MMP-9-mediated cardiac fibroblast activation contributes to sepsis-associated cardiac dysfunction in mice

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Abstract

Aims Cardiac dysfunction is a frequent and severe complication of septic shock and contributes to the high mortality of sepsis. Although a number of mechanisms have been suspected to be responsible for sepsis-associated cardiac dysfunction, the precise cause(s) remains unclear to date. We tested the hypothesis that cardiac fibroblasts may play a critical role as a disease modifier involved in sepsis-associated cardiac dysfunction.

Methods and results Human cardiac fibroblasts (HCFs) culture *in vitro* were exposed to lipopolysaccharide (LPS; 1 μg/ml) for 24 h. LPS increased the mRNA expression levels of matrix metalloproteinase (MMP)-9, but not MMP-2, as assessed by real-time PCR, and caused a specific increase in the band corresponding to MMP-9, but not MMP-2, as measured by gel zymography. LPS also up-regulated the mRNAs of pro-inflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α, in HCFs, an effect which was slightly but significantly reduced by the MMP inhibitor ONO-4817. Western blotting and immunocytochemistry showed that LPS-induced expression of α-smooth muscle actin, a classical marker for myoblast differentiation, was abrogated when MMP-9 small interfering RNA was transfected into HCFs. In cecal ligation and puncture-induced septic mice, perivascular and interstitial cardiac fibrosis was prevented by ONO-4817. Echocardiography demonstrated that septic mice had profound cardiac dysfunction, which was significantly improved by ONO-4817.

Conclusions We suggest that cardiac fibroblasts are important in inflammation and fibrosis in the heart through active participation of MMP-9, leading to cardiac dysfunction, that would affect the outcome of sepsis syndrome.

Keywords Cardiac fibroblast, MMP-9, Inflammation, Cardiac fibrosis, Sepsis

1. Introduction

Sepsis and especially its life-threatening complication, septic shock, are major causes of death in intensive care units, despite advancements in antimicrobial therapy.[1] Cardiac depression is a well-recognized manifestation of organ dysfunction in sepsis. Due to the lack of a generally accepted definition and the absence of large epidemiologic studies, its accurate frequency is uncertain. However, clinical studies report that sepsis-induced cardiac dysfunction occurs in approximately 40 to 50% of patients with prolonged septic shock and is associated with increased mortality.[2] Interestingly, cardiac contractility is found to be reduced even in septic patients in the absence of changes in ventricular preload or afterload.[3] Although studies using animal models of sepsis have extensively explored the etiology of cardiac dysfunction, the underlying mechanisms linked to effective methods for preventing and/or treating sepsis-induced cardiac dysfunction have remained elusive.

It has been shown that bacterial endotoxin lipopolysaccharide (LPS) causes cardiac dysfunction by enhancing cardiac-derived inflammatory mediator expression, associated with the release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and overproduction of nitric oxide (NO).[4,5] In this regard, cardiac fibroblasts may serve critical roles as intermediate sensors and amplifiers of inflammatory signals from immune cells and myocytes in response to LPS, through production of cytokines and NO,[6] since they make up 60-70% of the total cell number in the heart. Furthermore, cardiac fibroblasts play a central role in synthesizing extracellular matrix (ECM) proteins, and their activation and transition into a myofibroblastic phenotype, which are controlled by a variety of stimuli, including cytokines, could contribute to the development of cardiac fibrosis and adverse ventricular remodeling.[6] Thus, the genesis of reduced cardiac function during sepsis

may involve the abnormal responses of cardiac fibroblasts.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are able to degrade numerous ECM components and several intracellular proteins. MMPs, such as MMP-9, are involved in many pathological conditions, including inflammation and heart failure.[7,8] The pathogenic role of MMPs in sepsis has so far been suggested. In sepsis, circulating MMP-2 and MMP-9 were elevated in both animal models and patients,[9-11] and elevated serum MMP-9 levels in septic patients were correlated with the severity and mortality of the disease.[12] Despite these insights, no study examined whether MMPs are potentially involved in cardiac dysfunction during sepsis.

In the present study, we hypothesized that cardiac fibroblasts may emerge as a disease modifier due to MMP-mediated regulation of inflammatory and fibrotic remodeling in the heart, that would affect the progress, severity, and outcome of sepsis. Accordingly, we initially examined the responses of human cardiac fibroblasts (HCFs) to LPS and the involvement of MMPs in those responses. To our knowledge, our further study is the first to investigate the effects of MMP inhibition on functional and histological derangements in the hearts of mice with cecal ligation and puncture (CLP)-induced sepsis that has high clinical relevance to humans.[13]

2. Materials and methods

2.1. HCF cultures and treatments

Primary HCFs from the adult ventricle were purchased from Cell Applications Inc. (San Diego, CA, USA). Cells were grown in Fibroblast Growth Medium in a 5% CO_2 humidified incubator kept at 37^{o}C. Cells of passage 3-6 were used when 80% confluent. Then, cells were harvested and seeded into six-well plates. LPS (List

Biological Laboratories, Campbell, CA, USA) was given at a dose of 1 μg/ml. ONO-4817 (Ono Pharmaceutical Co., Osaka, Japan) was used as a synthetic MMP inhibitor. ONO-4817 has a broad inhibitory spectrum for MMPs, including MMP-2, -3, -8, -9, -12, and -13, but not MMP-1 and -7.[14] Cells were usually pre-incubated for 30 min with ONO-4817 at a concentration of 1 or 10 μM, which remained in the medium during exposure of cells to LPS. MMP-9 small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The negative control (sc-37007) consists of a 20-25 nucleotides scrambled sequence, which does not target any known mRNA. Introduction of siRNAs into cells was performed in siRNA Transfection Medium (Santa Cruz Biotechnology) according to the manufacturer's protocol. MMP-9 siRNA (80 pmol) or control siRNA was transfected one day before LPS exposure.

2.2. RNA extraction and quantitative real-time PCR

Total RNA was isolated from HCFs or cardiac tissue with an RNeasy Mini Kit (Qiagen, Tokyo, Japan). RNA was reverse-transcribed to cDNA, and real-time PCR analyses were performed as described previously[15] using Takara RNA PCR kit (Takara Shuzo, Ohtsu, Japan). The sequences of specific primer pairs for target genes are available upon request from a website of Takara Bio Inc. (http://www.takara-bio.co.jp/).

2.3. Western blot analysis

Cells were harvested and lysed in 300 μl of RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, pH 7.4; Thermo, Rockford, IL, USA) containing protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) on ice. The lysates were centrifuged at 18,000 × *g* for 10 min at 4°C and the resulting

supernatants were collected. The proteins in the supernatant were measured using BCA Protein Assay Kit (Thermo). The supernatants (10 μg of protein) were run on 10% polyacrylamide gel and electrotransferred onto polyvinylidine fluoride filter membrane. To reduce nonspecific binding, the membrane was blocked for 90 min at room temperature in Odyssey blocking buffer, followed by overnight incubation with anti-MMP-9 (Millipore, Billerica, MA, USA), anti-α-smooth muscle actin (α-SMA; Sigma-Aldrich, St. Louis, MO, USA) , or anti-GAPDH (Millipore) antibody at 4^{o}C. The membrane was washed four times with PBS with 0.1% Tween 20 and incubated with goat anti-rabbit IRDye 680, goat anti-mouse IRDye 800 CW, or goat anti-chicken IRDye 680 diluted in 1:1,000~15,000 in Odyssey blocking buffer for 120 min at 38^{o}C in dark. After being washed six times in PBS with 0.1% Tween 20, the blots were visualized using the Odyssey Infrared imaging system from LI-COR.

