Recombinant Human Annexin A5 Inhibits Proinflammatory Response and Improves Cardiac Function and Survival in Mice With Endotoxemia*

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Objectives: Annexin A5 is a 35-kDa protein with high affinity binding to negatively charged phospholipids. However, its effects on sepsis are not known. Our aim was to study the effects of annexin A5 on myocardial tumor necrosis factor-α expression, cardiac function, and animal survival in endotoxemia.

Design: Prospective experimental study.

Setting: University laboratory.

Subjects: Adult male C57BL/6 mice.

Interventions: Mice were challenged with lipopolysaccharide (4 or 20 mg/kg, i.p.) to induce endotoxemia with and without recombinant human annexin A5 treatment (5 or 10 μg/kg, IV). Cytokine expression and cardiac function were assessed, and animal survival was monitored.

Measurements and Main Results: Treatment with annexin A5 inhibited myocardial mitogen-activated protein kinase, and nuclear factor-κB activation in mice with endotoxemia. Furthermore, annexin A5-treated animals showed significant reductions in myocardial and plasma levels of tumor necrosis factor-α and interleukin-1β while cardiac function was significantly improved during endotoxemia. Additionally, 5-day animal survival was significantly improved by either an immediate or a 4-hour delayed annexin A5 treatment after lipopolysaccharide challenge. Importantly, annexin A5 dose-dependently inhibited lipopolysaccharide binding to a toll-like receptor-4/myeloid differentiation factor 2 fusion protein.

Conclusions: Annexin A5 treatment decreases cytokine expression and improves cardiac function and survival during endotoxemia. These effects of annexin A5 are mediated by its ability to inhibit lipopolysaccharide binding to toll-like receptor-4, leading to reductions in mitogen-activated protein kinase and Akt signaling. Our study suggests that annexin A5 may have therapeutic potential in the treatment of sepsis. (*Crit Care Med* 2014; 42:e32–e41)

Key Words: annexin A5; cardiac function; sepsis; tumor necrosis factor-α

Sepsis is a systemic inflammatory response to infection with an estimated 18 million cases annually worldwide and is a leading cause of in-hospital death (1, 2). Mortality rate is 30–40% in severe sepsis and 40–80% in septic shock (3). More than 750,000 people have severe sepsis each year in the United States (3–5). Hospitalizations for sepsis have doubled over the last 10 years and have overtaken those for myocardial infarction (4, 6). Sepsis is characterized by excessive proinflammatory cytokine production in response to bacterial infection. Initiation of the host's innate immune response is mediated through activation of pattern recognition receptors such as toll-like receptor (TLR) 2 and 4 for Gram-positive and -negative bacterial infections, respectively (7, 8). Lipopolysaccharide (LPS) released from Gram-negative bacteria binds to TLR4 in a CD14 and LPS binding protein-dependent manner. Activation of TLR4 upon LPS binding initiates a signaling pathway that leads to activation of mitogen-activated protein kinases (MAPKs) and production of inflammatory cytokines including tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) (7, 8). TNF-α is a major contributing factor to cardiac dysfunction leading to high mortality in severe sepsis and septic shock (9–12).

*See also p. 219.

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Despite decades of research, there is no specific treatment for sepsis. To date, the only sepsis specific therapy approved by the U.S. Food and Drug Administration is the recombinant activated protein C (Xigris, Eli Lilly, Indianapolis, IN), which is a potent anticoagulant and profibrinolytic agent (13). However, the inherent risk of severe bleeding due to its strong anticoagulant properties results in a contraindication for many patients (14). After the recent Prospective Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis and Septic Shock (PROWESS-SHOCK) trial demonstrated no survival benefit of Xigris over placebo (15), Eli Lilly announced voluntary withdrawal of Xigris from all markets in 2011 (16). With increasing number of sepsis cases every year due to an ageing population and high mortality associated with this disease (3–5), there is a pressing need to find a safe and effective therapy for sepsis (1, 2).

Annexin A5 (Anx5) is a 35-kDa phospholipid binding protein and a member of the 13 annexin protein family (17). It binds to anionic phospholipids in particular phosphatidylserine on the plasma membrane in a calcium-dependent manner and has antiapoptotic and anticoagulant properties by forming a protective 2D crystallized shield over the surface of cells where phosphatidylserine is exposed (18). This protective shield sequesters the phospholipid sites and decreases their ability to initiate phagocytosis or thrombosis. Furthermore, Anx5 has been shown to interact with cell receptors and inhibit their function by binding to leucine-rich repeats through a conserved N-terminal sequence (19). Interestingly, the LPS binding region of the extracellular domain of TLR4 receptors contains 21 separate leucine-rich repeats (20). It is possible that Anx5 may inhibit LPS binding to TLR4 receptors via its interaction with the leucine-rich repeats, leading to decreases in downstream MAPK signaling. In the present study, we hypothesized that Anx5 improves cardiac function and animal survival during sepsis by inhibiting LPS binding to the TLR4/myeloid differentiation factor 2 (MD-2) receptor complex. A mouse model of endotoxemia was employed to simulate sepsis. Our results showed that treatment with Anx5 decreased myocardial TNF-α expression and improved cardiac function and survival in mice with endotoxemia. Our study suggests that Anx5 may have novel therapeutic potential in sepsis.

MATERIALS AND METHODS

Animals

The investigation conforms with the *Guide for the Care and Use of Laboratory* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). Use of animals was approved by the Animal Use Subcommittee at the University of Western Ontario, Canada. C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). A breeding program was carried out at the Lawson Health Research Institute animal care facility to produce offspring. Adult male mice (3–4 months old) weighing 21–26 g were studied.

Experimental Protocols

Mice were randomly assigned to the following groups: saline (control, $n = 27$), recombinant human Anx5 ($n = 24$), LPS ($n = 26$), and LPS plus recombinant human Anx5 treatment group ($n = 26$). LPS extracted from *Salmonella typhosa* (Cat. L7136, Sigma-Aldrich, St. Louis, MO; 4 mg/kg, i.p.) was administered to simulate sepsis. Mice were treated with an injection of Anx5 (BioVision, Mountain View, CA; 5 μg/kg, IV) immediately after LPS administration. Four hours after LPS administration, mice were anesthetized with an i.p. injection of ketamine and xylazine mixture, and in vivo cardiac function was measured using a Millar pressure-conductance catheter (Millar Instruments, Houston, TX) (21–23). Mice were killed, blood was drawn, and hearts were isolated. Ex vivo cardiac function was also measured using a Langendorff heart preparation (24, 25). At the end of cardiac function measurements, plasma and hearts were stored in a –80°C freezer for cytokine expression analysis. To measure MAPK phosphorylation, mice were killed after 30 minutes of LPS with and without Anx5 treatment.

