*Biomedical Research 2017; 28 (19): 8601-8607* *ISSN 0970-938X*
*www.biomedres.info*

# Appropriate transient receptor potential vanilloid 4 (Trpv4) inhibition protects mice against lipopolysaccharide stress.

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## Abstract

**Background: TRPV4 as a therapeutic target was used in endotoxemia research. However, conflicting results have been reported. It was reported that HC067047 as a TRPV4 inhibitor caused a reduction in LPS-induced mortality. Oppositely, TRPV4 inhibited with HC06047 could not attenuate LPS-induced symptoms and exaggerated pathology. The mechanism of conflict is still unknown.**
**Material and methods: We assessed the pathological state in their experiments and further assessed the levels of TRPV4 expression with their respective treatment. Then the cell models with various levels of TRPV4 expression were used to explore the mechanism of conflicting results. The effect of TRPV4 expression on the apoptosis was observed under same LPS-dose stress. Meanwhile, F-actin contents were assessed with FITC-phalloidine staining.**
**Results: Our experiments verified their results and further showed that TRPV4 expression differed between the two reports. We speculated that the different TRPV4 expression might have caused the conflicting results. Cell models of varying TRPV4 expression included normal TRPV4 expression and overexpression models. Results showed that TRPV4 overexpression significantly increased the apoptosis when compared with normal expression cells with the same lipopolysaccharide dose ($P<0.05$). HC067047 enhanced apoptosis in normal expression cells ($P<0.05$) and reduced apoptosis in overexpression cells ($P<0.05$). Research about the mechanism showed that HC06047 blocked TRPV4 signals, caused actin stress fiber accumulation, and induced apoptosis in normal expression cells. Oppositely, HC06047 inhibited relatively overactive TRPV4, attenuated excessive actin depolymerization, and reduced apoptosis ratios in overexpression cells.**
**Conclusion: Underactive and/or overactive TRPV4 may be inhibitory to maintenance of cell function against lipopolysaccharide -induced stress.**

**Keywords:** TRPV4, Apoptosis, HC06047, Lipopolysaccharide, Actin cytoskeleton.

*Accepted on December 22, 2017*

## Introduction

Transient Receptor Potential Vanilloid 4 (TRPV4) is a non-selective cation channel and is widely expressed in mammalian tissues [1]. It is known as an osmosensor [2], a thermosensor [3], and/or a chemoreceptor [4] and appears to play an important role in a multitude of disease processes [5,6]. TRPV4 as a therapeutic target has been investigated in septic diseases [5].

Sepsis is characterized by increasing vascular permeability and reducing blood pressure [7]. TRPV4 is expressed in the vasculature [8]. It has been reported that TRPV4 can influence the septic process and may be a potential therapeutic target [5]. Interestingly, there are conflicting results on the potential role of TRPV4 in sepsis pathogenesis [5,9,10]. Dalsgaard et al. reported that TRPV4 inhibition with HC067047 reduced mortality in a Lipopolysaccharide (LPS)-induced sepsis model [10]. In contrast, Sand et al. found that TRPV4 inhibited with HC067047 could not attenuate LPS-induced symptoms and exaggerated pathology [9]. The LPS doses used by Dalsgaard et al. and Sand et al. were 50 mg/kg LPS and 12.5 mg/kg, respectively. The two groups used the same dose of TRPV4 inhibitor with 10 mg/kg HC067047. The same dose of HC67047 used in different LPS-dose induced mice might have caused conflicting results. However, the mechanism behind the conflicting results is still unclear.

We assessed the pathological state in their experiments in order to examine the reasons behind this discrepancy. 12.5 mg/kg LPS did not kill the experimental mice [9], but Dalsgaard et al. reported that all of the mice died three days after 50 mg/kg LPS treatment [10]. It has been suggested that different LPS doses can induce different levels of sepsis. The principal aspect of sepsis is a systemic inflammatory response to infection. TRPV4 activators are produced in inflammatory processes [11,12]. The activators induced an increase in TRPV4

expression levels [13] and enhanced TRPV4 activation levels [14,15]. Therefore, the level of TRPV4 activation with 50 mg/kg LPS-induced mice should be higher than that in 12.5 mg/kg LPS-induced mice. The 10 mg/kg dose of HC067047 inhibited the relatively low TRPV4 activation in 12.5 mg/kg LPS-induced mice and might induce underactive TRPV4. The same dose of HC067047 suppressed the relatively high TRPV4 activity in 50 mg/kg LPS-induced mice and reduced TRPV4 activation levels. This finding suggests that the same dose of HC067047 in different inflammation levels is inappropriate. However, there is little information about the importance of TRPV4 activation levels.

