Macrophage migration inhibitory factor mediates late cardiac dysfunction after burn injury

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WILLIS, MONTE S., DEBORAH L. CARLSON, J. MICHAEL DIMAIO, MICHAEL D. WHITE, D. JEAN WHITE, GLENN A. ADAMS, IV, JURETA W. HORTON, AND BRETT P. GIROIR. Macrophage migration inhibitory factor mediates late cardiac dysfunction after burn injury. Am J Physiol Heart Circ Physiol 288: H795–H804, 2005. First published September 23, 2004; doi:10.1152/ajpheart.00189.2004.—We have recently demonstrated that macrophage migration inhibitory factor (MIF) is a myocardial depressant protein and that MIF mediates late, prolonged cardiac dysfunction after endotoxin challenge in mice. Because many factors, including endotoxin, have been implicated in the pathogenesis of cardiac dysfunction after burn injury, we tested the hypothesis that MIF might also be the mediator of prolonged cardiac dysfunction in this model. At 4 h after 40% total body surface area burn in anesthetized mice, serum MIF levels increased significantly compared with baseline (2.2-fold). This increase was accompanied by a significant decrease in cardiac tissue MIF levels (2.1-fold decrease compared with controls). This pattern was consistent with MIF release from preformed cytoplasmic stores in the heart and other organs. To determine whether MIF mediates cardiac dysfunction after burn injury, mice were pretreated with anti-MIF neutralizing monoclonal antibodies or isotype control antibodies. Beginning 4 h after burn injury (and continuing through 48 h), burned mice demonstrated a significantly depressed left ventricular shortening fraction of 38.6 ± 1.8%, compared with the normal controls (56.0 ± 2.6%). Mice treated with anti-MIF displayed an initial depression of cardiac function similar to nontreated animals but then showed rapid restoration of cardiac function with complete recovery by 24 h, which persisted for the duration of the protocol. This study is the first to demonstrate that MIF mediates late, prolonged cardiac dysfunction after burn injury and suggests that MIF blockade should be considered a therapeutic target for the treatment of burn injury.

cytokine; endotoxin; innate immunity; sepsis; lipopolysaccharide

BURN INJURY RESULTS IN CARDIAC injury and depression characterized by severe systolic and diastolic ventricular dysfunction (37, 42, 50, 57). These contractile deficits have been reported to appear as early as 2 h after burn injury (28) and to persist for 2–7 days. Several recent studies have elucidated the early molecular events leading to cardiac dysfunction, including activation of an endotoxin signaling pathway including the toll-like receptor 4 (tlr-4), IL-1 receptor-associated kinase-M, and NF-κB in response to translocated gut factors (12, 45, 52, 53). Whereas endotoxin signaling through the tlr-4 receptor represents the initial pathway of burn injury-associated cardiac dysfunction, the final common cytokine pathways may involve a number of potential mediators, including TNF-α, IL-1β, and IL-6 (35). Experimentally, when TNF-α bioactivity is inhibited, burn-induced cardiac dysfunction is ameliorated (27).

Macrophage migration inhibitory factor (MIF) was identified nearly 40 years ago as one of the first cytokines having the activity of inhibiting the migration of macrophages in vitro (34). Over the years, numerous activities have been attributed to MIF such as modulation of TNF-α release, cell proliferation, and apoptosis (34). MIF is a unique cytokine for several reasons. It is a constitutively expressed protein found ubiquitously in cells throughout the body and is secreted from lysosomal compartments through nonclassical (nonendoplasmic reticulum) pathways (22). A putative receptor has not clearly been defined, and the in vivo physiological relevance of its tautomerase and oxidoreductase enzymatic activities is unknown (34). Recently, the cytokine MIF has been implicated in the immune pathogenesis of experimental sepsis in animals (34). Specifically, antibodies directed against MIF have been shown to improve survival significantly in lethal models of sepsis (cecal ligation and puncture), even when administered up to 8 h after the insult (10). We have recently demonstrated that MIF is synthesized by cardiac myocytes, it is released from cytoplasmic stores after endotoxin challenge, and is responsible for the late and prolonged cardiac dysfunction seen in experimental endotoxemia (26). This cardiac sparing effect may, at least in part, account for the significant survival advantages seen in murine sepsis models (including cecal ligation and puncture) when MIF activity is neutralized in vivo (5, 6, 8, 10, 11).