To study the effects of recombinant human Anx5 on animal survival during endotoxemia, mice were randomly assigned to the following three treatment groups and survival was monitored for 5 days:

1. LPS (20 mg/kg, i.p.) treatment alone ($n = 22$).
2. Immediate Anx5 treatment group ($n = 16$): An LPS (20 mg/kg, i.p.) injection was followed by an immediate administration of recombinant human Anx5 (10 μg/kg, IV).
3. Delayed Anx5 treatment group ($n = 23$): 4 hours after an LPS (20 mg/kg, i.p.) injection, recombinant human Anx5 (10 μg/kg, IV) was administered.

Nuclear Factor (NF)-κB Activity Assay

NF-κB binding activity was determined using the NF-κB p65 Transcription Factor Assay Kit (Abcam, Toronto, Canada) according to the manufacturer's instructions. Nuclear proteins (50 μg) isolated from the left ventricular (LV) myocardium were incubated in a 96-well plate precoated with a specific double-stranded (ds) DNA sequence containing the NF-κB response element. The transcription factor bond to the specific dsDNA was detected by rabbit anti-p65 antibody and followed by a goat anti-rabbit horseradish peroxidase (HRP) secondary antibody. Absorbance was measured at 450 nm using SpectraMax M5 (Molecular Devices, Sunnyvale, CA) microplate reader. Wells with competitor dsDNA served as nonspecific binding controls.

LPS/TLR4 Binding Activity Assay

An enzyme-linked immunosorbent assay (ELISA)-based LPS binding assay was performed to study the effects of recombinant human Anx5 on the binding activity of biotinylated LPS (biotin-LPS) to a designed TLR4/MD-2 fusion protein as previously described (26, 27). The TLR4/MD-2 fusion protein is His tagged on the C-terminus and Flag tagged on its N-terminus and contains the extracellular domain of mouse TLR4 and mouse MD-2, which are fused with a flexible linker (26). The p3xFlag-CMV-8 expression vector containing TLR4/MD-2 complementary DNA sequence

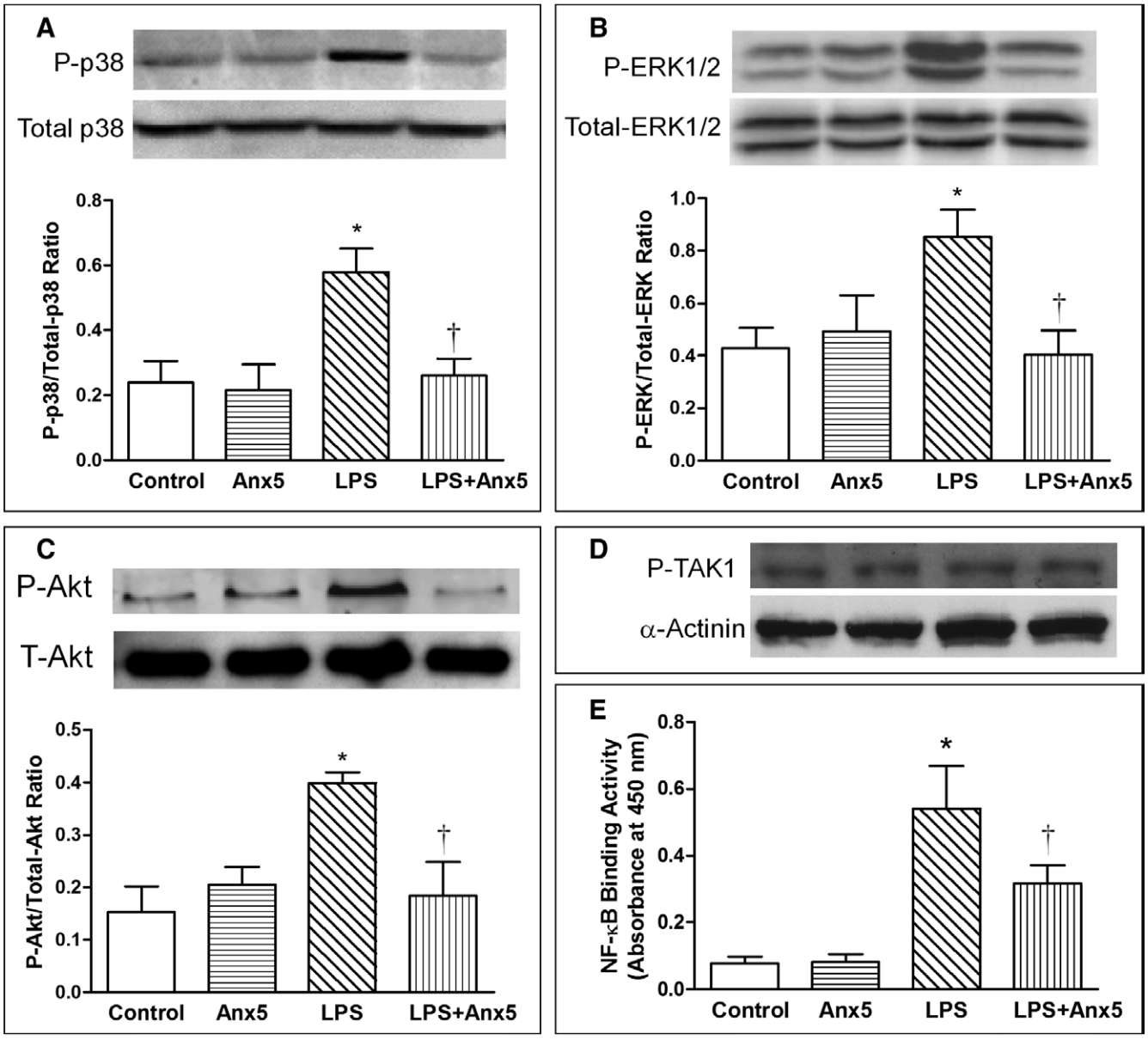

Figure 1. Annexin A5 (Anx5) decreases myocardial mitogen-activated protein kinase phosphorylation and nuclear factor (NF)-κB activation during endotoxemia. Mice were treated with saline (control, 100 μL, i.p.), Anx5 (5 μg/kg, IV), lipopolysaccharide (LPS) (4 mg/kg, i.p.), or LPS plus Anx5 for 30 min. Phosphorylation of myocardial p38 (**A**), ERK1/2 (**B**), Akt (**C**), and TAK1 (**D**) by Western blot analysis. **E**, NF-κB binding activity in the nuclear proteins isolated from the heart was determined using an enzyme-linked immunosorbent assay kit. *$p < 0.01$ versus control; †$p < 0.01$ versus LPS; $n = 4$–6 per group for A–C and E, $n = 2$ per group for D.

