Cellular and molecular mechanisms underlying LPS-associated myocyte impairment

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Tavener, Samantha A., and Paul Kubes. Cellular and molecular mechanisms underlying LPS-associated myocyte impairment. Am J Physiol Heart Circ Physiol 290: H800–H806, 2006.—Recently we reported that Toll-like receptor 4 (TLR4)-positive immune cells of unknown identity were responsible for the LPS-induced depression of cardiac myocyte shortening. The aim of this study is to identify the TLR4-positive cell type that is responsible for the LPS-induced cardiac dysfunction. Neither neutrophil depletion alone nor mast cell deficiency had any impact on the impairment of myocyte shortening during LPS treatment. In contrast, LPS-treated, macrophage-deficient mice demonstrated a partial reduction in shortening compared with saline-treated, macrophage-deficient mice. Because the removal of macrophages could only partially restore myocyte shortening, we also investigated the effects of removing both neutrophils and macrophages on myocyte shortening. Interestingly, endotoxemic, neutrophil-depleted, and macrophage-deficient mice had completely restored myocyte shortening. Because both macrophages and neutrophils can produce nitric oxide (NO) and TNF-α, we examined LPS-treated inducible NO synthase knockout (iNOSKO) mice and TNF receptor (TNFR)-deficient mice. Eliminating both TNFR1 and TNFR2 was required to restore myocyte shortening during LPS treatment, whereas iNOS deficiency had no effect. These data suggest that macrophages and to a lesser degree neutrophils cause cardiac impairment, presumably via TNF-α.

LPS is a cell membrane component shed from gram-negative bacteria and is a major contributor to the development of sepsis (9), primarily via Toll-like receptor 4 (TLR4). To date, most studies have examined cardiac responses after animals were treated with LPS with clear evidence that LPS has myocardial depressive properties (15, 34, 41). Recently, we uncovered that myocytes per se are not the direct target of LPS-induced responses observed in the heart over the first few hours of endotoxemia. Rather, TLR4-positive leukocytes are responsible for LPS-induced myocyte dysfunction (52). This was done by making chimeric mice where either the bone marrow cells or myocytes expressed TLR4. Only mice with TLR4 on immune cells demonstrated cardiac impairment in response to LPS. However, the identity of the bone marrow-derived cells was not identified. In the heart there are a number of infiltrating and resident cells, and those immune cells have been shown to express TLR4: neutrophils (28, 33, 43), mast cells (18, 29, 50), and macrophages (28, 33).

Neutrophils or other circulating leukocytes (monocytes) may be responsible for LPS-induced myocyte dysfunction because they are known to rapidly infiltrate cardiac tissue during inflammation. Accumulation of neutrophils in the endotoxemic heart has been reported (13, 41). In fact, a study (15) using Langendorff perfused hearts demonstrated that hearts perfused with leukocyte-depleted, endotoxemic blood had no reduction in pressure generation compared with perfusion with normal endotoxemic blood. In addition, blocking general leukocyte recruitment or leukocyte activation prevented LPS-induced cardiac dysfunction (41, 58), although which leukocytes were responsible was unclear. In vitro studies illustrate that activated neutrophils adhere to myocytes (39, 48) and increase oxidative and nitrosative stress within the cardiac myocyte, ultimately resulting in myocyte dysfunction (11, 39). These studies suggest, albeit indirectly, that neutrophils and/or other circulating leukocytes may be involved in sepsis-associated cardiac dysfunction.

Despite mast cells being involved in inflammation via the production of various mediators, there is no evidence that these cells contribute to LPS-induced myocyte dysfunction. An in vitro study (16) demonstrated that culturing myocytes with mast cell granules can elevate cardiomyocyte apoptosis, suggesting mast cells can certainly cause myocyte damage. During ischemia-reperfusion injury, mast cells (and the release of TNF-α) were suggested to be key for the recruitment of damaging neutrophils and thus the development of myocardial infarct (12). In addition, the stabilization of mast cells with lodoxamide reduced infarct size during ischemia-reperfusion injury (19). During sepsis, the removal of dipeptidyl peptidase I from mast cells was shown to increase survival (26). Clearly, mast cells can release injurious molecules during infection or ischemia-reperfusion to induce damaging processes.