Because MIF was recently reported to be increased in the serum of rats after burn injury (31), we similarly hypothesized that burn trauma might also be associated with a release of MIF from the heart. We further hypothesized that, similar to the endotoxin model, MIF might be a mediator of prolonged cardiac failure after burn injury in mice (56). If MIF were proven to be a mediator of burn-induced cardiac injury, antibody therapy directed against this cytokine might be a candidate for testing in future clinical trials.

MATERIALS AND METHODS

Antibodies and Cytokines

A polyclonal rabbit anti-rat MIF IgG (Torrey Pines BioLabs, Houston, TX) was used for Western immunoblot and immunohistochemistry. This antibody has been shown to cross-react with murine MIF and was prepared as previously described (26). A purified rabbit IgG (Torrey Pines BioLabs) was used as an isotype control in

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immunohistochemistry experiments. A polyclonal goat anti-rabbit IgG-horseradish peroxidase (HRP) (Bio-Rad, Hercules, CA) was used as a secondary antibody for Western immunoblots and was stored at 4°C. Two monoclonal mouse anti-mouse (and human) MIF IgG1 antibodies (XIV.15.5 and III.D.9, provided by Cytokine Pharma-Sciences, King of Prussia, PA) and a monoclonal mouse IgG1 isotype control antibody (HB-49, provided by Cytokine PharmaSciences) were used in the echocardiographic studies and in the Langendorff studies. In vivo neutralization of MIF activity by both the XIV.15.5 and III.D.9 antibodies has been previously demonstrated (5, 17, 19, 26, 33, 36).

Animals and Burn Injury

Male C57BL/6J mice ages 6–10 wk (Jackson Labs, Bar Harbor, ME) were maintained in a specific pathogen-free environment. Commercial chow and tap water were made available ad libitum. All animal protocols were reviewed and approved by the University of Texas Southwestern Medical Center Institutional Animal Care Advisory Committee and were in compliance with the rules governing animal use as published by the National Institutes of Health. Mice were subjected to a 40% total body surface area burn injury as previously described (56). Briefly, mice were anesthetized with isoflu- rane (1–2%) with 2.5 l/min oxygen to effect. Hair was then removed from the back and sides by using a surgical preparation blade and 70% ethanol. Brass probes heated to 100°C in boiling water were then applied in pairs (total of 8 probe surface areas) on the animal’s side and back for 5 s. Alternatively, sham mice received anesthesia and were shaved but not given the burn injury. Intraperitoneal injection of lactated Ringer (2 ml) with buprenorphine hydrochloride (Buprenex, 0.1 ml per mouse) was given immediately following the procedure. The four burn injury groups received either MIF antagonist or burn injury only, three mice per group.

Western immunoblot and serum cytokine detection. In the first set of experiments, 30 mice were given a burn injury and 6 mice underwent a sham burn procedure. At each time point (sham, 4, 8, 12, 24, and 48 h), six mice were anesthetized, bled, and killed. The hearts were immediately harvested and flash frozen in liquid nitrogen and stored at −80°C for later processing and analysis by Western immunoblotting. Whole blood was collected by retro-orbital bleeding, processed for serum immediately, and stored at −80°C as previously described (26) to be assayed at a later time for cytokines. In additional experiments, 18 samples (6 time points × 3 mice per group) were run in parallel on two gels, transferred in the same transblot apparatus at the same time, and exposed to film in parallel to ensure comparative conditions.

