Effects of soluble TNF receptor treatment on lipopolysaccharide-induced myocardial cytokine expression

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Kadokami, Toshiaki, Charles F. McTierman, Toru Kubota, Carole S. Frye, George S. Bounoutas, Paul D. Robbins, Simon C. Watkins, and Arthur M. Feldman. Effects of soluble TNF receptor treatment on lipopolysaccharide-induced myocardial cytokine expression. Am J Physiol Heart Circ Physiol 280: H2281–H2291, 2001.—Tumor necrosis factor (TNF)-α plays a key role in the pathogenesis of septic shock syndrome, and myocardial TNF-α expression may contribute to this pathophysiology. We examined the myocardial expression of TNF-α-related cytokines and chemokines in mice exposed to lipopolysaccharide (LPS) and tested the effects of anti-TNF therapy on myocardial cytokine expression. Cytokine mRNA levels were measured by RNase protection assay, and protein levels in the plasma and myocardium were assessed by enzyme-linked immunosorbent assays. LPS (4 μg/g body wt ip) induced marked cytokine expression, including TNF-α, interleukin (IL)-1β, IL-6, and monocyte chemotactic protein (MCP)-1, in both the plasma and myocardium. Pretreatment with adenovirus-mediated TNF receptor fusion protein (AdTNFR1; 109 plaque-forming units iv) decreased plasma cytokine levels. In contrast, whereas myocardial IL-1β expression was also suppressed, expression of IL-6 and MCP-1 was not inhibited by AdTNFR1. In summary, anti-TNF treatment differentially altered the cytokine expression in the plasma and myocardium during endotoxemia. Inability to block myocardial expression of IL-6 and MCP-1 suggests a possible mechanism for the failure of anti-TNF therapies in the treatment of endotoxin shock.

adenovirus; chemokine; endotoxin shock; tumor necrosis factor-α

SEPTIC SHOCK, triggered by products of infectious agents, causes substantial morbidity and mortality characterized by a hypotensive state accompanied by inadequate tissue perfusion, metabolic acidosis, and coagulopathy (6). Evidence suggests that tumor necrosis factor (TNF)-α, which is a proinflammatory cytokine with pleiotropic biological effects (60), is a key mediator of the septic shock syndrome induced by either lipopolysaccharide (LPS) or bacterial superantigens (6, 38, 40, 50). Indeed, in response to LPS, TNF-α is produced in large amounts earlier than any other cytokine (6), and, given as a purified preparation, TNF-α evokes most of the effects of LPS in animals including fever, shock, and death (50). Furthermore, mice lacking TNF receptor type 1 (TNFR1) are resistant to septic shock (43, 45).

Given that TNF-α plays a key role in the pathogenesis of septic shock syndrome, anti-TNF therapy was expected to provide protection against the toxicity of TNF-α and reduce mortality. However, the results of currently tested anti-TNF therapies for endotoxemia have been controversial. In some (3, 17, 39) but not all (13, 33) experimental studies, anti-TNF therapies ablated TNF-α bioactivity and reduced mortality after LPS injection. Moreover, anti-TNF antibodies can convert a nonlethal model of endotoxemia into a lethal one (12). In one clinical study (14), administration of a monoclonal antibody to TNF-α in patients with septic shock limited hypotension. However, treatment with the TNFR:Fc fusion protein in patients with septic shock failed to reduce mortality, and higher doses appeared to be associated with increased mortality (16).

Elevated plasma levels of TNF-α have been reported in a variety of cardiovascular diseases, including acute myocarditis (35), cardiac allograft rejection (8), myocardial infarction (36), and congestive heart failure (32, 34), as well as in endotoxemia (17, 51, 54). Recent studies have demonstrated that the heart can produce TNF-α in response to pathological stresses (24, 49) and endotoxin (18, 23). In addition, transgenic mice overexpressing cardiac TNF-α develop myocardial hypertrophy and dilatation, interstitial infiltrates and fibrosis, attenuation of adrenergic responsiveness, and robust expression of a variety of TNF-α-responsive proinflammatory cytokines and chemokines, including interleukin (IL)-1β and monocyte chemotactic protein (MCP)-1 (29, 31). These observations led us to hypothesize that the inability of anti-TNF therapy to improve
survival during endotoxemia is due to an inability to suppress the expression of non-TNF-α endotoxin-responsive cytokines and chemokines in the heart.

