**Recombinant Human Annexin A5 Inhibits Pro-inflammatory Response and Improves Cardiac Function and Survival in Mice with Endotoxemia**

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**Supplemental Methods:**

## Hemodynamic Measurements. After 4 hours of LPS and/or recombinant human annexin A5 treatment, mice were anaesthetized with ketamine (50 mg/kg) and xylazine (12.5 mg/kg). A Millar pressure-conductance catheter (Model SPR-839, Size 1.4F) was inserted into the right carotid artery and advanced into the LV to measure hemodynamic parameters as we previously described ([1](#_ENREF_1)).

 ***Isolated Mouse Heart Preparation.*** After 4 hours of LPS and/or recombinant human annexin A5 treatment, mice were sacrificed. Mouse hearts were isolated and perfused in a Langendorff system to measure cardiac function as we previously described ([2](#_ENREF_2)). Contractile force and heart rate were measured a force-displacement transducer (FT03, Grass Instrument, West Warwick, RI).The heart work was calculated by multiplying the force (g) by the heart rate (beats/min) and normalized to heart weight.

***Real-Time RT-PCR.***TNF- and IL-1β mRNA levels in the LV myocardium and cardiomyocytes were determined by real-time RT-PCR using SYBR Green as we previously described ([3-5](#_ENREF_3)). The primers for TNF- were sense 5’ CCG ATG GGT TGT ACC TTG TC 3’; and antisense,5’ GGGCTG GGT AGA GAA TGG AT 3’. The primers for IL-1β were sense 5’ ACA AGG AGA ACC AAG CAA CGA C 3’ and antisense 5’ GCT GAT GTA CCA GTT GGG GAA C 3’. 28S rRNA was used as a loading control using primers for sense 5′ TTG AAA ATC CGG GGG AGA G 3′ and antisense 5′ ACA TTG TTC CAA CAT GCC AG 3′. Samples were amplified for 35 cycles using MJ Research Opticon Real-Time PCR machine (South San Francisco, CA). Levels of TNF- and IL-1β relative to 28S rRNA were obtained using a comparative CT method as our previous report ([6](#_ENREF_6)).

# *Measurement of TNF-α and IL-1 Protein Levels.* Myocardial and plasma TNF-α protein levels were measured using a mouse TNF-α ELISA kit (eBioscience, San Diego, CA) as described in our previous reports ([2](#_ENREF_2), [7](#_ENREF_7)). The LV myocardial tissues were homogenized in PBS. After centrifugation, the supernatant was collected for protein concentration and TNF-α ELISA. Myocardial TNF-α measurements were standardized with protein concentrations of each sample. Plasma IL-1 protein levels were determined using an IL-1 ELISA kit (eBioscience, San Diego, CA).

***Determination of p38, ERK1/2 and Akt Phosphorylation.*** Phosphorylated/total p38, ERK1/2 and Akt protein levels in heart tissues were measured by western blot analysis. Briefly, 50 μg of protein was separated by SDS-PAGE. Proteins were then transferred to nitrocellulose membranes and blots were probed with antibodies against p38 (1:800, Cell Signaling, Danvers, MA), phosphorylated p38 (Thr 180/Tyr 182, 1:800, Cell Signaling), ERK1/2 (1:800, Cell Signaling), phosphorylated ERK1/2 (Thr 202/Tyr 204, 1:800, Cell Signaling), Akt (1:1000, Cell Signaling), phosphorylated Akt (Ser 473, 1:500, Santa Cruz, CA), or phosphorylated TAK1 (Thr 184/187, 1:500, Cell Signaling). Blots were probed with horseradish peroxidase-conjugated secondary antibodies (1:2000, BioRad, Hercules, CA). Protein bands were detected using an enhanced chemiluminescence method and quantified by densitometry.

# *Adult Cardiomyocyte Culture.* Cardiomyocytes were isolated from the hearts of adult C57BL/6 mice. Hearts were mounted on a Langendorff apparatus and perfusedwith digestion buffer containing 45 μg/mL of liberase blendzyme IV (Roche) as we described ([5](#_ENREF_5)). The rod-shaped myocytes were then plated on laminin-coated 35-mm dishes at a density of 50 cells/mm2 and cultured for 6 hours at 37°C in a 2% CO2 incubator. This was followed by 4 hours of LPS (2.5 μg/ml) treatment with or without recombinant human annexin A5 (1.0 μg/ml).

**References:**

1. Xiang FL, Lu X, Hammoud L, et al. Cardiomyocyte-specific overexpression of human stem cell factor improves cardiac function and survival post myocardial infarction in mice. Circulation 2009;120:1065-1074.

2. Peng T, Lu X, Feng Q. Pivotal role of gp91phox-containing NADH oxidase in lipopolysaccharide-induced tumor necrosis factor-alpha expression and myocardial depression. Circulation 2005;111:1637-1644.

3. Burger DE, Xiang FL, Hammoud L, et al. Erythropoietin protects the heart from ventricular arrhythmia during ischemia and reperfusion via neuronal nitric-oxide synthase. J Pharmacol Exp Ther 2009;329:900-907.

4. Geoghegan-Morphet N, Burger D, Lu X, et al. Role of neuronal nitric oxide synthase in lipopolysaccharide-induced tumor necrosis factor-alpha expression in neonatal mouse cardiomyocytes. Cardiovasc Res 2007;75:408-416.

5. Zhang T, Lu X, Li J, et al. Inhibition of Na/K-ATPase promotes myocardial tumor necrosis factor-alpha protein expression and cardiac dysfunction via calcium/mTOR signaling in endotoxemia. Basic Res Cardiol 2012;107:1-12.

6. Hammoud L, Xiang F, Lu X, et al. Endothelial nitric oxide synthase promotes neonatal cardiomyocyte proliferation by inhibiting tissue inhibitor of metalloproteinase-3 expression. Cardiovasc Res 2007;75:359-368.

7. Peng T, Lu X, Lei M, et al. Inhibition of p38 MAPK decreases myocardial TNF-alpha expression and improves myocardial function and survival during acute endotoxemia in mice. Cardiovasc Res 2003;59:893-900.