There are a few studies describing a role for the monocyte/macrophage lineage to reduce cardiac myocyte shortening. One in vitro study (2) illustrated that myocytes treated with supernant from activated macrophages could reduce shortening. Another in vitro study (46) demonstrated that the adhesion of monocytes via ICAM-1 on cardiac myocytes was shown to reduce shortening. To date, a role for macrophages in LPS-induced cardiac dysfunction per se has not been tested. However, with the availability of macrophage-deficient mice [colony stimulating factor 1 (CSF-1) deficient], this is now possible.

Macrophages, mast cells, neutrophils (1, 3, 37), as well as cardiac myocytes (8, 21, 59), can all release many mediators to...

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affect heart function. TNF-α has been one of the more avidly studied molecules in endotoxemia and sepsis. In vitro studies showed the treatment of myocytes with TNF-α alone can reduce myocyte shortening (23, 24). In addition, infusion of TNF-α showed depression of left ventricular function and myocyte shortening (6). The importance of TNF-α becomes even more evident with anti-TNF therapy demonstrating a decrease in myocardial infarct size and an effective reduction in mortality in septic animal studies (7). Furthermore, inhibition of TNF-α with TNF-binding proteins restored the dysfunctional left ventricular developed pressure (LVDP) in endotoxemic rats (31). Although cardiac myocytes express both TNF receptors TNFR1 and TNFR2 (53), it remains unclear which receptor is important.

This study identifies the specific cell type (primarily the monocyte/macrophage lineage) as responsible for LPS-induced myocyte dysfunction. This occurred due to TNF-α, via both TNFR1 and TNFR2.

MATERIALS AND METHODS

Animals and experimental protocol. Male C57BL/6 mice were purchased from Charles River (Montreal, Quebec, Canada), and male mast cell-deficient (WBB6F1/J-kitW/W−/−), macrophage-deficient (B6.C3Fe a/a-Csf1r−/−), iNOS-knockout (C57Bl6/X129Sv/Ev), TNFR1 (B6.129-Tnfrsf1a tm1Mak/J), TNFR2 (B6.129S7-Tnfrsf1b tm1Msw/J), and TNFR1/2-deficient (B6.129-Tnfrsf1a tm1Msw/J Tnfrsf1b tm1Msw/J) mice and their respective control littermates were purchased from Jackson (Bar Harbor, ME). No differences in the proportion of myeloid or other circulatory leukocytes were detected in the TNFR-deficient mice relative to wild-type controls (32, 42), and the mast cell-deficient mice had no mast cells detected in various organs, including the heart (22). All 152 mice were maintained in a pathogen-free facility until 6–10 wk old, at which time the mice were used. Mice received LPS (10 mg/kg, Escherichia coli serotype 0127:B8) intraperitoneally to induce endotoxemia, a dose demonstrated to ensure cardiac dysfunction without any mortality (39). After 4 h, mice were anesthetized and hearts excised for myocyte isolation. All experimental protocols were reviewed and approved by the University of Calgary Animal Resource Center and conform to the guidelines established by the Canadian Council for Animal Care.