To test this hypothesis, we neutralized TNF-α bioactivity by injecting mice with a replication-deficient recombinant adenovirus encoding a 55-kDa soluble TNF receptor (AdTNFR1) before LPS challenge. This anti-TNF treatment reduced the expression of TNF-α and TNF-α-responsive proinflammatory cytokines and chemokines in plasma during endotoxemia but was ineffective in attenuating the myocardial expression of the endotoxin-responsive cytokines IL-6 and MCP-1.

MATERIALS AND METHODS

Animal Preparation

Male 3-mo-old FVB mice (29.3 ± 1.8 g body wt) bred from an in-house colony were used for protocol 1. Male 3-mo-old TNFR1-deficient mice created on a C57BL/6 background (Jackson Laboratory) (43) were used for protocol 2. Age- and sex-matched wild-type mice served as controls. Detailed descriptions about the protocols are shown in Experimental Design. Animals were housed in cages at 20–22°C with a 12-h:12-h light-dark cycle. Animals were allowed free access to water and laboratory chow throughout the experimental period. The mice were utilized according to protocols approved by the Institutional Animal Care and Use Committee, University of Pittsburgh.

Recombinant Adenoviruses

AdTNFR1 (26), encoding the extracellular domain of human 55-kDa TNFR coupled with a mouse IgG heavy chain (42), was used in the present study. The original viruses were generously provided by Dr. Bruce Beutler, University of Texas Southwestern Medical Center. The viruses were propagated in 293 cells, purified by cesium chloride density centrifugation, and stored in aliquots at −80°C as previously described (26). The virus titer was equal to the optical density at 260 nm (OD260) divided by 9.09 × 10−12 particles/ml. One hundred particles were assumed to be one plaque-forming unit (pfu). After injection, the majority of virus is extracted by the liver, with subsequent hepatic production and release of soluble receptor into the peripheral circulation (26). Intravenous injection of 10⁹ pfu of AdTNFR1 has been shown to inhibit TNF activity in plasma for up to 6 wk (26). This dose of AdTNFR1 could effectively abrogate the changes of myocardial inflammation and cardiac-specific gene expression in our transgenic mice with cardiac-specific overexpression of TNF-α (29).

Experimental Design

The experimental design sought to examine whether treatment with soluble TNFR1 could ameliorate the expression of cardiac cytokines during endotoxemia in mice. To this end, we performed two experiments.

Protocol 1. Mice were divided into two groups as follows: 1) animals injected with 10⁹ pfu of AdTNFR1 through the retroorbital venous plexi 1 wk before LPS exposure and 2) control animals not injected with AdTNFR1. We used the LPS of Escherichia coli 0127 (Sigma), which strongly stimulates TNF-α production in rat and human cardiomyocytes (55, 57). Animals were given a single intraperitoneal injection of LPS (4 μg/g body wt) as a 1 μg/μl solution in physiological saline. At 0.5, 2, and 24 h after LPS injection, the animals were euthanized. After the ventricular weight was determined, excised ventricles were snap-frozen in liquid nitrogen for RNA and protein analysis. In some of the animals euthanized at 2 h after LPS challenge, hearts were perfused with 2% paraformaldehyde and then processed for immunohistochemical analysis as described in Immunohistochemistry. Plasma was also collected for assessment of cytokines and soluble TNFR1. The hearts and plasma from age- and sex-matched mice exposed to saline (instead of LPS) with or without AdTNFR1 pretreatment served as controls.

Protocol 2. TNFR1-deficient mice and wild-type controls were also injected with LPS the same way as protocol 1. Animals were divided into two groups, and each group was given either a low (4 μg/g body wt) or high (40 μg/g body wt) dose of LPS, respectively. At 2 h after injection, the animals were euthanized, and cardiac tissue and plasma samples were harvested as described in Protocol 1.

