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Oscillating flow promotes inflammation through the TLR2–TAK1–IKK2 signalling pathway in human umbilical vein endothelial cell (HUVECs)

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ABSTRACT

Background: Oscillatory shear stress (OSS) occurs in areas where atherosclerosis is prevalent. Toll-like receptor 2 (TLR2) has been associated with mechanical-stress-mediated activation of signalling pathways that may lead to inflammation, apoptosis, and atherosclerosis. Nonetheless, the mechanism underlying the connection between TLR2 and OSS is not fully understood. The purpose of this study was to investigate the link between OSS and TLR2 in human umbilical vein endothelial cell (HUVECs).

Methods: Monolayer endothelial cells were stimulated or not stimulated by OSS. Protein expression was determined by western blotting and immunofluorescent staining. Endothelial function was assessed by using dihydroethidium assay, RT-PCR, immunofluorescent staining and western blotting. The carotid artery of rats was ligated for 1 week, and a section exposed to OSS was excised and analysed.

Results: In vitro, the expression of TLR2 in HUVECs was activated by OSS. Additionally, OSS increased apoptosis, inflammatory changes, and oxidative stress in HUVECs, and these effects were reversed by down-regulation the expression of TLR2. We proved that OSS regulates the inflammatory response of endothelial cells through the TLR2–TAK1–IKK2 pathway. In the rats with carotid artery ligation, TLR2, TAK1 and phospho-IKK2 amounts increased at the site of OSS.

Significance: According to our results, the OSS-mediated HUVECs injury may be associated with an increase in TLR2 expression. Accordingly, strategies designed to reduce TLR2 expression or inhibit TLR2 activation may be an effective approach to reduce the incidence of atherosclerosis.

1. Introduction

Endothelial shear stress (ESS) is the tangential component of the mechanical stress exerted on endothelial cells by flowing blood. In the endothelial cell membrane, a variety of membrane proteins (such as receptor tyrosine kinases, G protein coupled receptors, integrins, and glycocalyx) and ion channels sense the mechanical signal change of ESS [1] and send a signal into the cell to activate multiple signalling pathways, regulate cell function, and maintain vasodilatation function [1]. Oscillatory flow not only shows a significant decrease in ESS but also involves forward and reverse blood flow [2–6]. Oscillatory flow occurs in areas susceptible to atherosclerosis e.g. the curved inner side of an artery, the narrow downstream, the branched lateral wall, and adjacent areas of branch openings [7–9]. Oscillating flow and unidirectional blood flow probably have different effects on endothelial cells. Few investigators have studied the specific pathogenesis of oscillating shear stress (OSS). There is experimental evidence that in comparison with unidirectional shear stress, OSS induces a decrease in eNOS mRNA and in protein amounts within 24 h [10]. The latest literature suggests that oscillating flow can also increase the uptake of lipids by endothelial cells [11]. Nonetheless, the biomechanical and molecular biological mechanisms are still unclear. Furthermore, the possible role of oscillating flow in the progression of atherosclerosis is still rarely studied.

Toll-like receptors (TLRs, receptors of innate immunity), are known as receptors that recognize pathogen-associated molecular patterns. Recent reports suggest that Toll-like receptor 2 (TLR2) may act as an important cell surface mechanosensor. When atheroprotective laminar shear stress is tested the mRNA and protein levels of TLR2 significantly increase [12–14]. Studies have shown that the expression of TLR2 in endothelial cells is related to the formation, development, and vascular occlusion of atherosclerosis. At the same time, hyperlipidaemia can also

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promote the expression of TLR2 [15–17]. Other studies have revealed that TLR2 signalling has an important function in atherosclerosis and protects against both foreign pathogens and endogenous inflammatory ligands [18]. These data suggest that TLR2 may be associated with mechanical-stress-mediated activation of signalling pathways that may lead to inflammation, apoptosis, and atherosclerosis.

Transforming growth factor kinase 1 (TAK1) is a member of the mitogen-activated protein kinase kinase kinase family, which is functionally located upstream of mitogen-activated protein kinase and IKB kinase. Experiments indicate that shear stress can protect endothelial cells by reducing the expression of TAK1.

Endothelial activation, dysfunction, and damage are known as the key initial events in the pathogenesis of atherosclerosis. OSS, TLR2, and TAK1 all participate play a role in the development of atherosclerosis in some way. The relations between OSS and TLR2, TLR2 and TAK1 are rarely studied. Our study was aimed to determine whether OSS activates the innate immune response, whether it causes inflammatory changes in endothelial cells, and via which signalling pathway it promotes inflammation.

2. Materials and methods

2.1. Animals and treatment

All the animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (Nanjing, China). Sprague–Dawley rats (6 weeks old) were obtained from the Department of Laboratory Animals of Nanjing Medical University. All the rats were kept at room temperature (25 °C ± 2 °C), and on a 12/12 h-light/dark cycle and had unrestricted access to food and water. We used a cast model to study the direct effects of OSS in vivo. The cylindrical hollow cast was applied to the carotid artery, creating a fixed geometry where the upstream region was exposed to low shear stress (LSS), the vascular region within the cast was exposed to high shear stress (HSS), and the downstream portion of the cast received OSS [19–21]. A week later, carotid ultrasonography was performed, and tissues were taken. We used the left carotid artery as the experimental group and the right carotid artery as the control group. We also included the left carotid artery of another rat as a sham group.

2.2. Cell culture and treatment

HUVECs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, 10% of foetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator at 37 °C and 5% CO_2. A parallel-plate flow channel (Think Nature, Shanghai, China) was employed to provide a stable fluid. A direction control instrument changed the direction of the fluid at 1 Hz. Monolayer endothelial cells are subjected to mechanical forces of ± 4 dyn/cm^2 in vitro [22,23]. Our cells were transferred to DMEM without serum and incubated for 120 min for flow experiment.