Murine ventricular myocyte isolation. Ventricular myocytes were isolated as previously described (39), and unless otherwise stated, all chemicals were purchased from Sigma-Aldrich. Briefly, 6- to 16-wk-old, male, septic (LPS, E. coli serotype 0127:B8, 10 mg/kg ip, 4 h), wild-type (C57BL/6), neutrophil-depleted, mast cell-deficient or macrophage-deficient mice were anesthetized with methoxyflurane (Metafane; Janssen Pharmaceuticals). Mice were then cervically dislocated, and hearts were removed and placed in ice-cold Tyrode buffer containing (in mmol/l) 139.9 NaCl, 5.4 KCl (BDH), 1.0 Na2HPO4 (BDH), 5.0 HEPES, 10.0 glucose, and 1.0 MgCl2, containing 1.0 CaCl2. Hearts were then cannulated via the aorta for retrograde perfusion of the coronary arteries with Tyrode buffer containing 1 mmol/l CaCl2 at 2 ml/min for 3 min at 37°C and subsequently perfused with buffer containing no CaCl2 for 5 min. Final perfusion buffer contained 40 μmol/l CaCl2, 20 μg/ml collagenase (Yakult Pharmaceuticals), and 4 μg/ml protease (type XIV bacterial) for 8–10 min. Ventricles were then minced and placed in Tyrode buffer containing 500 μg/ml collagenase, 100 μg/ml protease, and 2.5% bovine serum albumin and placed in a shaking water bath for 10–20 min to obtain a suspension of individual myocytes. Myocytes were then placed in DMEM (GIBCO) and were used within 4 h.

Cell shortening. The mechanical properties of the myocytes were measured by using a video-based, edge-detection system (IonOptix, Milton, MA), as previously described (52). Isolated myocytes were allowed to adhere to a glass coverslip in a Warner chamber that was mounted on the stage of an inverted microscope. Myocytes were perfused with 1 ml/min Tyrode buffer and field stimulated at 1 Hz using a threshold voltage of +10%. Shortening and relengthening were recorded with the soft-edge software (IonOptix). Nonsimultaneously contracting myocytes were selected randomly but had to respond to electrical stimulation and have increased shortening in response to a β-adrenergic agonist isoproterenol (0.1 μmol/l, Sigma). It is our experience that myocytes that do not meet these criteria usually die during the protocol, making the data acquisition impossible (52). Therefore, we selected the healthiest myocytes from both the untreated and LPS-treated groups.

Neutrophil depletion. C57BL/6 (wild-type) and macrophage-deficient and their respective control mice received anti-neutrophil antibody (RB6–8C5) intraperitoneally at 150 μg/mouse for 24 h before experimentation, as we have previously described (5, 17) and as per manufacturer’s instructions. This treatment removed >90% of the neutrophils from each strain of mice and did not significantly alter the blood levels of other leukocyte populations. In addition, RB6–8CS treatment has been shown to substantially reduce cardiac neutrophil accumulation (14).

Statistical analysis. Statistical significance between data was assessed using a t-test with a Bonferroni correction for multiple comparisons. Statistical significance was set at P < 0.05.

RESULTS

Neutrophils are not required for LPS-induced myocyte dysfunction. Neutrophil-depleted mice were generated by using a 24-h anti-neutrophil antibody treatment (Becton Dickinson Pharmingen), which was shown to deplete neutrophils by at least 90% in this study and in previous work (5, 17). These mice were also treated with either saline or LPS for 4 h, at which point cardiac myocytes were isolated and cell shortening was measured (Fig. 1). Myocyte shortening from saline-treated, wild-type (5.2 ± 0.7%) and neutrophil-depleted mice (7.1 ± 1.5%) was not significantly different. Cardiac myocytes from LPS-treated, wild-type and neutrophil-depleted mice demonstrated significantly depressed shortening to 2.6 ± 0.3% and 2.5 ± 0.3%, respectively. Despite the elimination of neutrophils, a profound reduction in myocyte shortening remained, illustrating that neutrophils alone are not responsible for the LPS-induced cardiac dysfunction.

