International Journal of Medical Microbiology 309 (2019) 151340

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Chlamydia pneumoniae infection promotes vascular smooth muscle cell migration *via* c-Fos/interleukin-17C signaling

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ARTICLE INFO

Keywords:
Chlamydia pneumoniae
Atherosclerosis
Interleukin-17C
c-Fos
Vascular smooth muscle cell
Migration

ABSTRACT

Chlamydia pneumoniae (*C. pneumoniae*) infection is associated with the initiation and progression of atherosclerosis. The migration of vascular smooth muscle cell (VSMC) from the media to the intima is a key event in the development of atherosclerosis. Interleukin-17C (IL-17C) could enhance cell migration ability. The aim of our study is to investigate the role of IL-17C in *C. pneumoniae* infection-promoted VSMC migration, thereby possibly accelerating atherosclerosis. We firstly demonstrated that *C. pneumoniae* infection significantly increased IL-17C expression in VSMCs in the atherosclerotic lesion area from ApoE deficient mice. Our *in vitro* study further showed that IL-17C is required for *C. pneumoniae* infection-promoted VSMC migration, and its expression could be regulated by c-Fos through phosphorylating extracellular signal-regulated kinase (ERK). Unexpectedly, in the present study, we also found that IL-17C is critical for *C. pneumoniae* infection-induced c-Fos activation. c-Fos expression and activation induced by the exposure to recombinant IL-17C were markedly suppressed in the presence of the ERK inhibitor PD98059. These results suggest a possible positive feedback between c-Fos and IL-17C after *C. pneumoniae* infection. Taken together, our results indicate that *C. pneumoniae* infection promotes VSMC migration *via* c-Fos/IL-17C signaling.

1. Introduction

Atherosclerosis is a slowly progressing chronic inflammatory disorder of the arteries (Hansson, Hermansson, 2011). *Chlamydia pneumoniae* (*C. pneumoniae*) infection is associated with the initiation and progression of atherosclerosis (Belland et al., 2004; Sakurai-Komada et al., 2014). *C. pneumoniae* is sufficient to exacerbate atherosclerosis in ApoE deficient ($ApoE^{-/-}$) mice (Sorrentino et al., 2015). The migration of vascular smooth muscle cell (VSMC) from the media to the intima is a key event in the development of atherosclerosis (Lacolley et al., 2012; Bennett et al., 2016). Although our previous studies indicated that *C. pneumoniae* infection could promote VSMC migration (Wang et al., 2013b; Ma et al., 2015), the exact mechanisms have not yet been fully elucidated.

Interleukin (IL)-17C, a novel member of the IL-17 cytokine family (Song et al., 2014), has emerged as an important proinflammatory cytokine that is required to efficiently control bacterial and fungal infections, such as *Citrobacter rodentium* (Song et al., 2011), *Helicobacter pylori* (Tanaka et al., 2017), *Staphylococcus aureus* (Roth et al., 2014) and *Candida albicans* (Huang et al., 2016). IL-17C is produced by distinct cell sources, such as epithelial cells (Kusagaya et al., 2014), keratinocytes and endothelial cells (Johnston et al., 2013). But recently, Butcher et al. (2016) reported that VSMC was prominent source of IL-17C in aorta. Excitingly, Ding et al. (2017) found that IL-17C could enhance the migration ability of peripheral blood leukocytes. Toll-like receptor 2 (TLR2) agonists upregulated IL-17C expression in colon epithelial cells (Ramirez-Carrozzi et al., 2011). Our previous study found that *C. pneumoniae* infection promoted VSMC migration *via* TLR2 signaling (Wang et al., 2013b). Therefore, we hypothesized that *C. pneumoniae* infection might induce VSMC migration through IL-17C. Then, how does *C. pneumoniae* infection affect the expression of IL-17C in VSMCs?

The FBJ osteosarcoma oncogene c-Fos is a component of activator protein 1 (AP-1), which is the most powerful transcriptional factors of the immune system and plays an important role in inflammation (Chinenov, Kerppola, 2001; Shaulian, Karin, 2002; Hop et al., 2018) and the development of a variety of cardiovascular diseases (Zhang et al., 2013; Palomer et al., 2015; Zhu et al., 2017). c-Fos has been

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https://doi.org/10.1016/j.ijmm.2019.151340
Received 24 January 2019; Received in revised form 8 July 2019; Accepted 18 August 2019

shown to be involved in regulating the expression of inflammatory cytokines. Zickler et al. (2018) reported that tumor necrosis factor-α (TNF-α) promoted IL-6 expression in VSMCs through c-Fos. Importantly, Wang et al., 2013a, also found that *C. pneumoniae* infection could activate c-Fos in human coronary artery endothelial cells. Therefore, *C. pneumoniae* infection might also regulate the expression of IL-17C through activating c-Fos.

In the present study, accordingly, we demonstrated that *C. pneumoniae* infection upregulated the expression of IL-17C, thereby promoting VSMC migration. Moreover, our further study suggested a possible positive feedback between c-Fos and IL-17C *via* phosphorylation of extracellular signal-regulated kinase (ERK) in this process.

2. Materials and methods

2.1. Reagents

Rat recombinant IL-17C (rIL-17C) protein was purchased from Creative Biomart (USA). Anti-c-Fos, anti-pSer32c-Fos, anti-alpha-smooth muscle actin (anti-α-SMA) and anti-β-actin antibodies were obtained from CST (USA), and anti-pThr325c-Fos antibody was obtained from Bioss (China). Rabbit anti-IL-17C and Histone H3 polyclonal antibodies were purchased from ABclonal (China). Alexa Fluor 594 labeled goat anti-rabbit IgG and Alexa Fluor 488 labeled goat anti-mouse IgG antibodies were purchased from Invitrogen (USA). An inhibitor (PD98059) of ERK phosphorylation and Chromatin immunoprecipitation (CHIP) assay kit were purchased from CST (USA). SYBR Green detection chemistry was obtained from Promega (USA). NE-PER nuclear and cytoplasmic extraction reagents, rat Stealth RNAi™ IL-17C siRNA, c-Fos siRNA and Lipofectamine RNAiMAX were purchased from Thermo Fisher Scientific (USA). A-c-Fos [Ser32A], A-c-Fos [Thr325A], c-Fos and empty control plasmids were designed by GENECHEM (China). AmaxaTM Basic NucleofectorTM Kit was purchased from Lonza Cologne GmbH (Germany).