Inhibitors for TLR2 (CU CPT) and TAK (5O-7Z) were used, both inhibitors were originated from MCE company. According to the operating instructions, the concentration of 50-7z was 8.1 nmol, CU CPT was 0.58 μmol [24–26]. Cells were preincubated with the inhibitors for 1 h prior to stimulated by OSS.

2.3. Expression vectors and transient transfections (RNA interference)

The homo TLR2, TAK1, IKK2 and negative control small interfering RNA (siRNA) were purchased from Gene Pharma (Shanghai, China). The nucleotide sequences were as follows:

Homo TLR2 siRNA, 5′-GCCCUCUCUACAAACUUUATT′ (sense)
5′-UAAAGUUUGUAGAGAGGGCTT-3′ (antisense);
Homo TAK1 siRNA, 5′-AAAGTGGCTTATCTTACACTGGA-3′ (sense)
5′-AAAATCCAGTGTAAGATAAGCCA-3′ (antisense);

Homo IKK2 siRNA, 5′-CAGCUUUAAGUUUAAGAUGUCAUCCAG-3′ (sense)
5′-GGAUGACAUCUUAAACUUAAAGCTG-3′ (antisense);
The negative control siRNA with scrambled sequence was
5′-UUCUCCGAACGUGUCACGUTT-3′ (sense)
5′-ACGUGACACGUUCGGA GAATT-3′ (antisense).
The cells transfected with nonspecific target siRNA served as controls.

2.4. Western blot analysis (WB) and reagents

After treatment with the oscillatory flow, the cells were lysed, and the lysates were diluted with loading buffer (5×)and boiled for 8 min. Total protein was isolated by electrophoresis in a polyacrylamide gel (8% to 15%), which was then transferred to a polyvinylidene difluoride membrane. A primary antibody was incubated with the membrane at 4 °C overnight, and a secondary antibody was incubated at room temperature for 2 h. Primary antibodies against TLR2(cat. # ab16894 1:500), TAK1 (ab109526 1:1000), phospho-(p-)4-IKK1 + 2(ab194528 1:1000), and p-IKK2 (ab194845 1:1000) were purchased from abcam (USA); primary antibodies of TAK1 (5206 s 1:1000), IKK2 (8943 s 1:1000) as well as secondary antibody (7074 s; 1:5000) were purchased from Cell Signalling Technology (Massachusetts, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, GB11002 1:1000) was acquired from Servicebio (Wuhan, China).

2.5. Flow cytometry

A total of 1 × 10^6 cells were collected after being exposed to OSS or after no exposure, then washed three times. Flow cytometry is then prepared by following steps: firstly, the cells were resuspended in binding buffer which Annexin VFITC and PI were added. Secondly, the mixtures were incubated for 15 min at room temperature in the dark. Finally, the mixtures were added binding buffer and shaken slightly. The cells were subjected to flow cytometry within 1 h. The data were analysed by using FlowJo software.

2.6. TUNEL staining

Cells were washed with PBS thrice after being fixed with 4% neutral paraformaldehyde for 15 min. Then, we exposed the cells to 0.2% Triton X-100 for 10 min before treating them with a tunnel staining kit (Vazyme Biotech) according to the manufacturer's instructions. The cells were cultured with DAPI (Beyotime Biotechnology) for another 5 min after being treated with the TDT mixture for 2 h. All fluorescent images were examined via fluorescence microscopy and photographed.

2.7. Quantitative real-time PCR

Total RNA was extracted using TRIzol from cells after the OSS was applied or not. The primer sequences for the real-time PCR assays were listed as follows:

ICAM-1 Forward: 5′-CAGTGACCATCTACAGCTTTCCGG-3′
Reverse: 5′-GCTGCTACCACAGTGATGACAA-3′
VCAM-1 Forward: 5′-GATACAACCGTCTTGGTCAGCCC-3′
Reverse: 5′-CGCATCCTTCAACTGGGCCTT-3′
GAPDH Forward: 5′-AGAAGGCTGGGGCTCATTTG-3′
Reverse: 5′-AGGGGCCATCCACAGTCTTC-3′

2.8. Quantification of reactive oxygen species (ROS)

ROS levels were measured by means of dihydroethidium (DHE; Sigma-Aldrich) fluorescence under a fluorescence microscope. The endothelial cells were incubated with DHE for 30 min at 37 °C in the dark. ROS were quantified by the ImageJ software.

2.9. Immunofluorescent staining

After stimulation by OSS, the cells were washed three times with PBS and fixed with 4% neutral paraformaldehyde at room temperature for 15 min. Then, the cells were washed with PBS and permeabilised with 0.2% Triton X-100 for 20 min. After blockage with 3% BSA for 1 h, the cells were incubated with a primary antibody overnight at 4 °C. After labelling with FITC/CY3 antibody, the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Finally, fluorescence images were acquired by means of a confocal laser-scanning microscope. DAPI (c1006) was purchased from Beyotime (Shanghai, China).

2.10. Statistical analysis

The data are shown as mean ± standard error of mean (SEM) from at least three different experiments, and were analysed in SPSS 20.0 software. The difference between groups was determined by one-way ANOVA with the Bonferroni correction or Paired *t*-test.

3. Results

3.1. OSS induces the expression of TLR2 in HUVECs

The shear stress range of the vascular bed is 10–70 dyne/cm^2, but the shear stress in the curved and bifurcated regions of arteries (these regions are prone to atherosclerotic lesions) is much lower, approximately 4 dyne/cm^2. The apparatus we used was designed by *Nature Think* (Shang Hai China). A pump (a) draws fluid from the liquid storage bottle (b) at a constant speed to provide a stable fluid supply. A direction control instrument (c) closes and opens different hoses at 1 Hz, thus forming a roundtrip fluid on the cell surface of chamber (d) (Fig.1A).