was transfected in HEK 293 cells using lipofectamine 2000 (Invitrogen, Burlington, ON, Canada). The expression of the fusion protein was confirmed by Western blot analysis using an anti-His antibody. The expressed protein was purified by nickel-nitrilotriacetic acid magnetic beads (Qiagen, Toronto, ON, Canada). The purified TLR4/MD-2 fusion protein was then added to the anti-Flag antibody-coated 96-well plates (Sigma, Oakville, ON, Canada), which was followed by incubation with biotin-labeled LPS (1 μg/mL) in the presence of unlabeled LPS (100 μg/mL) or increasing concentrations of recombinant human Anx5 (0.5, 5, and 50 ng/mL) for 2 hours at room temperature. The binding activity of biotin-labeled LPS to TLR4/MD-2 fusion protein was assessed through streptavidin-conjugated HRP with tetramethylbenzidine as a chromogenic substrate, which was read at 450 nm using a microplate reader (SpectraMax M5, Molecular Devices).

Dot Blot Analysis

Binding of Anx5 to LPS was studied using a dot blot method previously described (28). LPS from *S. typhosa* (Cat. L7136, Sigma) and *Pseudomonas aeruginosa* (Cat. L9143, Sigma) was applied to polyvinylidene difluoride (PVDF) membrane and incubated for 1 hour in a wet chamber. The blots were washed and incubated with 3% bovine serum albumin (BSA) to block nonspecific binding. The blots were then incubated with Anx5

(2 μg/mL) in the presence of 5 mM $CaCl_2$ for 1 hour. Primary rabbit anti-Anx5 antibody was applied and followed by IRDye 680RD goat anti-rabbit secondary antibody. Signals were detected using Li-Cor Odyssey CLx infrared imaging system (Mendel Scientific, Guelph, ON, Canada).

Coimmunoprecipitation

Coimmunoprecipitation of TLR4 and Anx5 was performed using the Dynabead Protein G Immunprecipitation kits (Invitrogen, Carlsbad, CA). Briefly, the magnetic dynabeads were suspended in an antibody-binding buffer with 20 μg of TLR4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour. Myocardial tissues were homogenized and 1 mg of protein from each sample was used. Human recombinant Anx5 (500 ng) was added to the tissue samples and incubated with the TLR4-coated beads for 2 hours. The samples were eluted from the dynabeads and equally loaded on a 10% polyacrylamide gel. Western blotting was performed to detect Anx5 and TLR4 using anti-Anx5 (1:2,000, Biovision, Mountain View, CA) and anti-TLR4 antibodies (1:2,000, Santa Cruz Biotechnology), respectively.

Statistical Analysis

All results are expressed as mean ± SEM. For multigroup comparisons, one-way analysis of variance (ANOVA) was employed, and when overall ANOVA result was significant, Tukey's pair-wise comparisons were performed. Animal survival was analyzed by the method of Kaplan-Meier followed by Log-rank test. Statistical significance was assigned when a p value was less than 0.05.

For hemodynamic measurements; isolated heart preparation; real-time reverse transcriptase-polymerase chain reaction (RT-PCR); TNF-α and IL-1β ELISA; phosphorylation of p38, ERK1/2, and Akt; and adult cardiomyocyte culture, please see **supplemental data** (Supplemental Digital Content 1, http://links.lww.com/CCM/A732).

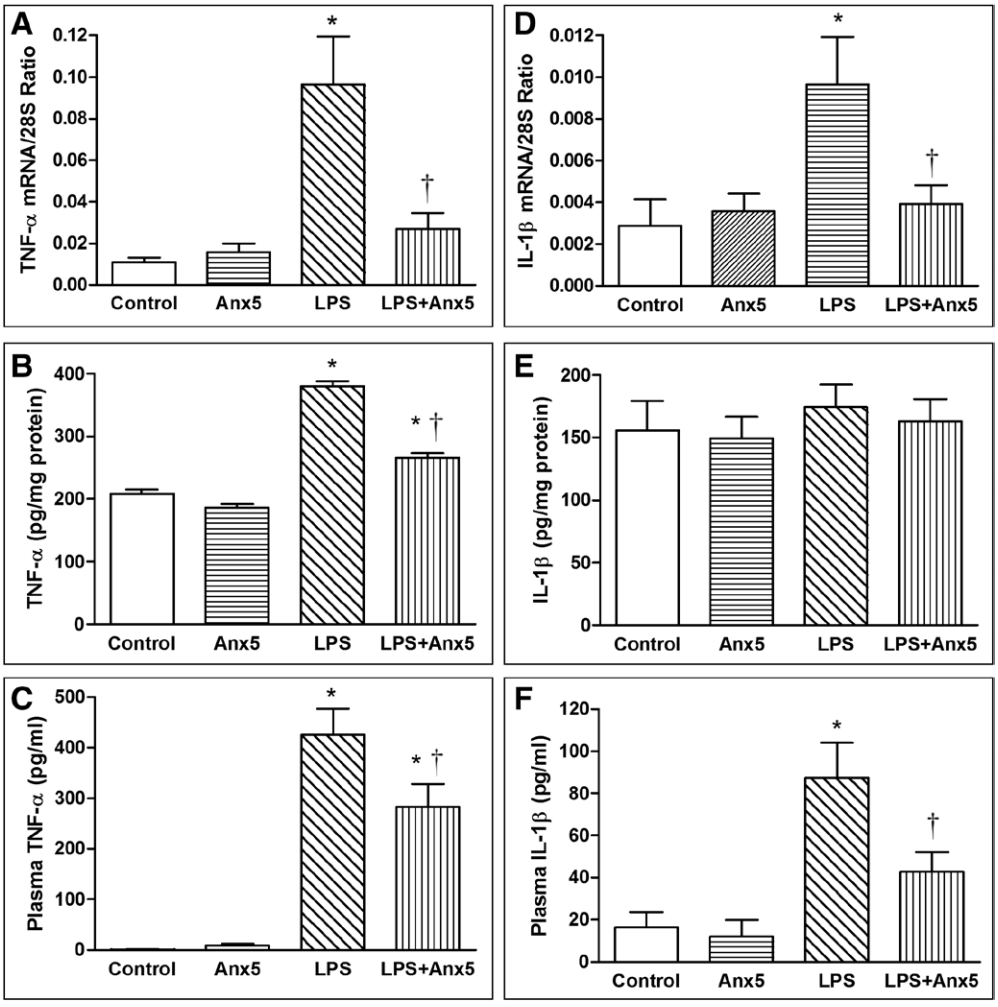

Figure 2. Effects of annexin A5 (Anx5) on tumor necrosis factor (TNF)-α and interleukin (IL)-1β production in endotoxemic mice. Mice were treated with saline (control, 100 μL, i.p.), Anx5 (5 μg/kg, IV), lipopolysaccharide (LPS) (4 mg/kg, i.p.), or LPS plus Anx5 for 4 hr. Treatment with Anx5 significantly decreased myocardial TNF-α messenger RNA (mRNA) (**A**) and protein (**B**) and myocardial IL-1β mRNA (**D**) during endotoxemia. No significant changes in myocardial IL-1β protein levels (**E**) were observed among all four groups. **C** and **F**, Plasma levels of TNF-α and IL-1β were significantly decreased by Anx5 treatment during endotoxemia. *$p <$ 0.01 versus control; †$p < 0.05$ versus LPS; $n = 8$–12 per group.