2.2. C. pneumoniae infection of mice

C. pneumoniae strain AR-39 (ATCC 53592) was propagated in HEp-2 cells (ATCC CCL-23) and purified by gradient centrifugation as previously described (Wang et al., 2017). ApoE$^{-/-}$ mice were obtained from the Experimental Animal Centre of Military Medical Science Academy (China) and were bred and housed in a specific-pathogen-free, temperature-controlled environment with 12 h light/dark cycles, and received food and water *ad libitum*in Tianjin Medical University Animal Center. The animal-use-protocol listed below has been reviewed and approved by the Animal Ethical and Welfare Committee (AEWC) of Tianjin Medical University (TMUaMEc2018007). 6-week-old male ApoE$^{-/-}$ mice (18–20 g) were randomized into two groups, which were fed Western-type diet (MD12017, containing 40 kcal% fat, 1.25% cholesterol and 0.5% cholic acid) for 6 weeks. The mice were inoculated intranasally with *C. pneumoniae* strain AR-39 at a titer of 2×10^7 inclusion forming unit (IFU)/ml for 3 times at the age of 6, 8 and 10 weeks. At the end of the experiment, the mice were sacrificed after being anaesthetized with isoflurane. The hearts from the mice were embedded in Tissue-Tec optimal cutting temperature moulds at −80 °C after fixing and dehydrating.

2.3. Immunofluorescence staining

The fluorescent double-staining was performed to detect the expression levels of IL-17C and c-Fos in VSMCs in the aortic root from the ApoE$^{-/-}$ mice. Aortic roots sections were fixed in 4% paraformaldehyde solution, and then were incubated with rabbit anti-c-Fos antibody and mouse anti-α-SMA antibody or rabbit anti-IL-17C antibody and mouse anti-α-SMA antibody overnight at 4 °C, followed with Alexa Fluor 594-labeled goat anti-rabbit IgG and Alexa Fluor 488-labeled goat anti-mouse IgG antibodies for 1 h. Immunofluorescence was viewed under an Olympus FV10i laser scanning confocal microscope. Images were analyzed by ImagePro image analysis software.

Table 1
Primer sequences.

Gene	Sequence (5'-3')
IL-17A	F: : 5'- ACA GTG AAG GCA GCG GTA CT -3' R : 5'- GCT CAG AGT CCA GGG TGA AG -3'
IL-17C	F : 5'- ATC GCA TCG ACA CAG ATG AG -3' R : 5'- CGT CGT CTC AGC ACC AGT AG -3'
IL-17E	F : 5'- GTA CCA GGC TGT TGC GTT CT -3' R : 5'- GGG GAA TTC TTG TTG TTT GC -3'
IL-17F	F : 5'- TGG TCA AGT CTC TGC TGC TG -3' R : 5'- ACA GAA ATG CCC TGG TTT TG -3'

2.4. C. pneumoniae infection of VSMCs

Rat VSMCs were isolated from the thoracic aorta of 8-week-old male Sprague-Dawley rats by the explant method and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 25 μg/ml vancomycin and 10 μg/ml gentamicin as previously described (Wang et al., 2013b). VSMCs were seeded at a density of 5×10^5 cells per well into 6-well plates, and were infected with *C. pneumoniae*at an infectious dose of 5×10^5 IFU/ml.

2.5. Quantitative real-time PCR analysis

Total RNA from cultured rat VSMCs was extracted with TRIzol reagent according to the manufacturer's instructions. The mRNA expression levels of IL-17A, IL-17C, IL-17E and IL-17 F were detected by using SYBR Green detection chemistry, and β-actin was used as an internal control. Primer sequences were listed in Table 1. PCR was performed on the Bio-Rad real-time PCR system. The amount of each gene in each sample was relatively quantified using threshold cycle values.

2.6. Western blot analysis

Total proteins from the cultured rat VSMCs were prepared according to the established methods (Wang et al., 2013b; Ma et al., 2015). The nuclear proteins were isolated from the cultured rat VSMCs by using NE-PER nuclear and cytoplasmic extraction reagents. Identical quantities of proteins were separated by 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes. Then, they were respectively incubated with rabbit polyclonal anti-IL-17C, anti-c-Fos, anti-pSer32c-Fos, anti-pThr325c-Fos and anti-β-actin antibodies overnight at 4 °C, and the blots were incubated with goat anti-rabbit secondary antibody or goat anti-mouse secondary antibody for 2 h at room temperature. After washing, the bands were detected using a chemiluminescent HRP substrate kit.

2.7. Cell migration assay

Transwell migration assay was performed as previously described (Wang et al., 2013b). Briefly, rat VSMCs were infected with *C. pneumoniae* for 16 h and then were trypsinized and plated in the upper chamber at the density of 1.5×10^4 cells per well. Cells were subsequently incubated at 37 °C for 8 h. The number of cells migrated through the pores was counted in nine randomly chosen fields under an inverted phase-contrast microscope (magnification ×100).

2.8. Transfection with siRNA

24 h before cell transfection, 1×10^6 rat VSMCs per well were seeded into the 6-well plates with DMEM supplemented with 10% FBS

