Follistatin-Like Protein 1 Promotes Mechanical Ventilation-Induced-NACHT, LRR and PYD Domains-Containing Protein 3 Inflammasome in Human Pulmonary Microvascular Endothelial Cells

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To the Editor: The inflammasome, a multiprotein oligomer which regulates the innate immune functions by activating caspase-1 and catalyzing the hydrolysis and secretion of interleukin (IL)-1β and IL-18, is an important mediator of ventilator-induced lung injury (VILI).[1] The NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) initiates the inflammatory response by assembling with the ASC and caspase-1 to form the inflammasome and then activating pro-caspase-1 and the release of IL-1β and IL-18.[2] That study also showed the involvement of NLRP3 inflammasome in VILI. Follistatin-like protein 1 (FSTL-1) is a secreted glycoprotein which regulates cell proliferation, differentiation, apoptosis, and metabolism. FSTL-1 upregulates the expression of pro-inflammatory cytokine and inflammatory response genes through the activation of NLRP3 in monocytes and macrophages.[3] Although both FSTL-1 and NLRP3 inflammasome are activated in VILI, their exact relationship is unclear. The aim of this study was to determine whether FSTL-1 enhances the inflammatory response by activating NLRP3 inflammasome.

Human pulmonary microvascular endothelial cells (HPMECs) were plated at the density of 5×10^5 cells/ml on either culture dishes or collagen I-coated flexible bottom BioFlex plates in endothelial cell medium with 5% fetal bovine serum, 1% endothelial cell growth factor, and 1% penicillin/streptomycin solution. The cells were maintained at 37°C under 5% CO_2 and pH 7.4. FSTL-1 small interfering RNA (siRNA) was transiently transfected into the HPMECs using INTERFER in and different dilutions of the siRNA.

The confluent HPMECs stably transfected with the FSTL-1 siRNA, or treated for 2 h with 10 μmol/L of the NLRP3 inhibitor MCC950, were exposed to cyclic stretch using a FX-5000T Flexercell Tension Plus system (Flexcell International, McKeesport, PA, USA) equipped with a 25-mm BioFlex loading station. The cyclic stretch pattern had a frequency of 0.5 Hz for 30 cycles/min and a stretch-to-relaxation ratio of 1:1. Cyclic stretch was conducted at 8% or 20% of the change in the basement membrane surface area for 4 h. Nonstretched cells were used as controls.

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Following the cyclic stretch treatment, the culture medium was collected and centrifuged at 1500 × *g* for 3 min, and the levels of IL-1β, IL-18, and FSTL-1 in the supernatant were quantified using specific ELISA kits according to the manufacturer's instructions.

The levels of caspase-1 (p20) and NLRP3 proteins were quantified by Western blotting. Total cellular protein was extracted, and the concentration was determined using BCA kits. Equal amounts of protein from each sample were denatured and separated on an SDS-PAGE gel and subsequently transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). After blocking with 5% skimmed milk, the membranes were incubated overnight with the requisite primary antibodies at 4°C, followed by an hour-long incubation with the horseradish peroxidase-conjugated secondary antibody at 37°C. Positive bands were detected through chemiluminescence with the enhanced chemiluminescence system.

In the present study, we used HPMECs to study the underlying inflammatory mechanisms of VILI. Cyclic stretching of HPMECs with a stretch machine is a well-established method to simulate *in vivo* lung expansion and contraction.[4] We used 8% or 20% cyclic stretch and measured the ensuing inflammatory response. Cyclic stretch-induced inflammation, manifested as IL-1β and IL-18 secretion, compared to the control and 8% cyclic stretch groups, the levels of secreted IL-1β and IL-18 were significantly increased in the 20% cyclic stretch group after 4 h ($P < 0.05$). Therefore, 20% cyclic stretch for 4 h was used in the subsequent experiments.

