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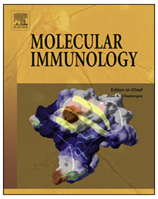

Fli-1 transcription factor regulates the expression of caspase-1 in lung pericytes

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ABSTRACT

Our previous data demonstrated that Friend leukemia virus integration 1 (Fli-1), an ETS transcription factor, governs pericyte loss and vascular dysfunction in cecal ligation and puncture-induced murine sepsis by regulating essential pyroptosis markers including caspase-1. However, whether Fli-1 regulates caspase-1 expression levels *in vitro* and how Fli-1 regulates caspase-1 remain unknown. Our present work further demonstrated that overexpressed Fli-1 significantly increased caspase-1 and IL-18 expression levels in cultured mouse lung pericytes. Bacterial outer membrane vesicles (OMVs) have been found to induce cell pyroptosis through transferring LPS intracellularly. Using OMVs to induce an *in vitro* model of pyroptosis, we observed that OMVs significantly increased protein levels of Fli-1 in mouse lung pericytes. Furthermore, knockdown of Fli-1 by siRNA blocked OMVs-induced caspase-1, caspase-11 and IL-18 expression levels. As caspase-1 was predicted as a potential target of Fli-1, we cloned murine caspase-1 promoter into a luciferase construct. Our data demonstrate for the first time that Fli-1 regulates caspase-1 expression by directly binding to its promoter regions measured by chromatin immunoprecipitation (ChIP) assay and luciferase reporter system. In summary, our findings demonstrated a novel role and mechanism of Fli-1 in regulating caspase-1 expression in lung pericytes.

1. Introduction

Pericytes are embedded in the basement membrane of the microvasculature which wrap around the microvascular endothelial cells (EC) (Armulik et al., 2005). LPS-induced pericyte loss has been associated with microvascular dysfunction and mortality; however, this loss is not caused by apoptosis (Zeng et al., 2016). Our previous study demonstrated that cecal ligation and puncture (CLP)-induced lung pericyte loss was attributed to pyroptosis (Li et al., 2018). Pyroptosis, an inflammatory form of programmed cell death, is dependent on caspases 1 and 11 and is accompanied by the release of pro-inflammatory cytokines including IL-18 (Aachoui et al., 2013; Cookson and Brennan, 2001; Hu et al., 2016; Aglietti and Dueber, 2017). Pyroptosis can be induced by intracellular stimulation with LPS (Pfalzgraff et al., 2017). Recent studies have demonstrated that Gram-negative bacteria outer membrane vesicles (OMVs), contain abundant LPS and can induce pyroptosis of host cells (Vanaja et al., 2016). In addition, OMVs induce inflammasome activation and pro-cytokine release in macrophages in a caspase-1 dependent manner (Cecil et al., 2017). As an inflammatory caspase and the most important pyroptosis marker, caspase-1 can be activated by inflammasomes, and processes pro-IL-18 into the active form and cleaves gasdermin D to trigger pyroptosis (Sun and Scott, 2016). Circulating microvesicular caspase-1 activity was higher in septic patients and plays a critical role in sepsis-induced endothelial cell injury (Exline et al., 2014; Mitra et al., 2018). Caspase-1-dependent pyroptosis of peripheral blood mononuclear cells predicts the development of sepsis in severe trauma patients (Wang et al., 2018). Inflammasome NLRP3/caspase-1 pathway also mediated cognitive deficits in a CLP-induced murine septic model (Fu et al., 2018). However, inhibition of caspase-1 by either antimicrobial cathelicidin peptide LL-

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37 or its inhibitor was associated with reduced inflammation, improved organ injury and increased survival in animal sepsis (Hu et al., 2016; Chen et al., 2016). Therefore, signaling pathways controlling caspase-1 expression may provide beneficial effects in sepsis.

Friend leukemia virus integration 1 (Fli-1), an ETS transcription factor, regulates a wide spectrum of biological processes including cancer development, fibrosis, vasculopathy and inflammation (Lou et al., 2017; Sato et al., 2014; Suzuki et al., 2012; Akamata et al., 2015; Theisen et al., 2016; Takahashi et al., 2017). Fli-1 is expressed in endothelial cells, macrophages, B cells and T cells, and regulates expression of several important cytokines and chemokines including monocyte chemoattractant protein-1 (MCP-1), IL-6, granulocyte colony stimulating factor (G-CSF) and CCL5 by directly binding to these respective promoters (Sato et al., 2014; Suzuki et al., 2012; Lennard Richard et al., 2016; Gao et al., 2016; Zhang and Watson, 2005; Zhang et al., 2008; Richard et al., 2013; Lennard Richard et al., 2014). Our previous study suggested that Fli-1 mediated lung pericyte loss and vascular function *via* regulating expression levels of essential pyroptosis markers including caspase-1 and IL-18 in CLP-induced septic mice (Li et al., 2018). However, the role and mechanism of Fli-1 in regulating caspase-1 expression in lung pericytes remains unknown. We hypothesized that Fli-1 transcription factor regulates caspase-1 gene expression in lung pericytes by directly binding and activating the caspase-1 promoter.