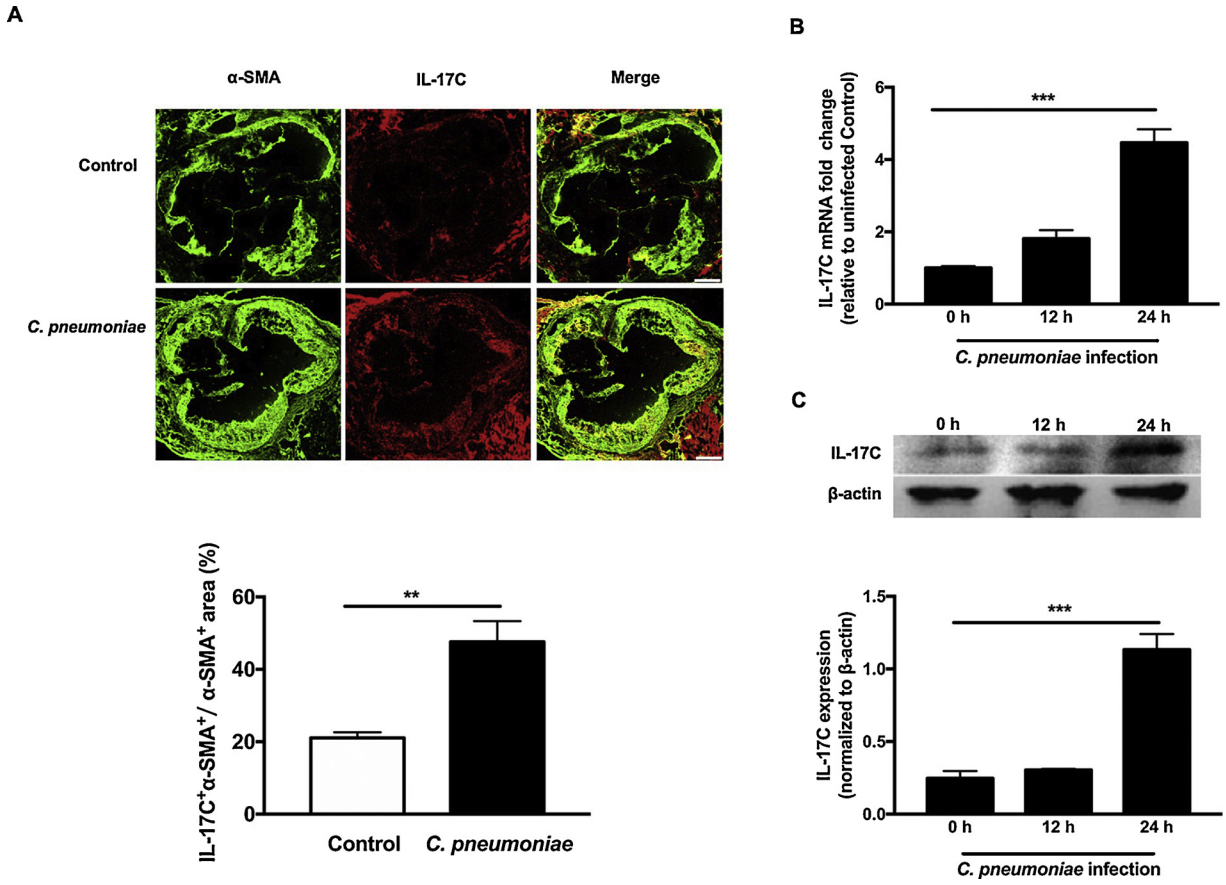

Fig. 1. *C. pneumoniae* infection induces the expression of IL-17C in VSMCs. (A) Representative images of aortic root sections stained with immunofluorescence. And the expression of IL-17C in VSMCs in the atherosclerotic lesions was visualized by colocalization of α-SMA (FITC, green) with IL-17C (phalloidin, Red). α-SMA$^+$ IL-17C$^+$/α-SMA$^+$ (%) represents the percentage of IL-17C expressed in VSMCs in the atherosclerotic lesion area. Scale bar: 100 μm, magnification ×10. $^{**}P < 0.01$ *vs* Control group. IL-17C mRNA (B) and protein (C) expression levels in VSMCs at 12 h and 24 h after *C. pneumoniae* infection were respectively detected by qRT-PCR and Western blot. $^{***}P < 0.001$ *vs* Control (0 h after *C. pneumoniae* infection) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

without antibiotics. Rat Stealth RNAi™ IL-17C siRNA, or c-Fos siRNA, or scramble RNA control was transfected into cells by using Lipofectamine RNAiMAX according to the manufacturer's instructions.

2.9. Plasmid construction and transient transfection

2 μg of c-FosSer32A (encoding an EGFP-c-Fos fusion protein that carries a serine-to-alanine substitution at codon 32-EGFP-c-FosS32A), or 2 μg of c-FosThr325A (encoding an EGFP-c-Fos fusion protein that carries a threonine-to-alanine substitution at codon 325-EGFP-c-FosT325A), or 2 μg of c-Fos (encoding an EGFP-c-Fos full length fusion protein), or 2 μg of empty vector was transiently transfected into rat VSMCs for 48 h using AmaxaTM Basic NucleofectorTM Kit according to the manufacturer's instructions. The expression levels of c-Fos and its phosphorylation at Ser32 and Thr325 were detected by Western blot. Transfection efficiencies evaluated by the percentage of cells that exhibited green fluorescence 48 h after transfection varied between 60% and 70%.

2.10. CHIP

CHIP assay was performed using a commercially available kit. The rat VSMCs were infected with *C. pneumoniae* for 24 h, and then were cross-linked by 1% formaldehyde solution. The extracted chromatin was digested and fragmented into 150–900 bp. Precleared supernatants were immunoprecipitated overnight at 4 °C using the anti-c-Fos or IgG antibody. The protein A/G plus agarose beads were added into samples for DNA enrichment. PCR was performed using the primers spanning the putative c-Fos binding site in rat IL-17C promoter (forward 5′-AAGTCCATCCACGGGTCCAG -3′, reverse 5′ -GCTGAACGGCTC TCT GCTTG -3′).

2.11. Statistical analysis

Data were presented as means ± standard error of means (SEMs). Data analyses were performed with SPSS 16.0 software. Statistical significance was determined using Student *t*-test or ANOVA followed by *Tukey's* test. $P < 0.05$ would be considered to be statistically significant.

3. Results

3.1. C. pneumoniae infection upregulates the expression of IL-17C inVSMCs

Previous study revealed the possible role of IL-17C in atherosclerosis (Butcher et al., 2016). Whether IL-17C is involved in *C. pneumoniae* infection-exacerbated atherosclerosis is unclear. VSMC is known to have important roles in atherosclerosis (Lacolley et al., 2012; Bennett et al., 2016). Therefore, we evaluated the effects of *C. pneumoniae* infection on the expression of IL-17C in VSMCs in atherosclerotic lesions by immunofluorescence staining. As shown in Fig. 1A, *C. pneumoniae* infection resulted in massive VSMC accumulation in the atherosclerotic lesion area, and IL-17C was also dramatically increased in the lesion area and shared the same area with VSMCs. In *vitro*, qRT-PCR and Western blot assays were performed to determine whether *C.*