RNase Protection Assay

Total RNA was extracted from frozen tissues with the use of an acid guanidinium thiocyanate-phenol-chloroform method (9). The concentration of RNA in each sample was assessed spectrophotometrically. To evaluate transcript levels of cytokines in the myocardium, a commercially available multiprobe RNase protection assay kit (RiboQuant, Pharmingen) was used, with the assay performed according to the manufacturer’s protocol. Briefly, a set of 13P-labeled RNA probes synthesized from DNA templates using T7 polymerase was hybridized with 5 μg of total RNA, after which free probes and other single-stranded RNA were digested with RNases. The remaining RNase-protected probes were purified, resolved on denaturing polyacrylamide gels, and quantified by Phospholmerager using ImageQuant software (Molecular Dynamics). The value of each hybridized probe was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) included in each template set as an internal control (arbitrarily set as equal to 1). The following template sets for murine cytokines were used in the present study: mCK-2b (No. 45051P), IL-12p35, IL-12p40, IL-10, IL-1α, IL-1β, IL-1 receptor antagonist (Ra), IL-18, IL-6, interferon (IFN)-γ, macrophage migration inhibition factor (MIF), L32, and GAPDH; mCK-3b (No. 45071P), TNF-β, lymphotoxin (LT)-β, TNF-α, IL-6, IFN-γ, IFN-β, transforming growth factor (TGF)-β1, TGF-β2, TGF-β3, MIF, L32, and GAPDH; and mCK-5 (No. 45026P), lymphotoxin, regulated upon activation normal T-cell expressed and secreted (RANTES), eotaxin, macrophage inflammatory protein (MIP)-1α, MIP-1β, IFN-γ-inducible protein (IP)-10, MCP-1, T-cell activation gene (TCA)-3, L32, and GAPDH.

Enzyme-Linked ImmunoSorbent Assay

Protein levels of cytokines were assessed using commercially available ELISA kits for mouse TNF-α, mouse IL-1β, mouse IL-6, mouse MCP-1, mouse IL-10, mouse IL-12p75, and human TNFR1 (Quantikine, R&D Systems). Plasma samples were measured at a dilution of 10⁻¹–10⁻³ for TNF-α, IL-1β, IL-6, and MCP-1 and 10⁻⁵–10⁻⁷ for TNFR1. Cytokines in the myocardium were measured as previously reported (30, 31). Briefly, frozen tissues (5–20 mg) were homogenized in 300–500 μl of ice-cold phosphate-buffered saline containing 1 mmol/l phenylmethylsulfonyl fluoride protease inhibitor (Sigma). After a brief centrifugation, samples were kept on ice for the duration of the assay. Total protein levels were quantitated using a commercially available assay (Bio-Rad Protein Assay, Bio-Rad Laboratories) with BSA (Sigma) as a standard. The same amount of protein
was applied for each immunoassay: 100 μg for TNF-α, IL-1β, IL-6, and MCP-1 and 1 μg for TNFRI. Cytokines provided by the manufacturer were used as a standard. All assays were done in duplicate. Results were analyzed spectrophotometrically at a wavelength of 450 nm with a microtitrater plate reader. The values are reported as picograms or nanograms per milligram of protein for tissue samples and nanograms or micrograms per milliliter for plasma samples.

**Immunohistochemistry**

To clarify which cell types of cardiac tissue are responsible for LPS-induced IL-6 production, we performed IL-6 immunostaining in some LPS-treated mice. Mouse hearts were perfused with 2% paraformaldehyde, followed by a 2-h postfix in the same fixative. After overnight cryoprotection in ice-cold 30% sucrose, the hearts were flash-frozen with liquid nitrogen-cooled isopentane. Cryostat (Microm) sections (6 μm) were cut and mounted onto Superfrost Plus slides (Fisher). Sections were then rinsed with PBS, followed by rinsing in a blocking buffer (0.5% BSA and 0.15% glycine in PBS) before treating with 5% normal goat serum for 30 min. Double-immunofluorescent detection was performed using a polyclonal rabbit anti-mouse IL-6 antibody (1:100, sc-7920, Santa Cruz) and a monoclonal rat anti-mouse CD45 antibody (1:100, 01111D, Pharmingen). Samples were treated with primary antibodies for 1 h at room temperature and then rinsed with blocking buffer. Fluorescent secondary antibodies included goat anti-rabbit IgG conjugated with CY3 (1: 3000, Jackson ImmunoResearch Laboratories) and goat antirat IgG conjugated with C59 (1:1000, Jackson ImmunoResearch Laboratories). Slides were treated with secondary antibodies for 1 h at room temperature and then sequentially rinsed with blocking buffer, followed by PBS before nuclei were labeled with Hoescht 33342 (Sigma). Slides were overslipped and viewed with an Olympus Provis AX70 fluorescent microscope. Images were collected with a cooled charge-coupled device camera (Optronics Magnifier) at a 12-bit gray depth and assembled in Photoshop (Adobe). No further post-processing or filtering of the images was performed.