Firstly, we proved that oscillatory flow can promote the expression of TLR2 at the protein level in endothelial cells, and this effect is time-dependent (0, 15, 30, 60, and 120 min) (Fig. 1B and C). Meanwhile, in the fluorescence experiment, the fluorescence intensity of TLR2 increased continually, which was consistent with the results of WB (Fig. 1D).

3.2. OSS can also affect the expression of inflammatory and apoptotic molecules in HUVECs

Then we found that caspase 3 was activated under the action of OSS. As shown in Fig. 2A, ECs were treated with OSS for different periods (0, 15, 30, 60, or 120 min). Expression levels of cleaved-caspase3 rose continuously. AS depicted in Fig. 2B, we also carried out RT-PCR and found that OSS can increase the expression of ICAM-1 and VCAM-1 at the mRNA level.

Inhibitor of the NF-kappa B kinase (IKK) plays an important part in innate immunity and adaptive immunity and is involved in apoptosis, inflammation, and necrosis. The IKK cytokine has been reported to have an antiapoptotic effect [27,28], but there are also studies showing that it can promote apoptosis [29–31]. TAK1 is a key signalling molecule of the NF-kB pathway. Similarly, TAK1 has different functions in different types of cells, and a TAK1 knockout exerts differential effects on NF-kB, p38, JNK, and ERK activation [32–34]. To test their participation in the process of OSS-induced expression of inflammatory and apoptotic molecules, protein expression levels of the above proteins were determined next. As illustrated in Fig. 2C, OSS activated the phosphorylation of IKK2, and the phosphorylation level increased with the time of stimulation. As shown in Fig. 2D, OSS markedly induced protein expression in a time-dependent manner. Flow cytometry and TUNEL were used to further verify whether OSS can increase apoptosis of endothelial cells (Fig. 2E and F).

3.3. The expression of TAK1 and IKK2 promoted by OSS is dependent on TLR2

To explore the relations among TAK1, IKK2, and TLR2, we applied TLR2-specific siRNA to reduce the expression of TLR2 and observed changes in its expression. We exposed the cells for an hour to the stationary condition, HSS, or OSS. As depicted in Fig. 3A, we found that OSS can increase the amounts of TLR2, TAK1, and p-IKK2, consistently with the data in Fig. 1. In contrast to previous studies, we failed to detect a protective effect of HSS, and the expression level of TAK1 was not reduced [35]. Nonetheless, in cells transfected with TLR2 siRNA, the increased levels of TAK1 expression and IKK phosphorylation were suppressed. This finding indicates that TAK1 and IKK2 were regulated by TLR2. As presented in Fig.3 B, immunofluorescence experiments indicated that when the expression of TLR2 decreased, the fluorescence intensity of TAK1 also decreased under the action of OSS. Next, we applied an inhibitor of TLR2, CU CPT, and got the same results (Fig. 3C). At this point, we can draw the conclusion that TLR2 functions upstream of TAK1 and IKK2 under OSS. We also found that up-regulation of ROS, ICAM-1 and VCAM-1 by OSS can be attenuated or cancelled out after transfection with TLR2 siRNA. (Fig. 3Dand E)

3.4. The up-regulation of p-IKK2 promoted by OSS is dependent on TAK1

To further elucidate the role of TAK1, we transfected endothelial cells with TAK1 siRNA. The results showed that OSS-induced p-IKK2 up-regulation was dramatically attenuated by TAK1 siRNA transfection (Fig. 4A). As shown in Fig. 4B, the fluorescence intensity of p-IKK2 also decreased under the influence of OSS in endothelial cells transfected with TAK1 siRNA. Next, we tested 5O-7Z, an inhibitor of TAK1, and obtained the same results (Fig. 4C). From the above results, we concluded that TAK1 is upstream of IKK2 under the influences of the OSS.

Then to determine whether there is an interaction among TAK1, IKK2, and TLR2, the endothelial cells were transfected with TAK1 and IKK2 siRNA. When the expression of TAK1and IKK2 decreased, the expression of TLR2 did not. We also found that OSS promoted endothelial-cell apoptosis through IKK2 but probably not TAK1 (Fig. 4D). ICAM-1 and VCAM-1 up-regulation by OSS was attenuated after transfection with TAK1 or IKK2 SiRNA (Fig. 4E).

3.5. The amounts of TLR2, TAK1, and p-IKK2 increased in vivo

Meanwhile, we also conducted the experiment on carotid artery ligation in rats. This experimental method is described before [21].The cylindrical hollow cast was applied to the carotid artery, creating a fixed geometry where the upstream region was exposed to LSS, the vascular region within the cast was exposed to HSS, and the downstream region of the cast received OSS (Fig. 5A). Ultrasound examination of the carotid artery in mice was performed 1 week after ligation, we detected significant OSS, back and forth in the ligated left carotid artery (Fig. 5B). The ligated artery was marked by hyperplasia of the smooth muscle layer (Fig. 5C). Various proteins in the endodermis, including TLR2, TAK1, p-IKK2, were significantly up-regulated compared with the control group and sham group (Fig.5D).

