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## LRP1-dependent BMPER signaling regulates lipopolysaccharide-induced vascular inflammation

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### Abstract

**Objective**—Bacterial endotoxin (lipopolysaccharide, LPS)-mediated sepsis involves dysregulated systemic inflammation, which injures the lung and other organs, often fatally. Vascular endothelial cells act as both targets and mediators of LPS-induced inflammatory responses. Dysfunction of endothelium results in increases of pro-inflammatory cytokine production and permeability leakage. Bone morphogenetic protein (BMP)-binding endothelial regulator (BMPER), an extracellular modulator of BMP signaling, has been identified as a vital component in chronic endothelial inflammatory responses and atherosclerosis. However, it is unclear whether BMPER also regulates inflammatory response in an acute setting such as sepsis. To address this question, we investigated the role of BMPER during LPS-induced acute lung injury.

**Approach and results**—Mice missing 1 allele of BMPER (BMPER<sup>+/-</sup> mice used in the place of BMPER<sup>-/-</sup> mice that die at birth) were used for LPS challenge. LPS-induced pulmonary inflammation and injury was reduced in BMPER<sup>+/-</sup> mice as shown by several measures, including survival rate, infiltration of inflammatory cells, edema and production of pro-inflammatory cytokines. Mechanistically, we have demonstrated that BMPER is required and sufficient for the activation of nuclear factor of activated T cells (NFAT) c1. This BMPER-induced

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### DISCLOSURES

None.

NFAT activation is coordinated by multiple signaling pathways, including BMP-independent LRP1-ERK activation, calcineurin signaling and LRP1 $\beta$ -mediated nuclear factor-45 (NF45) nuclear export in response to BMPER treatment.

**Conclusions**—We conclude that BMPER plays a pivotal role in pulmonary inflammatory response, which provides new therapeutic options against sepsis shock. The new signaling pathway initiated by BMPER/LRP1 axis broadens our understanding about BMPER's role in vascular homeostasis.

### Keywords

Bone morphogenetic protein-binding endothelial regulator; Nuclear factor of activated T cells; Low density lipoprotein receptor-related protein 1; NF45; Endothelial inflammation

### Subject codes

[138] Cell signaling/Signal transduction; [95] Endothelium/vascular type/nitric oxide

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## INTRODUCTION

Septic shock, induced in response to severe infection, remains a major cause of morbidity and mortality, despite of effective antimicrobial therapy. Severe sepsis is reported in 2% of patients admitted to the hospital. The number of cases exceeds 750,000 in the United States and 19 million worldwide per year<sup>1-3</sup>. Recent studies indicate that sepsis increases the risks of other complications such as impaired physical and neurocognitive function<sup>3</sup>. These clinical manifestations highlight a lack of understanding of pathways involved in sepsis and the demands for improved therapies. Impairment of pulmonary vascular integrity and endothelial dysfunction are key features observed in sepsis and other acute pulmonary dysfunctions including acute respiratory distress syndrome (ARDS), lung inflammation and ventilator-induced lung injuries<sup>4</sup>. In response to bacterial endotoxin lipopolysaccharide (LPS) in the blood, endothelial cells act as the first line of defense by recognizing invading pathogens through pattern recognition receptors and initiating inflammatory and coagulation cascades. Pro-inflammatory cytokines secreted from endothelial cells, which function in autocrine or paracrine loops, will further activate neutrophils, monocytes, tissue macrophages and/or endothelial cells. However, endothelial cell activation induced by LPS will eventually result in an increase in vascular permeability, allowing increased flux of proteins, fluid and immune cells across vessels into tissues. Therefore, maintenance of vascular endothelial integrity is crucial for vascular and tissue homeostasis. However, the underlying mechanisms mediating endothelial activation in response to LPS remain largely unknown.

Toll-like receptor 4 (TLR4) has been recognized as the major receptor for LPS. Ligation of LPS to TLR4 activates NF $\kappa$ B and nuclear factor of activated T cells (NFAT) pathways. In human umbilical vein endothelial cells (HUVECs), TLR4, MyD88 and Mal/TIRAP adaptor proteins are required for the activation of NF $\kappa$ B and production of interleukin-6 (IL-6)<sup>5</sup>. Animal studies with inactive NF $\kappa$ B signaling indicate that, in response to LPS-induced endotoxemia, NF $\kappa$ B activation acts as a quick adaptive response, which provides important

survival signal and maintains a normal but dynamic endothelial barrier function<sup>6</sup>. On the other hand, LPS activates transcriptional factor NFAT in endothelial cells, which results in increased expression of pro-inflammatory cytokines such as IFN- $\gamma$  and TNF $\alpha$ <sup>7</sup>. The activation of NFAT is mediated by reactive oxygen species (ROS)-driven Ca<sup>2+</sup> signaling pathway and TLR4 may be required for ROS generation<sup>7</sup>. In activated immune cells such as dendritic cells, CD14 is another receptor of LPS for NFAT activation, likely through Src-family kinase and phospholipase C $\gamma$ 2, and Ca<sup>2+</sup>/calcineurin signaling<sup>8</sup>. In endothelial cells, CD14 has also been suggested to function together with TLR4 for LPS-induced E-selectin and IL-6 production<sup>9</sup>. Although the exact roles of TLR4 and CD14 in LPS-mediated NFAT activation and endothelial inflammatory responses remain to be further clarified, NFAT activation is recognized as a crucial transcription factor controlling the expression of pro-inflammatory cytokines<sup>7</sup>.

Endothelial integrity and vascular homeostasis are tightly regulated by multiple signaling pathways such as bone morphogenetic protein (BMP) signaling<sup>10</sup>. BMP-binding endothelial regulator (BMPER), an extracellular regulator of BMPs<sup>11, 12</sup>, has been identified as an important regulator of vascular inflammation and atherosclerosis<sup>13-15</sup>. Knockdown of BMPER by its specific siRNA or its deficiency in BMPER<sup>+/-</sup> mice potentiates TNF $\alpha$  induced endothelial inflammatory responses<sup>14</sup>. This anti-inflammatory phenotype of BMPER is mediated by blocking BMP activity, which likely explains the atheroprotective function of BMPER<sup>15</sup>. However, it is not clear whether BMPER regulates inflammatory responses in endothelial cells in an acute setting, such as LPS challenge. We have recently demonstrated that LDLR-related protein (LRP) 1, a member of the LDL receptor family, is associated with BMPER and regulates angiogenesis during zebrafish vein development<sup>16</sup>. The mature form of LRP1 is a heterodimer composed of a 515-kDa  $\alpha$  chain (LRP1 $\alpha$ ), possessing four extracellular ligand binding domains, and an 85-kDa membrane-anchored cytoplasmic  $\beta$  chain (LRP1 $\beta$ ), which remains non-covalently associated with  $\alpha$  chain. LRP1 is an endocytic receptor for multiple signaling pathways and mediates their signals through its  $\beta$  chain, which interacts with many scaffolding proteins<sup>17-23</sup>. In addition, processed forms of LRP1 $\beta$  can also translocate into nucleus and regulate the enzyme activity of PARP1<sup>24</sup> and expression of PPAR target genes by acting as a PPAR $\gamma$  co-activator<sup>25</sup>. Although LRP1 is required for the endocytosis of BMPER signaling complex in endothelial cells, it remains elusive whether the coupling of BMPER to LRP1 may also initiate their own signaling events.

BMPER null mice die at birth<sup>11</sup>. In this study, we used mice with BMPER haploinsufficiency to study the effect of reduced BMPER expression on LPS-induced endothelial inflammation. Surprisingly, we observed that BMPER<sup>+/-</sup> mice exhibit reduced vascular inflammatory responses upon LPS treatment as shown by several parameters, including wet/dry lung weight ratio, pulmonary edema, survival rate and production of pro-inflammatory cytokines. Interestingly, BMPER activates NFATc1 in a LRP1-dependent but BMP-independent manner. Furthermore, the coordinative actions of LRP1/NF45 nuclear export and NFATc1 nuclear import may play a crucial role for BMPER-induced NFATc1 activation. These results indicate that inhibition of BMPER signaling limits endotoxemia-induced pulmonary inflammatory responses and that BMPER/LRP1 signaling axis may provide potential therapeutic targets against sepsis-induced acute lung injury.

## MATERIAL AND METHODS

Materials and Methods are available in the online-only supplement.