RESULTS

Anx5 Inhibits MAPK, Akt, and NF-κB Activation in Endotoxemia

To study the effects of Anx5 on myocardial MAPK and Akt phosphorylation, mice were randomly assigned to saline, recombinant human Anx5 (5 μg/kg, IV), LPS (4 mg/kg, i.p.), or LPS plus recombinant human Anx5 treatment groups. After 30 minutes of LPS and/or Anx5 treatment, mice were killed and hearts were isolated to assess p38 and ERK1/2 phosphorylation using Western blot analysis. Our data showed that myocardial p38, ERK1/2, and Akt phosphorylation was increased in mice with endotoxemia. Treatment with Anx5 inhibited LPS-induced p38, ERK1/2, and Akt phosphorylation to control levels ($p < 0.01$) (**Fig. 1*A*–*C***). TAK1 also known as MAP3K7 is involved in LPS-induced signaling in macrophages (29). However, TAK1 phosphorylation was not induced in the heart by LPS or affected by Anx5 treatment (**Fig. 1*D***). NF-κB, a downstream signaling molecule of MAPK and Akt, was activated in the heart

by LPS. Anx5 treatment significantly inhibited myocardial NF-κB activation during endotoxemia ($p < 0.01$) (**Fig. 1*E***).

Anx5 Inhibits TNF-α and IL-1β Expression in Endotoxemia

To assess TNF-α messenger RNA (mRNA) and protein expression, mice were treated with saline, recombinant human Anx5, LPS, or LPS plus recombinant human Anx5. Four hours after these treatments, myocardial TNF-α mRNA and protein levels were determined by real-time RT-PCR and ELISA, respectively. Results showed that both TNF-α mRNA and protein levels were significantly increased in the LV myocardium in LPS-treated mice compared with saline-treated controls ($p < 0.01$) (**Fig. 2, *A*** and ***B***). Treatment with Anx5 decreased TNF-α expression induced by LPS ($p < 0.05$) (Fig. 2, *A* and *B*). LPS also induced myocardial IL-1β mRNA expression, which was inhibited by Anx5 treatment ($p < 0.05$) (**Fig. 2*D***). However, myocardial IL-1β protein levels were not significantly different among all four groups (**Fig. 2*E***). Interestingly, both TNF-α and IL-1β plasma levels induced by LPS were inhibited by Anx5 treatment ($p < 0.05$) (**Fig. 2, *C*** and ***F***).

In order to determine the direct effects of Anx5 on TNF-α and IL-1β expression in cardiomyocytes, adult cardiomyocytes were isolated and cultured. Consistent with the in vivo data reported above, LPS significantly increased TNF-α and IL-1β mRNA levels measured by real-time RT-PCR in the cultured adult cardiomyocytes and the response was abrogated by recombinant human Anx5 treatment ($p < 0.05$) (**Fig. 3, *A*** and ***B***).

Anx5 Improves Cardiac Function in Endotoxemia

Four hours after treatment with saline, recombinant human Anx5, LPS, or LPS plus recombinant human Anx5, mice were anesthetized and cardiac function was measured using a Millar pressure-conductance catheter. Following LPS treatment, mean artery pressure, derivatives of left ventricular pressures (LV $+dP/dt_{max}$ and $-dP/dt_{min}$), pressure at maximal dP/dt ($P@dP/dt_{max}$), LV end systolic pressure (LVESP), LV end diastolic pressure, stroke work, and maximal power were significantly decreased ($p < 0.01$), whereas time constant of isovolumic relaxation (Tau) was significantly increased ($p < 0.01$). Treatment with Anx5 significantly increased LVESP and $P@dP/dt_{max}$ and decreased Tau in endotoxemic mice ($p < 0.05$) (**Table 1**). Importantly, LV $+dP/dt_{max}$ and $-dP/dt_{min}$ were significantly increased in LPS plus Anx5 group compared with the LPS treatment alone group ($p < 0.05$) (**Fig. 4**).

The ex vivo cardiac function was assessed using a Langendorff heart preparation after mice were treated with saline, recombinant human Anx5, LPS, or LPS plus recombinant human Anx5 for 4 hours. Results showed that rate of contraction ($+dF/dt_{max}$) and relaxation ($-dF/dt_{min}$), contractile force, and heart work were significantly decreased in LPS-treated group compared with control group ($p < 0.001$) (**Fig. 5*A*–*C***). Treatment with Anx5 in the endotoxemic mice restored cardiac function to control levels ($p < 0.01$) (Fig. 5*A*–*C*) without any significant changes in heart rate (p = not significant) (**Fig. 5*D***).