Immunohistochemistry

In the second set of experiments, six mice were given a burn injury and two mice underwent a sham burn procedure. Mice were killed, and hearts were immediately harvested and flash frozen in liquid nitrogen and stored at −80°C for later processing and analysis by Western immunoblotting. Whole blood was collected by retro-orbital bleeding, processed for serum immediately, and stored at −80°C as previously described (26) to be assayed at a later time for cytokines. In additional studies comparing serum IL-6 concentrations 12 h after burn injury with anti-MIF pretreatment or burn injury only, three mice per group were utilized.

Experimental Designs

Four experimental designs were employed in the present study: 1) Western immunoblot and serum cytokine detection; 2) immunohistochemistry; 3) echocardiography; and 4) Langendorff preparations.

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Echocardiography. In the third set of experiments, 24 mice (4 groups) were given a burn injury and 6 mice underwent a sham burn procedure. The four burn injury groups received either 1) monoclonal anti-MIF antibody III.D.9, 2) monoclonal anti-MIF antibody XIV.15.5, 3) an isotypic (IgG1) control monoclonal antibody (HB-49) intraperitoneally (100 μg in 200 μl PBS) 90 min before burn injury, or 4) burn injury alone, and serial echocardiography was performed at the time points 0, 4, 8, 12, 24, and 48 h after burn injury. Langendorff preparations. In the last set of experiments to assay cardiac function by Langendorff experiments, 5–11 mice per group were given either 1) sham burn procedure, 2) burn injury, 3) monoclonal anti-MIF antibody III.D.9 intraperitoneally 90 min before burn injury, or 4) an isotypic (IgG1) control monoclonal antibody (HB-49) intraperitoneally 90 min before burn injury. These mice were killed, and hearts were assayed for function 24 h after burn injury procedure, when protection with neutralizing antibodies was identified by echocardiography.

Protein Extraction and Western Blot Analysis

Hearts stored at −80°C were homogenized on ice in lysate buffer [10 mM HEPES, 2 mM EDTA, pH 7.4 with one Complete Mini-EDTA-Free protease inhibitor cocktail tablet per 10 ml of buffer; Roche Diagnostics, Mannheim, Germany]. The lysate was centrifuged at 14,000 rpm (Eppendorf 5415C, Hamburg, Germany) for 20 min at 4°C. The supernatant was removed and the protein concentration was quantified by using the Bio-Rad protein assay. Fifty micrograms of total protein (lysate) diluted in Laemmlli sample buffer (Bio-Rad) in a 1:1 ratio to a final volume of 10 μl were then resolved on a 12% SDS-polyacrylamide gel under reducing conditions. Prestained SDS-PAGE standards (Kaleidoscope Broad range, Bio-Rad Laboratories) were run with each gel to determine the approximate molecular weight of detected bands. The gel was transferred to a polyvinylidene fluoride membrane (New England Nuclear, Boston, MA) by using a mini transblot transfer apparatus (Bio-Rad) at 100 V for 70 min and was cooled with ice packs. For each experiment, all 18 samples (6 time points × 3 mice per group) were run in parallel on two gels, transferred in the same transblot apparatus at the same time, and exposed to film in parallel to ensure comparative conditions. The membrane was rewrapped with methanol, washed a minimum of three times with >100 ml water, and blocked (5% nonfat dry milk (Bio-Rad)-TBS-0.1% Tween 20 (TBS-T)) overnight at 4°C. The membrane was then incubated with the primary rabbit anti-MIF (1:1,250 dilution) for 2 h at room temperature in 5% milk-TBS-T and washed once for 15 min in TBS-T, followed by five washes (5 min each) in TBS-T. It was then incubated for 1 h with a HRP-conjugated goat anti-rabbit antibody in TBS-T (1:5,000) at room temperature, washed twice for 15 min, followed by five additional washes (5 min each) in TBS-T. To develop, 5 ml of enhanced chemiluminescence reagent (SuperSignal West Pico, Pierce, Rockford, IL) were placed on the polyvinylidene fluoride membranes for 5 min, and the resulting chemiluminescent reaction was detected by Kodak X-OMAT AR Film (Eastman Kodak, Rochester, NY).

