Neutralization of IL-18 attenuates lipopolysaccharide-induced myocardial dysfunction

CHRISTOPHER D. RAEBURN, CHARLES A. DINARELLO, MICHAEL A. ZIMMERMAN, CASEY M. CALKINS, BENJAMIN J. POMERantz, ROBERT C. McINTYRE, JR., ALDEN H. HARKEN, AND XIANZHONG MENG

Department of Surgery, University of Colorado Health Sciences Center, Denver, Colorado 80262

Received 20 January 2002; accepted in final form 24 April 2002

Lipopolysaccharide (LPS) depresses intrinsic myocardial contractility (1, 31, 35) and is believed to be an important factor contributing to myocardial dysfunction during sepsis. LPS induces a cascade of proinflammatory cytokines, and tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) synergistically depress myocardial function (4, 9, 16, 30). Whereas TNF-α is involved in LPS-induced myocardial dysfunction, we (22) reported a temporal discordance between myocardial TNF-α levels and the contractile dysfunction that occurs during endotoxemia. After LPS, myocardial dysfunction did not occur until TNF-α levels had returned to baseline. These observations suggest that TNF-α may provide an essential early signal, but other cardio depressant factors may more directly conspire to depress cardiac function. In addition to TNF-α and IL-1β, we have observed an essential role for both vascular cell adhesion molecule-1 (VCAM-1) (34) and intercellular adhesion molecule-1 (ICAM-1) (unpublished data) in LPS-induced myocardial dysfunction.

IL-18 is a proinflammatory member of the IL-1 superfamily (42). Similar to IL-1β, IL-18 is synthesized as an inactive precursor and is cleaved to its active form by caspase-1 (10, 13). IL-18 was initially recognized for its ability to induce interferon-γ (IFN-γ) and its capacity to induce Th1 responses (23, 28). However, IL-18 was subsequently found to play an important role in LPS-induced hepatotoxicity (41), which stimulated further study of the role of this cytokine in sepsis. Elevated levels of IL-18 occur in the serum (5, 12, 27) and bronchoalveolar lavage fluid (18) of septic patients, and in vitro studies (17) indicate that LPS induces IL-18 secretion in human monocytes. LPS has also been shown to induce IL-18 in murine macrophages (37) and lungs (2). Myocardial production of IL-18 during endotoxemia has not been reported.

IL-18 activates nuclear factor (NF)-κB (19), which is a transcriptional regulator of many proinflammatory cytokines and cellular adhesion molecules. In vitro studies have shown that IL-18 increases the production of TNF-α and IL-1β in murine macrophages (26) and human monocytes (33) and also induces the expression of ICAM-1 and VCAM-1 on endothelial cells (24, 43) and monocytes (15). In vivo studies have shown that neutralization (6, 25) or genetic absence (14) of IL-18 protects mice from lethal endotoxemia and from LPS-induced liver injury (6, 36, 41).

Specific blockade of IL-18 using IL-18 binding protein improves contractile function in human atrial strips after ischemia-reoxygenation (32). However, the specific role of IL-18 in myocardial dysfunction and the
complex cascade of cytokines and cellular adhesion molecules induced by LPS is unknown. Therefore, in the present study, we sought to determine the role of IL-18 in LPS-induced myocardial dysfunction and to examine its role in myocardial TNF-α and IL-1β production and ICAM-1/VCAM-1 expression during endotoxemia.

**METHODS**

**Materials.** The IL-18 antiserum was obtained from New Zealand White rabbits immunized by intradermal injections of murine IL-18 (PeproTech; Rocky Hill, NJ) with Hunter’s Titermax adjuvant. This antibody has been shown to inhibit LPS-induced IFN-γ production in vivo (8) and to protect mice from lethal Escherichia coli endotoxemia (25). Murine IL-18, TNF-α, and IL-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN). Rat anti-mouse VCAM-1 monoclonal antibody (MAb) (clone MVCAM-A429) was purchased from Endogen (Woburn, MA). Rat anti-mouse ICAM-1 MAb (clone KAT-1) and rat anti-mouse neutrophil p40 antigen MAb (clone 7/4) were purchased from Serotec (Oxford, UK). Rat IgG and Cy3-conjugated donkey anti-rat IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Rabbit anti-mouse ICAM-1/VCAM-1 MAb (clone KAT-1) and rat anti-mouse ICAM-1/VCAM-1/VCAM-3 MAb were purchased from Sigma (St. Louis, MO).