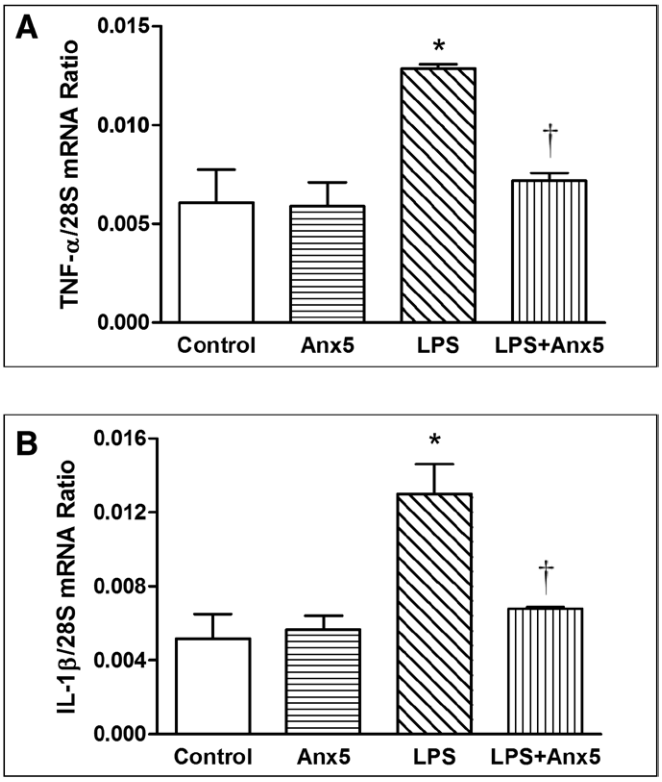

Figure 3. Annexin A5 (Anx5) inhibits tumor necrosis factor (TNF)-α and interleukin (IL)-1β mRNA expression in adult cardiomyocytes. Adult cardiomyocytes were cultured on 35-mm dishes. Cells were treated with lipopolysaccharide (LPS) (2.5 μg/mL) in the presence or absence of Anx5 (1 μg/mL) for 4 hr. TNF-α (**A**) and IL-1β (**B**) messenger RNA (mRNA) levels were determined by real-time reverse transcriptase-polymerase chain reaction analysis with 28S as a loading control. *$p < 0.01$ versus control, †$p < 0.05$ versus LPS; $n = 3$–4 independent experiments per group.

Anx5 Improves Animal Survival in Endotoxemia

To study the effects of recombinant human Anx5 on animal survival during endotoxemia, mice were treated with LPS alone, LPS with immediate Anx5 treatment, or LPS with delayed Anx5 treatment (equivalent of clinical treatment). Animal survival was monitored for 5 days (or 120 hr). Following an LPS injection, mice started to die at 12 hours with most of the death occurring within 48 hours, and by 120 hours, animal survival was reduced to 9% (**Fig. 6**). Treatment with Anx5 immediately after an LPS injection resulted in a 5.6-fold increase in animal survival to 50% ($p = 0.0047$). Importantly, a delayed Anx5 treatment also induced a 4.3-fold increase in mouse survival to 39% ($p = 0.0127$) (Fig. 6). These data show that treatment with Anx5 improves animal survival in endotoxemia.

Anx5 Inhibits LPS Binding to TLR4/MD-2 Fusion Protein

To study the effects of Anx5 on LPS binding to its receptor TLR4/MD-2 complex, a TLR4/MD-2 fusion protein was produced and an ELISA-based assay was employed as shown in **Figure 7*A***. Biotin-labeled LPS (1 μg/mL) strongly binds to the TLR4/MD-2 fusion protein, which was competitively inhibited

TABLE 1. In Vivo Hemodynamic Measurements in Mice With Endotoxemia

Variables	Saline	Anx5	LPS	LPS + Anx5
n	10	10	9	10
Heart rate, beats/min	412±23	399±8	474±19	486±19
Mean artery pressure, mm Hg	86±3	83±5	49±3[a]	59±4[a,b]
Left ventricular ejection fraction, %	63±6	65±7	49±4	54±7
Cardiac output, μL/min	5,188±834	3,925±563	3,153±786	4,386±928
Stroke work, mm Hg·μL	967±178	750±119	319±119[c]	486±156
Left ventricular pressure at maximal d*P*/d*t*, mm Hg	64±2	64±5	31±2[a]	40±3[a,b]
Left ventricular end systolic pressure, mm Hg	102±5	97±9	66±2[a]	77±4[a,b]
Left ventricular end diastolic pressure, mm Hg	6.9±0.9	7.2±1.0	3.6±0.2[a]	4.9±0.5[c]
Left ventricular end diastolic volume, μL	20±4	15±3	13±4	17±4
Time constant of isovolumic relaxation, ms	7.9±0.3	8.1±0.5	10.2±0.4[a]	8.6±0.6[b]
Maximal power, mW	6.1±1.1	4.4±0.8	2.0±0.5[a]	3.7±0.9

Anx5 = annexin A5, LPS = lipopolysaccharide.
[a]$p < 0.01$ versus saline.
[b]$p < 0.05$ versus LPS.
[c]$p < 0.05$ versus saline.

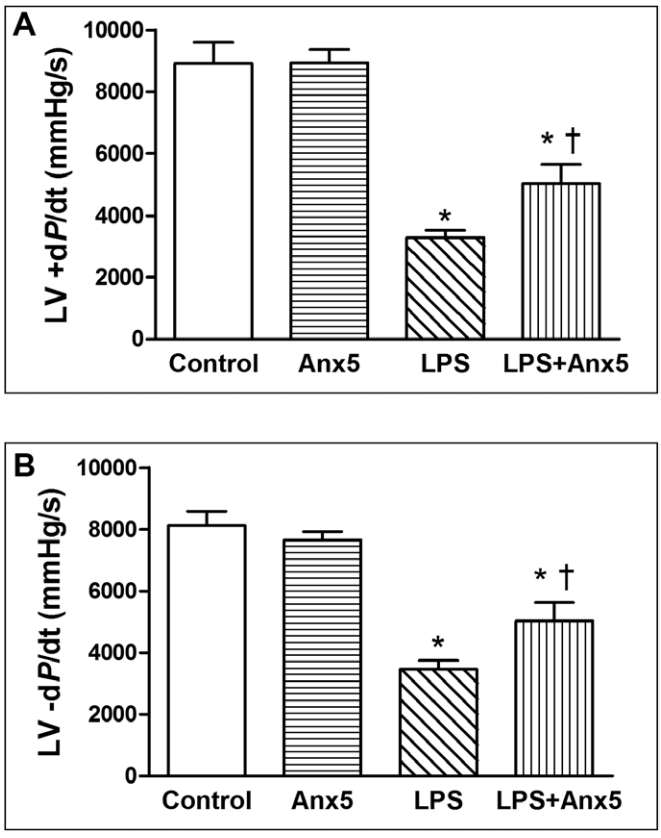

Figure 4. Annexin A5 (Anx5) improves in vivo cardiac function in mice with endotoxemia. Mice were treated with saline (control, 100 μL, i.p.), Anx5 (5 μg/kg, IV), lipopolysaccharide (LPS) (4 mg/kg, i.p.), or LPS plus Anx5 for 4 hr. Anx5 treatment significantly increased left ventricular (LV) +d*P*/d*t* (**A**) and −d*P*/d*t* (**B**) in mice with endotoxemia. *$p < 0.01$ versus control; †$p < 0.05$ versus LPS; $n = 9$–10 per group.

by 100 times unlabeled LPS (**Fig. 7*B***). Human recombinant Anx5 at increasing doses (0.5, 5, and 50 ng/mL) significantly inhibited biotin-labeled LPS binding to the TLR4/MD-2 fusion protein ($p < 0.001$) (Fig. 7*B*). These results show that Anx5 dose-dependently inhibits LPS binding to the TLR4/MD-2 receptor complex.