The quantification of the single band density with the approximate molecular mass of MIF (12.5 kDa) was determined using Quantity One software (version 4.4.0, Build 36, Bio-Rad) after conversion of radiographic film to TIFF files (8-bit gray scale) using a Scanjet 3400c (Hewlett-Packard, Palo Alto, CA) and reported in arbitrary units/mm².

Immunohistochemistry

Tissue was fixed in neutral buffered formalin, processed to paraffin, and subsequently immunostained at room temperature on a BioTek Solutions Technmate 1000 automated immunostainer (Ventana Medical Systems, Tucson, AZ) by using the Ultra-streptavidin biotin system with HRP and dianimobenzidine (DAB) chromogen (Signet Laboratories, Dedham, MA). Optimum primary antibody concentrations were predetermined by use of known positive control tissues [lipopolysaccharide (LPS)-challenged rat as previously described] (3). Paraffin sections were cut at 3 μm on a rotary microtome, mounted on positively charged glass slides (POP100 capillary gap slides, Ventana Medical Systems), and air-dried overnight. Sections were then deparaffinized in xylene and ethanol, quenched with fresh 3% hydrogen peroxide for 10 min to inhibit endogenous tissue peroxidase activity, and rinsed with deionized water. Sections were incubated in unlabeled blocking serum for 15 min to block nonspecific binding of the secondary antibody. All slides were then incubated at the same time.
isolated on a 1.2% agarose gel by use of GenElute Agarose Spin digestion (Fisher Scientific, Pittsburgh, PA), gel was purified and was prepared from an MIF-containing plasmid as previously de- and quantified by spectrophotometry. An MIF-specific Northern probe

cols, brought up in Tris-EDTA buffer, pH 7.0 (Ambion, Austin, TX),

mortar and pestle and placed in 2 ml TRIzol (Invitrogen, Carlsbad, CA) on a Luminex xMAP system (Luminex, Austin, TX) according to the manufacturer’s instructions. Sera from six mice per group per time point were assayed for mouse MIF by use of the Chemikine rat/mouse MIF EIA kit (Chemikon International, Temecula, CA) according to the manufacturer’s instructions. Briefly, 5 µl of standards, samples, or reaction buffer (blank) were added to each well in triplicate. Next, 100 µl of diluted MIF-HRP antibody conjugate were added to each well and allowed to incubate for 2 h at room temperature. Wells were then washed five times, and 100 µl of 3.3',5'-tetramethylbenzidine substrate were added and allowed to incubate in the dark for 30 min at room temperature. The stop reagent was added to each well and gently mixed, and the ELISA was read on an ELISA plate reader (EL 312e microplate reader, Bio-Tek Instruments, Winooski, VT) at 450 nm (630 nm background) within 30 min of completion of the assay.

Multiplex Cytokine Detection by LumineX

Serum inflammatory cytokine [IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ, TNF-α, and granulocyte-macrophage colony-stimulat-
ing factor (GM-CSF)] concentrations were determined by using the Mouse Cytokine Ten-Plex antibody bead kit (Biosource International, Camarillo, CA) on a LumineX xMAP system (LumineX, Austin, TX) according to the manufacturer’s instructions. Six mice per group per time point were assayed in duplicate. The plate was loaded onto the LumineX XYP platform, the instrument was set to remove 50 µl, and the total event count was set to equal 100 per bead set. At least 100 events (most >200) for each cytokine were collected in each sample to determine statistically significant results as previously described (24, 32, 55). Data were collected by using the LumineX Data Collector software. The concentrations of the lot-specific reconstituted standards used in each run were entered into the software, and the analyte concentrations for unknown samples were then extrapolated from the cytokine-specific standard curve by use of MasterPlex QT software (version 1.2.8.58, Mirai Bio, Alameda, CA). Final concentrations were multiplied by two to account for the initial dilution factor. No samples were detected that were higher than the standard curves for any cytokine.