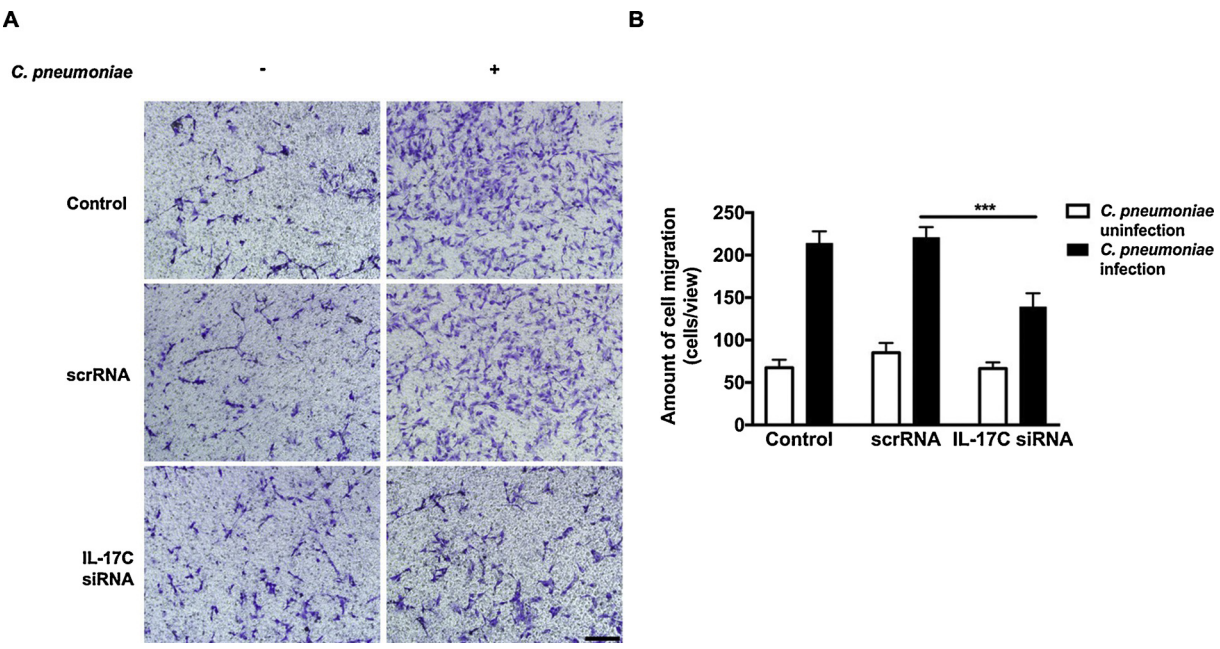

Fig. 2. The role of IL-17C in *C. pneumoniae* infection-induced VSMC migration. (A and B) Transwell assay was performed to assess VSMC migration ability. After the knockdown of IL-17C by transiently transfecting 20 nM IL-17C-siRNA into VSMCs for 48 h, the cells were infected with *C. pneumoniae* for 16 h. Cells were then trypsinized and seeded into the upper chamber at the density of 1.5×10^4 cells per well, and were subsequently incubated at 37 °C for 8 h. Cells which passed through the Transwell chamber were counted in five randomly chosen visual fields. Representative images are shown (magnification ×100). Scale bar: 20 μm. $^{***}P < 0.001$ *vs C. pneumoniae* infection group.

pneumoniae infection affected the IL-17C expression in VSMCs. The results showed that mRNA and protein expression levels of IL-17C were significantly upregulated at 24 h after *C. pneumoniae* infection (Fig. 1B and C). These data indicate that *C. pneumoniae* infection significantly upregulates the expression of IL-17C in VSMCs.

3.2. *C. pneumoniae infection promotes VSMC migration possibly through IL-17C*

IL-17C could significantly enhance the migration ability of peripheral blood leukocytes (Ding et al., 2017). Our previous studies found that *C. pneumoniae* infection could promote VSMC migration (Wang et al., 2013b; Ma et al., 2015). Therefore, we hypothesized that IL-17C might be involved in *C. pneumoniae* infection-promoted VSMC migration. The results from Transwell assay showed that rIL-17C stimulation significantly enhanced the migration of VSMCs (Fig. S1B). And IL-17C-siRNA-mediated knockdown of IL-17C (Fig. S2A) markedly suppressed the infection-promoted VSMC migration (Fig. 2A and B). Collectively, our results demonstrate that *C. pneumoniae* infection promotes VSMC migration possibly through IL-17C.

3.3. *C. pneumoniae infection upregulates IL-17C expression through activating c-Fos*

c-Fos is the key transcription factor to modulate proinflammatory cytokines on stimulation (Zickler et al., 2018; Hop et al., 2018). Therefore, we evaluated the protein expression of c-Fos in VSMCs from $ApoE^{-/-}$ mice with *C. pneumoniae* infection. Immunofluorescence staining results showed that the protein expression of c-Fos in the atherosclerotic lesion area was significantly upregulated by *C. pneumoniae* infection and shared the same area with VSMCs (Fig. 3A), revealing that *C. pneumoniae* infection significantly increased the expression of c-Fos in VSMCs in the atherosclerotic lesion area. Then, we detected c-Fos expression and activation in *C. pneumoniae*-infected VSMCs in *vitro*. Western blot results showed that the expression of c-Fos and phosphorylated c-Fos at Ser32 and Thr325 were all upregulated at 24 h after *C. pneumoniae* infection (Fig. 3B).

To further determine whether *C. pneumoniae* infection upregulated IL-17C expression through c-Fos, we detected IL-17C expression after the overexpression of c-Fos. And the results showed that expression of IL-17C was significantly upregulated by the overexpression of c-Fos (Fig. S3). And c-Fos-siRNA-mediated c-Fos knockdown (Fig. S2B) significantly suppressed the increase in IL-17C expression induced by *C. pneumoniae* infection (Fig. 3C). After *C. pneumoniae* infection, IL-17C expression was also significantly counteracted upon the inhibition of c-Fos phosphorylation at Ser32 (Fig. 3D) and Thr325 (Fig. 3E) respectively. These data indicate that *C. pneumoniae* infection promotes IL-17C expression in VSMCs through c-Fos.

3.4. *c-Fos regulates the expression of IL-17C through phosphorylating ERK but not binding to IL-17C promoter region directly after C. pneumoniae infection*

c-Fos could bind to the promoters of many inflammatory genes for stimulation-triggered inflammatory gene expression (Yang et al., 2016; Zickler et al., 2018). Therefore, a CHIP assay was performed to determine whether c-Fos regulated IL-17C expression by direct transcriptional regulation. As shown in Fig. 4A, IL-17C has three c-Fos putative binding sites in its promoter. Unfortunately, *C. pneumoniae* infection could not significantly increase the abundance of c-Fos on the promoter of IL-17C (Fig. 4B), indicating that c-Fos cannot regulate expression of IL-17C by direct transcriptional regulation in *C. pneumoniae*-infected VSMCs.