TRPV4 activation induced calcium ($Ca^{2+}$) influx [1]. The elevated $Ca^{2+}$ is partially involved in actin cytoskeleton reorganization and endothelial dysfunction [10]. This suggests that HC067047-induced TRPV4 inhibition reduces $Ca^{2+}$ influx, partially interferes with actin dynamics and might have contributed to the contradictory results. Furthermore, quantitative Real-Time Polymerase Chain Reaction (RT-PCR) verified that TRPV4 expression levels increased with LPS in a dose-dependent manner *in vivo*. Therefore, we created *in vitro* cell models in order to verify the mechanism. Human Embryonic Kidney (HEK) 293 cells were transfected with TRPV4 vectors as overexpression cell models. RT-PCR analysis showed that TRPV4 was normally expressed in HEK 293 cells. When the same stimulus was given, normal and overexpression cells formed different TRPV4 activation models. HC06047's effects on cell apoptosis were observed with the same LPS doses as described previously. The change in filamentous (F)-actin content was also assessed. It was found that HC067047 promoted cell apoptosis and enhanced F-actin contents in normal expression cells. Similarly, F-actin contents in TRPV4 overexpression cells treated with HC067047 increased, but the apoptosis ratio decreased. These findings demonstrated that TRPV4 inhibition in normal expression cells accumulated stress fibers, which might have promoted cell apoptosis [16]. The overactive TRPV4 in overexpression cells that induced excessive F-actin depolymerization might also have aggravated cell apoptosis [17].

## Material and Methods

### *Animal procedures*

Animal procedures and methods used in this study were approved by the Animal Ethics Committee of the Zhejiang University. Ten-week-old C57BL/6J male mice were used in this study. The weights of the mice ranged from 23 to 25 g. Animal administrations according to Sand et al. [9] and Dalsgaard et al. [10] are described briefly below. The LPS sepsis model was created by various intravenous (i.v.) LPS doses of 12.5 mg/kg or intraperitoneal (i.p.) doses of 50 mg/kg (O55: B5; Sigma Aldrich). The vehicles for LPS and HC067047 (Tocris Bioscience, bristol, UK) were normal saline solution (NaCl 0.9%) and DMSO (Sigma Aldrich), respectively. Mice were divided into various groups based on treatment methods: 1) controls, 12.5 mg/kg LPS only; 2) 12.5 mg/kg LPS+10 mg/kg HC067047 (i.p.) [9] 3) 50 mg/kg LPS only; and 4) 50 mg/kg LPS+10 mg/kg HC067047 (i.p.) [10]. At 16 h after endotoxemia induction, the induced injuries among the various groups were assessed.

### *Analysis of vascular permeability*

Evans Blue (EB) and formamide were purchased from Sigma (Sigma Aldrich, St. Louis, MO, USA). LPS-induced vascular leakage in the lungs was assessed by EB extravasation [18]. EB was dissolved in 0.9% saline (final concentration of 5mg/ml). Animals (n=8) were injected with 20 mg/kg EB in the caudal vein. At 1 h after the injection [19], mice were anesthetized and then perfused with phosphate-buffered saline from the left ventricle to remove intravascular EB. Mice were sacrificed during this procedure. The left lungs were excised, weighed and homogenized in formamide (1 ml formamide/100 mg lung). The homogenates were incubated on an oscillating table at 56°C for 48 h and centrifuged at 5000 g for 30 min. Optical density of the supernatant was measured at 620 nm with a spectrophotometer (Beckman Coulter, Brea, USA). EB concentrations were calculated from standard curve and measurements were performed in parallel.