Total RNA Isolation, MIF and β-Actin Probe Preparation, and Northern Blot Analysis

Hearts were ground to a fine powder in liquid nitrogen with a mortar and pestle and placed in 2 ml TRizol (Invitrogen, Carlsbad, CA). Total RNA was isolated according to the manufacturer’s protocol, brought up in Tris-EDTA buffer, pH 7.0 (Ambion, Austin, TX), and quantified by spectrophotometry. An MIF-specific Northern probe was prepared from an MIF-containing plasmid as previously described (26). The fragment was prepared by an EcoR1 and Not1 digestion (Fisher Scientific, Pittsburgh, PA), gel was purified and isolated on a 1.2% agarose gel by use of GenElute Agarose Spin

Columns (Supelco, Bellefonte, PA). The β-actin probe DNA fragment was purchased from Ambion. Both MIF and β-actin probes were labeled with 5 µl of [α-32P]dATP (3,000 Ci/mmol, 10 mCi/ml) (PerkinElmer, Boston, MA) by use of a Strip-EZ DNA probe synthesis kit (Ambion) and purified in ProbeQuant Microcolumns (Amer-

shaft Pharmacia, Piscataway, NJ) according to manufacturers’ protocols.

Northern blot analyses on all samples were run in parallel on two gels, transferred in the same apparatus at the same time, and exposed to film at the same time to ensure comparative conditions. Isolated total RNA (10 µg) was combined with formaldehyde loading dye (Ambion) at a ratio of 1:3 sample-loading dye according to the manufacturer’s protocols. Samples and RNA ladder were placed at 65°C for 10 min before electrophoresis and resolved on a 1.2% agarose gel with 1× Tris-acetate-EDTA buffer (Ambion) and transferred to a Hybond-N+ membrane (Amersham Pharmacia, Buckingham, UK). RNA was linked to the membrane for ~2 min by using a GS Gene Linker (Bio-Rad). The membrane was prehybridized in a hybridization oven (Sorvall Life Science, Greensboro, NC) in Perfect-Hyb Plus (Sigma, St. Louis, MO) with sheared, denatured salmon sperm DNA (100 µg/ml) for 1 h at 68°C, followed by the addition of ~0.1 µg probe labeled at >5×10^6 cpm/µg to the hybridization buffer and hybridized for 12 h at 68°C. Membranes were washed twice at 68°C in 2× SSC, 0.1% SDS for 1 h. The membrane was wrapped in Saran Wrap, and mRNA was detected by Kodak X-OMAT AR film after 24 h (Eastman Kodak). The same membrane was then reprobed in a similar manner with radiolabeled β-actin (0.1 µg probe labeled at >5×10^6 cpm/µg) (Ambion). Densitometry was performed as described above for the Western immunoblots. The β-actin mRNA bands served as a control against which to normalize the MIF mRNA densitometry.

Determination of Cardiac Dysfunction by Echocardiography

Echocardiograms to assess systolic function were performed using M-mode measurements. Mice were anesthetized with 5% isoflurane with 2.5 l/min O2 for 20 s (until unconscious) followed by 2% isoflurane and O2 for an average of 12–15 min. Hair was removed from the torso and upper abdomen by use of Nair hair remover and gauze after sitting for 3 min. Echocardiographic measurements were obtained on anesthetized mice ~5–8 min after induction as previously described (26). Echocardiography was performed using a Hewlett-Packard Sonos 5500 (Agilent Technologies, Andover, MA) with a frame rate of 300–500 frames/s in a random and blinded manner (acquisition and analysis). An S12 Ultraband 5.0 –12.0 (model 21380A) pediatric neonatal transducer (Agilent Technologies) was placed on the left hemithorax interfaced with a layer of US transmission gel (Aquasonic 100, Parker Laboratories, Fairfield, NJ). The two-dimensional parasternal short-axis imaging plane guided left ventricular (LV) M-mode tracings close to the papillary muscle level. Depth was set at a minimum of 2 cm with a sweep speed of 150 m/s.