2.4. Gel zymography

MMP activity was measured by zymography with gelatin (Nacalai Tesque) as the substrate. Gelatin (0.5 mg/ml) was added to 7.5% acrylamide polymerization gel mixture without reducing agent. Samples (3-5 μg of protein) were loaded without boiling. Gels were run at 15 mA/gel in the refrigerator. Following electrophoresis, the gels were soaked in 2.7% (W/V) Triton X-100, incubated overnight at 37^{o}C in activating buffer (50 mM Tris, 40 mM HCl, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% (W/V) Brij 35), and stained with Coomassie brilliant blue. Unstained, digested regions representing gelatinolytic activity were semiquantified by reverse image densitometry. MMP identify was determined by estimated molecular weights against prestained molecular weight marker (Cosmo Bio, Tokyo, Japan).

2.5. Immunofluorescence

HCFs were fixed using 10% formalin solution for 30 min and incubated with anti-mouse α-SMA antibody (Sigma-Aldrich) for 2 h, followed by exposure to the anti-mouse IgG conjugated to the high quality fluorophore Alexa Fluor 568 for 1 h. The nucleus was counterstained with Hoechst 33258 (Nacalai Tesque). Immunofluorescent images were observed using a fluorescence microscope.

2.6. Animal model of sepsis

All animal studies were conducted in accordance with the National Institute of Health Guidelines on the use of laboratory animal and with approval of the Care and Use Committee of the University of Toyama. Male BALB/c mice, 8-10 weeks of age, were quarantined in quiet, humidified, light-cycled rooms for at least 1 week before use. The surgical procedure to generate CLP-induced sepsis was performed as previously described.[16,17] Mice were anesthetized with 3-4% sevoflurane, and a middle abdominal incision was made. The cecum was mobilized, ligated, and punctured twice with a 21-gauge needle, allowing exposure of faces, the bowel was repositioned, and the abdomen was closed with sterile suture. Sham-operated control mice underwent the same procedure except for ligation and puncture of the cecum. In the MMP inhibitor-treated CLP group, ONO-4817 was orally administered to the animal via gavage as a single bolus at a dose of 100 mg/kg 2 h before and 30 min after CLP. Functional experiments and surgical manipulations, including tissue isolation, were performed one day after CLP. Mice were sacrificed under deep anesthesia with sevoflurane.

2.7. Histological examination

The hearts were fixed by immersion in 10% buffered formaldehyde overnight, embedded in paraffin, and cut into 5-μm-thick sections. After deparaffinization, slides were stained with hematoxylin and eosin by standard methods. In addition to staining with hematoxylin-eosin, azan staining was performed with aniline blue to identify fibrosis in the heart.

2.8. Echocardiographic measurements

Echocardiographic studies were carried out using a US animal-imaging system (Prospect; S-Sharp Corporation, Taipei, Taiwan) at a frequency of 40 MHz with a transducer (acoustic pressure: 0.59 MPa [MI = 0.09]) with a diameter of 7 mm and a fixed focus at 12 mm. The LV dimensions in diastole (LVDd) and systole (LVDs), percent fractional shortening (FS), and ejection fraction (EF) were estimated using the M-mode measurements.

2.9. Statistical analysis

Data are expressed as mean ± SEM. Statistical assessment of the data was made by Student's unpaired *t*-test or one-way ANOVA followed by Tukey's multiple comparison test. A significant difference was assumed to exist if the *P* value was less than 0.05.

3. Results

3.1. Effect of LPS on MMP expression and activity in HCFs

Figure 1A shows the time course of changes in MMP-9 mRNA levels in HCFs after LPS administration. MMP-9 mRNA levels were significantly elevated from

control between 6 and 24 h, with a peak level at 12 h. In contrast, LPS failed to up-regulate MMP-2 mRNA in HCFs (see Supplementary material online, Figure S1A). Furthermore, the mRNA level of transforming growth factor-β_1 (TGF-β_1), which stimulates myofibloblast formation, contributing to cardiac fibrosis and failure,[18] was unchanged when HCFs were challenged with LPS (see Supplementary material online, Figure S1B). On Western blots, the protein level of MMP-9 was increased by LPS challenge in a time-dependent manner (Figure 1B). MMP activity in the media of cultures treated with LPS was determined by gel zymography using gelatin as the substrate. In HCFs, specific band corresponding to the molecular weight of MMP-9 (92 kDa) was increased by treatment with LPS (Figure 1C), indicating that the LPS-stimulated increase in MMP-9 activity was at least in part due to increased MMP-9 synthesis. Notably, a 72 kDa band of MMP-2 was undetectable in any samples of LPS-stimulated HCFs. The MMP inhibitor ONO-4817 (10 μM) abrogated the LPS-induced increase in MMP-9 activity (Figure 1C).

3.2. Effect of ONO-4817 on LPS-induced inflammatory genes in HCFs

In HCFs, LPS greatly up-regulated mRNA levels of IL-1β, TNF-α, IL-6, and inducible NO synthase (iNOS), although the time course of changes in the expression after LPS was somewhat different among those inflammatory genes (Figure 2A). Treatment with ONO-4817 (10 μM) resulted in a slight but significant reduction in mRNA levels of IL-1β, TNF-α, and iNOS, with the exception of IL-6 (Figure 2B).

3.3. Effect of LPS on α-SMA expression in HCFs

In order to assess the time-dependent changes in α-SMA expression in HCFs after LPS challenge, immunoblots of α-SMA and GAPDH were performed and the ratio of

α-SMA to GAPDH was calculated. In cells cultured without LPS, the α-SMA content was not appreciably changed with time. On the other hand, LPS challenge showed significant increases in α-SMA between 12 and 24 h (Figure 3A).

To define the involvement of MMP-9 in α-SMA expression in HCFs exposed to LPS, the knockdown of MMP-9 gene expression was performed using siRNAs. α-SMA protein expression was inhibited by MMP-9 siRNA in a concentration-dependent manner and was strikingly repressed by 80 pmol MMP-9 siRNA, as revealed by immunoblot (Figure 3B). When the MMP-9 siRNA transfection experiments were performed at its concentration of 80 pmol, the ablation of MMP-9 by siRNAs resulted in a marked decline in α-SMA expression 12 h after LPS was applied to HCFs (Figure 3C). Control siRNA had no significant effect on LPS-induced α-SMA expression.

α-SMA immunocytochemistry showed that α-SMA-containing stress fibers developed 24 h after HCFs were challenged with LPS (Figure 3D). Cells with α-SMA organized into stress fibers are identified as myofibroblasts. The formation of stress fibers was strongly prevented by transfection of MMP-9 siRNA, but not of control siRNA. Treatment with ONO-4817 also had a preventive effect on stress fiber formation.