**Animals.** Male C57BL/6 mice, B6.129 wild-type mice (B6), and TNF-α knockout (TNF−/−) mice, 20–25 g body wt, were purchased from Jackson Laboratory (Bar Harbor, ME). Animals were acclimated for 1 wk after delivery in a 12:12-h light-dark cycle room and maintained on a standard pellet diet. All animal care in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1985).

**Experimental protocols.** Mice were injected with either LPS (0.5 mg/kg ip) or vehicle (normal saline). We have previously monitored the hemodynamic response in rats to this dose of LPS and observed profound reduction in cardiac contractility with minimal inotropic effect (21). We conducted this study to determine whether the anti-IL-18 antibody or NRS were contaminated by cytokines or other molecules. Therefore, all data represented herein were obtained at a LVEDP of 10 mmHg.

**Cytokine measurements.** For cytokine measurements, the myocardial tissue was weighed and then sequentially homogenized and snap-frozen in dry ice-chilled isopentane and stored at −70°C for immunofluorescent staining. The remainder of myocardium was placed into liquid nitrogen and stored at −70°C.

**Isolated heart perfusion.** Myocardial function was determined by an isovolumetric nonrecirculating Langendorff technique, as described previously (22). Isolated hearts were perfused with 37°C Krebs-Henseleit solution containing (in mmol/l) 11.0 glucose, 1.2 CaCl2, 4.7 KCl, 25 NaHCO3, 119 NaCl, 1.17 MgSO4, and 1.18 KH2PO4. Coronary perfusion pressure was maintained at 70 mmHg. The perfusion buffer was gassed with a mixture of 95% O2, 5% CO2 to achieve a PO2 of 450 mmHg, PCO2 of 40 mmHg, and pH of 7.4. A balloon was constructed of ultrathin latex and tested to ensure that the balloon was of high compliance. A balloon was deemed to have an acceptable compliance if it did not show any positive pressure when filled with 5 μl of water. The balloon was inserted into the left ventricle via the left atrium and inflated with water (5–8 μl) to achieve a left ventricular end-diastolic pressure (LVEDP) of 10 mmHg. Pacing wires were attached to the right atrium and hearts were paced at 300 beats/min. Coronary flow was quantified by collecting the effluent from the pulmonary arteries as it dripped from the heart. Myocardial temperature was maintained at 37°C. Left ventricular developed pressure (LVPD), its maximum and minimum first derivatives over time (+dP/dt and −dP/dt, respectively), and LVEDP were continuously recorded by a computerized pressure amplifier-digitizer (MacLab version 8, ADInstruments; Cupertino, CA). After a 20-min equilibration period, LVPD and ±dP/dt were determined at varied LVEDP levels (10, 15, and 20 mmHg). The degree of reduction in LVPD and ±dP/dt in LPS-treated hearts, compared with saline controls, was not influenced by variation of LVEDP from 10 to 20 mmHg. Therefore, all data represented herein were obtained at a LVEDP of 10 mmHg.