## RESULTS

### **BMPER haploinsufficiency ameliorates pulmonary vascular leakage and lung injury in response to LPS treatment**

Accumulated evidence suggests that BMPER protects endothelial cells from chronic inflammation and atherosclerosis by inhibiting BMP activity<sup>13–15</sup>. However, it is unknown how BMPER regulates acute inflammatory responses. Given that BMPER<sup>-/-</sup> mice die at birth<sup>11</sup>, we evaluated the inflammatory responses with BMPER<sup>+/-</sup> mice upon LPS challenge. First, we compared the lung injury-associated parameters, including pulmonary edema and capillary leakage, in BMPER<sup>+/-</sup> and BMPER<sup>+/+</sup> mice where BMPER protein levels were decreased in serum and multiple tissues (Figure SIA, B). When BMPER<sup>+/+</sup> control mice were challenged with a sub-lethal dose of LPS (10 mg/kg i.v.), they rapidly developed symptoms consistent with sepsis (e.g., lethargy, ocular discharge; data not shown). However, mice with BMPER haploinsufficiency (BMPER<sup>+/-</sup>) demonstrated less severe symptoms, compared to BMPER<sup>+/+</sup> mice. Extravasation of Evans blue dye (EBD) from circulation into the lung tissue is often used as an indicator of elevated capillary permeability. We observed that extravasated EBD content in the lung tissue of BMPER<sup>+/-</sup> mice was significantly less than that from BMPER<sup>+/+</sup> mice (Figure 1A, B). Similarly, the lung wet/dry weight ratio of BMPER<sup>+/-</sup> mice, following LPS injection, was significantly smaller than that of BMPER<sup>+/+</sup> mice (Figure 1C). In addition, when challenged with a lethal dose of LPS (15 mg/kg), all BMPER<sup>+/+</sup> mice died within 2 days while the majority of BMPER<sup>+/-</sup> mice recovered from this challenge with a 7-day survival rate at ~62.5% (Figure 1D). Then we evaluated histopathological changes of lung in mice following LPS-induced acute lung injury. Consistently, we observed a marked increase of inflammatory cell infiltration, interalveolar septal thickening, and interstitial edema in BMPER<sup>+/+</sup> mice following LPS challenge (Figure 1E). However, BMPER<sup>+/-</sup> mice showed alleviated phenotypes of lung injury and a much reduced lung injury score (Figure 1E, F). These data suggest that BMPER haploinsufficiency protects lung from endotoxemia-induced injury.

### **LPS-induced lung inflammation, apoptosis and permeability increase are attenuated in BMPER<sup>+/-</sup> mice**

Endotoxemia-induced lung inflammation is associated with increases in the production of cytokines and leukocyte infiltration into lung tissue. Therefore, we evaluated the levels of LPS-induced pro-inflammatory cytokines in the serum of BMPER<sup>+/+</sup> and BMPER<sup>+/-</sup> mice. IFN $\gamma$ , IL-6, TNF- $\alpha$ , IL-2 and IL-5 were induced in BMPER<sup>+/+</sup> mice by LPS treatment but their inductions were significantly decreased in BMPER<sup>+/-</sup> mice (Figure 2A–C and data not shown). To further assess the influence of BMPER haploinsufficiency on pulmonary inflammation, we determined in bronchoalveolar lavage fluid (BALF) several parameters of lung inflammation, including cell infiltration, myeloperoxidase (MPO) activity, protein content and the levels of IFN $\gamma$ , IL-6 and TNF- $\alpha$ . In response to LPS, both BMPER<sup>+/+</sup> and BMPER<sup>+/-</sup> mice exhibited increased leukocyte infiltration into BALF, assessed by

measuring the total recovered cell numbers of BALF. However, cell counts in BALF of BMPER<sup>+/-</sup> mice were significantly lower than those in BMPER<sup>+/+</sup> mice (Figure 2D). In addition, myeloperoxidase (MPO) activity was also dramatically lower in BMPER<sup>+/-</sup> mice, compared to BMPER<sup>+/+</sup> mice (Figure 2E). These results suggest that BMPER haploinsufficiency blocks leukocyte infiltration into alveolar space. Next, we measured total protein and cytokine levels in BALF and found that the protein level and individual levels of IFN $\gamma$ , IL-6 and TNF- $\alpha$  were significantly decreased in BMPER<sup>+/-</sup> mice (Figure 2F-I). Taken together, BMPER<sup>+/-</sup> mice demonstrate alleviated pulmonary inflammatory response upon LPS challenge, exhibiting reductions in leukocyte infiltration and pro-inflammatory cytokine production.

Reports show that pulmonary microvascular endothelial cell apoptosis also contributes to pulmonary microvascular barrier dysfunction and edema during sepsis<sup>26</sup>. Therefore, we tested whether BMPER deficiency results in endothelial cell apoptosis in response to LPS. As expected, LPS injection in BMPER<sup>+/+</sup> mice induced vascular cell apoptosis, as measured by TUNEL analysis (Figure SIIA, B). However, TUNEL positive cell number significantly decreased in BMPER<sup>+/-</sup> mice. This protective effect of BMPER haploinsufficiency was also observed in primary mouse lung microvascular endothelial cells (MLECs; Figure SIIC, D). We also assessed the effects of BMPER haploinsufficiency on endothelial permeability, we measured solute flux of FITC-Dextran across the endothelial monolayer. LPS treatment led to a 2.2-fold increase in paracellular permeability (Figure SIII E). However, this LPS-mediated permeability was partially inhibited in BMPER knockdown MLECs. Surprisingly, the protein level of claudin 5, a key determinant of endothelial permeability, was not affected by BMPER treatment (Figure SIIIF), suggesting that BMPER-dependent permeability change is likely mediated through other mechanisms. Taken all together, these data suggest that BMPER deficiency reduces endothelial cell apoptosis and permeability in response to LPS.

### **BMPER is required for LPS-induced NFATc1 activation in endothelial cells**

Host inflammatory response, rather than the nature of pathogen, is the primary determinant for the outcome of sepsis patients<sup>3</sup>. Endothelial cell is one of the major cornerstones of the innate immune responses by initiating a cascade of signaling responses to defend the pathogen<sup>3</sup>. Our group and other laboratories have demonstrated that BMPER is a key regulator of endothelial cell function<sup>12-16, 27-30</sup>. In particular, BMPER can be induced by inflammatory-regulatory stimuli, such as oscillatory shear stress and mevastatin, and inhibits endothelial inflammatory responses<sup>13, 15</sup>. BMPER haploinsufficiency inhibition of LPS-induced lung injury was unexpected because of the known anti-inflammatory role of BMPER in chronic inflammatory responses. Therefore, these data suggested that BMPER might regulate acute and chronic inflammatory responses through distinctive pathways. We tested whether BMPER plays a role in LPS signaling pathway in endothelial cells. By using MLECs, we investigated whether NFAT, an important transcription factor of LPS-induced acute inflammatory responses in endothelial cells<sup>7</sup>, can be regulated by BMPER. As expected, LPS increased NFAT activity as measured by NFAT reporter luciferase activity (Figure 3A). However, when BMPER expression in MLECs was knocked down with its specific siRNA (Figure SIIIA), we observed a ~two-fold decrease in NFAT activation,

compared to control MLECs (Figure 3A). These data suggest that BMPER is required for NFAT activation in response to LPS.