3.4. Profibrotic mediators in CLP-induced septic mice

When polymicrobial sepsis was induced by CLP in mice, cardiac ventricular expression of MMP-9 mRNA was significantly up-regulated (Figure 4A). Furthermore, significant increases in the mRNA levels of MMP-2 and TGF-β_1 in ventricles of septic mice were observed. In light of the failure of HCFs to produce these profibrotic genes in response to LPS, they may be the genes expressed by

myocytes or coronary vascular cells. Additionally, septic ventricles showed increased mRNA expression of type I collagen, which is critically involved in the development of myocardial fibrosis.[19] The transcript levels of MMP-9, MMP-2, TGF-β_1, and type I collagen were also found to be increased in atrial tissues of septic mice (see Supplementary material online, Figure S2A).

The activities of MMPs in cardiac tissues of sham-operated control and CLP-induced septic mice were evaluated by gelatin zymography. Specific gelatinolytic bands corresponding to the molecular weights of MMP-9 (92 kDa) and MMP-2 (72 kDa) were obviously increased in both ventricular (Figure 4B) and atrial (see Supplementary material online, Figure S2B) tissues of septic mice.

3.5. Effect of ONO-4817 treatment on cardiac fibrosis in CLP-induced septic mice

Figure 5 shows representative photomicrographs of Azan-stained left ventricular sections from sham-operated control, CLP, and ONO-4817-treated CLP mice. Azan staining revealed that perivascular and interstitial fibrosis were evident in the hearts of CLP-induced septic mice. The development of cardiac fibrosis was prevented by ONO-4817 treatment.

3.6. Effect of ONO-4817 treatment on cardiac dysfunction in CLP-induced septic mice

As shown in the representative tracing images (Figure 6A), echocardiography demonstrated that the LVDd was significantly decreased without changing the LVDs in CLP-induced septic mice, in comparison with sham-operated control mice (Figure 6B and C). Consequently, septic mice had LV dysfunction as measured by LV ES and EF (Figure 6D and E). Treatment with ONO-4817 blunted the decrease in the LVDd in

septic animals. Furthermore, ONO-4817-treated septic mice exhibited significantly improved cardiac function as shown by LV ES and EF. There was no significant difference in heart rate between the three groups (sham: 368 ± 39, CLP: 332 ± 69, ONO-4817-treated CLP: 268 ± 19, n = 5 for each group).

4. Discussion

Cardiac fibroblasts are recognized as the cell type primarily responsible for homeostatic maintenance of ECM in the normal heart, but behave as mediators of inflammatory and fibrotic myocardial remodeling in the diseased heart.[6] Although not immune cells, such as macrophages and lymphocytes, the capacity of fibroblasts to produce cytokines, chemokines, and other pro-inflammatory mediators locally in tissues indicates that fibroblasts are an important participant in inflammation. In this study, we observed that cardiac fibroblasts exhibited high-level expression of pro-inflammatory genes in response to LPS. LPS also promoted the production of a substantial amount of MMP-9 in cardiac fibroblasts. MMP-9 released from cardiac fibroblasts appeared to positively regulate pro-inflammatory gene expression in their own, since the MMP inhibitor ONO-4817 slightly but significantly down-regulated the expression levels of pro-inflammatory genes in HCFs. This suggests that MMP-9 may contribute to cardiac inflammation by exacerbating production of pro-inflammatory cytokines and mediators in cardiac fibroblasts.

When the heart was subjected to chronic cardiac volume or pressure overload, exogenous physiological forces are converted into intracellular signals that stimulate the differentiation of cardiac fibroblasts into myofibroblasts.[20] This differentiation process is characterized by fibroblastic expression of α-SMA,[21] an actin isoform strongly

associated with increased contractile force.[22] Thus, cardiac fibroblasts respond to mechanical loading by a switch to a myofibroblastic phenotype in which cells express α-SMA. α-SMA expression in fibroblasts is also modulated by cytokines such as TGF-β_1.[23] We found that LPS treatment resulted in a significant increase in α-SMA content in HCFs. The LPS-induced up-regulation of α-SMA protein expression was largely blocked by MMP-9 siRNA transfection, indicating that at least part of LPS effect on α-SMA content was due to increased production of MMP-9. Furthermore, our α-SMA immunocytochemistry showed that α-SMA-containing stress fibers were strikingly formed when MCFs were given LPS. Transfection of MMP-9 siRNA and treatment with ONO-4817 prevented α-SMA stress fiber formation. This clearly implies that MMP-9 inhibition leads to a dramatic failure of cardiac fibroblasts to transform into a myofibroblastic phenotype. As α-SMA is an important contractile protein in fibroblasts[22] as well as a phenotypic marker for myofibroblasts,[21] the regulation of α-SMA by MMP-9 in cardiac fibroblasts is of pathological importance because of the pivotal role of myofibroblasts in synthesizing ECM proteins[24] and their contribution to cardiac fibrosis.[25]

In CLP-induced septic mice, coronary perivascular fibrosis and interstitial myocardial fibrosis appeared prominently. Although organ fibrosis is generally a pathological condition associated with chronic inflammation,[26,27] the present study suggests that systemic sepsis can lead to rapid pathological fibrosis. This may be the result of aberrantly-activated cardiac fibroblasts, many of which have the phenotypic characteristics of myofibroblasts, leading to promoting cardiac fibrosis under septic conditions. Additionally, their ability to up-regulate MCP-1 production, which could be demonstrated in LPS-challenged HCFs, may contribute to the development of cardiac fibrosis in sepsis, inasmuch as the MCP-1 pathway can regulate profibrotic

monocyte/macrophage activity.[28] Treatment with ONO-4817 evidently reduced coronary perivascular fibrosis and interstitial myocardial fibrosis in CLP-induced septic mice, suggesting the involvement of MMP-9 in the contribution of cardiac fibroblasts to sepsis-associated cardiac fibrosis.

The development of cardiac fibrosis would be responsible for abnormal myocardial stiffness with impaired cardiac function. Indeed, echocardiography showed that CLP-induced sepsis resulted in limited left ventricular diastolic performance, leading to depressed cardiac function as demonstrated by low EF and FS. However, more preserved cardiac function was observed when CLP-induced septic mice were treated with ONO-4817. The beneficial effect of ONO-4817 on sepsis-associated cardiac dysfunction can be, at least in part, attributed to the prevention of cardiac fibrosis development.