**Immunofluorescent staining.** Indirect immunofluorescent detection and localization of ICAM-1, VCAM-1, and neutrophils were performed as described previously (39). Transverse sections (5 μm thick) of ventricular myocardium were cut with a cryotome (International Equipment; Needham Heights, MA) and then dried at room temperature for 2 h. The sections were treated with a mixture of 30% methanol and 70% acetone at room temperature for 10 min and washed with PBS. The sections were then fixed in PBS-buffered 3% paraformaldehyde at room temperature for 10 min and washed with PBS. Each subsequent incubation was performed at room temperature. To block nonspecific binding sites, the sections were incubated for 30 min with 10% donkey serum in PBS. The sections were then incubated with a primary antibody (diluted 1:200 for ICAM-1 and VCAM-1 and 1:100 for ICAM-1 in PBS).
and 1.500 for neutrophils in PBS containing 1% bovine serum albumin (BSA) for 60 min. After being washed three times with PBS, the sections were incubated for 45 min with Cy3-labeled donkey anti-rat IgG (1:250 dilution with PBS containing 1% BSA). After thorough washes with PBS, sections were counterstained with fluorescein-labeled wheat germ agglutinin (5 μg/ml, for cell surface staining) and bisbenzimide (2.5 μg/ml, for nuclear staining). The sections were mounted with aqueous antif doubling media. To ascertain the specificity of the primary antibody, adjacent sections were incubated with nonspecific rat IgG (diluted 1:200 in PBS containing 1% BSA) in replacement of the primary antibodies and then processed in identical conditions. Microscopic observation and photography were performed with a Leica DMRXA microscope.

Image quantification. ICAM-1 and VCAM-1 images were quantitated with SlideBook version 2.6 software (Intelligent Imaging Innovations; Denver, CO). Eight random images were taken from each myocardial section. Imaging was performed at ×40 magnification (1,020 × 1,020 pixels/image). All images were taken while blinded to both the specimen and the Cy3 channel. Images were masked to exclude 95% of nonspecific fluorescence as determined from images of myocardium incubated with nonspecific rat IgG. Images were analyzed with the use of SlideBook to determine the mean area (μm²) and mean intensity and to calculate the integrated intensity (product of area and mean intensity).

Myocardial neutrophil number was determined by counting all nucleated cells with Cy3 fluorescence present on a myocardial section. The number of neutrophils per section was divided by the calculated area of the myocardial section and reported as the number of neutrophils per millimeter squared. This method of assessing tissue neutrophil accumulation has previously been shown to closely correlate with myeloperoxidase activity (40).

Statistical analysis. All data are expressed as means ± SE. Statistical significance of differences between groups was determined by analysis of variance and verified by a Bonferroni-Dunn post hoc test. Statistical analysis was performed using StatView version 5.0 (Abacus Concepts; Calabasas, CA).

Effect of neutralization of IL-18 on LPS-induced myocardial dysfunction. We (22, 34) examined the time course of myocardial dysfunction in mice during endotoxemia and found the maximal depression in function to occur at 6 h after LPS. Therefore, to examine the role of IL-18 in LPS-induced myocardial dysfunction, hearts were studied at 6 h after LPS. Compared with saline controls, LVDP was reduced by 38% after LPS (36.3 ± 1.9 vs. 59.1 ± 2.7 mmHg, n = 5, Fig. 1). Pretreatment of mice 30 min before LPS with NRS had minimal influence on LPS-induced myocardial dysfunction; however, pretreatment with IL-18 neutralizing antibody nearly abrogated the dysfunction (Fig. 1). Coronary flow was not different between groups (data not shown).

Myocardial IL-18 content after LPS. To determine the effect of LPS on myocardial tissue IL-18 content, mice were injected intraperitoneally with either vehicle (saline) or LPS. Hearts were harvested at 2, 4, and 6 h after LPS and homogenized to determine myocardial IL-18 content by ELISA. Compared with vehicle control, a twofold increase in myocardial IL-18 content was observed at 4 h after LPS (Fig. 2).

To determine whether TNF-α was required for the LPS-induced increase in myocardial IL-18 levels, TNF−/− mice were also injected with LPS and compared with B6 mice. Similar to C57BL/6 mice, myocardial IL-18 levels in LPS-treated TNF−/− mice were lower than in wild-type mice. However, 4 h after LPS, myocardial IL-18 levels were lower in TNF−/− compared with wild-type mice (1.9 ± 0.4 vs. 3.4 ± 0.8 pg/mg protein, n = 5, Fig. 3). This difference was significant by post hoc Fisher’s analysis but not by Bonferroni-Dunn.