### **BMPER activates NFATc1 signaling**

To understand the underlying molecular mechanisms responsible for BMPER's action, we performed gene profiling analysis with BMPER treated endothelial cells and searched for changes in gene expression. Microarray analysis of endothelial cells treated with BMPER or control revealed that BMPER elicits either up-regulation or down-regulation of genes that are not associated with BMP transcriptional regulation (Figure SIIIB). To further examine these observations, we used TRANSFAC analysis to map transcription factor binding sites upstream of the identified BMP-independent BMPER-regulated genes. Interestingly, we found that a number of genes regulated by BMPER contain NFAT consensus binding sites including phospholipase C $\beta$ 1 and NFATc1, which itself is a member of NFAT transcription factors. There are mainly five members of NFAT transcriptional factors that are evolutionarily related to the REL- NF- $\kappa$ B family of transcription factors: NFAT1 (also known as NFATc2 or NFATp), NFAT2 (also known as NFATc1 or NFATc), NFAT3 (also known as NFATc4), NFAT4 (also known as NFATc3 or NFATx) and NFAT5 (also known as tonicity enhancer binding protein; TonEBP). To examine the relationship between BMPER and NFAT, we assessed the induction of NFATs in response to BMPER treatment. By performing real-time PCR analysis, we demonstrated that NFATc1 was the most induced NFAT in response to BMPER treatment, with the peak at one hour (Figure SIIIC). Next, we tested whether BMPER could activate NFAT by performing the NFAT reporter assay in endothelial cells in response to BMPER. When mouse lung endothelial cells were treated with BMPER, we observed an increase in NFAT transcriptional activity (Figure 3B). Since NFATc1 needs to be dephosphorylated and then translocated from cytoplasm to the nucleus for fully activation, we performed fractionation assays to determine whether subcellular localization of NFATc1 can be regulated by BMPER. Indeed, we observed an increase of NFATc1 accumulation in the nucleus after BMPER treatment for 15 to 60 minutes (Figure 3C, D). Immunostaining also confirmed the nuclear translocation of NFATc1 in response to BMPER in endothelial cells (Figure III). A large number of NFAT target genes including IFN $\gamma$  are reported<sup>31, 32</sup>. Given that IFN $\gamma$  levels in BALF and serum were decreased in BMPER $+/$ - mice (Figure 2A, G), we tested whether NFATc1 is required for IFN $\gamma$  induction in MLECs. Importantly, BMPER treatment dramatically increased the mRNA level of IFN $\gamma$  in MLECs Figure 3E. However, knockdown of NFATc1 by its specific siRNA significantly blocked the induction of IFN $\gamma$  Figure 3E. Using chromatin immunoprecipitation (ChIP) assays, we discovered that the association of NFATc1 with the promoter of IFN $\gamma$  was increased in response to BMPER treatment (Figure SIIIE). Taken together, these data suggest that BMPER is not only necessary but also sufficient for NFATc1 activation and the induction of its target gene IFN $\gamma$  in endothelial cells.

### **LRP1 mediates NFAT activation induced by BMPER**

To determine how BMPER activates NFATc1, inhibitors of different signaling pathways were used to dissect downstream signaling pathways. Importantly, we showed that cyclosporin A (CsA; calcineurin inhibitor) and U0126 (MEK1/ERK inhibitor) blocked the activation of NFAT upon BMPER treatment (Figure 4A). In addition, the inhibition of



calcineurin activity by CsA improved the survival rate of BMPER<sup>+/+</sup> mice upon LPS challenge at 15 mg/kg (Figure SIVA), which phenocopies the protective effect of BMPER haploinsufficiency (Figure 1D). However, Smad6, which is the inhibitor of the BMP's downstream signaling mediators Smad1, 5 and 8, failed to inhibit NFAT activation (Figure 4A), suggesting that BMPER activates NFAT through BMP-independent pathways. We have recently demonstrated that LRP1 is associated with BMPER and is required for BMPER's regulatory effects on angiogenesis<sup>16</sup>. Given that the intracellular domain of LRP1 $\beta$  contains multiple serine, threonine and tyrosine residues that can be phosphorylated by PKA or Src<sup>23, 33</sup>, we hypothesized that BMPER may initiate signaling cascades directly through LRP1. Treatment with BMPER at increased dosages promoted ERK activation. However, ERK activation induced by BMPER was not inhibited by BMP4 neutralizing antibody (Figure 4B, SIVB), suggesting that BMPER may induce ERK activation in a BMP4-independent manner. On the other hand, LRP1 knockdown dramatically decreased ERK activation upon BMPER treatment in MLECs that have been transiently transfected with LRP1 siRNA or mouse endothelial cells being stably transfected with LRP1 shRNA (EC<sub>50</sub> is 51.20 nM; Figure 4C, SIVC), suggesting that LRP1 is required for BMPER-induced ERK activation. Given that ERK pathway is required for BMPER-induced NFAT activation, we then tested whether LRP1 is required for NFAT activation. Indeed, LRP1 knockdown significantly inhibited NFAT reporter activity in response to BMPER in MLECs (Figure 4D). More importantly, IFN $\gamma$  mRNA levels were increased by BMPER, LPS or their combined treatments. These increases were inhibited by LRP1 knockdown in MLECs (Figure 4E). Taken for all, these results suggest that ERK activation mediated by LRP1 and calcium dependent calcineurin pathways are required for NFAT activation upon BMPER treatment. Many NFAT regulatory elements can also be regulated by NF $\kappa$ B. For example, CD28 response element of the IL-2 promoter contains a dimeric NFAT/NF $\kappa$ B site, which may bind both NFAT and NF $\kappa$ B and function cooperatively with AP-1 protein that is associated with the adjacent AP-1 site<sup>34, 35</sup>. Therefore, we tested whether BMPER can also activate NF $\kappa$ B transcriptional activity. In response to BMPER treatment, NF $\kappa$ B transcriptional activity was increased, determined by NF $\kappa$ B reporter assays (Figure SIVD). However, LRP1 knockdown by its specific siRNA decreased BMPER-induced NF $\kappa$ B activity. It suggests that BMPER/LRP1 axis can also activate NF $\kappa$ B transcriptional activity.

#### **NF45 is associated with LRP1 $\beta$ and involved in NFAT activation**

LRP1's  $\alpha$  chain (LRP1 $\alpha$ ) is responsible for extracellular ligand binding and  $\beta$  chain (LRP1 $\beta$ ) for transducing signals. Additionally, LRP1 $\beta$  can be further processed<sup>36</sup> and translocated to nucleus, where it interacts with nuclear proteins such as poly(ADP-ribose) polymerase-1 (PARP-1)<sup>24</sup> and PPAR $\gamma$ <sup>25</sup> to regulate cell cycle progression and gene transcription. Interestingly, we observed that LRP1 $\beta$  and its processed intracellular domain (~12 kDa) were localized in the nucleus at basal condition and their levels were decreased following the treatment of DAPT, a  $\gamma$ -secretase inhibitor (an inhibitor of presenillin-dependent  $\gamma$ -secretase activity, to generate the intracellular domain of LRP1 (~12 kDa) from the ~25 kDa processed form<sup>24, 36</sup>; VA-B). It is unclear why DAPT decreased LRP1 $\beta$  level in the nucleus. However, upon DAPT treatment, the level of the ~25 kDa fragment of LRP1 was increased in the cytosolic fraction, suggesting that LRP1 $\beta$  processing takes place at basal condition in a  $\gamma$ -secretase dependent manner. Because we have demonstrated that

LRP1 is required for BMPER-induced ERK activation (Figure 4C), we wanted to determine if the nuclear LRP1 $\beta$  might play a role in BMPER-induced NFAT activation. To address this question, we started a search for LRP1 $\beta$ -interacting proteins as shown in our previous publication<sup>24</sup>. First, we performed liquid chromatography associated mass spectrometry (LC-MS/MS) analysis to identify LRP1 $\beta$ -associated proteins in HEK 293 cells (Figure 5A). Nuclear factor 45/ interleukin binding factor 2 (NF45/ILF2) was among proteins that co-immunoprecipitated with Flag-LRP1 $\beta$  (Figure 5A). NF45 is a nucleic acid-binding protein that regulates cellular gene expressions involved in DNA metabolism, transcription, translation, RNA export and microRNA biogenesis<sup>37–41</sup>. In addition, NF45 is considered as a transcriptional activator of IL-2 gene<sup>42</sup>. Previous reports show that NF45 and NFAT bind to the same promoter region of IL-2 promoter. However, they have different consensus binding sequences (TGTTTAC for NF45 and TGGAAAAT for NFAT)<sup>43</sup>. NF45, unlike NFAT, is associated with the IL-2 ARRE-2 site even at basal condition<sup>43</sup>. To date, it remains unknown whether this interchangeable binding of NF45 and NFAT transcriptional complex is important for IL-2 transcriptional activation. To study the role of NF45 in BMPER-induced gene expression, we first confirmed the interaction between overexpressed Flag-LRP1 $\beta$  and NF45 in HEK 293 cells (Figure 5B). Moreover, their endogenous association was detected in MLECs and blocked by the inhibitors of PKA- PKI, Src (PP2) or their combined treatments (Figure 5C, SVC), suggesting that the interaction of LRP1 $\beta$  with NF45 requires LRP1 $\beta$  phosphorylation by PKA and Src. Furthermore, both immunofluorescence imaging and subcellular fractionation analysis demonstrated that LRP1 $\beta$  and its processed intracellular domain, and NF45 were co-localized mainly in the nucleus of MLECs at basal condition (Figure 5D, E). Surprisingly, cytosolic levels of NF45, LRP1 $\beta$  and its intracellular domain, but not the processed form of LRP1 $\beta$  at 25 kDa, were increased in response to BMPER treatment (Figure 5E), suggesting that BMPER induces the cytosol translocation of NF45, LRP1 $\beta$  and its intracellular domain, but not the LRP1 processing. To further test whether NF45 translocation takes place in LPS-induced inflammatory setting, we performed immunofluorescent studies in MLECs and lung sections. In LPS-treated MLECs, NF45 positive signals in the cytosol fraction were significantly increased (Figure SVD, E). However, the increased signals were blocked in BMPER siRNA transfected MLECs. Additionally, 70.6% of NF45 signals were localized in cytosol of pulmonary vascular cells in BMPER<sup>+/+</sup> mice upon LPS injection (Figure SVF, G). However, the cytosol-localized NF45 signals were decreased to 55.1% in BMPER<sup>+/-</sup> mice. All these results indicate that NF45 was translocated from the nucleus to cytoplasm in response to LPS and its translocation was inhibited by BMPER deficiency, which supports the hypothesis that NF45 nuclear export is regulated by LPS through a BMPER-dependent pathway.