AP-1 complex mediates inflammatory signaling *via* MAP3K8/ERK phosphorylation (Wang et al., 2013a; Wei et al., 2017). Accordingly, we detected the phosphorylation of ERK in VSMCs with *C. pneumoniae* infection. Our results showed that ERK phosphorylation was significantly enhanced at 12 h and 24 h after *C. pneumoniae* infection (Fig. 4C). And ERK phosphorylation was also significantly enhanced in response to c-Fos overexpression (Fig. 4D). We further found that after the inhibition of ERK phosphorylation by PD98059, the increased IL-17C expression induced by overexpressing c-Fos was significantly

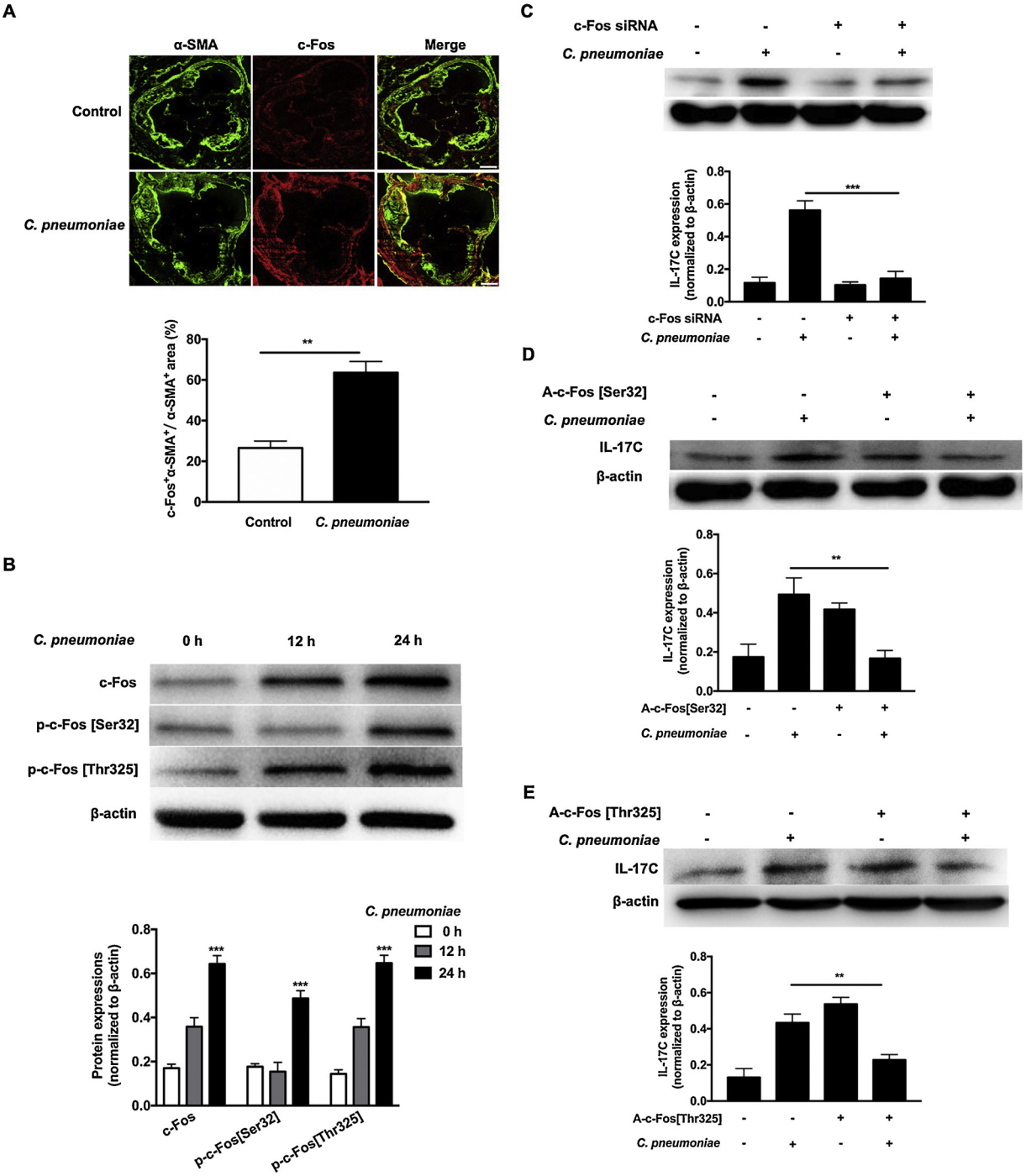

Fig. 3. *C. pneumoniae* infection upregulates IL-17C expression through activating c-Fos. (A) Representative images from aortic root sections were used for immunofluorescence staining. And the expression of c-Fos in VSMCs in the atherosclerotic lesions was visualized by colocalization of α-SMA (FITC, green) with c-Fos (phalloidin, Red). $\alpha\text{-SMA}^{+}\text{c-Fos}^{+}/\alpha\text{-SMA}^{+}$ (%) represents the percentage of c-Fos expressed in VSMCs in the atherosclerotic lesions. Scale bar: 100 μm, magnification ×10. $^{**}P < 0.01$ *vs* Control group. (B) c-Fos expression and phosphorylation at Ser32 and Thr325 were detected by Western blot in VSMCs at 12 h and 24 h after *C. pneumoniae* infection. $^{***}P < 0.001$ *vs* Control (0 h after *C. pneumoniae* infection). (C) After the knockdown of c-Fos by transiently transfecting 60 nM c-Fos-siRNA into VSMCs for 48 h, the cells were infected with *C. pneumoniae* for 24 h. Western blot were performed to detect IL-17C expression. $^{***}P < 0.001$ *vs C. pneumoniae* infection group. (D and E) A-c-Fos [Ser32A]: c-FosSer32A (encoding an EGFP-c-Fos fusion protein that carries a serine-to-alanine substitution at codon 32-EGFP-c-FosS32A), A-c-Fos [Thr325A]: c-FosThr325A (encoding an EGFP-c-Fos fusion protein that carries a threonine-to-alanine substitution at codon 325-EGFP-c-FosT325A). A-c-Fos [Ser32A], A-c-Fos [Thr325A] and empty control plasmids were respectively transfected into VSMCs in nucleofector solution for 48 h, and then cells were infected with *C. pneumoniae* for 24 h. Western blot were performed to detect IL-17C expression when phosphorylation of c-Fos at Ser32 and Thr325 were respectively inhibited in VSMCs. $^{**}P < 0.01$ *vs C. pneumoniae* infection group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