### *Caspase-3/7 activity assay*

Caspase-3/7 protease activity in lung tissue was measured using the caspase-3/7 activity apoptosis assay kit (Sangon, Shanghai, China) according to the manufacturer's instructions. Briefly, after homogenization of lung tissue in cell lysis buffer, homogenates were centrifuged for 1min at 10,000X g. The supernatant with equal amounts of protein was incubated with (Z-DEVD)2 R110 substrates for caspase-3/7 and reaction buffer for 90 min at room temperature while being protected from light. Caspase-3/7 activity was assessed according to the fluorescence intensity at Ex/Em=490/525 nm using an Infinite® 200 Pro NanoQuant microplate reader (Tecan, Mannedorf, CH). Data were normalized against the control [20].

### *RT-PCR analysis*

Total lung RNA was isolated with Trizol (Invitrogen), followed by using RNA prep pure Cell/Bacteria Kit (Tiangen Biotech, Qiagen, China) according to the manufacturer's protocols. The RNA (0.5 mg) was then subject to reverse transcription (RT) using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Subsequently, the products of the RT reactions were amplified using Mastercycler ep realplex (Eppendorf AG) on 96-well plates with the THUNDERBIRDTM SYBR® qPCR Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Fragments of TRPV4 or β-actin were amplified with the following primers: 1) TRPV4 forward (5'-ATGGCAGATCCTGGTGATGG-3'; 2) TRPV4 reverse (5'-GGAACTTCATACGCAGGTTTGG-3') [21]. 3) β-actin forward (5'-TCCTCCCTGGAGAAGAGCTA-3'); and 4) β-

actin reverse (5'-TCAGGAGGAGCAATGATCTTG-3') [22]. Data analyses were all performed using the $2^{-\Delta\Delta CT}$ method [22].

### *Vector construction*

Using the previous total RNA, complementary DNA (cDNA) was synthesized with the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo, Waltham, USA) according to the manufacturer's instructions. Full-length TRPV4 (NCBI accession number AF263521) cDNA was amplified by using forward primer (5'-TGACAGATCTATGGGTGAGACCGTGGGCCA-3') and reverse primer (5'-TGACAAGCTTCTACAGTGGTGCGTCCTCCG-3') [23]. The amplified cDNA was subcloned into the BglII and HindIII restriction sites of the pEGFPN1 vector (NEB, Beverly, MD, USA) to be used for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (TF3-dUTP, red fluorescence). EGFP was replaced by mCherry in order to distinguish the green fluorescent of FITC-phalloidin. The mCherry fragment was amplified by using forward primer (5'-TCCCCCGGGATGGTGAGCAAGGGCGAG-3') and reverse primer (5'-ATTTGCGGCCGCCTTACTTGTACAGCTCGTC-3'). The reverse TRPV4 cDNA primer was redesigned in order to avoid frameshift (5'-TGACAAGCTTATCTACAGTGGTGCGTCCTC-3'). The mCherry fragment was then subcloned into the SmaI and NotI restriction sites of the pEGFPN1 vector. Vector base sequences were verified by automated nucleotide sequencing.

### *Vector transfection and HEK cell treatment*

HEK 293 cells were stably transfected with empty vectors (negative control) and TRPV4 vectors (over-expression). Lipofectamine 2000 (Invitrogen, Carlsbad, USA) was used according to the manufacturer's instructions. Briefly, cells were plated in a petri dish containing culture medium and grown to confluence. The vectors were diluted in 250 µl Opti-MEMI (Reduced Serum Medium, Invitrogen). Lipofectamine 2000 was diluted in 250 µl Opti-MEMI, and mixtures were then incubated for 5 min. The two mixtures were combined together and incubated at room temperature for an additional 30 min. The total combined mixture was added to cells containing 2 ml complete medium and incubated for 24 h. The appropriate concentrations of LPS and HC067047 were selected for the experiments [9,24]. Cells were then treated with different agents for 16 h: 1) control; 2) 200 µg/ml LPS; or 3) 200 µg/ml LPS+10 µg/ml HC067047.