![Fig. 1. Shortening of ventricular myocytes from saline- and LPS-treated, wild-type (WT) and anti-neutrophil antibody (ANS)-treated mice. *P < 0.05 compared with saline-treated controls. n = minimum number of 4 mice or 11 myocytes/group.](http://www.ajpheart.org)
Does LPS-induced myocyte impairment involve cardiac mast cells? Another cell type present within the milieu of the endotoxemic heart is the cardiac mast cell. Cardiac myocytes were isolated from saline- and LPS-treated mast cell-deficient mice and littermate controls, and subsequent myocyte shortening was recorded (Fig. 2). The saline-treated, mast cell-deficient and control mice had similar myocyte shortening. Significantly depressed shortening was observed in the LPS-treated controls (2.1 ± 0.2%) and in LPS-treated, mast cell-deficient myocytes (1.4 ± 0.2%). Consequently, the removal of the mast cells does not restore myocyte shortening, suggesting that this cell type is not responsible for LPS-induced myocyte dysfunction.

Macrophages are partially involved in myocyte dysfunction associated with LPS treatment. Another candidate that may be responsible for LPS-induced myocyte impairment is the macrophage. Cardiac myocytes were isolated from saline- and LPS-treated macrophage-deficient and littermate control mice, and then myocyte shortening was examined (Fig. 3). Myocytes from the saline-treated, macrophage-control and saline-treated, macrophage-deficient mice had similar shortening. The LPS-treated, macrophage controls demonstrated significantly reduced myocyte shortening of 1.7 ± 0.4%. In contrast, the myocyte shortening of LPS-treated, macrophage-deficient mice was not significantly different from either saline-treated, macrophage-control or -deficient mice.

Both macrophages and neutrophils are required for complete LPS-induced myocyte depression. Because the absence of macrophages in the heart during endotoxemia only partially restores myocyte shortening, it is possible that another cell type may be contributing to myocyte dysfunction. Macrophage-deficient mice and littermate controls were treated with anti-neutrophil antibody for 24 h and then with saline or LPS for the last 4 h. The heart was then excised, and the cardiac myocytes were isolated. Saline-treated myocytes from neutrophil-depleted, macrophage-sufficient and -deficient mice demonstrated shortening of 6.2 ± 1.1% and 5.1 ± 1.3%, respectively (Fig. 4). Myocyte shortening from neutrophil-depleted, LPS-treated macrophage control mice was identical to the shortening in the LPS-treated, macrophage-control mice (Fig. 3), further demonstrating no negative effects of neutrophil depletion alone. However, neutrophil depletion in macrophage-deficient mice did not reduce myocyte shortening at all (5.3 ± 1.5%) after LPS treatment. The value is significantly higher than the neutrophil-depleted, LPS-treated, and macrophage-control mice.

Could the production of mediators, such as nitric oxide, be responsible for LPS-associated myocyte impairment? Macrophages and neutrophils will generate reactive nitrogen species, like nitric oxide (NO), which may react with other molecules and be damaging to both invading organisms and resident tissue. Cardiac myocytes from saline and LPS-treated inducible NO synthase (iNOS) knockout (iNOSKO) mice were isolated, and shortening was analyzed (Fig. 5). Myocyte shortening from the saline-treated, wild-type and iNOSKO mice were identical, whereas myocytes from LPS-treated, wild-type and iNOSKO mice demonstrated significantly depressed shortening of 2.6 ± 0.3% and 2.3 ± 0.3%, respectively. Thus NO may not be responsible for depressed myocyte shortening associated with LPS treatment in our model.

TNF-α can cause myocyte dysfunction. Although the literature suggests that TNF-α in vitro can cause myocyte depression, the effects of TNF-α on myocytes during endotoxemia are unclear. Additionally, the importance of TNFR1 and TNFR2 signaling for myocyte dysfunction are also unresolved. Cardiac myocytes were isolated from saline- and LPS-treated

![Fig. 2. Shortening of ventricular myocytes from saline- and LPS-treated, mast cell-control and mast cell-deficient mice. *P < 0.05 compared with saline-treated controls. n = minimum number of 3 mice or 9 myocytes/group.](image)