In the meantime, we observed the translocation of NFATc1 from cytoplasm to nucleus (Figure 3C, D). Therefore, we hypothesize that NF45 may negatively regulate NFAT activity by competing to bind the promoter region of NFAT target genes. As expected, knockdown of NF45 by its specific siRNA significantly increased NFATc1 chromatin occupancy, and its promoter activity at basal condition and in response to BMPER treatment (Figure 5F, SVIA). In addition, when LRP1 was knocked down in MLECs, both BMPER-induced NF45 nuclear export and NFATc1 cytoplasmic-nuclear shuttling were inhibited (Figure SVIB-E). When LRP1  $\alpha$  chain was blocked by its specific antibody 8G1, BMPER-induced NF45 nuclear



export was also blocked (Figure SVIF, G), suggesting that two signaling pathways, including the membrane LRP1-associated LRP1 $\beta$  signaling and nuclear LRP1 $\beta$ -NF45 signaling, are interdependent. However, exact mechanisms by which these two pathways are integrated remain unclear and will become one of our future research directions. Nevertheless, we conclude that NF45 acts as a repressor for NFAT activation and its nuclear export with LRP1 $\beta$  may contribute to BMPER-induced activation of NFATc1.

Taken all together, our results indicate that BMPER acts as a pro-inflammatory stimulus during acute lung injury. Its action requires coordinative actions of multiple signaling cascades, including the calcium-dependent calcineurin and LRP1-dependent ERK activation, the cytosolic translocation of NF45 and nuclear translocation of NFATc1, which eventually lead to the activation of NFATc1 and induction of its downstream inflammatory target genes (Figure 6). Our study provides BMPER signaling as a potential therapeutic target in preventing sepsis-induced pulmonary inflammation and injury.

## DISCUSSION

The roles of BMPER in inflammation have been examined in several mouse models of inflammation including thioglycollate-induced peritonitis and high fat diet induced atherosclerosis<sup>14, 15</sup>. The studies suggest that BMPER plays an anti-inflammatory role through modulating BMP activity and thereby blocking TNF- $\alpha$  or oscillatory shear-mediated induction of adhesion molecules such as ICAM-1 and VCAM1. In this study, we have demonstrated for the first time a pro-inflammatory role of BMPER in LPS-induced acute lung injury. BMPER<sup>+/-</sup> mice exhibit alleviated responses upon LPS challenge. Our biochemical studies suggest that BMPER regulates inflammatory responses through NFAT pathway, which plays important roles in endothelial inflammation. In addition, we provide evidence suggesting that BMPER may initiate its own signaling pathways via LRP1, which expands our understanding in the roles of BMPER in vascular homeostasis.

How BMPER may play a pro-inflammatory role in LPS-induced acute lung injury? Our gene expression profiling analysis provided us a clue by suggesting that BMPER may regulate NFAT transcriptional activity. We then confirmed that BMPER is not only required but also sufficient for NFAT activation, which plays an essential role in pro-inflammatory cytokines production in endothelial cells during LPS-induced endotoxemia<sup>7</sup>. This BMPER-induced signaling event involves coordinative actions of multiple pathways. As we propose in the working model (Figure 6), BMPER activates ERK pathway in a LRP1-dependent manner, which is required for NFAT activation. Although it remains to be further determined how calcium/calcineurin signaling is activated by BMPER, the studies with calcineurin inhibitor cyclosporine suggest that calcium/calcineurin is also required for BMPER-induced NFAT activation. In addition, we discovered that LRP1 $\beta$  is associated with NF45 in the nucleus at basal condition. Upon BMPER stimulation, they are translocated to cytoplasm in a timely fashion when NFATc1 is translocated into nucleus. Given that NFATc1 and NF45 are associated with the same promoter regions of their genes, these observations indicate that NF45 may suppress NFAT transcriptional activity at basal condition. Upon the stimulation, the replacement of NF45 by NFAT transcriptional complex could be allowed in a fast fashion, which may explain the quick transcriptional events for pro-inflammatory cytokines.

These timely reactions of endothelial cells, acting as the first line of defense, are essential for fighting against septic shock and the resolution of the acute inflammation.

We and others have identified both pro- and anti-BMP activities of BMPER<sup>11, 12, 44–51</sup>, resulting in some controversy as to whether BMPER is a BMP agonist or antagonist. To reconcile this issue, we have extensively analyzed the biochemical events resulting from BMPER's interaction with BMP4 and identified a concentration-dependent switch of BMPER from pro- to anti-BMP signaling, which is modulated by the relative concentration of BMPER to BMP4<sup>16</sup>. Specifically, when BMPER is at molar concentrations lower than BMP4 (sub-stoichiometric), BMP4 activity is enhanced, leading to increased phosphorylation of Smad1/5/8 in mouse endothelial cell. However, when BMPER is at molar concentrations higher than BMP4 (supra-stoichiometric), BMP4-mediated Smad1/5/8 phosphorylation is attenuated. Both the activation and inhibition of BMP4 signaling by BMPER require endocytosis of BMPER/BMP4 complex through a clathrin-dependent endocytosis pathway, suggesting that endocytosis of BMPER/BMP4 complex is involved in BMPER-mediated BMP4 signaling. This provides molecular mechanisms to support a role of BMPER in fine tuning BMP signaling at the single cell level. However, it may not necessarily explain all the pro- and anti-BMP phenotypes in different animal models. In this study, we provide strong evidence suggesting that BMPER, besides acting as an extracellular modulator of BMP, may also initiate its own signaling through LRP1 in endothelial cells. Even though this adds another layer of complexity to BMPER's functional roles, it provides feasible explanations for BMPER's different, sometimes even controversial, loss-of-function phenotypes in different animal models.

LPS-induced NFAT pathway plays a pivotal role in vascular endothelial activation. In endothelial cells, TLR4, and likely CD14 too, are main receptors of LPS. However, it is unclear whether TLR4 and/or CD14 are required for NFAT activation. In this study, we have discovered that BMPER is required and sufficient for NFAT activation. It will be interesting to determine how BMPER regulates LPS-induced NFAT activation. LRP1 has been reported to transactivate Trk receptors through a Src family kinase-dependent pathway<sup>52</sup>. It is likely that crosstalks may also exist between LRP1 and TLR4, CD14 and/or their downstream mediators, which coordinately regulate NFAT activation. The detailed protein-protein interactions responsible for this crosstalk will become one of our further research directions.