The up-regulation of genes specific to fibrosis, including MMP-2, MMP-9, TGF-β_1, and type I collagen, was found not only in the ventricles but also in the atria from CLP-induced septic mice, suggesting that sepsis can intensify the profibrotic signaling pathway to promote the development of atrial fibrosis. Atrial fibrosis is a common feature of clinical atrial fibrillation.[29] It should be noted that severe sepsis is associated with an increased risk of new-onset atrial fibrillation.[30,31] New-onset atrial fibrillation is associated with higher mortality and prolonged hospitalization during severe sepsis. However, further research is required into exploiting the putative role of atrial fibrosis in sepsis-associated atrial fibrillation. A recent study indicates that the changes in atrial expression and function of ion channels may also serve as one of the mechanisms underlying atrial fibrillation in sepsis.[32]

Previous data emphasizes the role of MMPs, including MMP-9, in the pathology of sepsis and its potential as a therapeutic target. An ameliorative effect of the

synthetic broad spectrum MMP inhibitor GM-6001 has been demonstrated in LPS-induced endotoxemia: It inhibits plasma levels of TNF-α and increases the survival of LPS-treated mice.[33] In CLP-induced septic rats, lung tissue levels of MMP-2 and MMP-9 are significantly increased, and COL-3, a chemically modified tetracycline known to inhibit MMP-2 and MMP-9, can reduce acute lung injury with improved survival.[34] The gelatinase B-deficient mice have been shown to be resistant to LPS-induced shock, which implies that specific MMP-9 inhibition may protect against septic shock.[35] Moreover, it has been documented that the peptidomimetic gelatinase B inhibitor Regasepin 1 is very effective in a mouse model of LPS-induced shock.[36] Those reports, together with the results of our present study, suggest that inhibitors of MMPs, including MMP-9, would constitute interesting pharmacological probes that may potentially become useful life-saving agents in septic shock.

To our knowledge, this is the first study to demonstrate that activated cardiac fibroblasts may play a contributory role in sepsis-associated cardiac dysfunction. Cardiac fibroblasts actively participated in cardiac inflammation by generation of cytokines and other pro-inflammatory mediators. Furthermore, cardiac fibroblast differentiation into the myofibroblast phenotype could promote cardiac fibrosis development. Such performances of activated cardiac fibroblasts were due largely to the involvement of MMP-9. MMP-9 secreted from activated cardiac fibroblasts up-regulated the expression levels of pro-inflammatory genes in cardiac fibroblasts and promoted myofibroblast transformation during sepsis. Thus, cardiac fibroblasts are important in inflammation and fibrosis in the heart through active participation of MMP-9, leading to cardiac dysfunction, that would have a negative impact on the outcome of sepsis syndrome.

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Conflict interest: none to declare

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Figure legends

Figure 1 MMP-9 expression and activity in HCFs. (*A*) Time course of changes in MMP-9 mRNA expression after LPS challenge (n = 3). Expression was normalized to GAPDH. ***Significant difference from time 0 (P<0.001). (*B*) Western blots of MMP-9 protein expression after LPS challenge. GAPDH served as loading control. This experiment was repeated twice. (*C*) In the top trace, representative zymograph depicting the effect of LPS on MMP-9 activity in the media of cultures with or without ONO-4847 treatment. Molecular weight (MW) standards are in the first lane. Summary data are shown in the bottom (n = 4). Values are expressed as ratio vs control. *Significant difference from control (P<0.05).

Figure 2 Inflammatory genes in HCFs. (*A*) Time course of changes in mRNA levels of IL-1β, TNF-α, IL-6, and iNOS after LPS challenge (n = 3). Expression was normalized to GAPDH. **, *** Significant difference from time 0 (P<0.01, P<0.001). (*B*) Effects of ONO-4817 (1 and 10 μM) on mRNA levels of IL-1β, TNF-α, IL-6, and iNOS at 6 h after LPS challenge (n = 5). The data are presented as ratio vs LPS alone, after being expressed relative to GAPDH. #, ##Significant difference from LPS alone (P<0.01, P<0.001).

Figure 3 α-SMA expression in HCFs. (*A*) Time course of changes in α-SMA protein expression in the presence or absence of LPS (n = 6). Expression was normalized to GAPDH. *, **Significant difference from the respective value without LPS (P<0.05, P<0.01). In the bottom, typical Western blots are shown. (*B*) Transfection of MMP-9 siRNA concentration-dependently decreased MMP-9 protein expression 12 h after LPS

challenge. (*C*) Effect of transfection of MMP-9 siRNA (80 pmol) or control siRNA on α-SMA expression 12 h after LPS challenge (n = 4). GAPDH served as loading control. (*D*) Cells were fixed and stained with α-SMA antibody. LPS challenge for 24 h led to a myofibroblastic phenotype characterized by organized α-SMA-containing stress fibers. The effects of transfection of MMP-9 siRNA (80 pmol), transfection of control siRNA, or treatment with ONO-4817 (10 μM) on LPS-induced stress fiber formation are depicted as representative images from two independent experiments in which the same results were obtained. Nuclei were counterstained with Hoechst 33258 dye (*blue*).

Figure 4 Profibrotic gene expression and MMP activities in ventricular tissues of sham-operated control and CLP-induced septic mice. (*A*) mRNA expression levels of MMP-9, MMP-2, TGF-β_1, and type I collagen (n = 4-5). Expression was normalized to GAPDH. *, **, ***Significant difference from the respective sham-operated control. (*B*) Gelatin zymogram of ventricular protein extracts. Molecular weights (MW) standards are in the first lane. Shown are representative images from two independent experiments in which the same results were obtained.