### *Detection of apoptotic cells by TUNEL staining*

The apoptosis of treated HEK cells was assessed with TUNEL Apoptosis Assay Kit (Sangon, Shanghai, China) according to the manufacturer's protocols. Briefly, Cells were fixed with 4% paraformaldehyde for 20 min and permeabilized for 2 min. The cells were then underwent TUNEL reactions at 37°C for 60 min. Hoechst 33258 staining was performed at room temperature for 10 min. The numbers of TUNEL-positive cells and total cell nuclei in normal expression cells were counted. In overexpression cells, we counted the TUNEL-positive cells and total transfected cell nuclei. Eight fields per sample were observed [25]. The apoptotic index (AI, % apoptotic cells) was presented as a percentage of TUNEL-positive cells to total cells [25]. All images were taken on Zeiss LSM 510 laser confocal microscope (Zeiss, Jena, Germany) with 40X-objective.

### *F-actin staining and confocal microscopy*

The actin cytoskeleton was stained with FITC-phalloidin (Actin-Tracker Green Kit, Beyotime, Nanjing, China) according to the manufacturer's protocol. Briefly, the treated cells were fixed in 3.7% formaldehyde without methanol for 10 min at room temperature. Cells were then incubated with FITC-phalloidin for 60 min in the dark. After Hoechst 33258 staining was performed, the fluorescence intensity of 60 cells from each sample was detected using a Zeiss LSM 510 laser confocal microscope (Zeiss, Jena, Germany) with a 63x-objective [26]. Fluorescent intensity data in each experiment were normalized against the control. In each experiment, cells were cultured on the same chamber slide and processed concurrently under the same conditions for comparison. The laser power, magnification, and other conditions were kept constant for all of the dishes in each experiment.

### *Statistical analysis*

Statistical calculations were performed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Biological triplicates were performed, and values are presented as mean ± Standard Error of the Mean (SEM). Student's paired t-tests and one-way ANOVA were applied for statistical analysis of results. A p-value<0.05 was considered significant.

## Results

Lung injury with 10 mg/kg HC067047 treatment was enhanced in 12.5 mg/kg LPS-induced mice and attenuated in 50 mg/kg LPS-induced mice.

EB extravasations were significantly increased in LPS-administered groups when compared with the control group. In the 12.5 mg/kg LPS-induced mice, 10 mg/kg HC067047 significantly induced EB extravasations compared to those without HC067047 treatment (t=2.50, P=0.03; Table 1). In contrast, 10 mg/kg HC067047 pretreatment significantly attenuated EB extravasations in 50 mg/kg LPS-induced mice (t=3.08, P=0.01; Table 1). This demonstrated that 10 mg/kg HC067047 increased pulmonary vascular permeability induced

by low doses of LPS and reduced the permeability in high-dose LPS-induced mice.

*Table 1. The pathological indicators of LPS-induced mice.*

| Index | Control | 12.5 mg/kg LPS | 12.5 mg/kg LPS+HC067047 | 50 mg/kg LPS | 50 mg/kg LPS+HC067047 |
|---|---|---|---|---|---|
| EB (μg/ml) | 0.19 ± 0.04 | 0.61 ± 0.08* | 0.70 ± 0.08 | 1.72 ± 0.12* | 1.55 ± 0.10 |
| Caspsae-3/7 activity normalized to control | 1.00 ± 0.07 | 2.01 ± 0.37* | 2.54 ± 0.41 | 5.02 ± 0.45* | 4.4 ± 0.43 |
| Relative TRPV4 mRNA level | 1.01 ± 0.13 | 2.92 ± 0.24 | 3.07 ± 0.027 | 5.46 ± 0.37 | 5.17 ± 0.36 |

*P<0.05, when compared with the group with HC067047 treatment.

### *HC067047 enhanced the apoptosis in 12.5 mg/kg lps-induced mice, but attenuated in 50 mg/kg lps-induced mice*

The apoptosis-related caspase-3/7 factor plays an important role in the process of apoptosis [27]. To further determine whether HC067047 affected LPS-induced injury, we measured caspase-3/7 activity in lung. Results showed that10 mg/kg HC067047 treatment induced an increase in caspase-3/7 activity in 12.5 mg/kg LPS-induced mice, the relative fluorescence intensity in mice with HC067047 was stronger than that without HC067047 (t=2.71, P=0.02; Table 1). In contrast, 10 mg/kg HC067047 pre-treatment resulted in a marked reduction in the level of cleaved caspase-3/7 in 50 mg/kg LPS-induced mice. The relative fluorescence intensity in mice treated with HC067047 was lower than that in mice without HC067047 treatment (t=2.82, P=0.01; Table 1). These data suggest that 10 mg/kg HC067047 could therapeutically reduce high-dose LPS-induced cell apoptosis but increased apoptosis in the low-dose LPS-induced inflammatory state; the changes in injury also agreed with two previous studies [9,10].