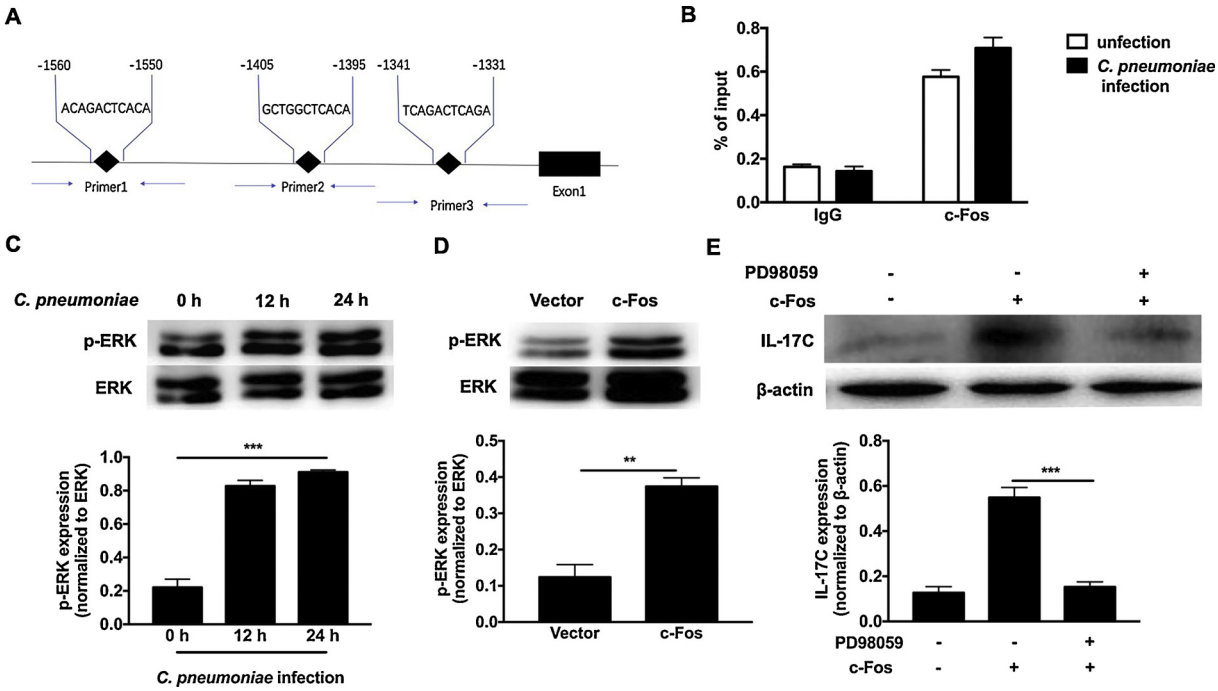

Fig. 4. c-Fos regulates *C. pneumonaie* infection-induced expression of IL-17C through phosphorylating ERK but not directly binding to IL-17C promoter region. (A) The schematic model for the localization of three putative c-Fos binding sites (-1560 to -1550, -1405 to -1395, -1341 to -1331) in the rat IL-17C promoter. (B) VSMCs were infected with *C. pneumoniae* for 24 h. CHIP assays were performed using anti-c-Fos and IgG antibodies, and the mRNA levels of immunoprecipitated rat IL-17C promoter region spanning the putative c-Fos site (-1560 to -1550) were measured by PCR. Input DNA was shown as control. (C) p-ERK was detected by Western blot in VSMCs at 12 h and 24 h post-infection. ***$P < 0.001$ *vs* Control (0 h after *C. pneumoniae* infection). (D) Cells were transfected with c-Fos plasmids for 24 h. p-ERK was detected by Western blot. **$P < 0.01$ *vs* Control group. (E) Cells were pretreated with PD98059 (an inhibitor of p-ERK) with an effective dose at 20 μM, and then were transfected with c-Fos plasmids for 24 h. IL-17C expression was detected by Western blot. ***$P < 0.001$ *vs* c-Fos overexpression group.

suppressed in VSMCs (Fig. 4E). Taken together, c-Fos regulates the expression of IL-17C through phosphorylating ERK but not directly binding to the IL-17C promoter after *C. pneumoniae* infection.

3.5. IL-17C is critical for C. pneumoniaeinfection-induced c-Fos activation

IL-17C could activate the downstream AP-1 for host defense and autoimmune inflammation (Song et al., 2016). Whether IL-17C could regulate c-Fos protein expression and phosphorylation in VSMCs after *C. pneumoniae* infection remains unknown. Our results showed that the increased c-Fos expression and phosphorylation at Ser32 and Thr325 induced by *C. pneumoniae* infection in whole cell lysates (Fig. 5A) and nuclear fractions (Fig. 5B) could be significantly suppressed after the knockdown of IL-17C. Furthermore, c-Fos expression and phosphorylation at Ser32 and Thr325 were significantly increased both in whole cell lysates (Fig. S4A) and nuclear fractions (Fig. S4B) after rIL-17C stimulation. Taken together, these results suggest that *C. pneumoniae* infection-induced c-Fos expression and phosphorylation in VSMCs can be regulated by IL-17C.

ERK phosphorylation could upregulate c-Fos expression (Li et al., 2017). Therefore, we wondered whether IL-17C could regulate c-Fos expression through phosphorylating ERK. Our data showed that ERK phosphorylation was dramatically upregulated by rIL-17C stimulation (Fig. 5C). And the ERK inhibitor PD98059 diminished rIL-17C-induced c-Fos expression and phosphorylation at Ser32 and Thr325 (Fig. 5D). These data suggest that IL-17C is critical for c-Fos expression and phosphorylation by phosphorylating ERK.

4. Discussion

IL-17 family members including IL-17A, IL-17C, IL-17E and IL-17 F are all associated with atherosclerosis (Jeon et al., 2015; Mantani et al., 2015; de Boer et al., 2010). We found that IL-17A, IL-17C, IL-17 F mRNA expression levels were significantly increased in VSMCs at 24 h after *C. pneumoniae* infection, and IL-17A expression was the highest and IL-17C expression was only second to IL-17A (Fig. S5). Accumulating studies have demonstrated that IL-17A plays an important role in high-fat diet-induced and *C. pneumoniae* infection-accelerated atherosclerosis (Chen et al., 2010; Di Pietro et al., 2013). However, few have focused on the role of IL-17C in *C. pneumoniae* infection-accelerated atherosclerosis.