![Fig. 3. Shortening of ventricular myocytes from saline- and LPS-treated, macrophage-control and macrophage-deficient mice. *P < 0.05 compared with saline-treated controls. n = minimum number of 4 mice or 12 myocytes/group.](image)

![Fig. 4. Shortening of ventricular myocytes from saline- and LPS-treated, macrophage-sufficient, ANS-depleted mice and macrophage-deficient, ANS-depleted mice. *P < 0.05 compared with saline-treated controls. †P < 0.05 compared with LPS-treated, neutrophil-depleted, macrophage-deficient mice. n = minimum number of 3 mice or 9 myocytes/group.](image)
TNFR1-deficient and TNFR2-deficient mice, and myocyte shortening was examined (Fig. 6). Similar myocyte shortening was demonstrated for all saline-treated groups. Furthermore, all groups showed equally depressed myocyte shortening with LPS treatment. Because both receptors have been reported to be involved in various TNF-α-induced effects, cardiac myocytes from double-deficient (TNFR1/2−/−) mice were isolated, and shortening was examined (Fig. 7). Myocytes from the saline-treated, wild-type and TNFR1/2−/− mice demonstrated shortening of 5.1 ± 0.7% and 6.8 ± 0.8%, respectively, whereas myocytes from the LPS-treated, wild-type mice have depressed shortening of 2.6 ± 0.3%. Myocyte shortening from the LPS-treated, TNFR1/2−/− mice had comparable shortening with the saline-treated group (5.1 ± 0.7%), suggesting that TNF-α is required for LPS-induced myocyte impairment.

**DISCUSSION**

Recently, we reported a role for TLR4 positive immune cells rather than parenchymal cells (myocytes or fibroblasts) in LPS-induced myocyte dysfunction (52). Yet, the TLR4-positive immune cell type that was responsible for myocyte impairment during endotoxemia was not addressed. Our results herein show that neither neutrophils nor mast cells alone are central in the depression of myocyte shortening during LPS treatment. In contrast, macrophages appear to play a significant role. Interestingly, LPS-treated, neutrophil-depleted, and macrophage-deficient mice had completely restored myocyte shortening. Although macrophages and neutrophils can both produce NO (4, 24) and TNF-α (37, 54), the elimination of both TNFR1 and TNFR2 was required to restore myocyte shortening during LPS treatment, whereas removal of only one TNFR or iNOS had no effect on the depressed myocyte shortening.

Although mast cells are not often considered to be important immune cells in endotoxemia or septicemia, they have been reported to be the primary source of TNF-α in the first few hours of infection in the murine peritoneal cavity (10, 25). Therefore, we hypothesized that mast cells known to reside in the heart (20, 49) and to harbor cytokines (12) were the cells involved in myocyte dysfunction. We made use of the well-established mouse system, the W/Wv mice lacking c-kit, a critical receptor for the mast cell growth factor. These mice lack mast cells in essentially all tissues, including the heart (35). Despite this deficiency, we observed absolutely no improvement in the impaired myocyte shortening associated with LPS.

Unlike studies with general leukocyte-depletion protocols, there is little direct evidence that specific removal of neutrophils can alleviate cardiac depression during endotoxemia. In fact, neither vinblastine nor anti-neutrophil antibody treatment had any effect on the reduction in LVDP induced by LPS (40). This group also showed that blocking ICAM-1 in endotoxemic mice improved LVDP but did not block neutrophil accumulation. In agreement, another study (27) using nonspecific neutrophil depletion (cyclophosphamide) was unable to show a reduction in cardiac damage during E. coli-induced multiple organ failure in guinea pigs. Therefore, improved cardiac function was suggested to be independent of neutrophil accumulation. These studies support our data that show LPS-treated, neutrophil-depleted mice still have impaired myocyte function.