To further determine interactions between Anx5 and TLR4 receptors, coimmunoprecipitation was performed. Myocardial tissues from saline- and LPS-treated mice were homogenized and incubated with Anx5. Magnetic dynabeads were coated with anti-TLR4 antibody to pull down TLR4 and blot for Anx5. Recombinant human Anx5 was used as a positive control. Results showed that Anx5 was coimmunoprecipitated with TLR4 in myocardial samples from both saline- and LPS-treated mice (**Fig. 7*C***). The data indicate a strong interaction between Anx5 and TLR4 in the myocardium.

To investigate if recombinant Anx5 binds to LPS, a dot blot analysis was performed. Anx5 (0.5 μg, positive control), BSA (10 μg, negative control), LPS from *S. typhosa* (5 and 10 μg), and LPS from *P. aeruginosa* (5 and 10 μg) were loaded to a PVDF membrane and incubated with Anx5 (2 μg/mL) in the presence of 5 mM $CaCl_2$ for 1 hour. An anti-Anx5 antibody was applied and recombinant Anx5 showed a very strong signal. However, no signals were detectable for BSA or LPS (**Fig. 7*D***), indicating that annexing A5 does not bind to LPS from either strain of Gram-negative bacteria.

DISCUSSION

The present study examined the effects of recombinant human Anx5 in a mouse model of endotoxemia induced by LPS to

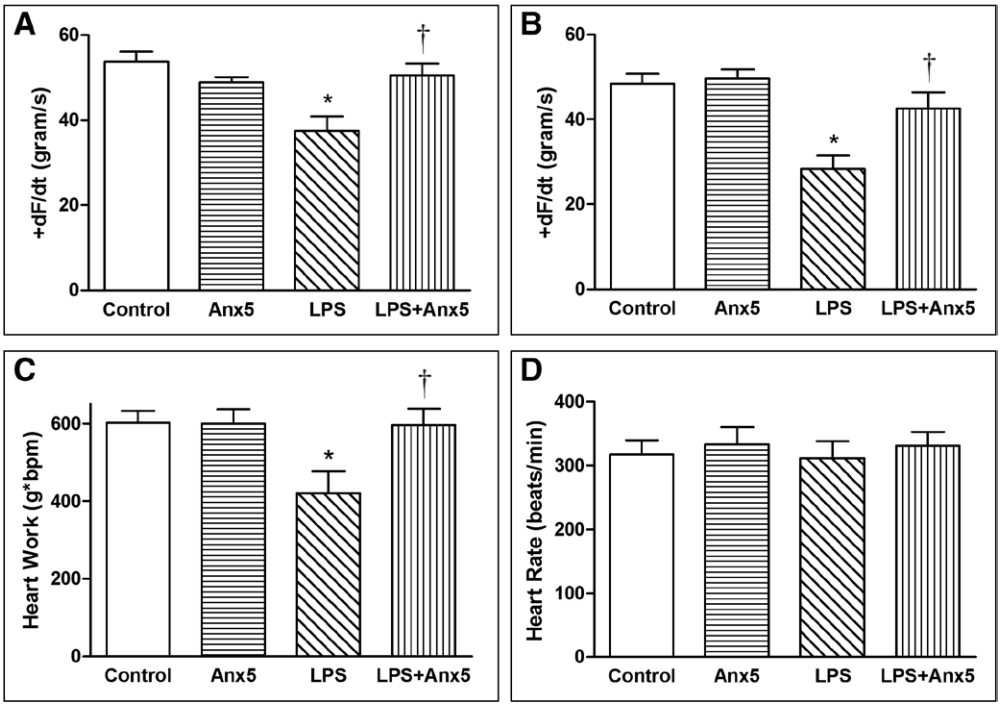

Figure 5. Annexin A5 (Anx5) improves ex vivo cardiac function in mice with endotoxemia. Mice were treated with saline (control, 100 μL, i.p), Anx5 (5 μg/kg, IV), lipopolysaccharide (LPS) (4 mg/kg, i.p.), or LPS plus Anx5 for 4 hr. Anx5 treatment significantly increased +d*F*/d*t* (**A**), −d*F*/d*t* (**B**), and heart work (**C**) without significant changes in heart rate (**D**) in mice with endotoxemia. *$p < 0.01$ versus control; †$p < 0.05$ versus LPS; $n = 4$–7 per group. bpm = beats/min.

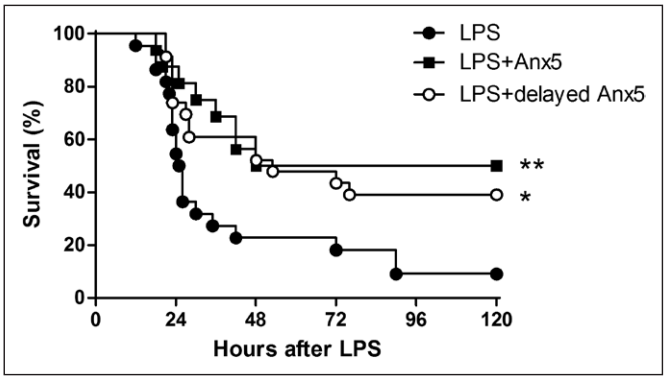

Figure 6. Annexin A5 (Anx5) improves survival in mice with endotoxemia. Kaplan-Meier survival curves of mice treated with lipopolysaccharide (LPS) alone (9%, $n = 22$) (*filled circles*), LPS followed immediately by Anx5 (50%, $n = 16$) (*filled squares*), or delayed Anx5 treatment after 4 hr of LPS administration (39%, $n = 23$) (*open circles*). Doses for LPS and Anx5 were 20 mg/kg, i.p., and 10 μg/kg, IV, respectively. A single injection of Anx5 was applied to all mice in both immediate and delayed treatment groups. Animal survival was significantly increased after either immediate or delayed Anx5 treatment. *$p = 0.0127$, **$p = 0.0047$ versus LPS group by Log-rank test.

simulate severe sepsis in humans (24, 30). We demonstrated for the first time that recombinant human Anx5 treatment decreases cytokine expression and improves cardiac function and animal survival during endotoxemia. We further showed that these beneficial effects of Anx5 are mediated by inhibiting LPS binding to the TLR4/MD-2 receptor complex, leading to reductions in MAPK, Akt, and NF-κB signaling (**Fig. 8**). Our study suggests that Anx5 may have novel therapeutic potential in clinical treatment of sepsis.