4. Discussion

New insights into mechanosensors in vascular endothelial cells in recent years have uncovered the potential links between shear stress and cell surface components. There are many potential mechanosensors such as integrins, ion channels, NADPH oxidase, receptor tyrosine kinases, and G protein coupled receptors. Studies have shown that shear stress can regulate the expression of an endothelial-cell surface receptor, AT1R, and further regulate intracellular signalling pathways [23,36]. In addition, recently, in vitro studies revealed that shear stress transports molecules into cells via glycocalyx, an extracellular

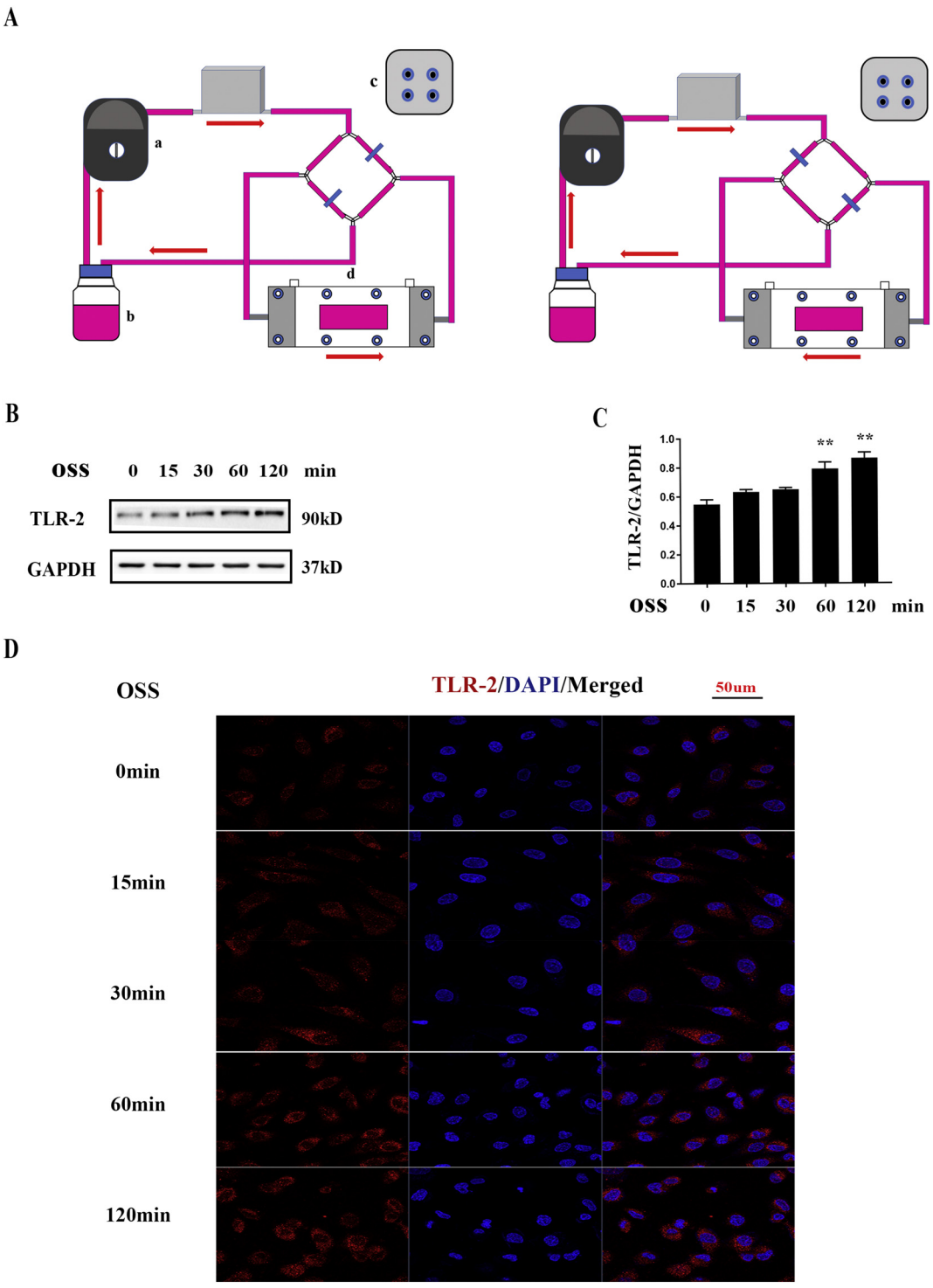

Fig. 1. OSS induced the expression of TLR2 in HUVECs. OSS stimulated the endothelial cells via time-dependent patterns. A: The parallel-plate flow chamber. The pump (a) draws fluid from the liquid storage bottle (b) at a constant speed to provide a stable supply of the fluid. Direction control instrument (c) closes and opens different hoses at 1 Hz frequency, generating a roundtrip fluid on the cell surface of chamber (d). B and C: OSS induced the protein expression of TLR2. D: The immunofluorescence assay was performed to detect the fluorescence intensity of endothelial cells stimulated by OSS. Data are shown the mean ± SEM. $^{*}P < 0.05$ vs. 0 min, $^{**}P < 0.01$ vs. 0 min.

substance [37–39]. Nonetheless, the function of most components on and outside the cell membrane under shear stress is still rarely studied. TLR2, an important protein molecule involved in nonspecific immunity, is also a bridge between nonspecific immunity and specific immunity. The reaction of TLR2 to mechanical stressors recently began to attract attention, but its function under shear stress is not known in ECs. Oscillatory flow occurs mainly near the outer wall of the main bifurcation of vessels and near the inner wall along the curved segments, where plaques are more likely to form [40–43]. In this study, we demonstrated that OSS can activate the TAK1-IKK2 axis by increasing the expression of TLR2 to promote endothelial inflammation (Fig. 6). We next confirmed that OSS can increase the protein amounts of TLR2, TAK1, and p-IKK2 in rats with left carotid artery ligation.