Whereas myocardial IL-18 levels in LPS-treated TNF−/− mice were increased slightly compared with saline controls, myocardial IL-18 content of B6 mice was increased slightly compared with saline controls (0.8 vs. 1.1 ± 0.2 pg/mg protein, Fig. 3). In saline controls, myocardial IL-18 levels were not different between TNF−/− and wild-type mice. However, 4 h after LPS, myocardial IL-18 levels were lower in TNF−/− compared with wild-type mice (1.9 ± 0.4 vs. 3.4 ± 0.8 pg/mg protein, n = 5, Fig. 3). This difference was significant by post hoc Fisher’s analysis but not by Bonferroni-Dunn.
saline controls, the increase did not reach statistical significance.

**Effect of neutralization of IL-18 on LPS-induced myocardial TNF-α production.** We then sought to determine whether protection against LPS-induced myocardial dysfunction afforded by neutralization of IL-18 is associated with attenuation in myocardial TNF-α content. TNF-α was below detection in vehicle-injected controls but reached peak levels at 1 h after LPS (7.7 ± 1.6 pg/mg protein). Pretreatment of mice 30 min before LPS with either NRS or neutralizing IL-18 antibody had no effect on myocardial TNF-α content at 1 h (7.7 ± 2.4 and 6.4 ± 1.1 pg/mg protein, respectively) (Fig. 4). Plasma concentrations of TNF-α were below detection in vehicle-injected controls but were elevated at 1 h after LPS (2,855 ± 391 pg/ml). Pretreatment with either NRS or neutralizing IL-18 antibody did not reduce LPS-induced elevation in plasma TNF-α concentration (2,454 ± 269 and 2,288 ± 146 pg/ml, respectively).

**Effect of neutralization of IL-18 on LPS-induced myocardial IL-1β levels.** To determine whether neutralization of IL-18 influences LPS-induced myocardial IL-1β production, we first determined the time of maximal IL-1β levels in the heart after LPS. Compared with control myocardium, LPS greatly increased myocardial IL-1β content at 4 h (132.2 ± 7.5 vs. 2.7 ± 1.4 pg/mg protein, *P* < 0.001, Fig. 5). In contrast to TNF-α, pretreatment of mice with IL-18 neutralizing antibody attenuated LPS-induced myocardial IL-1β production by 65% at 4 h (48.7 ± 3.5 pg/mg protein, *P* < 0.001, Fig. 5). Pretreatment of mice with NRS had no effect on myocardial IL-1β production.

**Effect of neutralization of IL-18 on LPS-induced myocardial ICAM-1 and VCAM-1 protein expression.**

We previously observed that neutralization of either VCAM-1 (34) or ICAM-1 (unpublished data) attenuates LPS-induced myocardial dysfunction. Therefore, immunofluorescence was used to examine the effect of neutralization of IL-18 on the expression of these two adhesion molecules. At 6 h after LPS, the integrated intensity of myocardial ICAM-1 increased eightfold compared with vehicle control. Pretreatment of mice with neutralizing IL-18 antibody attenuated LPS-induced myocardial ICAM-1 expression by 50% (Fig. 6). Similarly, LPS induced a greater than fourfold increase in the integrated intensity of VCAM-1 compared with vehicle control, but neutralization of IL-18 attenuated VCAM-1 expression by 36% (Fig. 7). Pretreatment with NRS did not influence LPS-induced myocardial ICAM-1 or VCAM-1 expression.
**Effect of neutralization of IL-18 on LPS-induced myocardial neutrophil accumulation.** LPS-induced injury in many organs is associated with the accumulation of neutrophils. We therefore investigated whether neutralization of IL-18 attenuates LPS-induced myocardial neutrophil accumulation. With the use of immunofluorescence, myocardial neutrophil number increased by fivefold at 6 h after LPS compared with vehicle control (13.3 ± 1.5 vs. 2.9 ± 1.5 neutrophils/mm², P < 0.01). Pretreatment of mice with IL-18 neutralizing antibody attenuated LPS-induced myocardial neutrophil accumulation by 55% compared with pretreatment with NRS (Fig. 8).