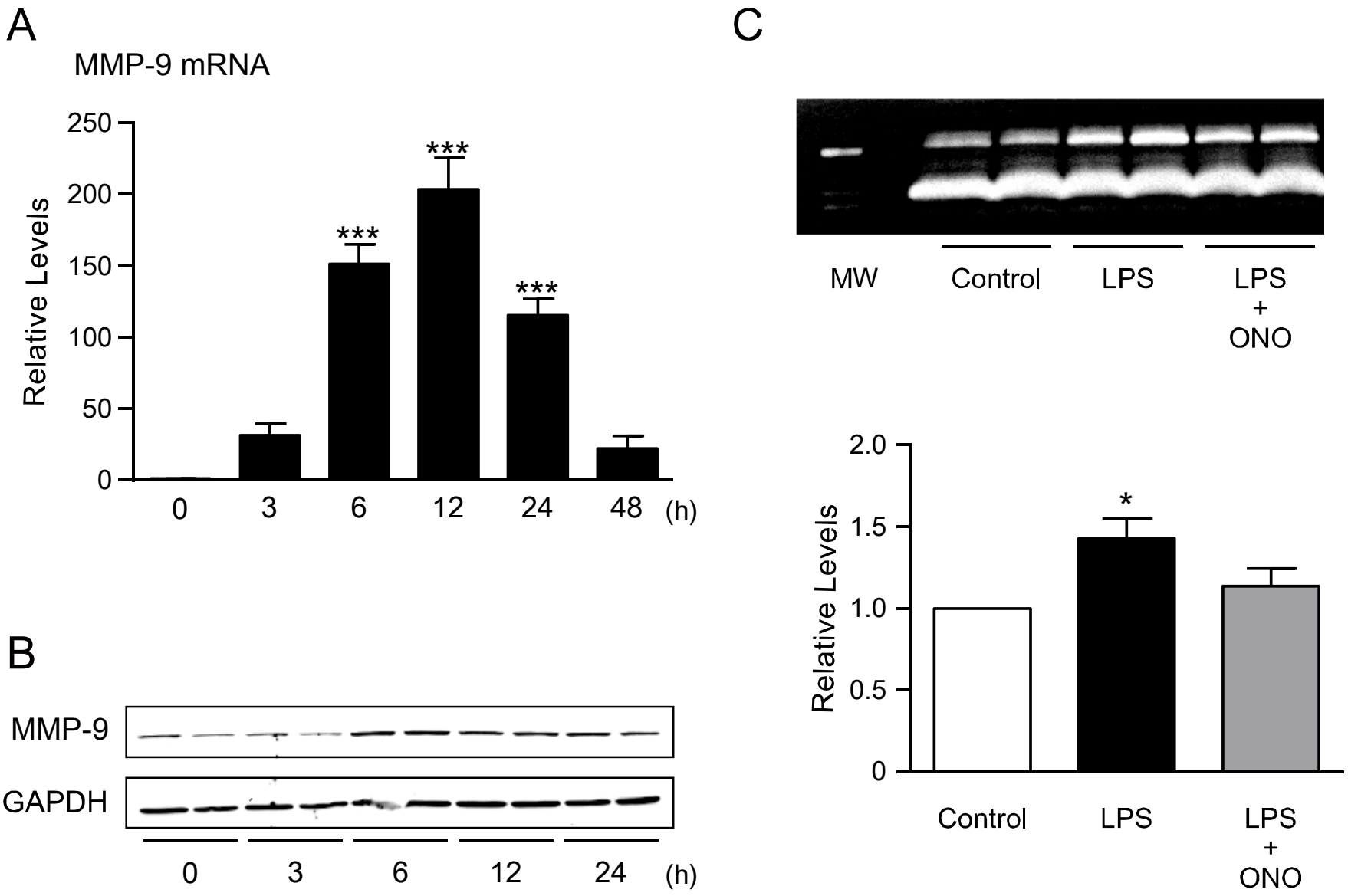
Figure 5 Hematoxylin & esosin and Azan stained left ventricular sections from sham-operated control (*A*), CLP (*B*), and ONO-4817-treated CLP mice (*C*). *Perivascular* (*upper*) and *interstitial* (*lower*) fibrotic areas are shown (*blue*). Shown are representative images from two independent experiments in which the same results were obtained.

Figure 6 Transthoracic echocardiography assessment of the effect of ONO-4817

treatment on LV dysfunction in CLP-induced septic mice. (*A*) Representative echocardiographic images for each of the three experimental groups. (*B*)-(*E*): The averaged data of LVDd (*B*), LVDs (*C*), FS (*D*), and EF (*E*) in the three groups are presented as mean ± SEM (n = 5 per group). ***Significant difference from sham (P<0.001). ##Significant difference from CLP alone (P<0.01).

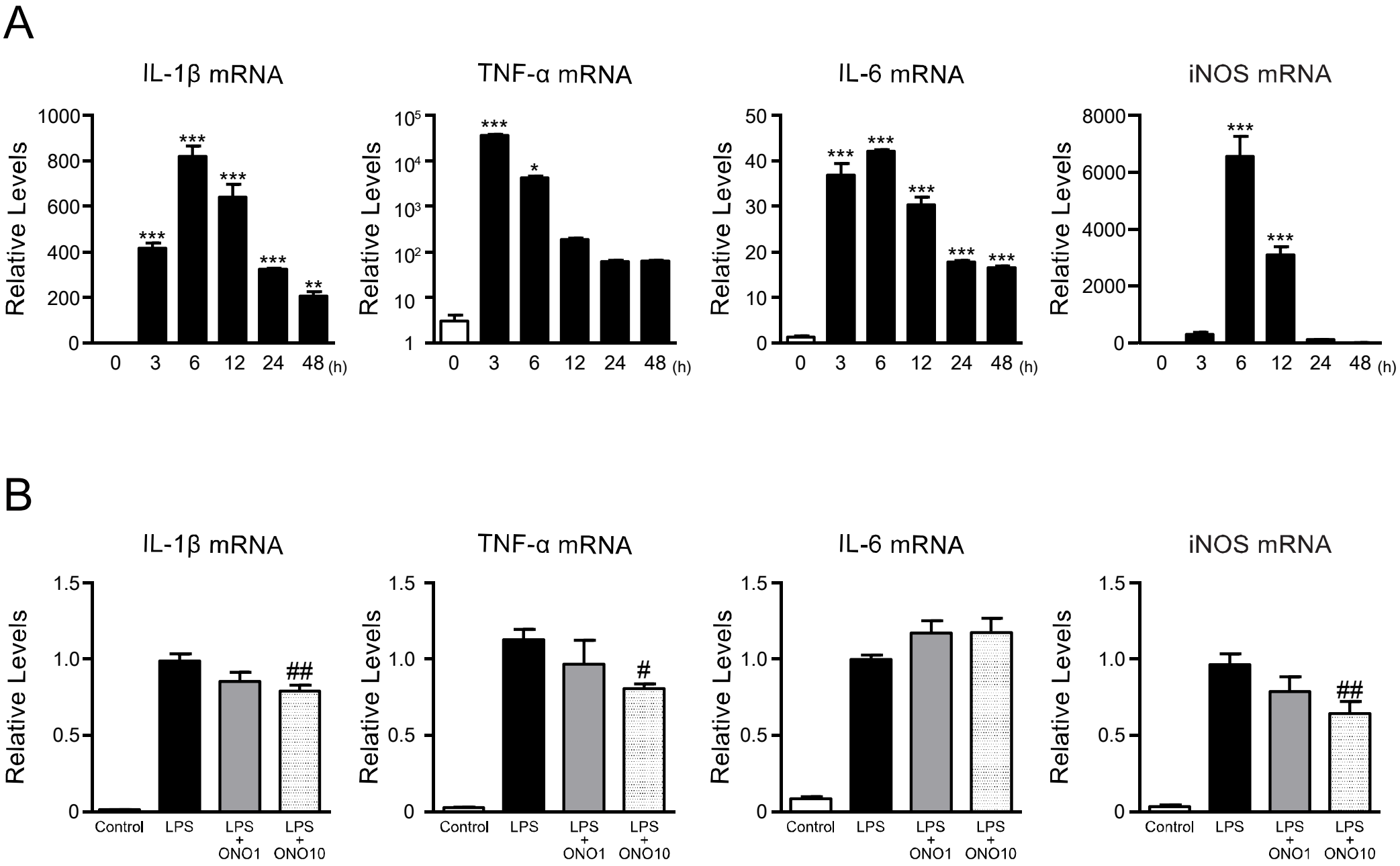
Figure(s)

Figure 1



Figure(s)