It is worth to note that the decrease of BMPER expression in the BMPER<sup>+/-</sup> mouse is not limited in endothelial cells. Since BMPER is a secreted protein, it can act on many different cell types. Additionally, inflammation is a multi-cellular process that involves not only endothelial cells but also other cells such as circulating leukocytes and lung epithelial cells. Even though our investigation has only been focused on lung endothelial cells, the role of BMPER in sepsis or other lung inflammatory pathogenesis may be more complicated than what we observed within this endotoxemia model. Previous studies show that the decreased BMPER expression following bleomycin-induced lung injury impairs epithelial barrier function and epithelial morphology<sup>53</sup>. It is very likely that BMPER haploinsufficiency also regulates functions of epithelial cells, neutrophils or other cell types during LPS-induced acute lung injury, which remains to be further investigated. Nevertheless, given that BMPER is accessible as a secreted protein, our findings shed light that BMPER may become a great

therapeutic target during endotoxemia, septic shock, and other pulmonary inflammatory conditions.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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### SOURCES OF FUNDING

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## ABBREVIATIONS

<b>BMPER</b>	bone morphogenetic protein- binding endothelial regulator
<b>BMP</b>	bone morphogenetic protein
<b>NFAT</b>	nuclear factor of activated T cells
<b>LRP1</b>	low-density lipoprotein receptor related protein 1
<b>LRP1<math>\beta</math></b>	low-density lipoprotein receptor related protein 1 $\beta$ chain
<b>MLEC</b>	mouse lung microvascular endothelial cells
<b>LPS</b>	lipopolysaccharide
<b>ARDS</b>	acute respiratory distress syndrome
<b>TLR</b>	toll-like receptor
<b>BALF</b>	bronchoalveolar lavage fluid
<b>MPO</b>	myeloperoxidase
<b>ROS</b>	reactive oxygen species
<b>IFN<math>\gamma</math></b>	interferon gamma
<b>TNF-<math>\alpha</math></b>	tumor necrosis factor alpha
<b>IL-6</b>	interleukin 6
<b>NF45</b>	nuclear factor 45
<b>ERK</b>	extracellular signal-regulated kinase

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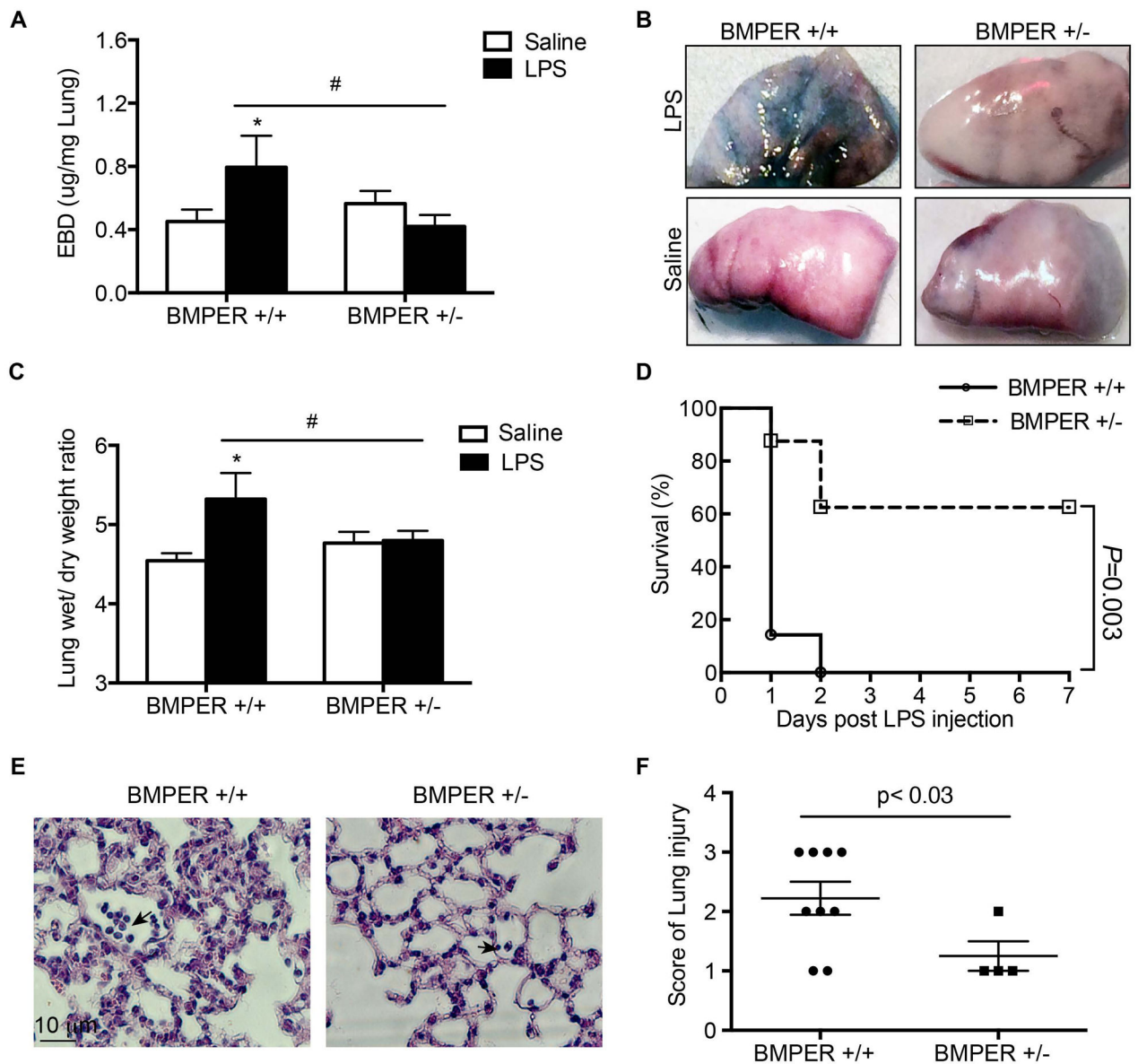
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**HIGHLIGHTS**

- In this study, we have demonstrated for the first time a pro-inflammatory role of BMPER in LPS-induced acute lung injury. BMPER<sup>+/-</sup> mice exhibit alleviated responses upon LPS challenge.
- Our biochemical studies suggest that BMPER regulates inflammatory responses through the activation of NFAT pathway, which plays important roles in endothelial inflammation.
- In addition, we provide data suggesting that BMPER may initiate its own signaling pathways via LRP1, which expands our understanding in the roles of BMPER in vascular homeostasis.



**Figure 1. BMPER haploinsufficiency ameliorates pulmonary vascular leakage and lung injury in response to LPS treatment**

**A–C**, Capillary leakage (**A–B**) and edema (**C**) are markedly improved in BMPER<sup>+/-</sup> mice versus BMPER<sup>+/+</sup> mice. BMPER<sup>+/-</sup> or BMPER<sup>+/+</sup> mice were injected i.v. with a sub-lethal dose (10 mg/kg) of LPS and 16 hours later with Evans Blue dye (EBD; 1% w/v). Mice were sacrificed 30 minutes later. Representative lung tissues with extravasated EBD were photographed and shown in (**B**). \*  $P < 0.05$ , compared with mice injected with saline; #  $P < 0.05$ ;  $n = 5\text{--}6$ . **D**, Survival curves for BMPER<sup>+/-</sup> and BMPER<sup>+/+</sup> mice injected i.v. with a lethal dose of LPS (15 mg/kg).  $P = 0.003$ ;  $n = 7\text{--}8$ . **E**, representative photomicrographs of lung tissues stained with hematoxylin and eosin after LPS injection. LPS stimulated infiltration of inflammatory cells into lung interstitium and alveolar spaces as indicated by arrows, alveolar wall thickening, and intra-alveolar exudation. **F**, Lung tissues were screened for lung injury score.  $n = 9$  (BMPER<sup>+/+</sup>) and 4 (BMPER<sup>+/-</sup>). Analysis was two-way ANOVA followed by