**Statistics**

The results are presented as means ± SD. Statistical comparisons were performed with the use of analysis of variance with Student-Newman-Keuls post hoc test. Differences were considered significant at a value of P < 0.05.

**RESULTS**

Body weight and ventricular weight of the animals at the time of death were comparable, with no significant differences among any of the groups (data not shown).

**Adenovirus-Mediated Production of TNF Receptor Fusion Protein**

To confirm that the dose of AdTNFRI used in the present study (10⁶ pfu iv) was adequate to block local bioactivity of TNF-α in the myocardium, the plasma and myocardial levels of TNFRI in AdTNFRI-treated animals were assayed. As shown in Table 1, intravenous injections of 10⁶ pfu of AdTNFRI produced a substantial amount of TNFRI in both the plasma and myocardial tissue after 1 wk of treatment. Compared with the peak amounts of TNF-α protein expressed in

<table>
<thead>
<tr>
<th>Time After LPS Injection</th>
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<th>Plasma, μg/ml</th>
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<tr>
<td>No LPS injection</td>
<td>4</td>
<td>460.2 ± 104.6</td>
<td>77.0 ± 15.9</td>
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<tr>
<td>2h</td>
<td>6</td>
<td>376.3 ± 129.0</td>
<td>67.3 ± 17.5</td>
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<tr>
<td>24h</td>
<td>5</td>
<td>400.3 ± 119.0</td>
<td>73.2 ± 16.6</td>
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Values are means ± SD; n, no. of mice. LPS, lipopolysaccharide; TNFRI, tumor necrosis factor (TNF) receptor type I; AdTNFRI, adenovirus-mediated TNF receptor fusion protein.

**Expression of Cytokines in Myocardium in Response to LPS Administration**

Because TNF-α is known to induce the expression of other proinflammatory cytokines and chemokines that contribute to TNF-α-induced pathophysiology (60), we examined the expression of a panel of cytokines using a multiprobe RNase protection assay. Representative images of RNase protection assays are shown in Fig. 1 and quantitative results are summarized in Fig. 2. Intraperitoneal injection of LPS induced the expression of a group of cytokines (including TNF-α, TNF-β, IL-1α, IL-1β, IL-1Ra, IL-6, IL-10, IL-12, IL-18, TGF-β1, TGF-β2, TGF-β3, and LT-β) and a group of chemokines (including MCP-1, RANTES, MIP-1α, MIP-1β, MIP-2, eotaxin, and lymphotactin). In particular, the induction of TNF-α, IL-1β, IL-6, and MCP-1 expression in the myocardium in response to LPS challenge was robust. In contrast, MIF was constitutively expressed in the myocardium in mice without LPS treatment and was increased after LPS injection. Cytokines IFN-β, IFN-γ, IP-10, and TCA-3 were not detected in any samples regardless of LPS treatment. The myocardial mRNA expression of various cytokines were followed before LPS injection and at 0.5, 2.0, and 24 h after the injection. The maximal myocardial expression of TNF-α mRNA was observed 0.5 h after LPS treatment, whereas most other cytokines showed peak responses 2.0 h after LPS treatment. A significant exception was RANTES, which showed maximal expression 24 h after LPS injection (Fig. 1 and Table 1). Pretreatment with AdTNFRI partly but significantly reduced the myocardial expression of IL-10 and IL-12p40 gene expression, and these changes were not significantly affected by AdTNFRI (Fig. 2).