This is the first study indicating that OSS can activate TLR2 in

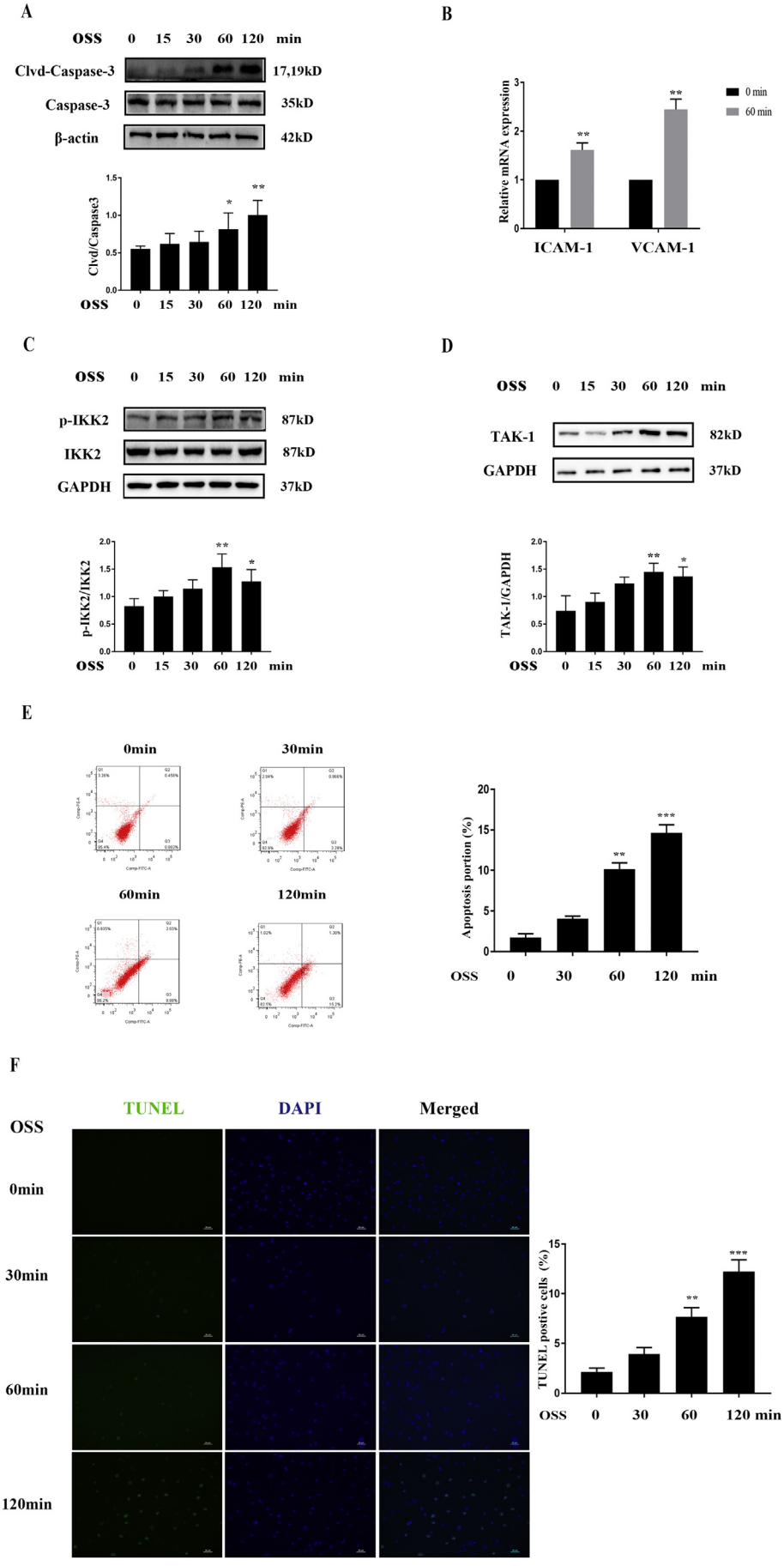

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Fig. 2. OSS affected the expression of inflammatory and apoptosis-related molecules in HUVECs. A: The protein amount of p-IKK2 increased. B: RT-PCR measured the mRNA expression of ICAM-1 and VCAM-1. C: Protein expression of TAK1 was increased. D. Cleaved-caspase 3 was up-regulated in a time-dependent manner. E and F. Flow cytometry and TUNEL were conducted to quantify apoptosis. Data shown are the mean ± SEM. $^{*}P < 0.05$ vs. 0 min, $^{**}P < 0.01$ vs. 0 min, $^{***}P < 0.05$ vs. 0 min.