Figure 2



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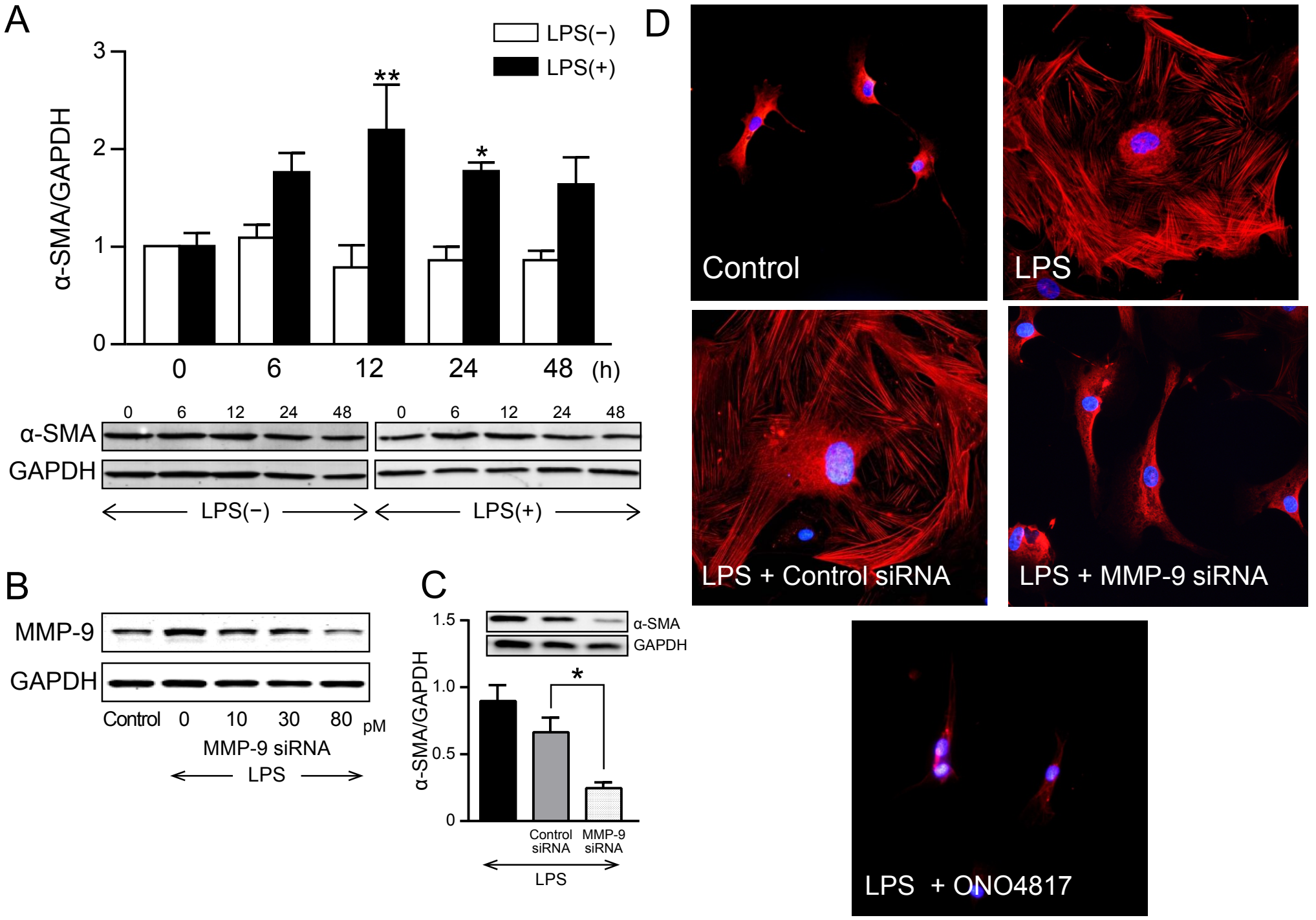

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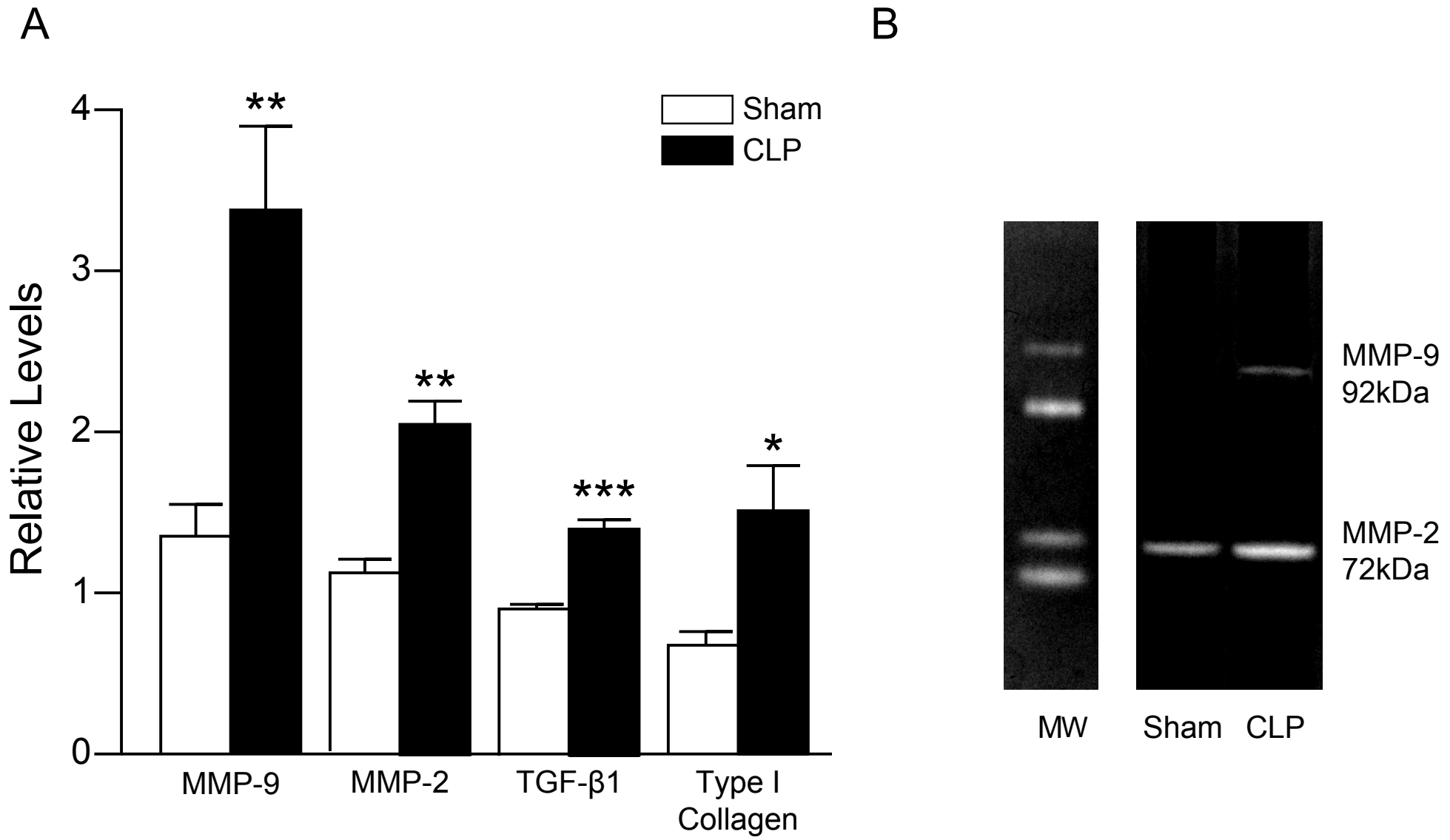