To confirm that the changes in mRNA reflected alterations at the protein level within the myocardium,
we measured the protein levels of six cytokines (TNF-α, IL-1β, IL-6, MCP-1, IL-10, and IL-12p75). These cytokines were chosen because of their synergistic effects with TNF-α as well as their independent effects on cardiomyocyte function and gene expression (7, 19, 25, 37, 44) or their well-established roles in the pathogenesis of endotoxin shock (21, 59). As shown in Fig. 3, myocardial TNF-α, IL-1β, IL-6, and MCP-1

Fig. 1. mRNA expression of cytokines in the myocardium in response to lipopolysaccharide (LPS) administration. A–C: representative images of multiprobe RNase protection assays: mCK-3b (A), mCK-2b (B), and mCK-5 (C). AdTNFR1, inoculation of adenovirus-mediated tumor necrosis factor (TNF) receptor type 1 (TNFR1) fusion protein (AdTNFR1) 1 wk before the LPS challenge. LT, lymphotoxin; IL, interleukin; TGF, transforming growth factor; MIF, macrophage migration inhibitory factor; GADPH, glyceraldehyde-3-phosphate dehydrogenase; IL-1Ra, IL-1 receptor antagonist; Ltn, lymphotactin; RANTES, regulated upon activation normal T-cell expressed and secreted; MIP, macrophage inflammatory protein; MCP, monocyte chemotactic protein.

Fig. 2. Time course of mRNA expression of the following cytokines in the myocardium in response to LPS stimulation with the presence (open bars) or absence (solid bars) of AdTNFR1 pretreatment: TNF-α (A), IL-1β (B), IL-6 (C), MCP-1 (D), IL-10 (E), and IL-12p40 (F). Values are expressed as means ± SD; n = 4–6 mice. *P < 0.05 vs. 0 h; †P < 0.05, AdTNFR1− vs. AdTNFR1+. 
proteins were not found in mice without LPS treatment but were abundant in the LPS-treated mice. One week of anti-TNF pretreatment with soluble receptor led to a moderate but significant decrease in the expression of IL-1β but not of IL-6 and MCP-1 proteins, consistent with studies assessing levels of mRNA. In fact, anti-TNF treatment actually increased the level of immuno-detectable TNF-α protein in the myocardium. IL-10 and IL-12p75 protein were also significantly increased by LPS treatment, although the amount of these two cytokines was much less than the amount of the other four cytokines examined. Cardiac IL-10 protein expression was significantly augmented by AdTNFR1 treatment, whereas IL-12p75 expression was not changed. LPS-induced cardiac cytokine expression was also examined in TNFR1-deficient mice (TNFR1−/−). As

Fig. 3. Effect of AdTNFR1 treatment on protein expression of the following cytokines in the myocardium in response to LPS stimulation: TNF-α (A), IL-1β (B), IL-6 (C), MCP-1 (D), IL-10 (E), and IL-12p75 (F). Values are expressed as means ± SD; n = 4–6 mice. *P < 0.05 vs. 0 h; †P < 0.05, AdTNFR1− vs. AdTNFR1+.

Fig. 4. Cardiac expression of the following cytokines in response to LPS treatment in TNFR1-deficient mice: TNF-α (A), IL-1β (B), and IL-6 (C). Bw, body weight. Values are expressed as means ± SD; n = 4–5 mice. *P < 0.05 vs. 0 h; †P < 0.05, wild-type controls vs. TNFR1-deficient mice with same treatment.
shown in Fig. 4, cardiac expression of TNF-α in response to LPS was similar to or somewhat higher in TNFR1-deficient mice compared with wild-type control mice (TNFR1+/+). In contrast, both IL-1β and IL-6 expression were significantly less in TNFR1-deficient mice. However, even in TNFR1-deficient mice, there were still substantial increases in IL-1β or IL-6 expression, ~50% of that reached in the wild-type LPS-treated mice.

Circulating Cytokine Levels after LPS Administration

We also examined the plasma protein levels of these four cytokines in response to LPS injection (Fig. 5). TNF-α, IL-1β, IL-6, and MCP-1 proteins were not found in the plasma of mice without LPS treatment but were abundant in the LPS-treated mice, which is similar to that observed for myocardial expression. The immunodetectable protein levels of plasma TNF-α were markedly increased by pretreatment with AdTNFR1. In contrast to the result from myocardium, IL-1β, IL-6, and MCP-1 proteins induced by LPS were significantly decreased by AdTNFR1. AdTNFR1 pretreatment again significantly augmented LPS-induced IL-10 expression, whereas IL-12p75 levels were markedly decreased.