In the present study, we demonstrated that *C. pneumoniae* infection significantly increased IL-17C expression in the atherosclerotic lesion area from ApoE$^{-/-}$ mice. Butcher et al. (2016) also reported that IL-17C was elevated in aortas of the atherosclerotic ApoE$^{-/-}$ mice and was produced primarily by aortic VSMCs. And our results further revealed that *C. pneumoniae* infection resulted in massive VSMC accumulation in the atherosclerotic lesion area, and IL-17C expression was dramatically increased in the VSMCs, suggesting a potential role of IL-17C in atherosclerosis accelerated by *C. pneumoniae* infection. How does IL-17C participate in this process?

It is well known that VSMC migration is essential to the development of atherosclerosis (Lacolley et al., 2012; Bennett et al., 2016). Our data showed that the ability of VSMC migration was enhanced by rIL-17C stimulation, and *C. pneumoniae* infection-promoted VSMC migration was significantly impaired after the silence of IL-17C expression. Although Ding et al. (2017) also demonstrated the role of IL-17C in cell migration in teleost, our results showed that IL-17C was involved in *C. pneumoniae* infection-induced VSMC migration in mammals for the first time, revealing the possible mechanism that IL-17C might participate in *C. pneumoniae* infection-accelerated atherosclerosis. Then, we wondered how *C. pneumoniae* infection affected IL-17C expression, thereby promoting VSMC migration.

c-Fos is a master inflammatory regulator and involved in multiple inflammatory responses (Lee et al., 2018; Kim et al., 2012). During bacterial infection, *Brucella abortus* stimulated c-Fos transcription and

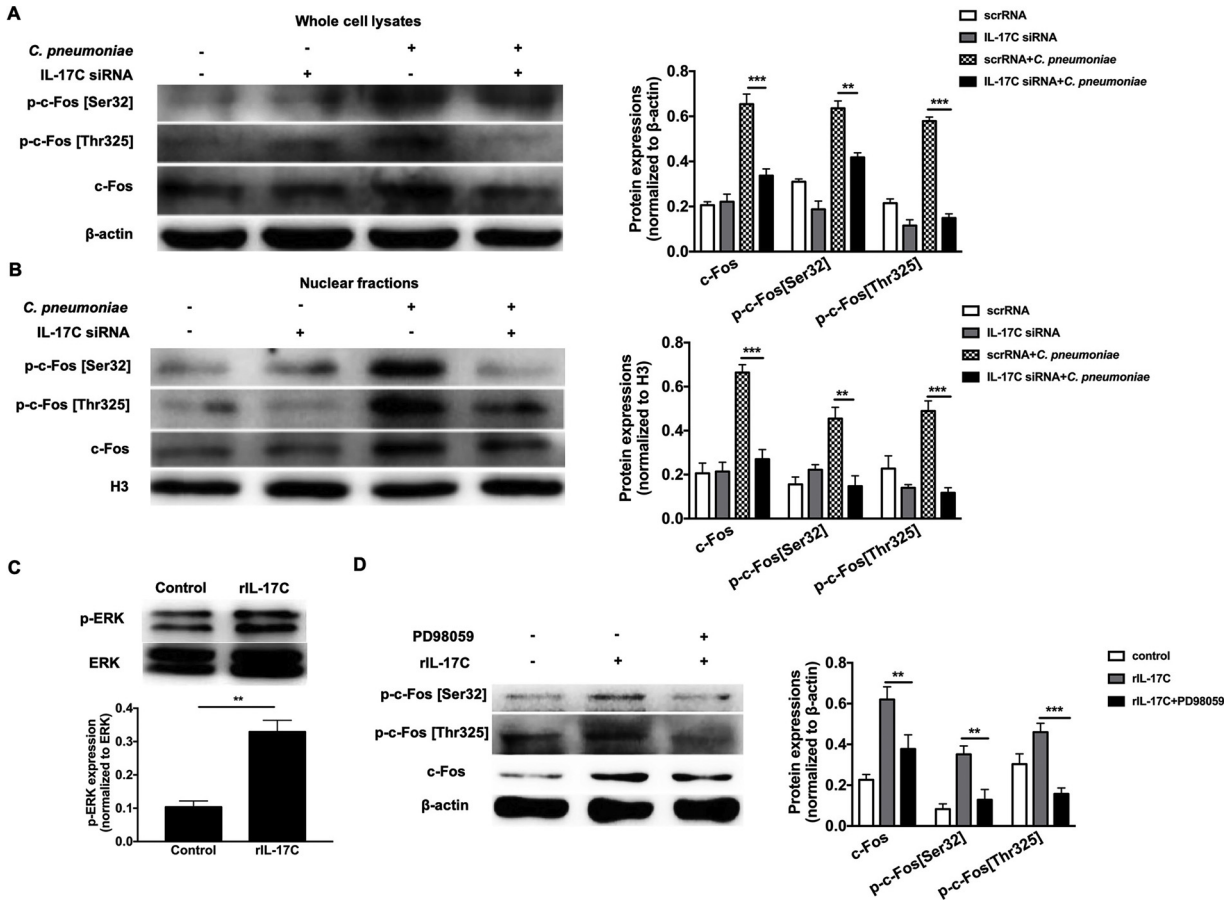

Fig. 5. IL-17C is critical for *C. pneumoniae* infection-induced c-fos activation. 20 nM IL-17C-siRNA was transiently transfected into VSMCs for 48 h, and then the cells were infected with *C. pneumoniae* for 24 h. c-Fos expression and phosphorylation at Ser32 and Thr325 in whole cell lysates (A) and nuclear fractions (B) were detected by Western blot. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs C. pneumoniae* infection group. (C) Cells were incubated with rIL-17C (100 ng/ml) for 24 h, and p-ERK was detected by Western blot. $^{**}P < 0.01$ *vs* Control group. (D) Cells were pretreated with PD98059 with an effective dose at 20 μM, and then the cells were incubated with rIL-17C. c-Fos expression and phosphorylation at Ser32 and Thr325 were determined by Western blot. $^{**}P < 0.01$; $^{***}P < 0.001$ *vs* rIL-17C stimulation group.