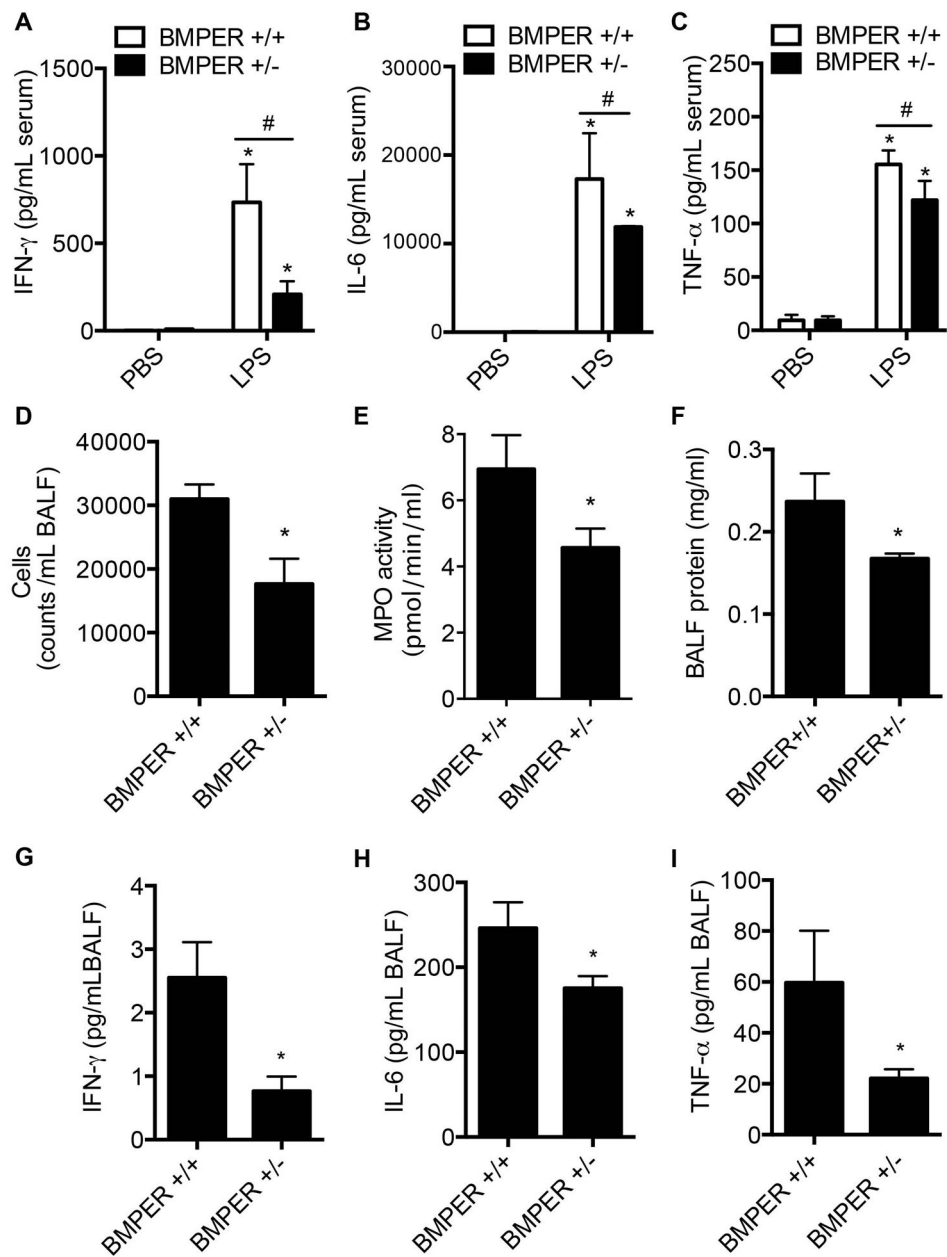
Fisher's LSD multiple comparison test (for **A**, **C**), log-rank test (for **D**) and unpaired Student's *t*-test (for **F**).

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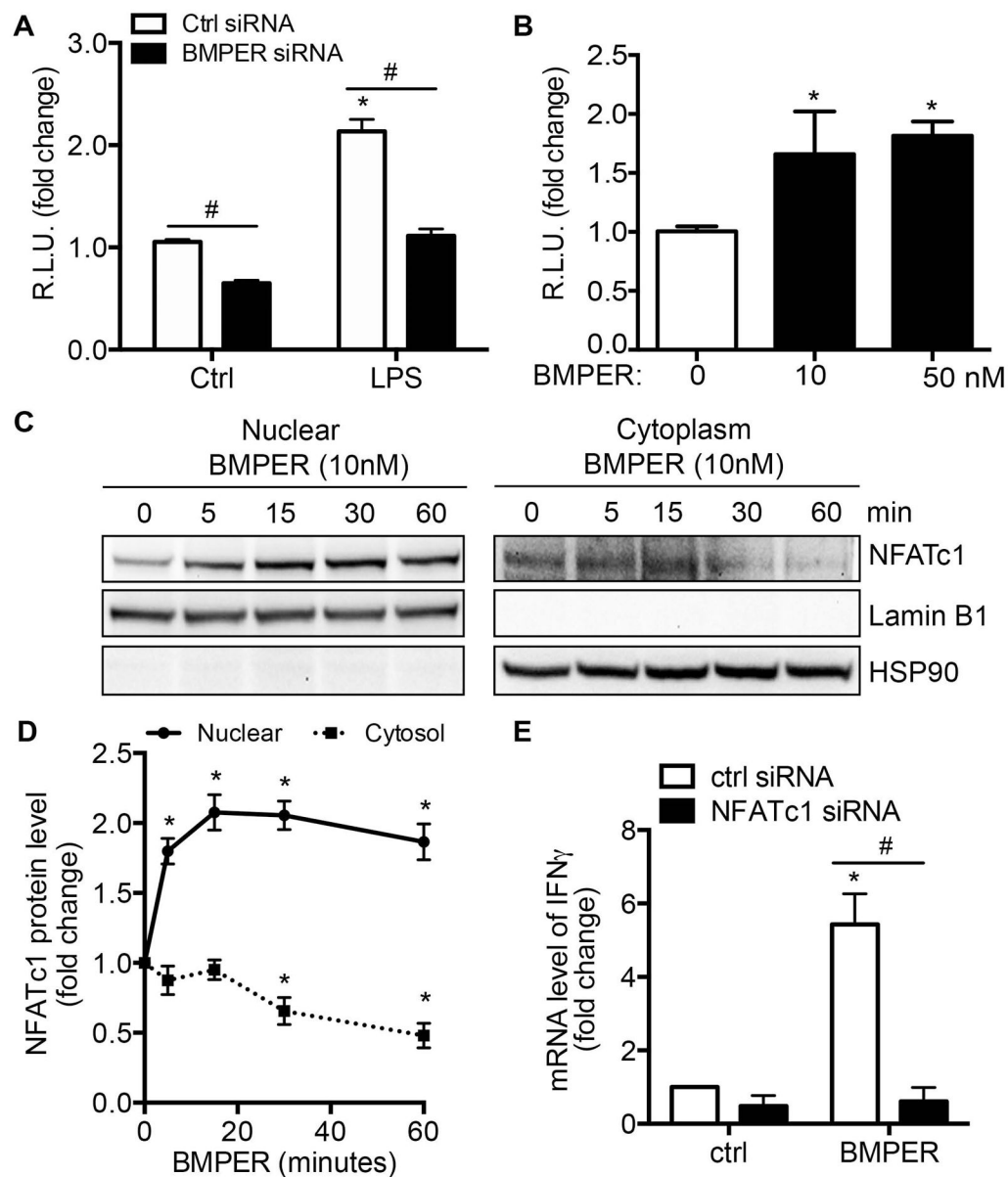
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**Figure 2. LPS-induced pro-inflammatory responses are attenuated in BMPER<sup>+/-</sup> mice**  
 BMPER<sup>+/+</sup> and BMPER<sup>+/-</sup> mice were treated with LPS (10 mg/kg i.v.) or PBS (for A–C) for 6 hours, indicated cytokine levels in serum (A–C) were measured. In BALF, total cell number (D), MPO activity (E), protein level (F), and indicated cytokine levels (G–I) were measured. \*  $P < 0.05$ , compared with same mice injected with PBS (A–C) or BMPER<sup>+/+</sup> mice (D–I); #  $P < 0.05$ ;  $n = 4\text{--}9$ . Analysis was two-way ANOVA followed by Fisher's LSD multiple comparison test (for A–C) and unpaired Student's  $t$ -test (for D–I).



**Figure 3. BMPER is required and sufficient to activate NFATc1**

**A**, BMPER knockdown blocks NFAT activation in response to LPS (10  $\mu$ g/ml). MLECs were transfected with a mixture of NFAT-responsive firefly luciferase and renilla constructs, and BMPER or control siRNA. One day later, cells were treated with LPS and the luciferase activity was measured after another day's incubation. **B**, BMPER increases transcriptional activity of NFAT in MLECs. MLECs were transfected with a mixture of NFAT-responsive firefly luciferase and renilla constructs. After 24 hours, cells were treated with BMPER or control for another 24 hours and the luciferase activity was measured. **C–D**, BMPER increases nuclear translocation of NFATc1. MLECs were treated with 10 nM BMPER for indicated time periods. Nuclear and cytoplasmic enriched fractions of cell lysates were used to determine the translocation of NFATc1. Lamin B1 (nuclear marker) and HSP90 (cytosol marker) immunoblotting were used to verify the purity of the fractions. NFATc1 protein

levels in nuclear and cytosol fractions were quantified and shown in **D, E**, NFATc1 is required for IFN $\gamma$  induction upon BMPER treatment. MLECs were transfected with NFATc1 or control siRNA. After 24 hours, cells were treated with BMPER at 10 nM or control and incubated for another 8 hours. Cells were harvested for real time PCR with IFN $\gamma$ -specific probe and primers. \*  $P < 0.05$ , compared to cells without LPS (**A**) or BMPER (**B, D, E**) treatment; #  $P < 0.05$ ; n=3~6. Analysis was two-way ANOVA followed by Fisher's LSD multiple comparison test (for **A, E**) and one-way ANOVA (for **B, D**).