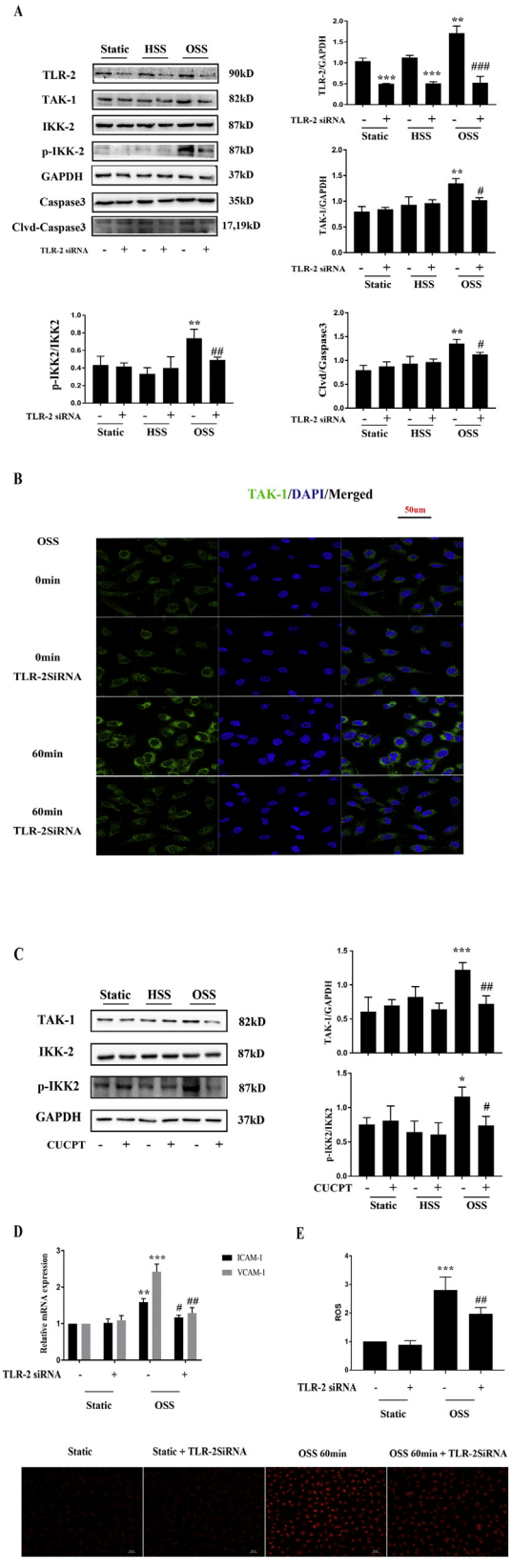
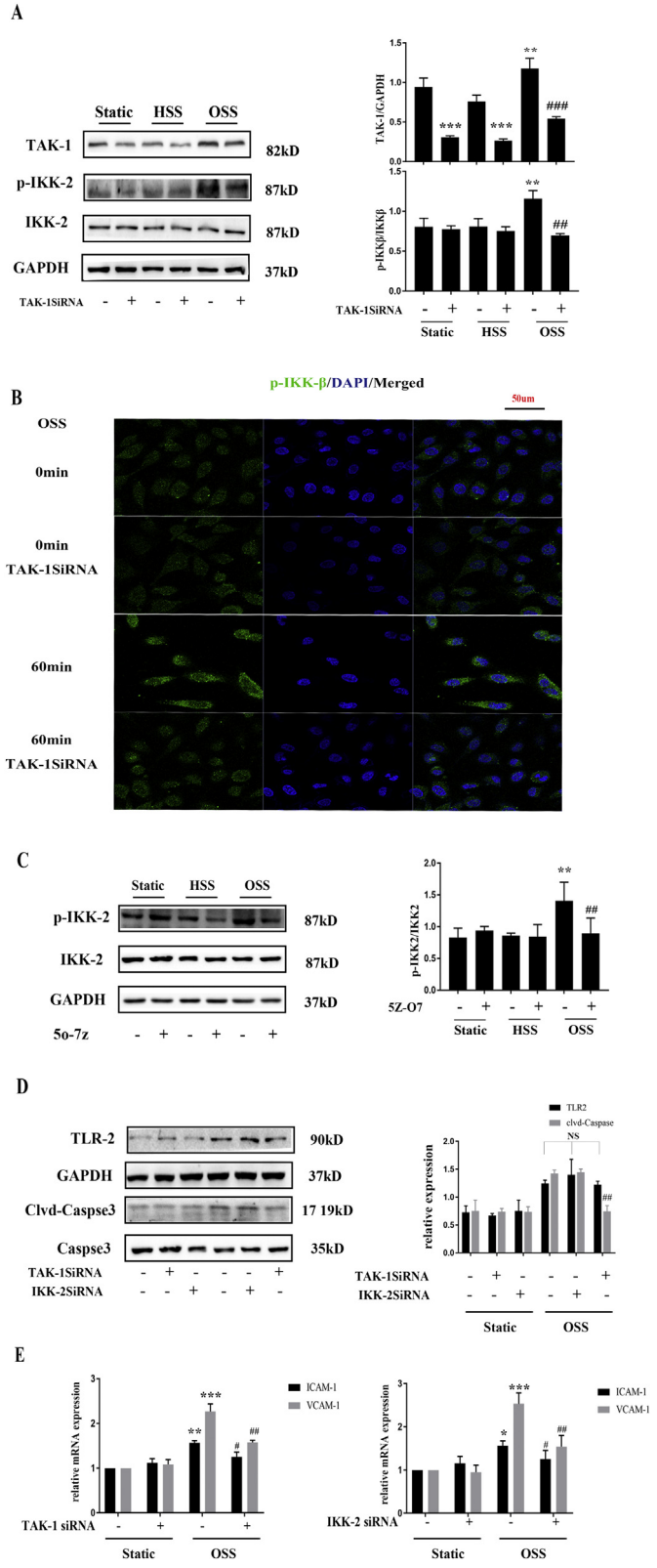


Fig. 3. The expression of TAK1 and IKK2 promoted by OSS is dependent on TLR2. ECs transfected with TLR2 siRNA or NC siRNA were either kept static or stimulated with HSS/OSS for 1 h. A: WB to assess the protein expression of TLR2, TAK1, IKK2, p-IKK2, caspase 3, and cleaved caspase 3. B: Changes in TAK1 fluorescence intensity were detected. C: WB for detecting the effect of CU CPT, an inhibitor of TLR2. D: RT-PCR for quantifying mRNA level of ICAM1 and VCAM1. E: DHE for quantification of ROS. Data are shown the mean ± SEM. $^{*}P < 0.05$ vs. static condition, $^{**}P < 0.01$ vs. static condition, $^{***}P < 0.001$ vs. static condition, $^{\#}P < 0.05$ vs. OSS, $^{\#\#}P < 0.01$ vs. OSS, $^{\#\#\#}P < 0.001$ vs. OSS.