2. Materials and methods

2.1. OMVs isolation and characterization

OMVs were purified from *E. coli* K12 as previously described (Vanaja et al., 2016). Briefly, the bacterial strain was grown in 600 ml of LB to an OD600 of 0.4-0.6 and centrifuged at 4000 g for 20 min at 4 °C. The bacteria-free supernatant was filtered through a 0.22 μm filter and OMVs were pelleted by ultracentrifugation at 100,000 g for 2 h at 4 °C. After removing the supernatant, OMVs were resuspended in 500 μl sterile PBS. The total protein concentration of the OMVs was measured by protein assay (Bio-Rad, Hercules, CA). The LPS concentration of OMVs was measured by Pierce LAL Chromogenic Endotoxin (Thermo Fisher Scientific, Rockford, IL). The size distribution and the total number of OMVs were analyzed by nanoparticle tracking analysis software (ZetaView 8.04.02).

2.2. Mouse lung pericyte isolation, culture and stimulation

Mouse lung pericytes were isolated as described previously (Hung et al., 2013, 2017). Briefly, single-cell preparations from whole lung digests were expanded, negatively selected by CD31, CD45 and CD326 magnetic beads (Miltenyi Biotec Inc., Auburn, CA), and positively selected by PDGFRβ magnetic beads (Miltenyi Biotec Inc., Auburn, CA). PDGFRβ positive lung pericytes were cultured in pericyte medium (ScienCell Research Laboratories, Carlsbad, CA) supplemented with pericyte growth supplement, 2% fetal bovine serum and 1% penicillin/streptomycin (ScienCell Research Laboratories, Carlsbad, CA). Pericytes were transfected with control plasmid or Fli-1 plasmid at different concentration (0.25–4 μg/ml) for 48 h. In another set of experiment, pericytes were transfected with Fli-1 siRNA or scrambled siRNA for 24 h and further stimulated with OMVs (25 μg/ml, containing 5.2 μg/ml LPS) for 16 h. Total RNA and protein were collected for further analysis. To determine intracellular LPS levels after OMVs treatment, the mouse pericytes were seeded into six-well plates and treated with 25 μg/ml OMVs (containing 5.2 μg/ml LPS) for 16 h. Then cells in each well were washed three times with PBS and lysed by 500 μl RIPA lysis buffer. The intracellular LPS in the lysate was measured by Pierce LAL Chromogenic Endotoxin (Thermo Fisher Scientific, Rockford, IL).

2.3. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured pericytes with RNeasy plus mini kit (Qiagen, Germantown, MD). cDNA was synthesized with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed by CFX96 Real-Time PCR system (Bio-Rad, Hercules, CA, USA) using SYBR Green PCR Kit (Qiagen, Germantown, MD) in a final reaction volume of 25 μl with each primer (Qiagen, Germantown, MD). Data were analyzed with $2^{-\Delta\Delta Ct}$ value calculation using GAPDH for normalization.

2.4. Western blot analysis

Lung pericytes were lysed with ice-cold RIPA lysis buffer (Cell Signaling, Danvers, MA). Western blot was performed as described (Li et al., 2011). All lysed samples were kept on ice for 30 min, and centrifuged for 10 min at 4 °C at 12,000 g. Cell lysates were subjected to 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 7% milk in TBST (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20) for 1 h. After washing with TBST twice, membranes were incubated with primary antibody overnight at 4 °C. Fli-1 primary antibody was provided by Dr. Xiankui Zhang (Medical University of South Carolina). Primary antibodies α-tubulin were from Cell Signaling. The membranes were washed twice with TBST and incubated with HRP conjugated secondary antibody in blocking buffer for 1 h. After washing three times with TBST, immunoreactive bands were visualized by incubation with ECL plus detection reagents (GE Healthcare, Waukesha, WI). The densitometry of bands was quantified with Image J2 software.

2.5. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using an anti-Fli-1 rabbit polyclonal antibody and normal IgG control (Cell Signaling, Danvers, MA) using EpiTect ChIP OneDay Kit (Qiagen, Germantown, MD) as described (Lou et al., 2017). The primers used in the ChIP assay are available upon request. Briefly, mouse lung pericytes (10^6) were cross-linked with 1% formaldehyde at room temperature and lysed in IP lysis buffer (Qiagen, Germantown, MD). DNA was sheared by sonication and immunoprecipitation was performed by using Fli-1 specific antibody and normal IgG control. After immunoprecipitation, the DNA was purified and amplified by PCR according to the manufacturer's instructions (Qiagen, Germantown, MD).