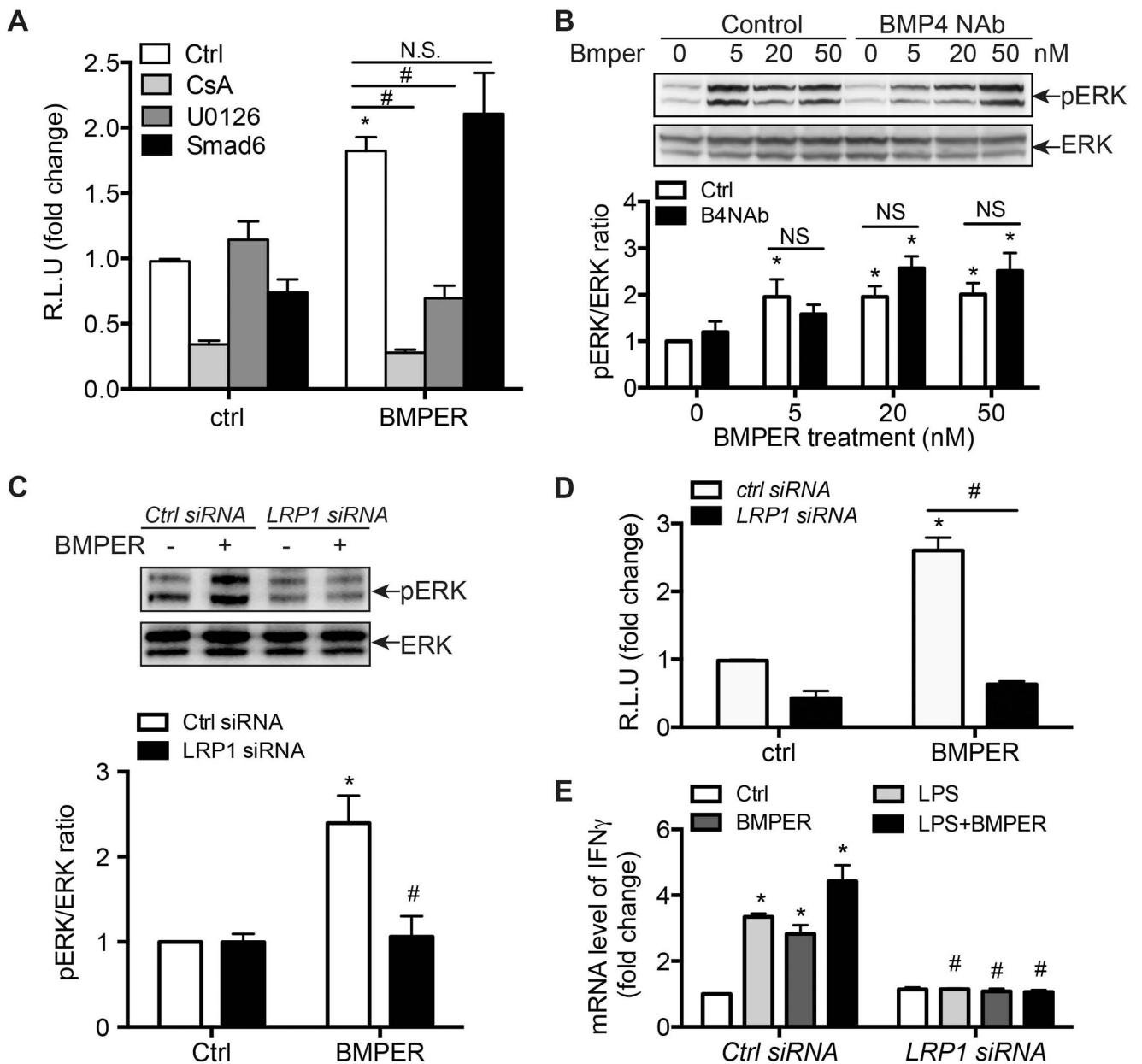
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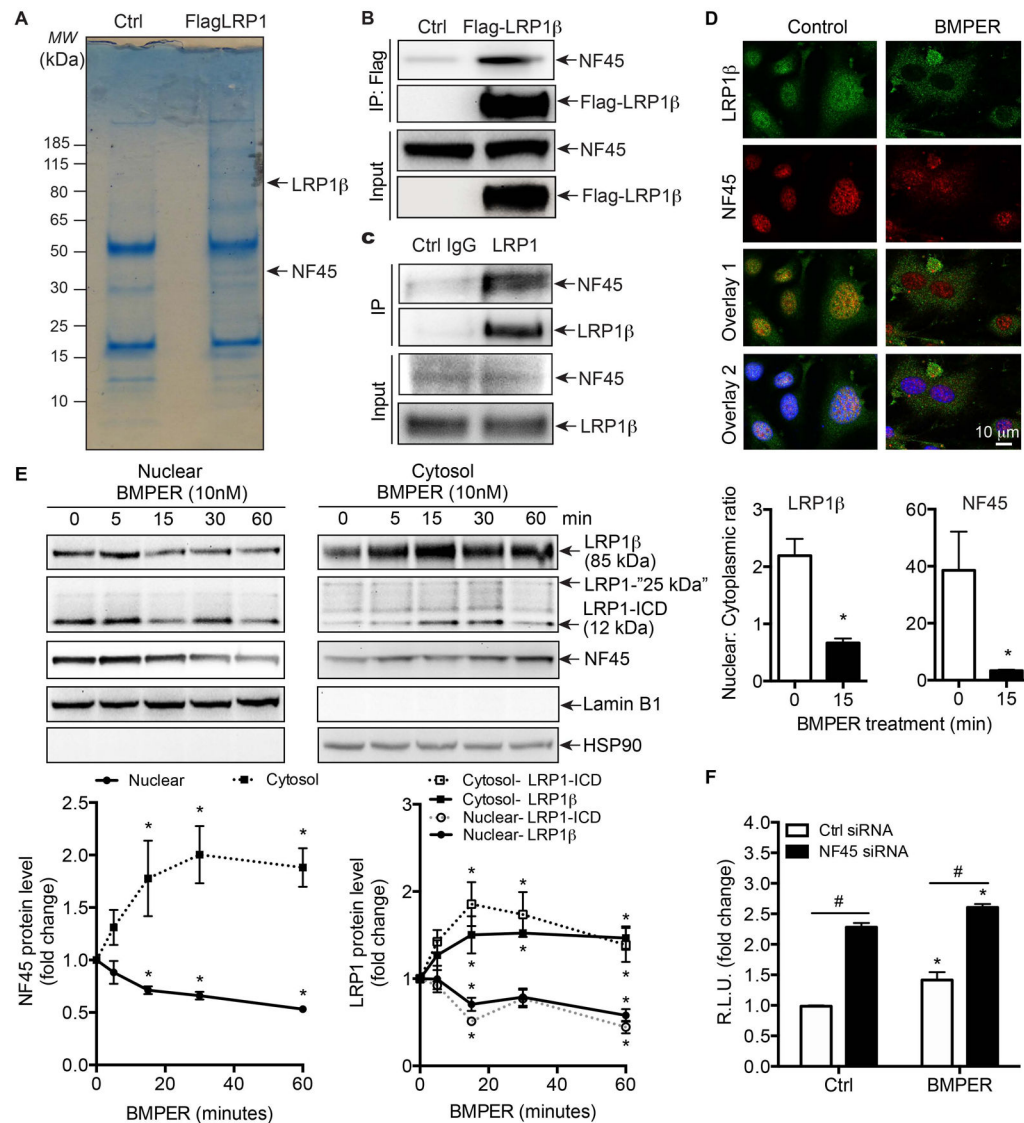




**Figure 4. LRP1 is required for BMPER induced NFAT activation**

**A**, Inhibitors of calcineurin and ERK pathways block NFAT activation upon BMPER treatment. MLECs were transfected with constructs of NFAT-responsive luciferase, renilla and inhibitory Smad-Smad6. After 24 hours, cells were pre-treated with cyclosporine A (CsA), U0126 for half an hour and then treated with BMPER or control. Luciferase activity was measured after another day's incubation. **B**, BMPER-induced ERK activation is not inhibited by BMP4 neutralizing antibody in MECs. Mouse cardiac-derived endothelial cells were treated with BMPER at indicated doses for 30 minutes. BMP4 neutralizing antibody (NAb; 40 ng/ml) was used to pretreat cells to block BMP4 activity (Figure SIVB). **C**, BMPER increases LRP1-dependent ERK activation. MLECs transiently transfected with LRP1 siRNA were treated with BMPER at 10 nM for 30 minutes, and then harvested for