HUVECs. Evidence that TLR2 is affected by mechanical factors, without ligands, has been initially demonstrated in endothelial cells stimulated by laminar shear stress. Under the shear stress of 15 dyn/cm^2, the expression of TLR2 decreases [14]. It was later reported that TLR2 is up-regulated when stimulated by centrifugal force in THP-1 cells [14]. Furthermore, mild stretch mechanical stimulation up-regulates TLR2 in epithelial human cells [13]. On the one hand, OSS-driven up-regulation of TLR2 were abolished by TLR2 siRNA and a suppressant. On the other hand, activation of VCAM1 and ICAM1 in response to OSS was similarly prevented by the TLR2 siRNA and a suppressant. The expression of TAK1 and IKK2 increased with duration of OSS stimulation. The effects of TAK1 and IKK2 on inflammation have been reported in other studies, and the influencing indicators are as follows: ICAM1, VCAM1, IL8, NO, SELE, CLL2 [32,33,45–50].

The literature shows that in different cell types (middle ear epithelium cells, HEK293 cells, and human primary bronchial epithelial cells), TLR2 as an upstream regulator of TAK1, regulates a variety of inflammatory markers [51,52]. Our results are in agreement with those of other studies. Under the action of OSS, the expression of TAK1 is regulated by TLR2; the expression of TAK1 is up-regulated and can be blocked by siRNA and inhibitors of TLR2. Recent literature indicates that laminar shear stress can counteract the up-regulation of TAK1 induced by TGF2 [35]. Our experimental results were different; after 1 h of laminar flow, there was no significant change in the expression of TAK1 in the static condition. This discrepancy may be related to the duration of the stimulation, and the protective effect of laminar flow may take longer to manifest itself. In cells except for neutrophilic granulocytes, the pathways of p38 MAPK and NF-κB completely blocked after a TAK1 knockout, which also leads to apoptosis [53,54]. Additionally, TAK1 is essential for the activation of NF-κB induced by LPS and TNF-α, and the regulation of the IKK subunit NEMO (IKKγ) has been identified as the main mechanism regulator of TAK1 in hepatocytes [55]. Loss of TAK1 in the skin proves that the TAK1–IKK–NF-κB pathway is the main regulatory factor for skin epidermal integrity and is crucial for evading skin cell death induced by TNF-α [56]. Our results are consistent with those of previous studies. Under the action of oscillating shear stress, the expression of IKK2 is regulated by TAK1; the expression of IKK2 is up-regulated and can be blocked by the siRNA and an inhibitor of TAK1.

There are many transcription factors that regulate TLR2, including sp1, IRF, NF-κB, AP-1 and so on. [14,44,57,58] We found that TLR2 showed significant changes after exposure to OSS for 1 h. The stimulation upregulated TLR2 so quickly. Similar results have been reported in the literature; TLR2 mRNA production peaked at 1 h after mechanical stimulation (5 min, 1.3 dyn/cm^2) and then decreased slowly. Centrifugation-mediated mechanical stress promoted the phosphorylation of TLR2 through c-Src [44]. It is to be expected whether c-Src or other transcription factors are involved in the regulation of OSS-induced TLR2.