Our data reveal that monocytes and macrophages contribute to myocyte impairment during endotoxemia. Interestingly, not all the myocyte dysfunction was alleviated, but the macrophage-deficient mice did have a small percentage of tissue macrophages remaining (57). However, these macrophages were nonfunctional [impaired ability to release TNF-α (56)].
and thus would be unlikely to cause LPS-associated myocyte dysfunction. Yet another possibility to explain the lack of complete inhibition of cardiac impairment is that neutrophils could contribute to the remaining myocyte depression during LPS treatment. Indeed, the removal of neutrophils in macrophage-deficient mice during endotoxemia exhibited normal myocyte shortening. Although neutrophil depletion alone could not alleviate any of the myocyte dysfunction, this may be due to the overwhelming arsenal of cytokines (e.g., TNF-α) produced by macrophages that mask the more subtle impairment induced by neutrophils per se. Indeed, neutrophils can also produce molecules like TNF-α but in barely detectable quantities. This would suggest that primarily macrophages but also neutrophils are required for LPS-induced myocyte dysfunction. Nevertheless, one cannot discount a role for neutrophils in this model of myocardial depression.

There have been a number of studies looking at the effects of NO on cardiac performance. In fact, Poon et al. (39) showed that extravascular neutrophils containing iNOS can adhere to myocytes and cause a rapid decrease in myocyte shortening, whereas neutrophils from iNOS-deficient mice were unable to cause myocyte damage. An important difference between the Poon et al. study and this study is the direct application of maximally activated neutrophils to myocytes in vitro in the former study. Clearly, under these conditions, neutrophils can produce sufficient NO to induce myocyte dysfunction. In contrast, in vivo, the neutrophils either were not sufficiently activated and/or did not have the same degree of access to myocytes as in vitro. Alternatively, macrophage iNOS may be a more relevant source, because macrophages in this study were required for myocyte dysfunction during endotoxemia. However, in our system, no benefit was observed in myocyte shortening from the endotoxemic iNOS-deficient mice, suggesting that despite the fact that neutrophils and macrophages have both been reported to have the capacity to upregulate iNOS, we could not observe NO-mediated myocyte dysfunction.

Our data suggest that TNF-α is the key mediator involved in the first 4 h of LPS-induced myocyte dysfunction. Both divergent and redundant roles for TNFR1 and TNFR2 have been suggested in some disease models. A study using TNFR1-deficient mice (36) or TNFR1-blocking monoclonal antibody (45) has demonstrated protection from lethal shock with the use of LPS and galactosamine. In contrast, an antibody that blocks TNFRII did not protect mice from lethal shock. Our data suggest that both TNFR1 and TNFR2 are involved in LPS-induced myocyte dysfunction, consistent with the observation that both TNFR1- and TNFR2-deficient mice are sensitive to LPS-induced mortality (36). In fact, both TNFR1 and TNFR2 have been reported as necessary in other biological responses, including TNF-mediated endothelial activation (44), expression of adhesion molecules (47), and TNF-induced cytotoxicity of tumor cells (44). Two different models, ligand passing and TNFR heterocomplexing, have been suggested to explain the cooperation between TNFR1 and TNFR2. The ligand passing model suggests that high-affinity TNFR2 (10− to 20-fold higher affinity than TNFR1) can rapidly associate and dissociate with TNF-α, thereby allowing a TNF-α association with TNFR1 (51). In contrast, the heterocomplexing model suggests that adjacent TNFR1 and TNFR2 can share downstream-signaling molecules (38), such that there exists a functional overlap.

In conclusion, macrophages appear to play an important role in LPS-induced myocyte dysfunction with a more minor contribution from infiltrating neutrophils. Although both macrophages and neutrophils can produce NO and TNF-α, we could demonstrate a role only for TNF-α in LPS-induced myocyte dysfunction in this model system. Intriguingly, the elimination of both TNFR1 and TNFR2 was necessary to restore myocyte shortening during LPS treatment. Therefore, both TNFR1 and TNFR2 are essential for myocyte dysfunction during endotoxemia.

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GRANTS

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