2.6. Reporter and expression constructs

Mouse caspase-1 (GeneID: 12362) was predicted as a potential target of Fli-1 analyzed by the Genomatix online software. We found 14 potential binding sites of Fli-1 in the mouse caspase-1 promoter. The −2410 to +30 region of the mouse caspase-1 promoter, which covers all the potential Fli-1 binding sites, was cloned into the pGL3 basic vector upstream of the luciferase gene. The mouse Fli-1 gene cloned into the pcDNA3.0 expression vector has been described previously (Lennard Richard et al., 2014).

2.7. Luciferase reporter assays

To measure the luciferase activity for the transient transfection experiments, the luciferase assay systems (Promega, Madison, WI) was employed. Briefly, mouse lung pericytes were transfected with caspase-1/pGL3 luciferase reporter construct (1 μg) along with increased amounts of Fli-1/pcDNA3.0 plasmid (0.25, 0.5, 1, 2 and 4 μg) for 48 h. Luciferase activity was determined by a plate reader (Biotek, Winooski, VT). All experiments were normalized using the fold activation of luciferase activity compared to the control luciferase reporter construct.

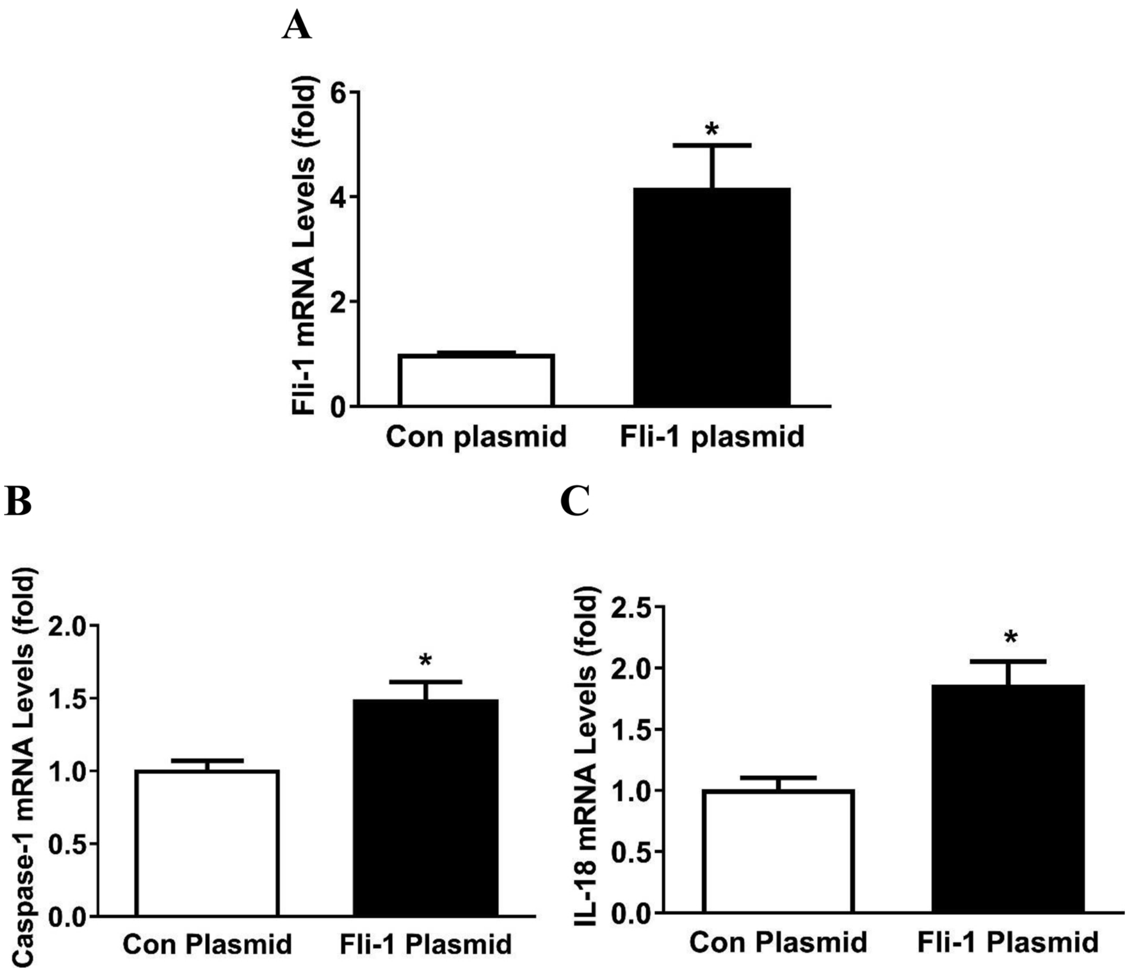

Fig. 1. Fli-1 overexpression increases caspase-1 in mouse lung pericytes. Lung pericytes were isolated from normal C57/BL6 mice and transfected with control or Fli-1 plasmid for 48 h. The mRNA levels of (A) Fli-1, (B) caspase-1 and (C) IL-18 were determined by Real-time PCR. *$p < 0.05$ compared to control plasmid group. Con: control.

2.8. Data analyses

Data are expressed as means ± standard error of the mean (SE). Statistical significance was determined by analysis of variance (ANOVA) with Fisher's probable least-squares difference test or Student's *t*-test using GraphPad Prism software. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Fli-1 regulates caspase-1 gene expression in mouse lung pericytes

Our previous study showed that Fli-1 regulates caspase-1 expression levels in lung pericytes of septic mice *in vivo* (Li et al., 2018). Here we sought to determine if Fli-1 regulates caspase-1 gene expression using an *in vitro* system. Isolated mouse lung pericytes were transfected with control or Fli-1 plasmid for 48 h. Fli-1 mRNA levels were significantly increased after transfection with Fli-1 plasmid ($p < 0.05$; Fig. 1A). Increased Fli-1 further significantly upregulates caspase-1 and IL-18 expression levels ($p < 0.05$; Fig. 1B–C).