Plasma cytokine levels in TNFR1-deficient mice after LPS challenge are summarized in Fig. 6. The increase of TNF-α levels in TNFR1-deficient mice after LPS treatment was significantly higher in TNFR1-deficient mice in both the low and high dosages of LPS. However, plasma levels of both IL-1β and IL-6 concentration were markedly suppressed in TNFR1-deficient mice. These observations were consistent with the previous report (45) and our present studies using AdTNFR1.

IL-6 Immunohistochemical Staining

Immunohistochemical staining of LPS-treated cardiac tissue was performed to determine the cell types that express IL-6 in response to systemic LPS challenge. Counterstaining with an anti-CD45 antibody was performed to distinguish cardiac infiltrating leukocytes from resident cardiac cells such as myocytes and fibroblasts. Representative images from LPS and saline-treated animals are shown in Fig. 7. Because both CD45-positive leukocytes and CD45-negative cardiac myocytes were positively stained with anti-IL-6 antibody in LPS-treated tissues, both of these cell types are likely sources of IL-6 production in response to LPS challenge.

DISCUSSION

While TNF-α has important direct biological functions and plays a key role in the pathogenesis of endotoxin shock syndrome, it can also induce the expression of “downstream” cytokines and chemokines that may contribute to TNF-α-induced pathophysiology (60). Furthermore, in endotoxin shock, different organs display diverse patterns of cytokine expression (18, 52). In
addition, while endotoxin may induce the expression of substances that alter cardiac function such as TNF-α and other cytokines and chemokines within the myocardium, endotoxin itself may have direct cardiodepressant effects (41). The diversity of both beneficial (3, 14, 17, 39) and antagonistic (12, 16) effects that may occur in endotoxemia consequent to anti-TNF-α therapies suggest a complex and incompletely understood role of TNF-α in this condition. One possible mechanism for the divergent responses elicited by anti-TNF-α therapy is that therapy with monoclonal anti-TNF antibody attenuates activation of the fibrinolytic system without influencing endotoxin-induced activation of the coagulation system, generating a potential enhancing effect on microvascular thrombosis in sepsis (53). In this study, we suggest that relative to systemic effects measured by LPS-induced serum cytokine levels, anti-TNF-α therapy fails to completely protect the myocardium from the detrimental effects elicited directly by LPS or other cytokines induced by LPS through non-TNF-dependent mechanisms.

Fig. 6. Plasma concentration of the following cytokines after LPS treatment in TNFR1-deficient mice: TNF-α (A), IL-1β (B), and IL-6 (C). Values are expressed as means ± SD; n = 4–5 mice. *P < 0.05 vs. 0 h; †P < 0.05, wild-type controls vs. TNFR1-deficient mice with same treatment.

Fig. 7. Immunohistochemical staining for IL-6 and CD45 in a LPS-treated mouse (A–C) and a saline-treated mouse (D–F) cardiac tissue. A and D: IL-6 staining; B and E: CD45 staining; C and F: merged imaging of IL-6 and CD45. Arrows indicate IL-6 and CD45 double-positive cells. Arrowheads indicate IL-6-positive/CD45-negative myocytes, which clearly displayed sarcomeric structures.
In this study, we utilized an anti-TNF therapy in which animals were injected intravenously with an adenoviral vector expressing a fusion protein of the extracellular domain of human TNFR1(p55) coupled with a mouse IgG heavy chain (26). The virus is rapidly taken up by the liver, which effectively becomes a production site for soluble TNFR1. A single intravenous injection allows sustained expression of a substantial amount of TNFR1 in the plasma, which permeates the extracellular space of multiple organs (27) including the heart, binds to TNF-α, and limits its ability to interact with cellular TNF receptors. The principle limitation of using recombinant adenovirus is that endogenous immunological activity limits the ability to reinject animals with AdTNFR1. Thus, after several months, biologically significant levels of TNFR1 cannot be found in either the plasma or tissues. However, as seen in our previous study, beneficial anti-TNF therapy with TNFR1, allowing low-level production of downstream cytokines.