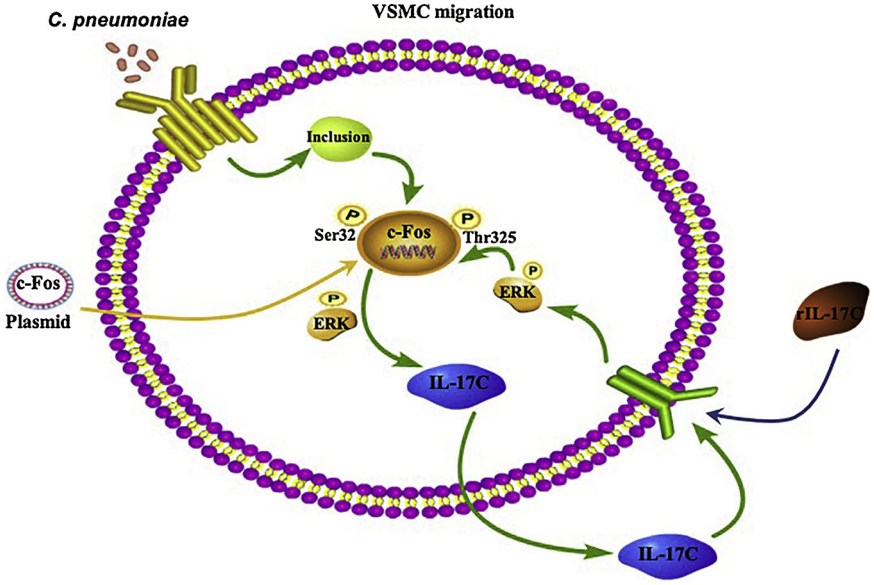

Fig. 6. A novel regulatory mechanism between c-Fos and IL-17C in *C. pneumoniae* infection-promoted migration of VSMC. *C. pneumoniae* infection of VSMCs could induce c-Fos expression and stimulate the phosphorylation of c-Fos at Ser32 and Thr325, and then upregulates the expression of IL-17C through phosphorylating ERK, thus promoting the migration of VSMC. In addition, rIL-17C could also enhance c-Fos expression and phosphorylation at Ser32 and Thr325 by phosphorylating ERK.

activation (Hop et al., 2018). Therefore, we firstly investigated the effect of *C. pneumoniae* infection on the expression and activation of c-Fos. Our results showed that *C. pneumoniae* infection could increase c-Fos expression, and stimulate its phosphorylation at Ser32 and Thr325 in VSMCs at 24 h after infection. Miller et al. (2000) also found that *C. pneumoniae* infection could activate AP-1 in VSMCs. In contrast, Krämer et al. (2015) identified that *C. pneumoniae* infection decreased the expression and phosphorylation of c-Fos in HEp-2 cells at 72 h after

infection. These different results may be based on a cell type-specific regulation of c-Fos signaling and function. Overexpression of c-Fos was found to regulate a long-term level of proinflammatory cytokines expression (Wang et al., 2013c; Sun et al., 2017). We found that the overexpression of c-Fos could increase the expression of IL-17C in VSMCs. Therefore, we further determined whether *C. pneumoniae* infection upregulated IL-17C expression through c-Fos. Our results showed that downregulation of c-Fos expression or inhibition the phosphorylation of c-Fos at Ser32 and Thr325 suppressed the upregulation of IL-17C expression induced by *C. pneumoniae* infection. Zickler et al. (2018) found that c-Fos mediated TNF-α-induced expression of IL-6 in VSMCs, which supported our results to some extent. How does c-Fos regulate IL-17C expression in VSMCs after *C. pneumoniae* infection?

c-Fos could bind to the inflammatory cytokine promoter for virus-triggered activation of gene expression (Yang et al., 2016). Wang et al. (2015) also showed that c-Fos binding sequence (5′- TGCCTCA-3′) in the rat and human Becn1/BECN1 promoters could promote Becn1/BECN1 transcription. Accordingly, we performed a CHIP assay to explore whether c-Fos regulated IL-17C expression by direct transcriptional regulation. Unexpectedly, we did not find that *C. pneumoniae* infection could significantly increase the abundance of c-Fos on the promoter of IL-17C, indicating that c-Fos might not regulate the expression of IL-17C *via* directly binding to IL-17C promoter region after *C. pneumoniae* infection. Such results made us wonder how c-Fos exactly regulated *C. pneumoniae* infection-induced IL-17C expression in VSMCs. Friedrich et al. (2015) found that TNF-α-stimulated IL-17C production was dependent on ERK activation in intestinal epithelial cells. Therefore, in our study, we determined whether c-Fos regulated the infection-induced IL-17C expression by phosphorylating ERK. Our data showed that *C. pneumoniae* infection significantly induced ERK phosphorylation, and overexpression of c-Fos could also enhanced ERK phosphorylation. We further found that inhibition of ERK phosphorylation by PD98059 significantly impaired IL-17C expression caused by overexpression of c-Fos, revealing that c-Fos may increase the infection-induced IL-17C expression by phosphorylating ERK.

Unexpectedly, in our study, we also found that the depletion of IL-17C inhibited c-Fos expression and phosphorylation at Ser32 and Thr325 after *C. pneumoniae* infection. And c-Fos expression and phosphorylation at Ser32 and Thr325 were all increased by rIL-17C stimulation. Based on our results, we propose a model: *C. pneumoniae* infection of VSMCs could induce c-Fos expression and stimulate the phosphorylation of c-Fos at Ser32 and Thr325, and then upregulates the expression of IL-17C through phosphorylating ERK, thus promoting VSMC migration. In addition, rIL-17C could also enhance c-Fos expression and phosphorylation at Ser32 and Thr325 by phosphorylating ERK. Therefore, there may be a positive feedback between c-Fos and IL-17C in this process (Fig. 6). Zhou et al. (2018) also found that c-Fos/microRNA-18a feedback loop modulated the development of human gliomas. Phosphorylation of c-Fos at Ser32 possibly regulated sphingosine kinase 1 expression in a positive feedback manner in the kidney under high glucose condition (Huang et al., 2014). Therefore, our data may elucidate a novel regulatory mechanism between c-Fos and IL-17C (Fig. 6).

In summary, our findings demonstrated the previously unknown roles of IL-17C in the migration of VSMCs induced by *C. pneumoniae* infection, which is crucial for the development of atherosclerosis. Moreover, our data may reveal a new regulatory mechanism between IL-17C and c-Fos to explain *C. pneumoniae* infection-induced VSMC migration.

Funding

This work was supported by grants from the National Natural Science Foundation of China, [81470551 and 30971225] Lijun Zhang.*, [81300206] to Lijun Zhang, [81700403] to Beibei Wang. Postgraduate Innovation Fund of 'The 13th Five-Year comprehensive investment', Tianjin Medical University, [YJSCX201704] to Ningbo Zheng, [YJSCX201819] to Guolin Miao.

Declaration of Competing Interest

No conflicts.

Acknowledgment

The authors highly appreciate the help of foreign expert Bronwyn Jean Tucker at Tianjin Medical University for critical reading of the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2019.151340.

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