### *TRPV4 expressions increased in 12.5 or 50 mg/kg lps-induced mice, but did not change with or without hc067047 treatment*

It has been reported that TRPV4 expression levels were up-regulated under inflammatory conditions [13]. Furthermore, TRPV4 expression levels in the 50 mg/kg LPS-induced mice were significantly higher than those in the 12.5 mg/kg LPS-induced mice (Table 1). This demonstrated that TRPV4 expression levels might be increased in a dose-dependent manner with certain LPS concentration ranges. HC067047 treatment did not significantly change TRPV4 expression levels whether in 12.5 mg/kg or in 50 mg/kg LPS-induced mice (t=1.17, P=0.26; t=1.59, P=0.13; respectively, Table 1). This finding suggested that HC067047 played a role *via* inhibition of TRPV4 activation rather than by suppressing its expression.

Next, cell models with different TRPV4 expression levels were created to verify our *in vitro* conclusions.

### *HC067047 enhanced the apoptosis in trpv4 normal expression cells, but attenuated the apoptosis in the trpv4 over expression cells*

RT-PCR analysis showed that TRPV4 was normally expressed in HEK 293 cells (data not shown). The apoptosis ratio as Apoptosis Index (AI) was used for statistical analysis. Results showed that apoptotic cells in all LPS-treated groups were more than those in controls (Table 2).

With HC067047 treatments, AIs were significantly increased in normal expression cells (t=3.30, P=0.03; Table 2), but it was obviously depressed in overexpression cells (t=4.06, P=0.01; Table 2). The results of negative controls (transfected with empty vector) were similar to the normal expression groups. HC067047's effects on LPS-induced *in vitro* injury were consistent with *in vivo* results. This verified that underactive or overactive TRPV4 increased the LPS-induced injury with the same LPS-dose administration.

*Table 2. The rate of apoptosis and F-actin content in cell models.*

| Index | Control | Normal expression | Normal expression +HC067047 | Negative control | Negative control +HC067047 | Over expression | Over expression +HC067047 |
|---|---|---|---|---|---|---|---|
| AI (%, apoptosis percent at 16 h) | 3.79 ± 0.8 | 31.4 ± 3.25* | 41.4 ± 4.12 | 30.9 ± 3.43* | 40.9 ± 4.00 | 66.8 ± 6.37* | 47.6 ± 5.16 |
| AI (%, apoptosis percent at 4 h) | 3.16 ± 0.6 | 10.9 ± 1.19 | 11.2 ± 1.13 | 10.6 ± 1.24 | 11.2 ± 1.28 | 12.1 ± 1.15 | 11.2 ± 1.14 |
| Relative F-actin content | 100.0 ± 1.3 | 60.12 ± 4.63* | 76.65 ± 6.17 | 60.87 ± 4.35* | 77.4 ± 6.22 | 51.92 ± 3.69* | 68.34 ± 5.21 |

*P<0.05, when compared with the group with HC067047 treatment.

### *HC067047 suppressed lps-induced actin depolymerization*

The actin cytoskeleton plays an important role in physiological function regulation [28]. TRPV4 regulates calcium influx and functions in part through modulation of actin dynamics [29]. In addition, apoptosis leads to cytoskeletal collapse [30]. To evaluate the effects of apoptosis on actin in cases in which the apoptosis ratios among each group were similar at 4 h after LPS treatment, F-actin contents in 60 cells per sample were assessed by examining fluorescence intensity of FITC-phalloidin. Results showed that F-actin contents in LPS-induced cells decreased when compared with the control (Table 2). In normal expression cells, the F-actin contents with HC067047 were higher than those without HC067047 (t=3.71, P=0.02; Table 2). HC067047 also induced an increase in F-actin content in negative controls (t=3.77, P=0.02; Table 2). Similarly, in TRPV4 overexpression cells treated with HC067047, F-actin contents were higher than those without HC067047 (t=4.45, P=0.01; Table 2).