3.2. Fli-1 regulates bacterial OMVs-induced caspase-1 expression in mouse lung pericytes

To further determine if Fli-1 regulates caspase-1 gene expression in activated lung pericytes *in vitro*, OMVs were isolated from *E.coli* K12 as previously described (Vanaja et al., 2016). The isolated OMVs were characterized by nanoparticle tracking analysis (NTA) with Zetaview PMX 120 (Particle Metrix, Meerbusch, Germany). The size distribution and the total number of OMVs were analyzed by nanoparticle tracking analysis software (ZetaView 8.04.02). We isolated 6.5×10^{10} particles (containing 0.3 mg protein and 62.5 µg LPS) from 600 ml bacterial supernatant with a concentration of 1.3×10^{11} particles/ml. More than 90% of OMVs are within 70–130 nm range (Fig. 2A). Bacterial OMVs were reported to induce pyroptosis by transferring LPS intracellularly with increased caspase-1 (Vanaja et al., 2016). We further determined intracellular LPS levels after OMV treatment, 25 µg/ml OMVs (containing 5.2 µg/ml LPS) were used to treat mouse pericytes for 16 h. Our results showed that 568 ± 32.4 ng/ml of intracellular LPS was detected in the 500 µl lysate from each well of pericytes. To investigate the role of Fli-1 in the regulation of caspase-1 expression under OMVs stimulation, we first determined the effect of OMVs on Fli-1 protein levels. Our data demonstrated that treatment with OMVs for 16 h significantly increased Fli-1 protein levels in mouse lung pericytes ($p < 0.05$; Fig. 2B). Lung pericytes were transfected with Fli-1 siRNA or scrambled siRNA and treated with bacterial OMVs (25 µg/ml) for 16 h. Fli-1 mRNA levels were significantly reduced by transfecting Fli-1 siRNA into pericytes (data not shown). Exposure of lung pericytes to OMVs significantly increased mRNA levels of pyroptosis markers including caspase-1, caspase-11 and IL-18, which were mitigated in cells transfected with Fli-1 siRNA ($p < 0.05$; Fig. 3A–C).