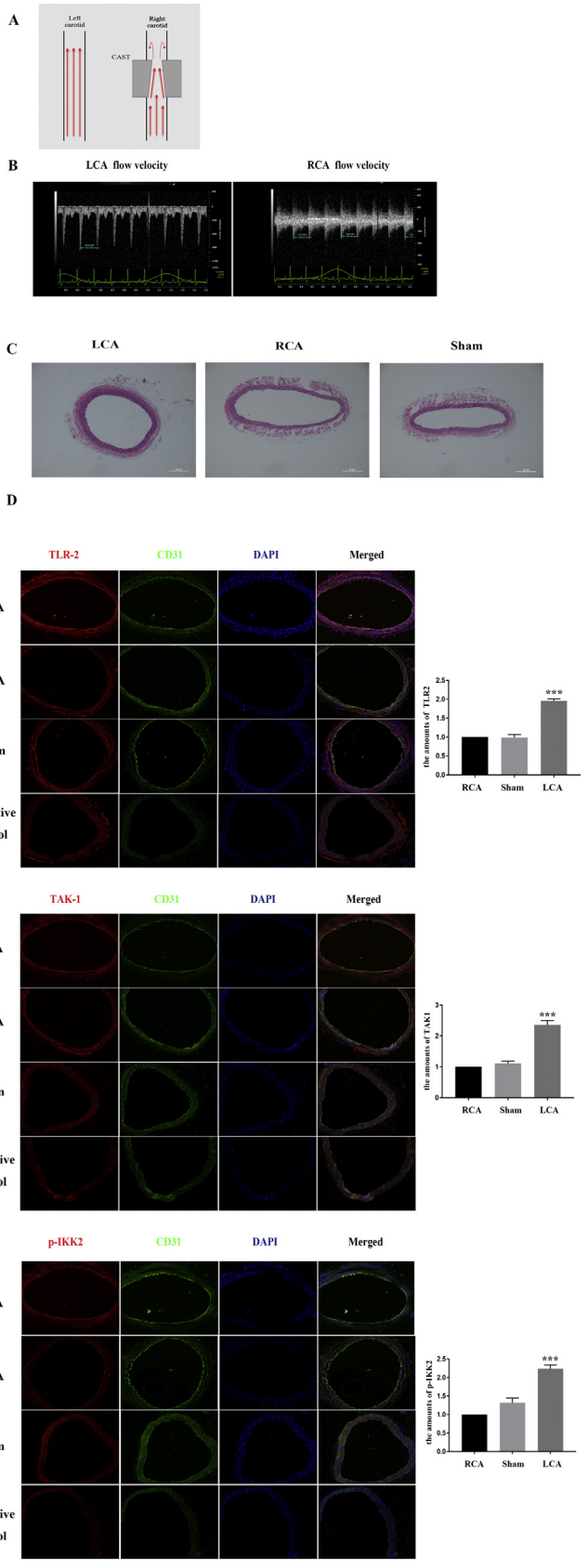


Fig. 4. The amount of p-IKK2 increased by OSS is dependent on TAK1. ECs transfected with TAK1 siRNA or NC siRNA, were either kept static or stimulated with HSS/OSS for 1 h. A: WB to detect the protein expression of, TAK1, IKK2, p-IKK2. B: Changes of TAK1 in fluorescence intensity were detected. C: WB for assessing the effect of 5O-7Z, an inhibitor of TAK1. D: A knockdown of TAK1 or IKK2 for evaluating the protein level of TLR2 and cleaved caspase 3. E: RT-PCR for measuring the mRNA levels of ICAM1 and VCAM1 when TAK1 and IKK2 were knocked down. Data are shown the mean ± SEM. $^{*}P < 0.05$ vs. static condition, $^{**}P < 0.01$ vs. static condition, $^{***}P < 0.001$ vs. static condition. $^{\#}P < 0.05$ vs. OSS, $^{\#\#}P < 0.01$ vs. OSS, $^{\#\#\#}P < 0.001$ vs. OSS.

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Fig. 5. The amounts of TLR2, TAK1, p-IKK2 increased in vivo. Twelve SD Rats (6 weeks old) were used, the left carotid artery served as in the experimental group, and the right carotid artery as the control group. (A) The cylindrical hollow cast was applied to the carotid artery, creating a fixed geometry where the upstream region was exposed to LSS, the vascular region within the cast was exposed to HSS, and the downstream portion of the cast received OSS. (B) Carotid artery ultrasonography measured carotid artery blood flow velocity ($n = 12$). (C) HE staining was performed to examine the structural changes in the experimental group and the other group. (D) Immunofluorescent staining was carried out to measure the fluorescence intensity of proteins during carotid artery ligation, in the control group, and in the sham group. We chose CD31 to mark vascular endothelial cells. $^{***}P < 0.001$ vs. RCA.

It has been widely documented that shear stress induces expression of inflammatory factors by endothelial cells. The main mechanisms include induction of MCP-1, TGF-2, and *E*-selectin and up-regulation of ICAM-1 and VCAM-1 [46,48–50]. Current data indicate that the expression of ICAM-1 and VACM-1 induced by OSS can be significantly inhibited by TLR2 siRNA, thus pointing to the key function of TLR2 in this process and revealing a new mechanism of shear stress-induced cell inflammation.

5. Conclusion

In summary, as depicted in Fig. 6, our results show that OSS activates TLR2 and induces ICAM-1 and VCAM-1 expression in a TAK1-and IKK2-dependent manner. OSS, through activation of TLR2 and TAK1, increases intracellular ROS formation. To the best of our knowledge, this is the first study to evaluate the causal link between OSS and TLR2, an important molecule of the immune system. OSS regulates TLR2 at such a fast speed, whether there is a potential transcription factor directly regulating its transcription and expression is worth further exploration. In stent restenosis occurs frequently in drug-eluting stents, and stent restenosis is mainly found at branch vessel openings, where OSS takes place. Our experimental results not only characterise the relation between shear stress and immune signals, but also implicate the molecular mechanism of oscillatory flow in restenosis in stents.

Ethical approval and consent to participate

Compliance with ethical guidelines.

Consent for publication

Not applicable.

Acknowledgements

Not applicable.

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Availability of data and materials

All relevant data are within the paper and its Availability of data and materials files.

Authors contribution

WF performed the in vitro experiment, analysed the echo data, and drafted the manuscript. WZM and PJQ performed the rat work, including feeding and coordinating the echography and harvest dates and times. XXR aided in collecting tissues from animals and analysing the data. GXF analysed the data and edited the manuscript. ZJJ and CSL designed and oversaw the study, and edited the manuscript. All authors read and approved the final manuscript.

Competing interests

Not applicable.

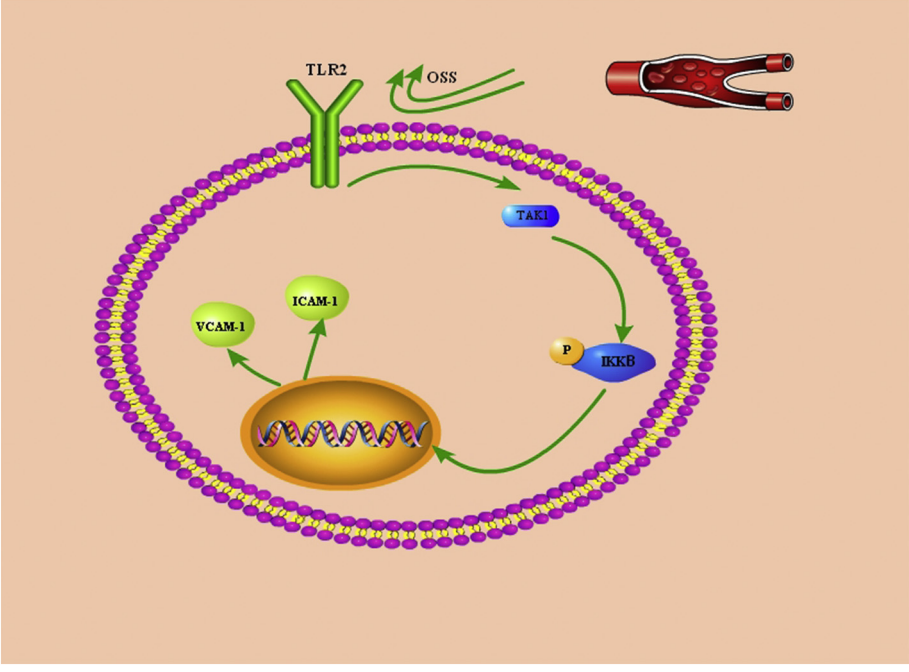

Fig. 6. Oscillating flow promotes inflammation through the TLR2–TAK1–IKK2 signalling pathway.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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