The NLRP3 inflammasome is a multiprotein complex made of specific NLR oligomers, procaspase-1 and ASC, which are known

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Table 1: Levels of IL-18, IL-1β, FSTL-1, NLRP3, and caspase-1 (p20) in HPMEC with different treatments

Groups	IL-18 (pg/ml)	IL-1β (pg/ml)	FSTL-1 (ng/ml)	NLRP3/GAPDH	Caspase-1 p20/GAPDH
Control	47.45 ± 6.63	36.80 ± 5.51	7.02 ± 0.58	0.30 ± 0.05	0.22 ± 0.07
8% CS	64.89 ± 7.04	54.61 ± 5.16	8.37 ± 0.83	0.37 ± 0.06	0.30 ± 0.05
20% CS	455.35 ± 26.95*,†	383.57 ± 18.83*,†	59.38 ± 3.54*,†	0.84 ± 0.08*,†	0.88 ± 0.07*,†
20% CS + MMC950	96.75 ± 10.30‡	85.32 ± 9.03‡	58.14 ± 2.52‡	0.39 ± 0.04‡	0.43 ± 0.05‡
20% CS + FSTL-1 siRNA	105.01 ± 10.61§	96.92 ± 8.44§	13.30 ± 0.94§,‖	0.43 ± 0.06§,¶	0.44 ± 0.04§,¶

Compared with the control group, *P<0.05; compared with the 8% CS group, †P<0.05; compared with the 20% CS group, ‡P<0.05, §P<0.05; compared with the 20% CS + MMC950 group, ‖P<0.05, ¶P>0.05. Data are representative of 3 independent experiments. IL: Interleukin; NLRP3: NACHT, LRR and PYD domains-containing protein 3; FSTL-1: Follistatin-like protein 1; HPMEC: Human pulmonary microvascular endothelial cell; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; CS: Cyclic stretch; siRNA: Small interfering RNA.

to mediate VILI. To determine whether the NLRP3 inflammasome is activated by cyclic stretching of HPMECs, we analyzed the expression of the NLRP3 and caspase-1 (p20) proteins. Compared to the unstretched control, 20% cyclic stretch upregulated both NLRP3 and caspase-1 (p20) ($P < 0.05$), indicating the activation of the NLRP3 inflammasome.

To further confirm whether the NLRP3 inflammasome mediated the cyclic stretch-induced inflammation in HPMEC, the cells were pretreated with MCC950, a potent, selective, and small molecule inhibitor of NLRP3. Pretreatment with MCC950 inhibited the cyclic stretch-induced expression of NLRP3 and caspase-1 (p20) in HPMECs, and subsequently, downregulated the levels of secreted IL-1β and IL-18. Taken together, the NLRP3 inflammasome facilitates the inflammatory response in HPMECs triggered by cyclic stretch.

FSTL-1 is a secretory protein[5] which is widely expressed during the early stages of lung development, and mainly confined to interstitial tissues in the later stage. Mechanical stretch could lead to an inflammatory imbalance, resulting in acute lung injury. Therefore, we next evaluated whether FSTL-1 played a role in the inflammatory process of VILI.

To determine whether FSTL-1 was involved in the cyclic stretch of HPMEC, we first analyzed the expression of FSTL-1 in the culture media. Compared to the control group, 20% cyclic stretch significantly increased FSTL-1 levels ($P < 0.05$) indicating that it could activate FSTL-1 in HPMECs [Table 1].

To further confirm whether FSTL-1 regulated the cyclic stretch-induced inflammation, FSTL-1 knockdown HPMEC cells were subjected to 20% cyclic stretch for 4 h. Compared to the wild-type cells, the expression of FSTL-1, IL-1β, and IL-18 were all decreased on FSTL-1 knockdown. These results suggested that FSTL-1 regulated the inflammatory response caused by cyclic stretch of HPMEC.

Based on these observations, we hypothesized that FSTL-1 might interact directly with NLRP3 and caspase-1 and promote IL-1β and IL-18 secretion in VILI. To determine the relationship between FSTL-1 and the NLRP3 inflammasome, specifically whether the inflammasome could be activated by FSTL-1, we analyzed their dynamics following FSTL-1 knockdown or NLRP3 inhibition. SiRNA-mediated FSTL-1 knockdown decreased its protein expression by 70% to 80%, and also significantly downregulated NLRP3 and caspase-1 (p20) ($P < 0.05$). In contrast, FSTL-1 expression was not significantly affected by MCC950 treatment ($P > 0.05$). Taken together, the activation of FSTL-1 may regulate the NLRP3 inflammasome-mediated secretion of IL-1β and IL-18 in VILI.

In conclusion, our findings showed that the cyclic stretch could increase the expression of FSTL-1 to activate NLRP3 inflammasome and to enhance IL-1β and IL-18 secretion in HPMECs.

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Conflicts of interest

There are no conflicts of interest.

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