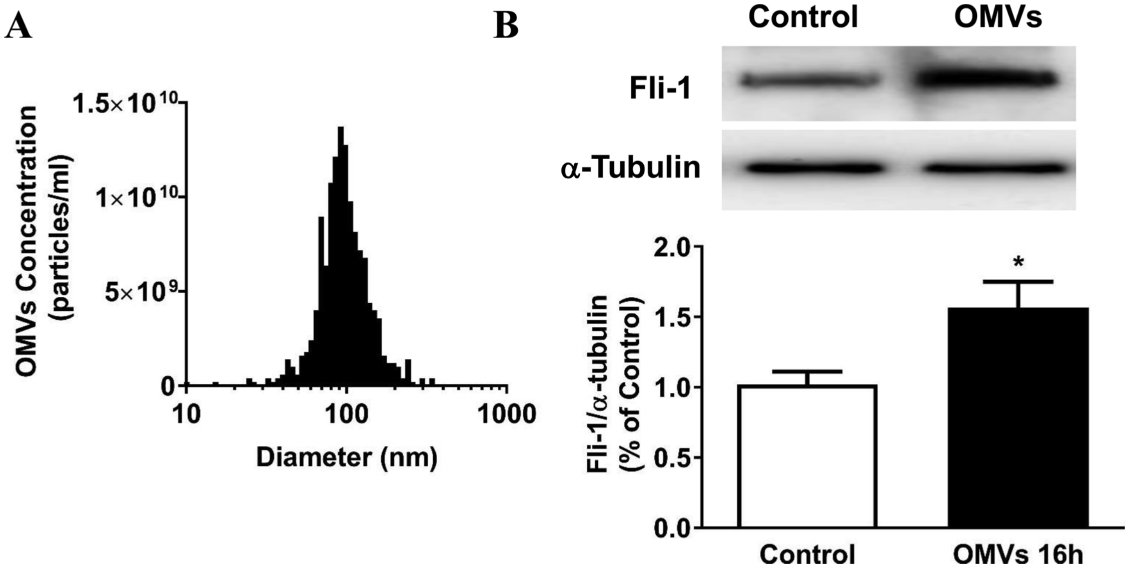

Fig. 2. Bacterial outer membrane vehicles (OMVs) increased Fli-1 protein levels in lung pericytes. (A) OMVs were purified from *E. coli* K12. The number of OMVs particles *vs.* particle size was generated by nanoparticle tracking analysis with ZetaView. Lung pericytes were stimulated with OMVs (25 μg/ml) for 16 h. (B) The effect of OMVs on Fli-1 protein levels were determined by western blot. N = 3 experiments. $*p < 0.05$ compared to control group.