To better elucidate the differential expression of cytokines and chemokines in the myocardium in response to LPS challenge with or without anti-TNF-α therapy, we used multiprobe RNase protection assay panels to assess the cytokine and chemokine expression in the ventricle and ELISA to assess the protein expression of cytokines and chemokines in the ventricle and plasma of the LPS-treated mice. The principal finding of these studies is that, whereas LPS induces a similar profile of cytokines and chemokines (TNF-α, IL-1β, IL-6, IL-10, IL-12, and MCP-1) in the plasma and myocardium, plasma and myocardial expression of these cytokines are differentially responsive to anti-TNF therapy.

In the present study, intraperitoneal administration of LPS to mice resulted in robust expression of a group of cytokines including TNF-α, IL-1β, IL-6, and MCP-1 in both the myocardium and plasma. Importantly, in the myocardium, there was a close correlation between the measure of cytokine mRNAs and proteins. Pre-treatment with soluble TNF receptor partially but significantly reduced plasma levels of IL-1β, IL-6, and MCP-1. In contrast, anti-TNF pretreatment significantly suppressed LPS-induced myocardial expression of IL-1β but had no effect on the production of IL-6 and MCP-1, suggesting that the myocardial induction of these two cytokines occurs either directly through LPS or through non-TNF-α-dependent pathways. To better assess the role of TNF-α in the cardiac response to LPS, we also treated mice lacking functional TNFR1 protein (TNFR1−/−) with LPS. LPS still induced an increase in plasma IL-1β and IL-6 levels, although at a level significantly lower than that observed in their wild-type littermates (TNFR1+/+), which is consistent with previous reports (43, 45). In particular, plasma IL-6 levels were reduced by ~80%, relative to those observed in LPS-injected TNFR1+/+ mice. However, the cardiac expression of IL-6 in LPS-injected TNFR1−/− mice was still ~50% of that observed in TNFR1+/+ mice, suggesting that the cardiac induction of IL-6 by LPS is not completely dependent on signals mediated through the TNFR1 receptor. While we formally cannot rule out the possibility that cardiac TNFR2 receptors in the TNFR1−/− mice mediate the induction of IL-6 after LPS challenge, studies (5, 28) in other tissue and cell types failed to find a significant role for TNFR2 in the production of IL-6 elicited by TNF-α.

Further evidence suggests that LPS stimulates cardiac expression of IL-6 and MCP-1 in a mechanism different from that induced directly by TNF-α. For example, transgenic mice (TNF1.6) that overexpress TNF-α in the myocardium (29) demonstrated elevated MCP-1 expression with no detectable expression of IL-6. Additionally, treatment of TNF1.6 mice with AdTNFR1 completely abrogated the myocardial expression of MCP-1. Therefore, it would appear that LPS augments the myocardial expression of IL-6 and MCP-1 independently of or synergistically with the expression of TNF-α. Thus, while the cardiac inflammation associated with myocarditis may be dependent on TNF-α signals mediated through TNFR1 (2), the cardiac toxicity arising from LPS challenge may be at least partially independent of TNF-α-mediated pathways. This finding might explain the inability of anti-TNF therapies to reverse the cardiotoxicity associated with endotoxemia because the myocardial expression of endotoxin-responsive cytokines (i.e., IL-6 and MCP-1) would be unabated and that of IL-1β only partially reduced.

The mechanisms by which LPS may induce cytokine expression in the various cells of the heart may include at least two pathways: one CD14 dependent and the other CD14 independent. CD14, a glycosyl-phosphati-
dyl inositol-anchored glycoprotein expressed in monocytes/macrophages and neutrophils, binds to the LPS/ LPS-binding protein (LBP) complex in serum to activate macrophages (58). Cardiac myocytes also express CD14 (10, 11). In addition, cells that do not express membrane-bound CD14 can still respond to the LPS/LBP complex by interacting with soluble CD14 (20). In the present study, immunohistochemical staining of IL-6 revealed that both myocytes and infiltrating inflammatory (+CD45) cells were the source of LPS-induced IL-6 in the mouse heart. This finding is consistent with other reports (22, 56) stating that myocytes, leukocytes, and endothelial cells all produce IL-6 on LPS challenge. However, this does not prove that this occurs through a CD14-mediated pathway. Indeed, the presence of a CD14-independent LPS signaling pathway has been proposed in various cell types including cardiac myocytes (11). The CD14-independent pathway seems to be evident only when higher
doses of LPS are administered. In the present study, however, two different doses of LPS injection resulted in the same cytokine expression profile in TNFR1-deficient mice. Taken together, these studies suggest that LPS-induced cardiac production of IL-6 is dependent on CD14 but not completely dependent on TNF-α. Interestingly, in decidual cells, a similar pattern of CD14-dependent LPS-induced cytokine production was observed (1); anti-TNF therapy significantly inhibited IL-1β but not IL-6 production in response to LPS stimulation.