A significant benefit of Anx5 treatment observed in our study is a 5.6-fold increase of animal survival in endotoxemia. This was observed when Anx5 was administered immediately after an LPS challenge. To further evaluate the therapeutic potential of Anx5 in sepsis, animals were treated with Anx5 4 hours after an LPS administration when clinical sepsis was well established with systemic inflammatory cytokine expression and significant impairment in cardiac function (Figs. 2–5). Importantly, the delayed Anx5 treatment still shows a 4.3-fold increase in animal survival in endotoxemia. This result is particularly relevant to clinical treatment after sepsis diagnosis is established in patients, which usually occurs hours or even days after a bacterial infection. Since concomitant Anx5 treatment with LPS shows a greater survival benefit, our study also suggests that Anx5 may prevent the development of sepsis.

Myocardial dysfunction is common in patients with severe sepsis and renders septic patients at high risk of developing multiple organ failure, which is associated with a high mortality (31). TNF-α is one of the proinflammatory cytokines responsible for cardiac dysfunction during sepsis (11, 32). In fact, cardiomyocytes synthesize TNF-α after LPS challenge (32–34) and high levels of TNF-α produced within the myocardium contribute to the development of cardiac dysfunction (34). In the present study, cardiac dysfunction induced by LPS was significantly improved after Anx5 treatment. To avoid the influences of cardiovascular reflex and loading conditions of the heart that may have on cardiac function measurements, an isolated Langendorff heart preparation was used. In agreement with the in vivo data, LPS-induced cardiac dysfunction was restored by Anx5 treatment. Thus, our results demonstrated that Anx5 improves cardiac function during endotoxemia in mice.

Previous studies have shown that annexin A1 has anti-inflammatory properties (35). Although Anx5 belongs to the same annexin superfamily as annexin A1, they are separate proteins encoded by distinct genes (17). In order to determine if Anx5 has anti-inflammatory effects, TNF-α and IL-1β levels were determined. Our results showed that treatment with Anx5 significantly decreased both mRNA and protein levels of TNF-α in the LV myocardium during endotoxemia. In

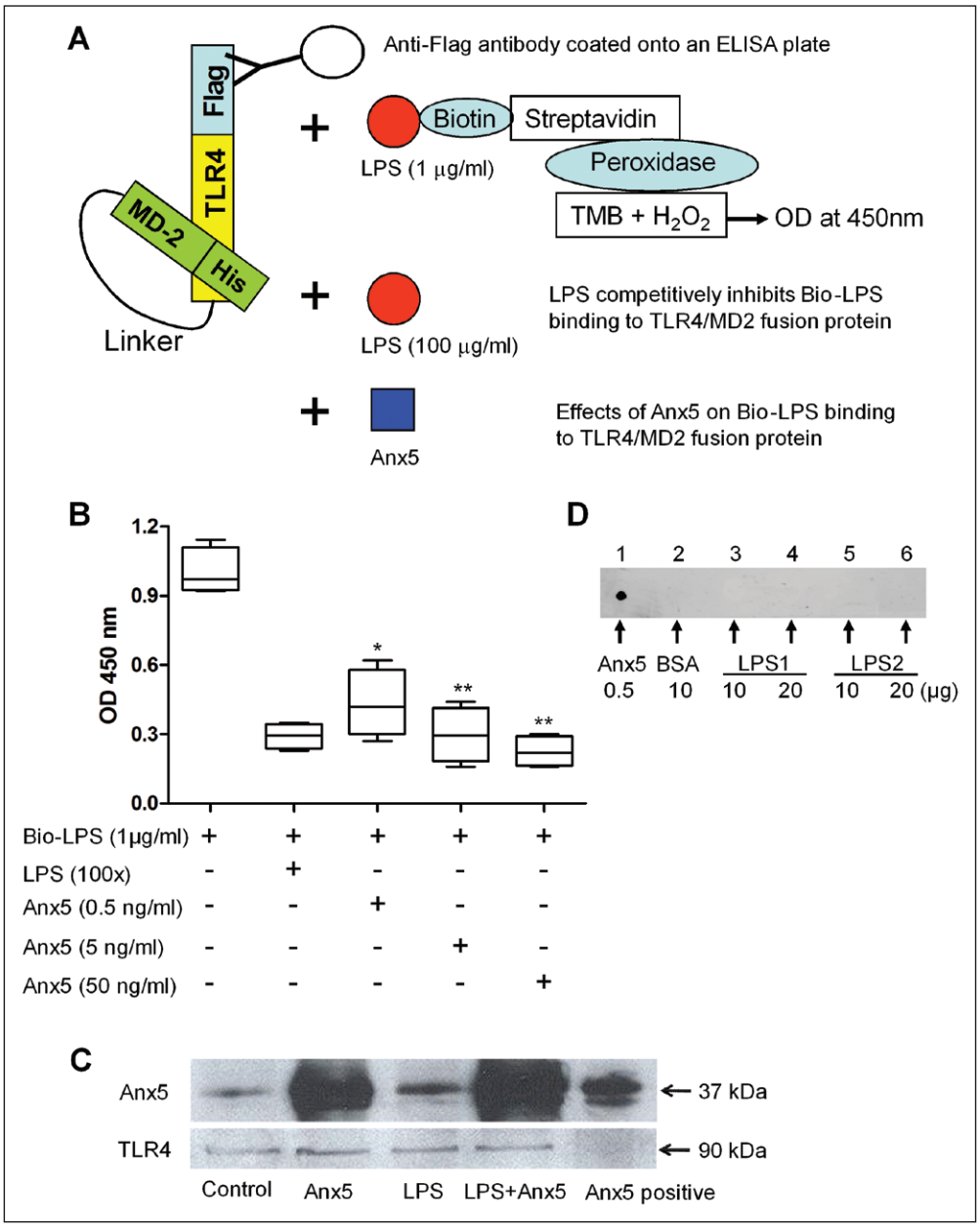

Figure 7. Annexin A5 (Anx5) inhibits lipopolysaccharide (LPS) binding to toll-like receptor-4 (TLR4)/myeloid differentiation factor 2 (MD-2) receptors. **A**, Enzyme-linked immunosorbent assay (ELISA)-based assay using a TLR4/MD-2 fusion protein. The TLR4/MD-2 fusion protein was coated on an ELISA microplate via anti-Flag antibody. The binding of biotinylated LPS (Bio-LPS) to the fusion protein was detected using the streptavidin-peroxidase system. A 100-fold excess of LPS (nonbiotinylated) was used to demonstrate specific and competitive inhibition of Bio-LPS binding to the TLR4/MD-2 fusion protein. Three doses of Anx5 were tested for their ability to inhibit the binding of Bio-LPS to the fusion protein. **B**, Quantification of optical density (OD) at 450 nm. Anx5 dose-dependently inhibited Bio-LPS binding to the TLR4/MD-2 fusion protein. **C**, TLR4 and Anx5 coimmunoprecipitation. Myocardial protein samples from saline- or LPS-treated mice were incubated with Anx5 (0.5 μg). TLR4 protein was pulled down using magnetic beads coated with anti-TLR4 antibody. This was followed by a Western blot analysis for Anx5 and TLR4. Anx5 positive indicates Anx5 positive control (20 ng) without myocardial proteins. **D**, Dot blot analysis of Anx5 and LPS binding. Bovine serum albumin (BSA) as negative control, recombinant Anx5 as positive control. LPS1 and LPS2 were LPS from *Salmonella typhosa and Pseudomonas aeruginosa*, respectively. Anx5 did not bind to LPS from either strain of bacteria. $^{*}p < 0.01$, $^{**}p < 0.001$ versus Bio-LPS alone. $n = 4$ per group. TMB = tetramethylbenzidine.