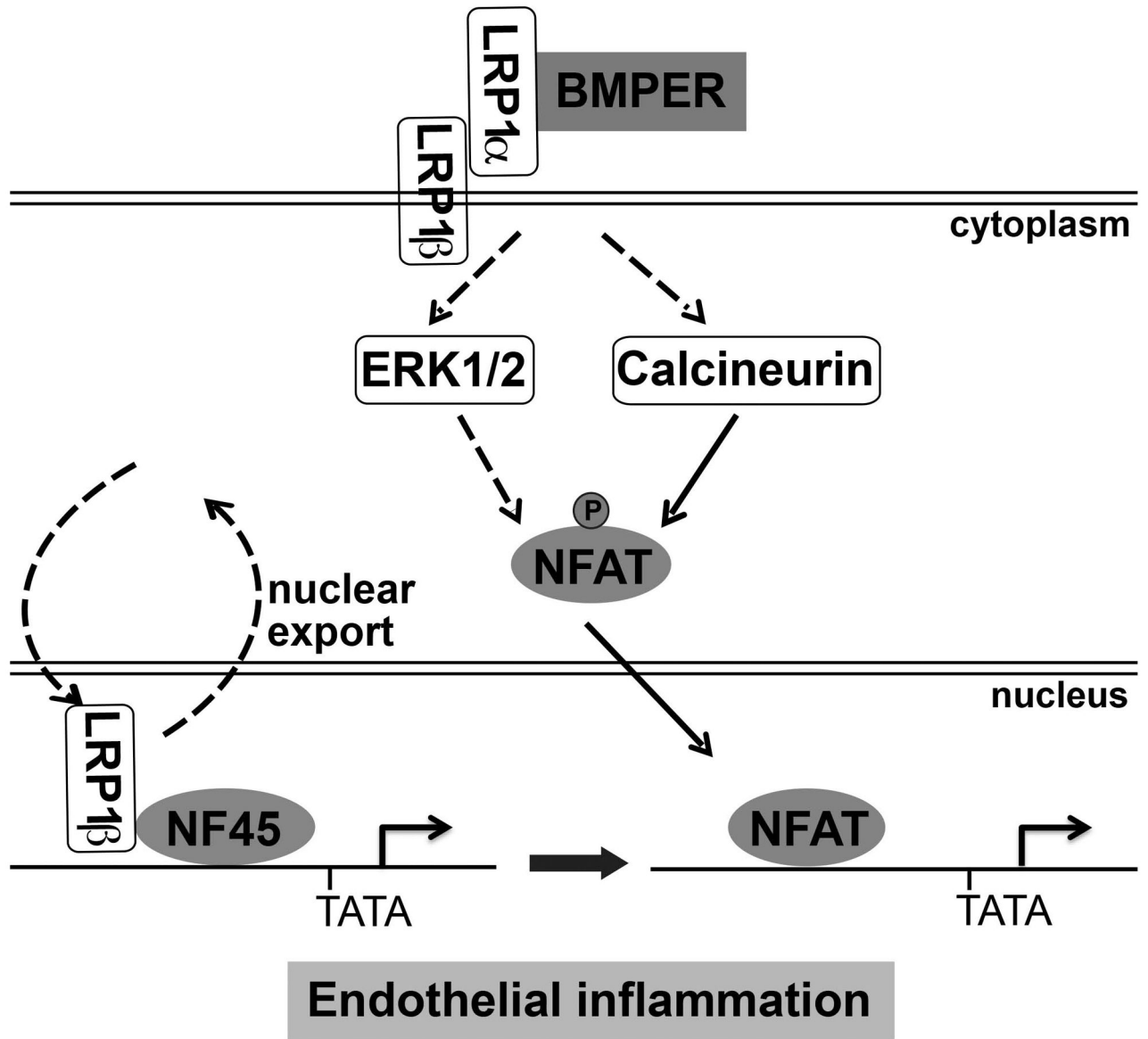
Western blotting. ERK activity was calculated by measuring the intensity of phospho-ERK and total ERK blots. **D**, LRP1 is required for NFAT activation induced by BMPER. MLECs were transfected with LRP1 or control siRNA, and NFAT-responsive luciferase and renilla constructs. After 24 hours, cells were treated with BMPER or control. Luciferase activity was measured after another day's incubation. **E**, LRP1 is required for IFN $\gamma$  induction upon LPS, BMPER, or their combined treatments. MLECs were transfected with LRP1 or control siRNA. After 24 hours, cells were treated with LPS at 10  $\mu$ g/ml, BMPER at 10 nM, their co-treatment, or control, and then incubated for another 8 hours. Cells were harvested for real time PCR with IFN $\gamma$ -specific probe and primers. \*  $P < 0.05$ , compared to control cells without BMPER or other indicated treatments; #  $P < 0.05$ , compared as indicated (for **A**, **C**, **D**) or to control siRNA transfected cells upon same treatments (for **E**); n=3~4. NA, not significant. Analysis was two-way ANOVA followed by Fisher's LSD multiple comparison test.



**Figure 5. NF45 is associated with LRP1β and involved in NFAT activation**

**A**, NF45 is a candidate protein associating with LRP1. Lysates of HEK 293 cells with stably transfected Flag-tagged LRP1 β chain (Flag-LRP1β) were immunoprecipitated with an anti-Flag antibody and stained with Coomassie blue. Positive bands are subjected to mass spectrometry analysis to identify interacting proteins. NF45 is one of these candidate proteins that are likely associated with LRP1. **B**, Flag-tagged LRP1β is associated with NF45 in HEK293 cells. Lysates of HEK 293 cells with stably expressing Flag-LRP1β were immunoprecipitated with an anti-flag resin and blotted with an anti-NF45 antibody. **C**, LRP1β is associated with NF45 in MLECs. Lysates of MLECs were immunoprecipitated with anti-LRP1β antibody or control IgG, and analyzed by Western blotting with an anti-NF45 antibody. **D–E**, LRP1β and NF45 are translocated from nucleus to cytoplasm upon BMPER treatment. MLECs were treated with BMPER at 10 nM for 15 minutes. After fixation, cells were stained with LRP1 (C-terminal) antibody (green), NF45 antibody (red) and DAPI (blue) for nucleus and representative pictures were shown in D. LRP1 (C-

terminal) antibody recognizes both LRP1 $\beta$  and its processed fragments at 25 kDa and 12 kDa intracellular domain. The relative intensity of LRP1 $\beta$  and its processed fragments, and NF45 in nuclear and cytoplasmic fractions was quantified and the ratios of nuclear to cytoplasmic signals were presented in **D**. \*  $P < 0.05$ , compared to control cells;  $n = 8 \sim 12$ . In addition, MLECs were treated with BMPER and fractionation assays was performed. Band intensity of LRP1 $\beta$ , its intracellular domain at 12 kDa (LRP1-ICD) and NF45 was calculated and normalized to the sample without BMPER treatment. The graph in **E** shows a decrease in nuclear NF45, LRP1 $\beta$  and LRP1-ICD protein levels but an increase in cytosolic NF45, LRP1 $\beta$  and LRP1-ICD. The protein level of the LRP1 $\beta$  processed form at 25 kDa was not changed in the cytosol in response to BMPER treatment. \*  $P < 0.05$ , compared to cells without BMPER treatment;  $n = 3$ . **F**, NF45 knockdown promotes NFAT activity. MLECs were transfected with a mixture of NFAT-responsive firefly luciferase and renilla constructs, and NF45 or control siRNA. One day later, cells were treated with BMPER at 10 nM and the luciferase activity was measured after another day's incubation. \*  $P < 0.05$ , compared to cells without BMPER treatment; #  $P < 0.05$ ;  $n = 3$ . Analysis was Student's *t*-test (for **D**), one-way ANOVA (for **E**) and two-way ANOVA followed by Fisher's LSD multiple comparison test (for **F**).



**Figure 6.** Schematic illustration to show how BMPER regulates NFATc1 activation via coordinative actions of LRP1, ERK, calcineurin and NF45.