**DISCUSSION**

In this study we observed that myocardial IL-18 content is increased by LPS and that LPS-induced myocardial dysfunction is attenuated by specific neutralization of endogenous IL-18. We also examined whether the protection against LPS-induced dysfunction provided by neutralization of IL-18 was associated with attenuation of other known cardiodepressant cytokines. Neutralization of IL-18 attenuated the increase in myocardial IL-1β content. In addition, the ICAM-1/VCAM-1 protein expression that occurs during endotoxemia was also decreased by neutralization of IL-18. In contrast, neutralization of IL-18 had no influence on LPS-induced myocardial TNF-α production. Gene deletion of TNF-α attenuated the LPS-induced increase in myocardial IL-18 levels, which suggests that the cardiodepressive role of TNF-α during endotoxemia may be mediated via induction of IL-18. IL-18 may, in turn, be a direct cardiodepressant or may mediate endotoxemic myocardial dysfunction via induction of and/or synergy with IL-1β, ICAM-1, and VCAM-1.

The IL-18 precursor is present in an inactive form, but after cleavage by caspase-1, biologically active IL-18 is released from cells (10, 13). Indeed, a basal level of IL-18 was detected in the myocardium of control mice with an ELISA that recognizes both pro-IL-18 and active IL-18. Whereas LPS induced a twofold increase in myocardial IL-18 content, the increase did not occur until 4 h after LPS; however, it is likely that some of the pro-IL-18 present in the myocardium was rapidly cleaved and secreted into the circulation as active IL-18 after LPS injection. Thus, although myocardial IL-18 content did not increase until 4 h after LPS, it is possible that an increase in the active form of IL-18 occurred earlier. The twofold increase in total myocardial tissue IL-18 observed in this study is similar to the degree of increase in lung and liver IL-18 content during endotoxemia reported by others (25).
Mice pretreated with NRS had similar LVDP compared with mice treated with LPS alone. In contrast, pretreatment with neutralizing antibody against IL-18 nearly completely abrogated LPS-induced myocardial dysfunction. Thus neutralization of IL-18 preserves myocardial function during endotoxemia. This finding is in agreement with other studies, which have demonstrated a protective effect of neutralization of IL-18 in LPS-induced hepatoxicity (6, 41), experimental colitis (38), postischemic acute renal failure (20), and ischemia-reoxygenation-induced myocardial contractile dysfunction (32). The results of this study suggest that IL-18 contributes as a significant cytokine in endotoxemic myocardial depression.

Neutralization of IL-18 attenuated LPS-induced myocardial dysfunction without reducing myocardial TNF-α production. Peak levels of myocardial IL-18 occurred at 4 h at which time TNF-α had returned to near baseline levels. Because the increase in myocardial IL-18 was delayed compared with myocardial TNF-α production, the increase in IL-18 could not be a signal for TNF-α production. Early activation of IL-18 is also unlikely a signal for TNF-α production because IL-18 neutralizing antibody was applied as a pretreatment. It is, however, not unexpected that neutralization of IL-18 protects against LPS-induced myocardial dysfunction without attenuating TNF-α because others have reported that neutralization of IL-18 protects against LPS-induced lung and liver (25, 36) injury without reducing tissue TNF-α levels. In addition, neutralization or genetic deficiency of IL-18 has also been reported to protect mice from lethal endotoxemia without reducing serum TNF-α levels (14, 26).

Meng et al. (22) reported a temporal discordance between myocardial TNF-α levels and contractile dysfunction during endotoxemia. After LPS, myocardial dysfunction did not occur until TNF-α levels had returned to baseline. Despite this observation, neutralization of TNF-α attenuated LPS-induced myocardial dysfunction. These data, as well as our current finding that neutralization of IL-18 attenuates LPS-induced myocardial dysfunction without attenuating myocardial TNF-α levels, suggest that the role of TNF-α in depressing myocardial function may lie in its induction of other downstream factors. This hypothesis was further examined by determining whether TNF-α was required for the LPS-induced increase in myocardial IL-18 levels. In TNF−/− mice, myocardial IL-18 levels were not significantly different between the saline control and LPS-treated groups. Compared with LPS-treated wild-type mice, myocardial IL-18 levels were decreased after LPS in TNF−/− mice. This suggests that TNF-α may regulate myocardial IL-18 production, and this may be a mechanism by which TNF-α contributes to myocardial dysfunction during endotoxemia. Similarly, neutralization of TNF-α was reported (7) to attenuate the induction of IL-18 by the T cell mitogen concanavalin A (Con A), and the reduction in IL-18 protected mice from Con A-induced hepatoxicty. However, TNF-α appears not to be the sole factor regulating myocardial IL-18 production during endotoxemia because TNF−/− did not completely eliminate LPS-induced myocardial IL-18 production. Furthermore, we have not examined whether TNF-α influences the activation of caspase-1 and subsequent cleavage of IL-18 to its active form.