## Discussion

TRPV4 is broadly expressed in mammalian tissues and plays important roles in many physiological processes [31]. It had been reported that excessive TRPV4 activation in sepsis contributes to lethal endothelial failure and pulmonary edema. TRPV4 inhibitors reduce hyper-inflammatory responses and increase cell survival during sepsis [28]. Sometimes, blockade of TRPV4 activity with HC067047 induces contradictory results [9,10]. Our study verified that HC067047 aggravated EB extravasation and caspase-3/7 activity in the lungs of 12.5 mg/kg LPS-induced mice (Table 1). In contrast, the same dose of HC067047 reduced EB extravasation and caspase-3/7 activity in lungs of 50 mg/kg LPS-induced mice (Table 1). EB dye has been widely used as a surrogate marker for lung injury [19]. Cleaved caspase-3/7 levels had a positive correlation with cell apoptosis levels [27]. Increasing cell apoptosis promotes EB extravasation [32]. However, at present, there are no relevant studies about the causes of conflicting results.

Our study showed that HC067047 as a selective TRPV4 inhibitor suppressed TRPV4 activation. TRPV4 is activated by the stimulating factors in inflammation [1,11]. TRPV4 expression levels were elevated in inflammatory diseases [13]. RT-PCR analysis showed that TRPV4 mRNA expressions were up-regulated by LPS in a dose-dependent manner (Table 1). Therefore, TRPV4 showed different activation levels with different LPS doses. It is inappropriate to use the same dose of HC067047 in different TRPV4 activity conditions. Under mild inflammatory conditions, TRPV4 is moderately activated. HC067047 at 10 mg/kg results in TRPV4 under activity, which may promote 12.5 mg/kg LPS-induced apoptosis. In contrast, the same dose of HC067047 was suitable to inhibit excessive TRPV4 activation under the fatal inflammatory conditions induced by 50 mg/kg LPS and thus, was capable of prolonging mice survival. Indeed, our *in vitro* experiments in various TRPV4 expression levels demonstrated that underactive or overactive TRPV4 aggravated apoptosis. The dynamic balance of the actin cytoskeleton between polymerization and depolymerization is very important for the LPS-induced cell survival [33]. TRPV4 activity induced actin depolymerization [10]. Hence, F-actin contents were assessed under different levels of TRPV4 activation. Our results indicated that HC067047 inhibited TRPV4-induced F-actin depolymerization. An increasing number of actin stress fibers have been shown to induce cell apoptosis [16]. Therefore, it seems that HC067047 aggravated LPS-induced apoptosis in normal expression cells.

Overactive TRPV4 aggravated LPS-induced actin depolymerization. In our study, HC067047 suppressed F-actin depolymerizationin overexpression cells 4 h after LPS treatment compared to those without HC067047 (Table 2). Furthermore, excessive actin depolymerization plays an important role in epithelial cell apoptosis and subsequent barrier dysfunction [17]. Our experiments also verified that excessive actin depolymerization could indeed increase the AIs of overexpression cells 16 h after LPS treatment (Table 2). Therefore, HC067047 suppressed overactive TRPV4, reduced excessive actin depolymerization, and then attenuated cell apoptosis. Furthermore, exocytosis and endocytosis depend on the perfectly tuned orchestration of intracellular calcium signaling with the actin cytoskeleton. Intracellular calcium is precisely regulated in a spatiotemporal manner through various $Ca^{2+}$ channels and membrane pumps. TRPV4 is one such channel. HC067047 interfered with actin dynamics and then suppressed TNF-α secretion in 50 mg/kg LPS-induced mice according to the results of Dalsgaard et al. TNF-α hyposecretion has been shown to reduce apoptosis *via* the death receptor pathway.

Hence, these conflicting results remind us that when TRPV4 is used as therapeutic target in the future, it should be examined whether TRPV4 activation levels can cause disease remission. Additional studies should look for methods which can be used to assess the relationship between the disease severity and TRPV4 activation levels. Then, the methods can guide a doctor to either activate or inhibit TRPV4.

## Conclusion

The conflicting results from two reports indicate that the same dose of HC067047 administration was inappropriate under the different levels of TRPV4 activation. TRPV4 regulation of the actin cytoskeleton was associated with different endotoxemia outcomes.

## Acknowledgements

We thank the Pei lab members for discussion and support.

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