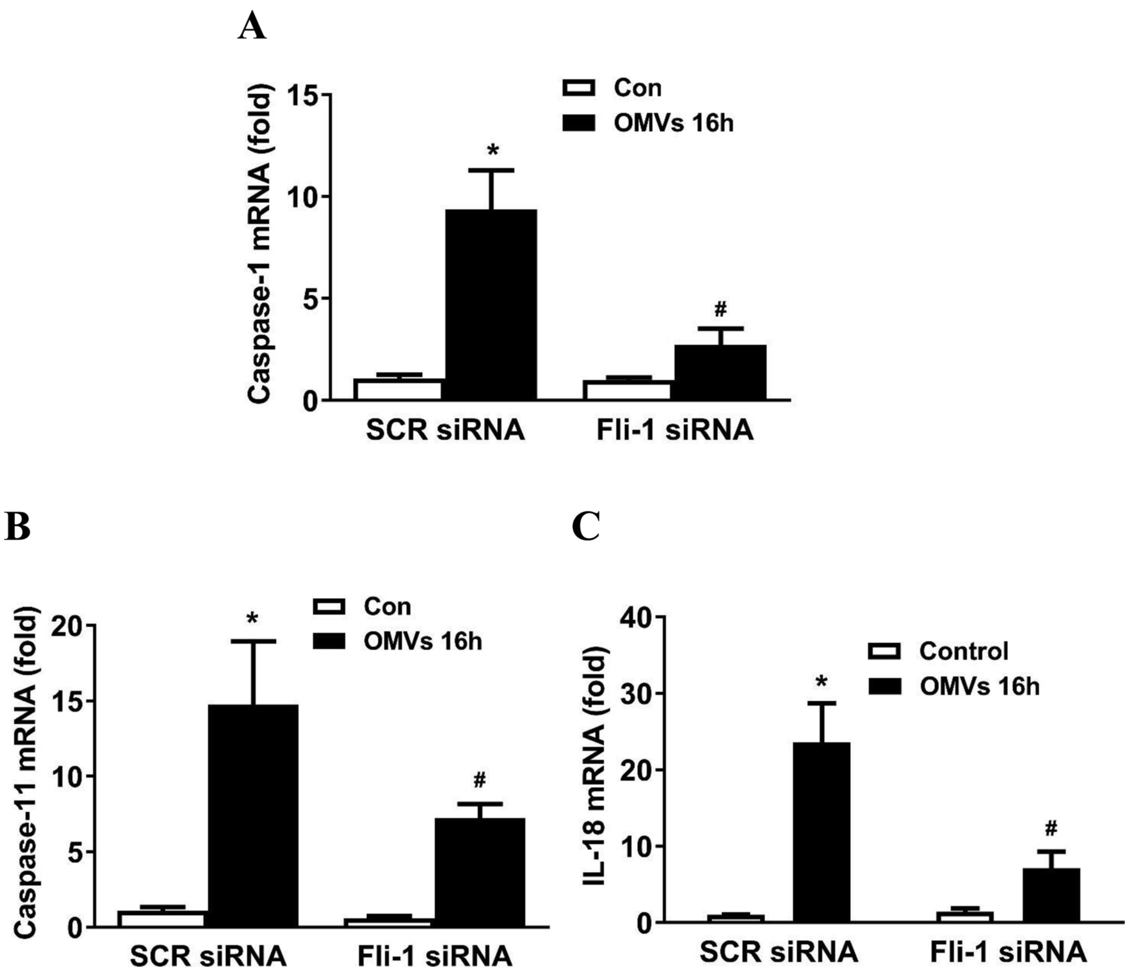

Fig. 3. Fli-1 regulates OMVs-induced caspase-1 expression in lung pericytes. Lung pericytes were transfected with Fli-1 specific siRNA or scrambled siRNA and further stimulated by OMVs (25 μg/ml, *E.coli* K12) for 16 h. The mRNA levels of (A) caspase-1, (B) caspase-11 and (C) IL-18 in lung pericytes were measured. N = 3 experiments. Data are expressed as means ± SE. $*p < 0.05$ compared to scrambled siRNA control group; $^{\#}p < 0.05$ compared to scrambled siRNA OMVs group. SCR: scramble.

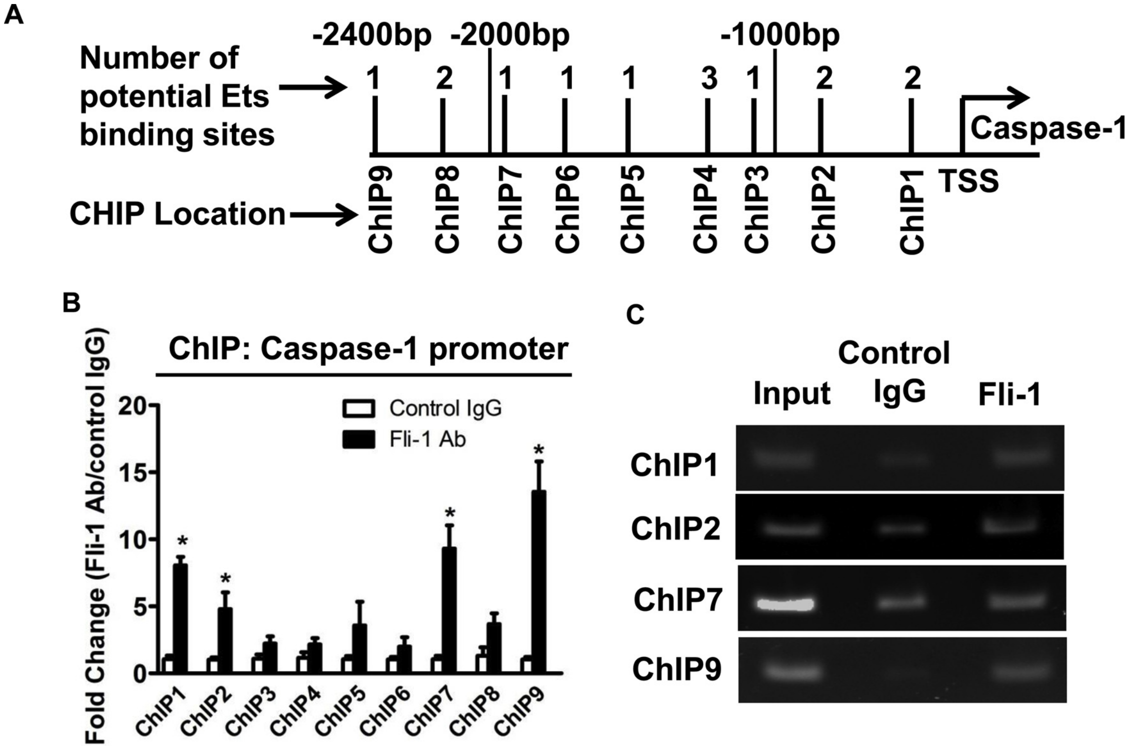

Fig. 4. Fli-1 regulates caspase-1 expression in lung pericytes by binding to its promoter. The potential Fli-1 binding sites on murine caspase-1 promoter region were determined by ChIP assay. A schematic diagram (A) showing the location of the fourteen putative Ets binding sites and nine primers designed for ChIP. ChIP analysis (B) of Fli-1 binding to the Caspase-1 promoter was performed. Representative agarose gel results (C) for ChIP1, 2, 7 and 9 were showed. N = 3 independent experiments. *$p < 0.05$ compared to control IgG group.