M-Mode Measurements

Data represent the average of nine selected cardiac cycles from at least two separate scans. End diastole was defined as the maximal LV diastolic dimension, and end systole was defined as the minimal dimension between anterior and posterior wall motion. Fractional shortening percent (FS%), a surrogate of systolic function, was calculated from LV dimensions as follows: FS% = (LVED – LVES)/LVED × 100 (as shown in Fig. 5, where LVED and LVES are LV dimensions at end diastole and end systole, respectively).

Ex Vivo Cardiac Function Determination by Langendorff

Mouse heart function was determined by the Langendorff assay procedure as previously described (56). Briefly, 200 units of heparin sulfate were injected intraperitoneally, and the mice were killed 20
Serum MIF, IL-6, and IL-12 Levels Are Modulated After Burn Injury

Serum MIF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ, TNF-α, and GM-CSF levels were assayed in serum from mice after burn injury (6 mice/group). Serum MIF levels were significantly increased at 4 h after burn injury (Fig. 3A). Serum IL-6 levels peak (182-fold baseline) at 24 h after burn injury (Fig. 3B). Serum IL-12 levels decrease maximally (0.7-fold baseline) at 8–24 h after burn injury (Fig. 3C). Pretreatment with anti-MIF (III.D.9) did not decrease serum IL-6 levels 12 h after burn injury (data not shown). No other cytokines of the 10 tested were detected after burn injury beyond baseline levels.

MIF mRNA in the Heart Significantly Increases by 8 h After Burn Injury

The levels of MIF mRNA were detected by Northern blot analysis from total RNA isolated from hearts of either sham mice or mice at 4, 8, 12, 24, and 48 h after burn injury. MIF mRNA is constitutively expressed in the heart; significant increases in mRNA levels initially occur at 8 h, and elevated mRNA levels persist for the remainder of the time course (48 h) (Fig. 4).

Anti-MIF Monoclonal Antibody Improves Cardiac Depression In Vivo

Serial echocardiography (M-mode) was performed on mice receiving burn injury and mice pretreated 90 min before burn injury with either of two anti-MIF antibodies, an isotype control, or no treatment (Fig. 5). At 4 and 8 h, the FS% values of all burn injury-treated mice were similarly depressed (56.2% control vs. 34.8% for burn injury), irrespective of anti-MIF treatment. At 12 h postburn injury, however, mice injected...
with either of the two monoclonal anti-MIF antibodies demonstrated statistically significant recovery of FS% compared with burn injury mice receiving either no treatment or an isotype antibody control (Fig. 5). By 24 h, the FS% of the treated mice was not significantly different from that of the controls, indicating complete resolution of the contractile defect. Throughout the 48 h, the FS% of sham mice did not change significantly, indicating that the testing regimen and anesthesia had no significant effect cardiac function. Finally, mice receiving isotype control antibodies did not demonstrate

Fig. 2. MIF, constitutively present in the heart, liver, spleen, lung, and kidney, is decreased after burn injury. Preformed MIF is present in the heart, kidney, and spleen (A, F, and K). Significant decreases in MIF can be identified by 8 h after burn injury in the heart, kidney, and spleen (B, G, and L). The decreases in the kidney are seen mainly in the outer cortex (white arrows). MIF concentration returns to baseline at 24 h in the heart and kidney (C and H), but not the spleen (M) as demonstrated by immunohistochemistry. A negative control (secondary antibody (Ab) without the primary anti-MIF antibody) consistently demonstrated that no background staining was present at each time point in each organ investigated as represented in the far right column (E, J, and O). Similar observations were made when a primary isotype control antibody and secondary antibody were used (D, H, and L). Magnification: ×100 (kidney, spleen), ×400 (heart).