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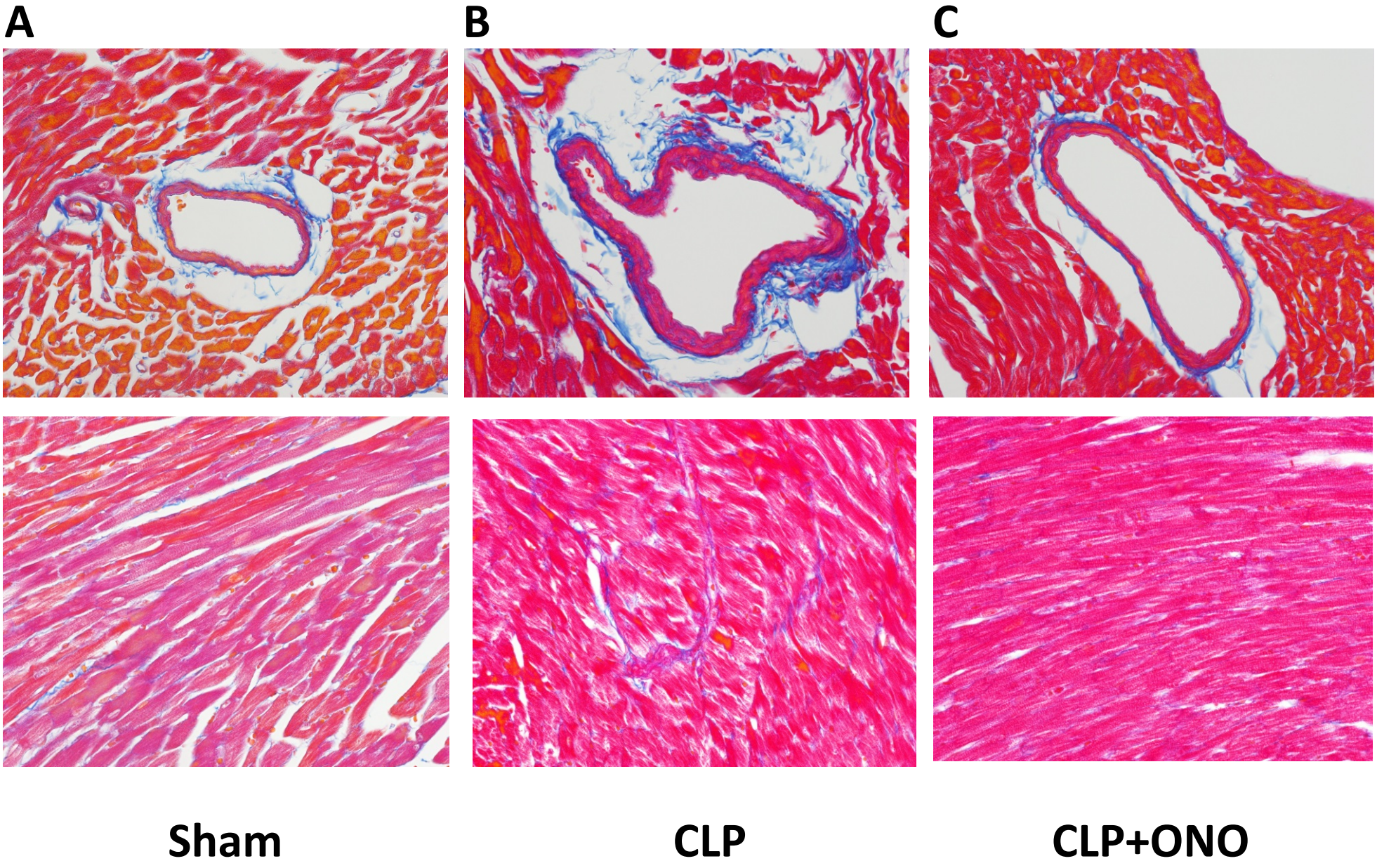
Figure 5



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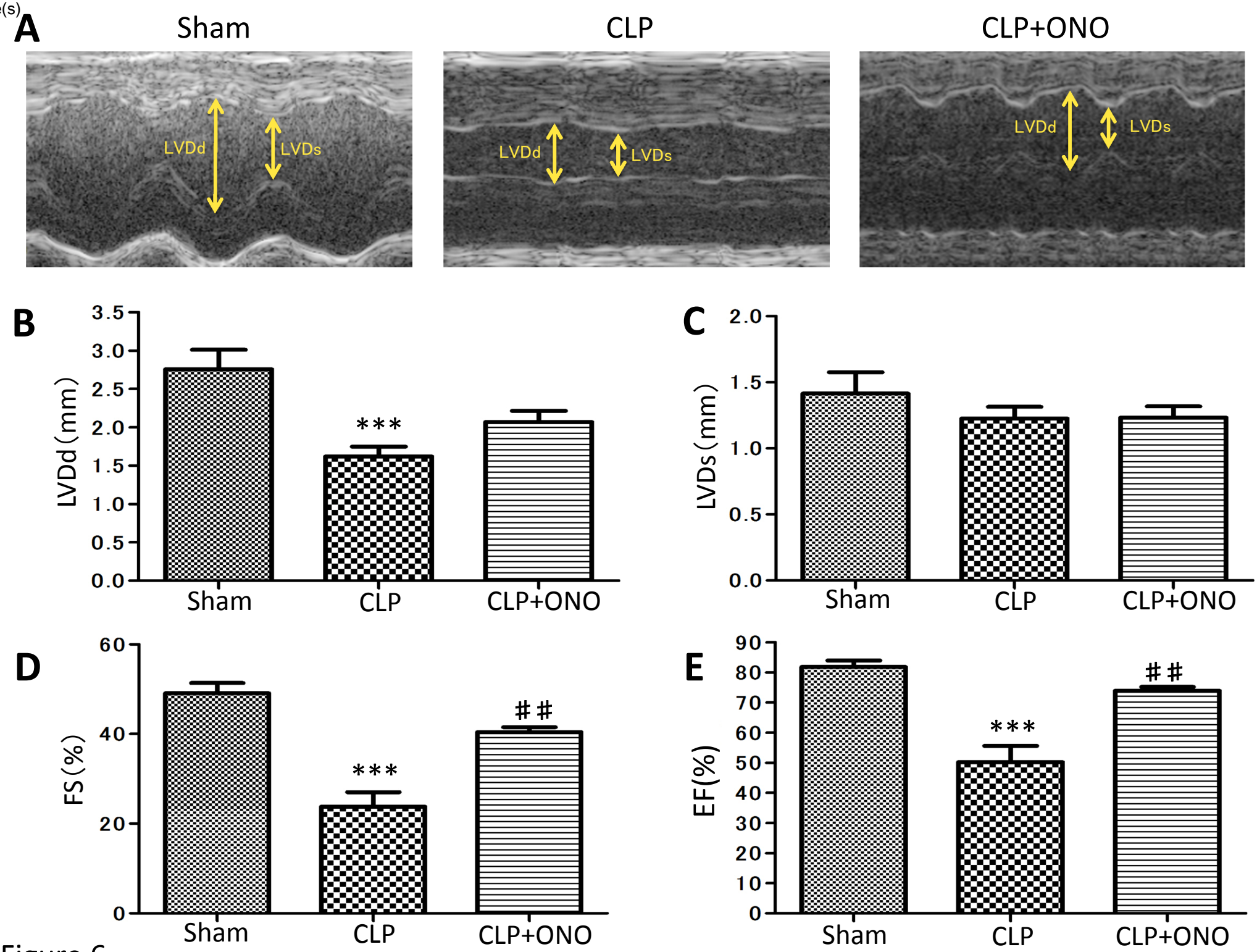