3.3. Fli-1 binds to the caspase-1 promoter in mouse lung pericytes

To determine if Fli-1 directly regulates caspase-1 expression levels, we used MatInspector Software (Genomatix) analysis to predict potential Fli-1 binding sites on the murine caspase-1 promoter. We identified 14 putative Fli-1 binding sites on the caspase-1 promoter and 9 pair primers were designed to cover these sites (Fig. 4A). After immunoprecipitation of the cultured lung pericytes with a Fli-1 specific antibody and normal IgG control, ChIP1, ChIP2, ChIP7 and ChIP9 were significantly enriched for Fli-1 specific antibodies (8.1 ± 0.6 fold for ChIP1, 4.8 ± 1.3 fold for ChIP2, 9.3 ± 1.7 fold for ChIP7 and 13.5 ± 2.3 fold for ChIP9, respectively, $p < 0.05$) compared to the IgG negative control (Fig. 4B–C).

3.4. Fli-1 drives transcription from the caspase-1 promoter

To further confirm that Fli-1 regulates the expression of caspase-1, transient transfection assays were performed. The Fli-1 transcription factor was transfected into mouse lung pericytes along with the mouse caspase-1 promoter/pGL3 reporter construct. We determined the Fli-1 protein expression levels after transfection by immunoblot. As shown in Fig. 5A, Fli-1 protein expression corresponds with increasing amounts of the Fli-1 plasmid transfected into the cells. Furthermore, the Fli-1 transcription factor strongly induced activation from the caspase-1 promoter in a statistically significant manner when compared to the activation of the reporter construct (Fig. 5B). The results demonstrate that Fli-1 drives transcription from the caspase-1 promoter in a concentration-dependent manner, with as little as 500 ng of Fli-1 plasmid needed to significantly activate transcription from the caspase-1 promoter.

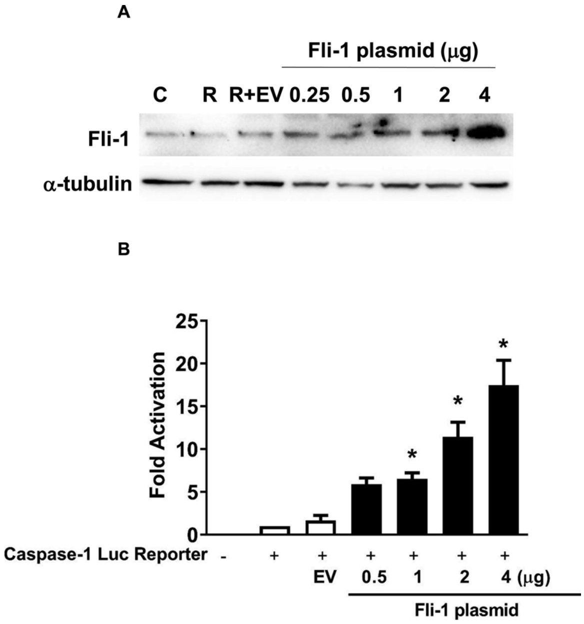

Fig. 5. Fli-1 drives transcription from the caspase-1 promoter. (A) Fli-1 protein concentrations after transfection into the pericytes. (B) Graph illustrating that Fli-1 drives transcription from the caspase-1 promoter in mouse lung pericytes. Transfections were carried out using increasing amounts of Fli-1 plasmid (0.5 μg, 1 μg, 2 μg and 4 μg) and mouse caspase-1 promoter/pGL3 reporter construct (1 μg). A luciferase assay was performed to determine activation from the caspase-1 promoter. Data presented are shown as fold activation over the activation of the empty reporter construct. N = 3 independent experiments. *$p < 0.05$ compared to the activation of the reporter construct. C: control; R: reporter; R + EV: reporter + Empty vector.

4. Discussion

Our previous data showed that Fli-1 regulates both mRNA and protein levels of caspase-1 *in vivo*. This study demonstrates several novel and important discoveries. First, Fli-1 regulates caspase-1 and IL-18 expression in cultured lung pericytes with or without stimulation *in vitro*. Secondly, we provide the first evidence that the Fli-1 transcription factor directly drives transcription from the murine caspase-1 promoter. Collectively, these findings reveal a novel mechanism for Fli-1 in the regulation of the inflammatory response and pyroptosis. Thus, Fli-1 may be a novel target for treating pyroptosis/inflammation-related diseases including sepsis