IL-1β production reached peak levels at 4 h after LPS, which coincides with the increase in myocardial IL-18. Of considerable importance was the observation that neutralization of IL-18 reduced myocardial IL-1β production. These findings suggest that IL-1β depresses myocardial function. Moreover, in vitro studies (4, 16) have demonstrated that TNF-α and IL-1β act synergistically to depress myocardial contractility. The results of the present in vivo study suggest that TNF-α may not be a direct cardiodepressant because myocardial TNF-α had returned to baseline at the time when myocardial dysfunction occurred. It is more likely that the importance of TNF-α is to act as an early signal during endotoxemia by inducing downstream cytokines rather than as a direct cardiodepressant. In contrast, both myocardial IL-18 and IL-1β levels are elevated at 4 h after LPS. These factors may contribute directly to myocardial depression.

Transgenic mice that specifically overexpress myocardial TNF-α develop cardiac dysfunction (3); however, simultaneous ICAM-1 gene deletion in this TNF-α-transgenic mouse model markedly improves cardiac function (11). These data suggest that ICAM-1 is an important mediator of the cardiac dysfunction induced by TNF-α. Similarly, we (34) have reported that VCAM-1 is required for LPS-induced myocardial dysfunction. In the present study, neutralization of IL-18 attenuated LPS-induced myocardial ICAM-1 and VCAM-1 expression, which corroborates previous reports (15, 24, 43) of induction of the expression of these adhesion molecules by IL-18. Thus attenuation of ICAM-1 and VCAM-1 expression by neutralization of IL-18 may have contributed to the protection against LPS-induced myocardial dysfunction.

We (34) reported that LPS-induced myocardial neutrophil accumulation is temporally associated with cardiac dysfunction and that neutralization of VCAM-1 attenuates both LPS-induced myocardial neutrophil accumulation and dysfunction. Similarly, the attenuation of ICAM-1 and VCAM-1 expression by neutralization of IL-18 was associated with a reduction in myocardial neutrophil accumulation. Whereas it is tempting to speculate that the reduction in myocardial neutrophils may have contributed to protection against LPS-induced myocardial dysfunction, further investigation is necessary to determine the role of neutrophils in endotoxemic cardiodepression.

In conclusion, neutralization of IL-18 attenuates LPS-induced myocardial dysfunction without reducing myocardial TNF-α production. Whereas this study does not
IL-8 ATTENUATES LPS-INDUCED DYSFUNCTION

discount TNF-α as an important cytokine in LPS-induced myocardial dysfunction, it suggests that TNF-α is likely an early initiator and not a direct end effector of endotoxic depression. This study does not suggest that IL-18 is the sole mediator of LPS-induced myocardial dysfunction but instead suggests that IL-18 plays an important role in this disorder likely via its induction of and/or synergy with IL-1β, ICAM-1, and VCAM-1. A limitation of this study is that the model used examines the role of IL-18 in LPS-induced myocardial dysfunction (in the absence of live infection), and thus the results cannot be directly extrapolated to live infection, sepsis-induced myocardial dysfunction. Further investigation of the role of IL-18 in sepsis-induced myocardial dysfunction using an animal model of sepsis such as cecal ligation and puncture is warranted.

The authors thank Lihua Ao for assistance in immunofluorescent staining of tissues and Sandor A. Falk for technical assistance in monitoring mean arterial pressures of mice during endotoxemia. This study was supported in part by National Institute of General Medical Sciences Grants GM-49222 and GM-08315.

REFERENCES