Fig. 3. Serum concentrations of MIF (ng/ml; A), IL-12 (pg/ml; B), and IL-6 (pg/ml; C) after burn injury. Data are expressed as means ± SE of 6–12 C57BL/6J mice as determined by ELISA or Luminex and were statistically analyzed using a 1-way ANOVA with a multiple comparison procedure employing the Bonferroni method to determine significance between groups (*P < 0.05 compared with baseline).
significant differences from animals undergoing burn injury alone, indicating specificity of the anti-MIF antibody effects.

**Anti-MIF Antibodies Improve Burn-Induced Cardiac Depression by Langendorff Preparation Ex Vivo**

We have previously demonstrated that burn injury induces pre- and afterload independent cardiac dysfunction at multiple time points after burn injury (51, 52, 56). Therefore, to further substantiate our findings in the in vivo echocardiography studies, we confirmed the effects of MIF neutralization using traditional Langendorff assays.

The responses of hearts to retrograde aortic perfusion at 1.5 ml/min from mice undergoing the sham operation, burn injury, or burn injury with pretreatment of anti-MIF antibodies were determined by a Langendorff analysis of heart function. Significant decreases in LV pressure, $dP/dt_{max}$, $dP/dt_{min}$, difference ratio, rate of LVP rise at developed pressure of 40 mmHg, time to peak pressure, time to 90% relaxation, and time to maximum $dP/dt$ were identified in mice 18 h after they underwent burn injury (with and without pretreatment of an antibody isotype control) (Table 1). Mice pretreated with the anti-MIF antibody III.D.9 undergoing burn injury were completely protected by 18 h (Table 1), whereas mice treated with the isotype control did not differ significantly from burn injury alone (data not shown).

Figure 6 illustrates heart function in sham mice, burn injury mice, and burn injury mice pretreated with the anti-MIF

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**Fig. 4.** Burn injury upregulates MIF mRNA expression in cardiac tissue significantly by 8 h. MIF and $\beta$-actin mRNAs were detected by using $^{32}$P-radiolabeled probes complementary to MIF and $\beta$-actin mRNAs. Each data point represents the mean density (in AU/mm$^2$ ± SE) of 3 independent Northern blot analysis experiments. A representative Northern blot analysis is shown below the graph. Normalized MIF was determined by multiplying the MIF density by the relative $\beta$-actin density present. A 1-way ANOVA and a multiple comparison procedure using the Tukey method were employed to determine statistical significance compared with baseline (*$P < 0.05$).

**Fig. 5.** Echocardiographic assessment of the effects of anti-MIF antibody therapy after burn injury demonstrates the cardioprotective effects of MIF blockade. Representative M-mode echocardiograms in wild-type mice at baseline and 48 h after burn injury (A and B, respectively). C and D depict representative echocardiograms in burn injury plus anti-MIF-treated mice at 4 and 48 h, respectively. A significant recovery of cardiac function (%fractional shortening, FS%) is observed in burn injury mice given anti-MIF antibodies preburn injury compared with burn injury mice pretreated with isotype control antibody or burn injury alone (E). Data from each group represent means ± SE of 9 cardiac cycles from 3 mice monitored at multiple time points. Cardiac function determined by echocardiography is expressed as %FS (LVED – LVES/LVED × 100) ± SE, where LVED and LVES are left ventricular (LV) dimension at end diastole and end systole, respectively, and was analyzed using a 1-way repeated-measures ANOVA and a multiple comparison procedure employing the Tukey test to determine significant differences between specific groups. *$P < 0.05$ compared with burn injury mice pretreated with isotype control antibody (and burn injury only). +$P < 0.05$ compared with mice undergoing sham procedure.
Table 1. In vitro stabilization data from isolated hearts in the Langendorff perfusion experiments 18 h after the burn injury