While LPS itself may have direct cardiotoxic effects independent of stimulation of cytokine production (41), IL-6 and MCP-1 are also clearly capable of contributing to cardiac dysfunction. IL-6 is induced in the plasma of patients with endotoxin shock (17, 54) and congestive heart failure (34) and is elevated in the myocardium in animal models of myocarditis or heart failure (46). Furthermore, IL-6 can directly diminish the contractile properties of isolated cardiomyocytes and cardiac tissues (15). In a less direct fashion, because MCP-1 is a prominent signal for the accumulation of monocytes (44), it may be a key mediator in the pathogenesis of myocardial inflammation leading to muscle dysfunction (25).

These studies do suggest that the LPS-induced level of IL-1β in the plasma and myocardium can be reduced by an anti-TNF-α therapy. This observation is consistent with a report (52) showing that injection of rodents with TNF-α can elicit a modest expression of IL-1β in some organs, and it would be reasonable that TNF-α may act synergistically with LPS to stimulate IL-1β expression. However, even though anti-TNF-α therapy reduced the LPS-induced serum level of IL-1β by almost 50% (to 13 ng/ml serum), these cytokine levels are still well above the concentration range capable of inducing alterations in gene expression and function in cultured cardiomyocytes (7, 37, 48). Thus the functional consequence of a partial reduction in IL-1β expression remains unclear.

In addition to the LPS-induced cytokines recognized for their direct adverse effects on the myocardium (IL-1β, IL-6, and TNF-α), IL-10 and IL-12 are also regulated by LPS exposure and play a prominent role in endotoxemia. IL-10 has been shown to be protective in murine endotoxemia (21, 47), whereas IL-12 plays an essential role in lethality in endotoxic mice (59). Although their expression levels were low, both cytokines were significantly induced by LPS challenge. Furthermore, AdTNFR1 treatment significantly increased the LPS-induced IL-10 protein levels in both the plasma and myocardium, consistent with previous findings that anti-TNF therapy accelerated the early peak but attenuated the delayed peak of IL-10 production induced by LPS (4). However, the cardiac levels of IL-10 transcripts were not augmented by blocking TNF-α bioactivity, suggesting a complex role for TNF-α, perhaps at the translational or protein processing level, in regulating IL-10 production. On the other hand, AdTNFR1 did not change LPS-induced IL-12 expression in cardiac tissue but markedly reduced the IL-12 plasma level. While a suppression of systemic IL-12 level may reduce the severity of endotoxia, the direct cardiac effects of altering cardiac levels of either IL-10 or IL-12 remain unknown.

TNF-α protein was elevated by LPS injection and further increased after the treatment with AdTNFR1. This phenomenon, also observed in our previous study (29) with TNF1.6 mice treated with AdTNFR1, may arise from several different mechanisms. First, a negative feedback mechanism present in the translation of TNF-α may have been uncoupled by anti-TNF treatment. Second, although TNF-α loses its bioactivity when bound by soluble TNFR1, it may also gain stability (39). Because ELISA measures both receptor-bound and free TNF-α, the major contribution to the increase might be due to soluble receptor-bound TNF-α. Whatever the mechanisms of increased TNF-α antigenicity in the mice treated with AdTNFR1, the biological effects of TNF-α were systemically attenuated by the treatment, as shown by the abrogation of expression of downstream cytokines in plasma.

In summary, anti-TNF treatment differentially altered the expression of key pro- and anti-inflammatory cytokines (IL-1β, IL-6, IL-10, IL-12, and MCP-1) in the plasma and myocardium during endotoxemia. Although we did not elucidate the entire mechanism of effects of blocking one inflammatory component in complex interactions within the cytokine network, the inability to block myocardial expression of IL-6, IL-12, and MCP-1 suggests a possible mechanism for the failure of anti-TNF therapies in the treatment of endotoxin shock syndrome.

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