addition to decreased myocardial TNF-α expression, plasma levels of TNF-α and IL-1β were also decreased by Anx5 treatment in the endotoxemic mice. Furthermore, Anx5 treatment inhibited TNF-α and IL-β expression in the cultured adult cardiomyocytes. These data suggest that Anx5 has an anti-inflammatory effect. As TNF-α is a major contributor to cardiac dysfunction during sepsis (8, 34), reductions of myocardial TNF-α expression may contribute to improvements in cardiac function and animal survival by Anx5 treatment in mice with endotoxemia.

We have previously demonstrated that LPS activates TLR4/MAPK/NF-κB signaling, leading to myocardial TNF-α expression (21, 36, 37). In addition, NF-κB can also be activated by PI3K/Akt signaling during LPS stimulation (25, 29, 38). In the present study, phosphorylation of p38, ERK1/2, and Akt as well as NF-κB activity induced by LPS were all inhibited by Anx5 treatment, suggesting that Anx5 impedes TLR4-mediated MAPK/NF-κB and PI3K/Akt/NF-κB signaling. Although TAK1 is an upstream signaling of NF-κB in macrophages during LPS stimulation (29), this is not the case in the heart because myocardial TAK1 phosphorylation was not increased during endotoxemia. It has been shown that Anx5 can interact with and inhibit cell receptor function by binding to leucine-rich repeats through a conserved N-terminal sequence (19). Because the extracellular domain of TLR4 receptors has 21 separate leucine-rich repeats (20), Anx5 may bind to leucine-rich repeats and inhibit LPS binding to the TLR4/MD-2 receptor complex. To this end, we performed an ELISA-based LPS binding assay using a TLR4/MD-2 fusion protein (26, 27). Our data showed that Anx5 dose-dependently inhibited LPS binding to the TLR4/MD-2 fusion protein. Furthermore, Anx5 was coimmunoprecipitated with TLR4, suggesting an interaction between Anx5

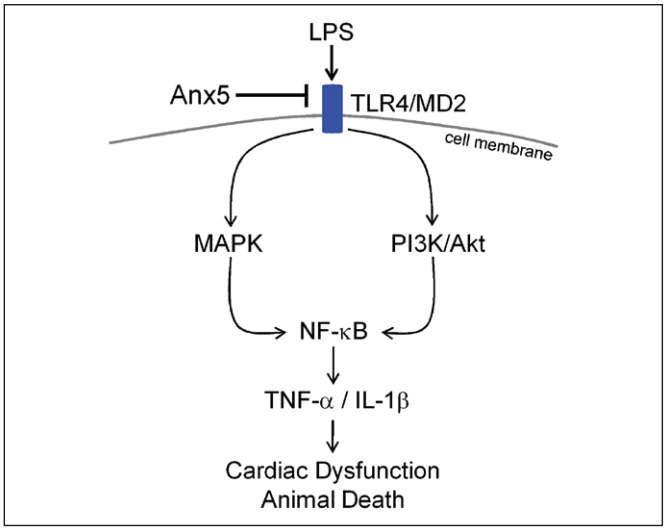

Figure 8. Proposed signaling mechanisms leading to cardiac dysfunction and animal death during endotoxemia and the effects of annexin A5 (Anx5). Lipopolysaccharide (LPS) binds to toll-like receptor-4 (TLR4)/myeloid differentiation factor 2 (MD-2) receptor complex and activates mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways, which induce myocardial tumor necrosis factor (TNF)-α and interleukin (IL)-1β expression via NF-κB. Treatment with Anx5 inhibits both MAPK and PI3K/Akt pathways during endotoxemia, leading to significant improvements in cardiac function and animal survival likely via inhibition of LPS binding to TLR4/MD-2 receptor complex.

and TLR4 receptors. The inhibition of LPS binding to TLR4/MD-2 receptors by Anx5 may decrease TLR4 signaling, leading to beneficial effects in endotoxemia.

A recent study by Rand et al (28) showed that Anx5 is able to bind to LPS from *P. aeruginosa*. The present study employed LPS from *S. typhosa* to induce endotoxemia. To confirm if Anx5 binds to LPS, a dot blot analysis was performed as previously described (28). Our data show that Anx5 does not bind to LPS from either strain of Gram-negative bacteria. The reason for the discrepancy between our data and the results by Rand et al (28) is not known. However, it is theoretically impossible to explain the beneficial effects of Anx5 by its binding to LPS as the doses of LPS used were more than 1,000-fold higher than those of Anx5 in our animal model. Notably, Anx5 also forms 2D arrays on cell membranes and promotes membrane repair (39). Sepsis increases endothelial permeability and causes sarcolemma damage (40, 41). Promotion of membrane repair may also represent another mechanism leading to the beneficial effects of Anx5 treatment in sepsis. Furthermore, Anx5 has antiapoptotic and anticoagulant properties (18). It should be noted that unlike Xigris, Anx5 does not have any thrombolytic effect (42). Thus, the risk of bleeding is likely much lower than Xigris during sepsis treatment.

In summary, the present study demonstrated a novel therapeutic effect of Anx5 in a mouse model of endotoxemia. Treatment with Anx5 decreases myocardial TNF-α expression and improves cardiac function and animal survival during endotoxemia. These beneficial effects of Anx5 are mediated at least in part through inhibition of LPS binding to the TLR4/MD-2 receptor complex, leading to decreases in NF-κB activation and cytokine expression via inhibition of MAPK and PI3K/Akt signaling pathways (Fig. 8). Our study suggests that Anx5 may have significant therapeutic potential in the treatment of sepsis. Given that Anx5 in similar doses (~10 μg/kg, IV) has been demonstrated to be safe for imaging studies in humans (43, 44), the proof of principle from our study can be easily translated to clinical studies to investigate the safety and efficacy of Anx5 in the treatment and prevention of sepsis.

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