Figure 6

Supplementary Material

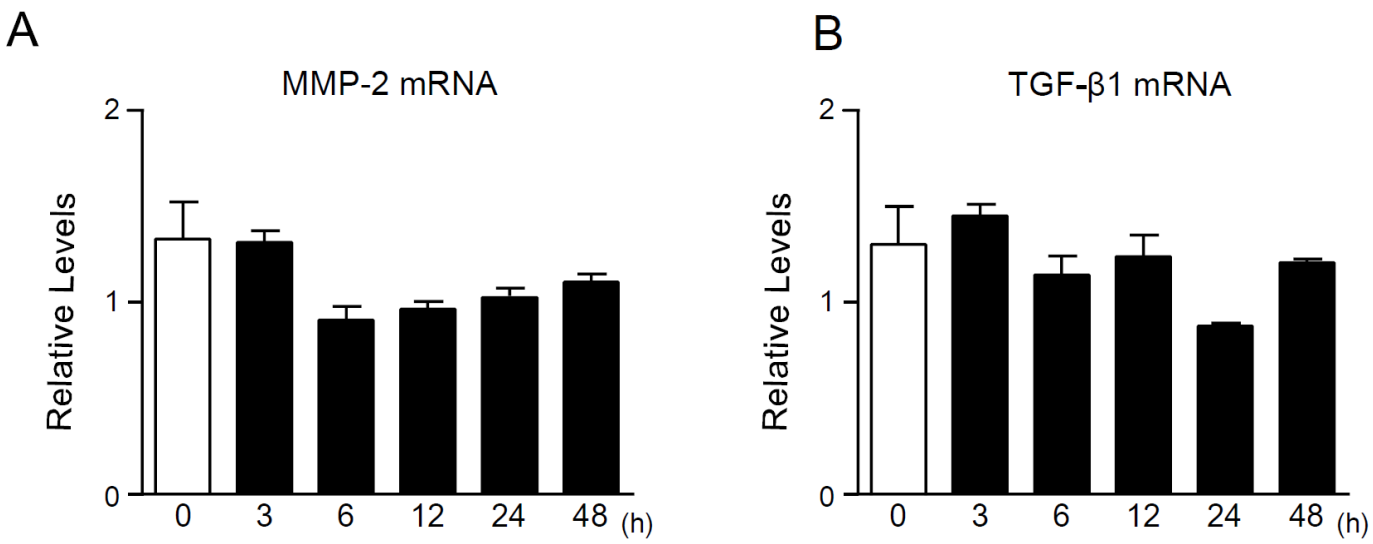

Figure S1. Lack of effect of LPS on mRNA levels of MMP-2 (*A*) and TGF-β_1 (*B*) in HCFs. The time course of mRNA levels after LPS is shown.
Expression was normalized to GAPDH, and the data represent the mean ± SEM (n=3).

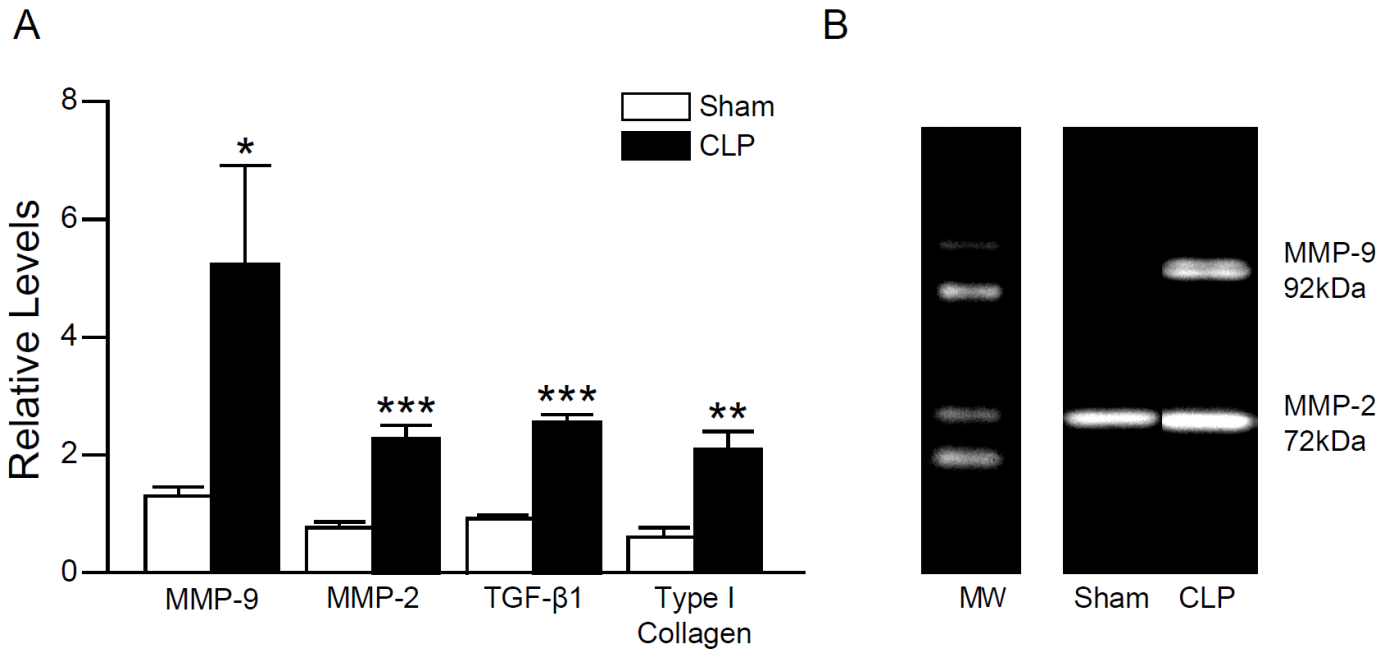

Figure S2. Cardiac atrial levels of mRNA expression of MMP-9, MMP-2, and TGF-β1 as well as type I collagen (*A*) and gelatin zymogram of atrial protein extracts (*B*) in sham-operated control and CLP-induced septic mice.
Expression was normalized to GAPDH, and the data represent the mean SEM (n=4-5). Significant difference from sham (*P<0.05, **P<0.01, ***P<0.001)