expression levels of ICAM-1, VCAM-1, Bcl2 and Bax (Fig. 7A). These results were consistent with observation in vitro. In that case, we continued to test p38 and ERK1/2/MAPK pathway. As expected, we found LPS treatment upregulated levels of p-p38 and p-ERK1/2 respectively. Of note, these effects can be reversed by ectopic expression of Fam46c and TAK-242. Collectively, Fam46c and TAK-242 reversed LPS induced apoptosis by downregulating ICAM-1, VCAM-1 and Bax via blocking p38 and ERK1/2/MAPK pathway (Fig. 7B).

4. Discussion

FAM46 has four members in humans and is a group activator of human NTase, which play crucial roles in various biological processes. However, the role of FAM46C in SIMD is still unclear. LPS has been demonstrated to stimulate cell adhesion molecule, such as VCAM-1 and ICAM-1, in mice renal diseases [29]. The upregulation of VACM-1 induced by LPS has been shown to be mediated through MAPKs [30,31]. However, the detail mechanisms underlying LPS-induced VCAM-1 expression and the role of FAM46C in SIMD remain unclear. In this study, we found that LPS-induced VCAM-1 expression was mediated through the p38 pathway, which also participated in LPS-induced apoptosis. Furthermore, FAM46C could reverse these effects induced by LPS (Fig. 8).

By the way, TLR4 plays a central role in LPS signaling and seems to be depended by the occurrence of SIMD. Collectively, we proposed FAM46C and TLR4 played role in sepsis. To address this concern, we evaluated and found downregulation of FAM46C but upregulation of TLR4 in sepsis patients (Fig. 1). Negative correlation between FAM46C and TLR4 was observed. Cell adhesion molecules, including ICAM-1 and VCAM-1, are increased in the plasma of children with sepsis-induced multiple organ failure which indicates potential involvement in sepsis [9]. LPS upregulated TLR4, ICAM-1, VCAM-1 and downregulated FAM46C in a dosage dependent manner (Fig. 2). These results implied crosstalk among FAM46C, TLR4 and cell adhesion molecules in sepsis. Further, we found that overexpression of FAM46C prohibited apoptosis induced by LPS, but knockdown exhibited the reverse effects which suggested the important role of FAM46C in sepsis (Figs. 3 and 5). Interestingly, cells treated with TAK-242, an inhibitor of TLR4 was found to attenuate apoptosis induced by LPS (Fig. 5). These results revealed that inhibition of TLR4 exhibited the similar effects as FAM46C.

As previous description, the expression of ICAM-1 and VCAM-1 were increased after treated with LPS (Fig. 2). That result was consistent with the published literatures [10,29]. Here we also shown that LPS induced expression of ICAM-1 and VCAM-1 were inhibited by FAM46C (Fig. 5). The MAPKs regulate many kinds of cell processes

including cell proliferation, differentiation survival and apoptosis. The best known MAPKs included ERK 1/2 and p38 [32]. Inhibition of phosphorylation of ERK1/2 induced by H(2)S formation is found in lung and liver 4 h after CLP, happens with activation of NF-kappa B [33]. Phosphorylation of ERK-1/2 is associated with the down-regulation of peroxisome proliferator-activated receptor (PPAR)-gamma during polymicrobial sepsis [34]. Mitogen-activated protein kinase phosphatase-1 (MKP1) could protect heart function in sepsis by attenuating ERK1/2 and p38 activation [35]. In this study, phosphorylation of ERK 1/2 and p38 were found to be increased by LPS, but these upregulations were attenuated by FAM46C (Fig. 4B). To explore underlying pathway involved, we blocked ERK1/2 and p38/MAPK pathway by treating cells with PD98059 and SB203580 respectively. We observed inhibition of ERK1/2 and p38/MAPK pathway could overcome apoptosis induced by LPS (Fig. 5). To validate our findings in vivo, we then introduced sepsis mouse model and evaluated how FAM46C and TLR4 affected sepsis. As we found in Fig. 6, we found LPS treatment induced severe edema and rupture in the myocardial fibers. However, overexpression of Fam46c or TLR4 inhibition by TAK-242 could reverse the morphological alterations induced by LPS. Consistently, we observed similar results in vitro (Fig. 7).

5. Conclusions

FAM46C could reverse apoptosis induced by LPS by downregulating ICAM-1, VCAM-1 and Bax via blocking p38 and ERK1/2/MAPK pathway in AC16 cells. Our study expanded knowledge about sepsis and provided new inspects to study the role of FAM46C. However, how TLR4 regulates expression of FAM46C still need to be cleared. By the way, findings should be introduced to clinical specimens to validate the possibility of clinical trials.

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Conflict of interest

The authors declare that they have no conflict interests.

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