Recent studies highlight a critical role of pyroptosis in sepsis. Sepsis-associated pyroptosis occurred widely in the various type of cells including peripheral blood mononuclear cells, neurons, macrophages, pericytes, endothelial cells and liver cells in patients and animal models (Li et al., 2018; Hu et al., 2016; Exline et al., 2014; Wang et al., 2018; Fu et al., 2018; Cheng et al., 2017). During sepsis, pyroptosis is required for defense against invasive pathogenic bacteria and microbial infections; however, when overactivated it can result in the inflammatory response, multi-organ dysfunction and septic shock (Gao et al., 2018; Pfalzgraff et al., 2017; Esquerdo et al., 2017). In addition, circulating microvesicular caspase-1 activity and IL-18 levels were significantly higher in septic patients (Exline et al., 2014; Esquerdo et al., 2017). However, inhibition of pyroptosis has been proven beneficial in reducing the inflammatory response, organ injury and mortality in animal models (Hu et al., 2016; Fu et al., 2018; Chen et al., 2016; Cheng et al., 2017). Consistently, our previous study demonstrated that inhibition of lung pericyte pyroptosis was associated with reduced lung vascular leak and decreased mortality in CLP-induced murine sepsis; this beneficial effect was mediated by knockout of Fli-1 in pericytes (Li et al., 2018). Our present data further showed that Fli-1 upregulated caspase-1 and IL-18 gene expression by directly binding to the caspase-1 promoter. Pericyte Fli-1 levels were elevated in CLP-induced septic mice (Li et al., 2018). Thus, the beneficial effects of Fli-1 pericyte knockout in sepsis may be partly attributed to attenuated caspase-1 expression. Similar, inhibition of caspase-1 was also found to improve sepsis outcomes in animal models (Hu et al., 2016; Fu et al., 2018; Chen et al., 2016). Therefore, Fli-1 is a potential novel target for inhibition of pyroptosis *via* controlling caspase-1 expression.

Besides pyroptosis, the uncontrolled inflammatory response is another hallmark of sepsis. LPS-induced overwhelming production of inflammatory mediators during sepsis was thought to be mediated by activation of the Toll-like receptor (TLR) 4 signaling pathway (Pfalzgraff et al., 2017; Palsson-McDermott and O'Neill, 2004). However, recent studies suggest that TLR4-independent recognition of intracellular LPS leads to non-canonical activation of the inflammasome and caspase-1 which, coupled with pyroptosis, triggers further inflammatory response (Pfalzgraff et al., 2017; Shi et al., 2014; Schmid-Burgk et al., 2015). Our previous data demonstrated that extracellular LPS increased Fli-1 expression levels in cultured lung pericytes; while inhibition of Fli-1 expression by transfecting Fli-1-specific siRNA blocked extracellular LPS-induced production of sepsis-related pro-inflammatory cytokines including G-CSF and IL-6 (Li et al., 2018). The present study further showed that intracellular LPS, the major components of OMVs, significantly upregulated Fli-1 protein levels in lung pericytes; while knockdown of Fli-1 expression attenuated OMVs-induced caspase-1 and IL-18 expression levels. Thus, inhibition of Fli-1 can block both extracellular and intracellular LPS-mediated inflammation in lung pericytes. Taken together, these findings provide new insights into the mechanisms by which Fli-1 exacerbates the inflammatory response: 1) Fli-1 transcription factor directly regulates the transcription of several important cytokines and chemokines including MCP-1, IL-6, G-CSF, chemokine C-X-C motif ligand 2 (CXCL2) and CCL5 through direct binding to their respective promoters (Lou et al., 2017; Sato et al., 2014; Suzuki et al., 2012; Lennard Richard et al., 2016, 2014); 2) Fli-1 mediates extracellular LPS-induced inflammatory response; 3) Fli-1 mediates intracellular LPS-induced inflammatory caspase-1 and IL-18 expression. Therefore, blockade of Fli-1 may exert multiple beneficial effects to inhibit inflammation during sepsis.

There are several limitations to our study. We demonstrated that both extracellular and intracellular LPS stimulate Fli-1 expression levels; however, the specific mechanism of how LPS induces Fli-1 expression needs further investigation. Although we found that Fli-1 transcription factor directly regulates caspase-1 gene expression by binding to its promoter, activated caspase-1 is the key factor for producing active IL-18 and inducing pyroptosis. Whether Fli-1 has the impact on activated caspase-1 remains unknown and could be an interesting topic for future study.

In summary, the Fli-1 transcription factor drives transcription from the murine caspase-1 promoter in a dose-dependent manner and affects the regulation of the pyroptosis and inflammation. Further, our previous study demonstrated that inhibition of Fli-1 reduces inflammatory response, attenuates vascular leak and increases survival in cultured lung pericytes and in CLP-induced sepsis (Li et al., 2018). Thus, we provide evidence that signaling pathways reducing Fli-1 expression may represent a novel potential way for inhibiting pyroptosis and inflammation during sepsis.

Competing interests

The authors declare no competing financial interests.

Acknowledgments

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