<table>
<thead>
<tr>
<th>Cardiac Function Tested</th>
<th>Sham</th>
<th>Burn Injury</th>
<th>Burn Injury + Anti-MIF</th>
<th>Burn Injury + Isotype Control (IgG1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>11</td>
<td>9</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>LVP, mmHg</td>
<td>96.5±1.5</td>
<td>63.2±5.3*</td>
<td>95.1±2.2*</td>
<td>75.6±5.2*</td>
</tr>
<tr>
<td>+dp/dt max, mmHg/s</td>
<td>2.217±0.44</td>
<td>1.631±0.40*</td>
<td>2.279±0.57*</td>
<td>1.910±0.68*</td>
</tr>
<tr>
<td>−dp/dt max, mmHg/s</td>
<td>1.855±0.45</td>
<td>1.187±0.70*</td>
<td>1.950±0.35</td>
<td>1.600±0.84*</td>
</tr>
<tr>
<td>DR</td>
<td>1.22±0.04</td>
<td>1.40±0.05*</td>
<td>1.17±0.02</td>
<td>1.40±0.06*</td>
</tr>
<tr>
<td>dPa0, mmHg/s</td>
<td>1.868±0.24</td>
<td>1.383±0.31*</td>
<td>1.903±0.36</td>
<td>1.680±0.73*</td>
</tr>
<tr>
<td>TPP, ms</td>
<td>82.0±2.5</td>
<td>72.8±2.5*</td>
<td>83.2±1.6</td>
<td>71.2±1.7*</td>
</tr>
<tr>
<td>RT90, ms</td>
<td>79.6±4.5</td>
<td>68.8±1.9*</td>
<td>79.7±1.6</td>
<td>68.0±3.6*</td>
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<tr>
<td>Time to maximum +dp/dt, ms</td>
<td>49.1±0.7</td>
<td>47.6±0.6*</td>
<td>52.7±0.9</td>
<td>49.4±1.3</td>
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<tr>
<td>Time to maximum −dp/dt, ms</td>
<td>50.1±0.5</td>
<td>45.7±1.3*</td>
<td>51.3±0.6</td>
<td>47.4±2.0*</td>
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<td>CPP, mmHg</td>
<td>89.2±4.9</td>
<td>91.0±6.3*</td>
<td>70.4±6.7</td>
<td>67.6±8.4</td>
</tr>
<tr>
<td>CVR, mmHg</td>
<td>59.4±3.3</td>
<td>60.6±4.2*</td>
<td>50.0±4.5</td>
<td>45.1±5.6</td>
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<tr>
<td>HR, beats/min</td>
<td>321±6</td>
<td>315±7*</td>
<td>327±12</td>
<td>347±22</td>
</tr>
</tbody>
</table>

Cardiac function values are expressed as means ± SE. LVP, left ventricular pressure; +/−dp/dt, positive and negative first derivative of LVP with respect to time, respectively; DR, differential ratio (+dp/dt/−dp/dt); dPa0, rate of LVP rise at developed pressure of 40 mmHg; TPP, time to peak pressure; RT90, time to 90% relaxation; CPP, coronary perfusion pressure; CVR, coronary vascular resistance; HR, heart rate. A repeated-measures ANOVA with a multiple comparison procedure employing the Bonferroni method was used to determine significant differences between groups (*P < 0.05 compared with sham control).

antibody III.D.9 or isotype control 18 h after the burn injury or sham procedure over a range of Ca2+ concentrations. Increases in perfusate Ca2+ concentrations resulted in incremental increases in contractile performance in all hearts (groups) tested. Mice undergoing burn injury (with or without pretreatment with the isotype control) demonstrated a downward shift in the LV pressure, +dp/dt max, and −dp/dt max function curves, demonstrating significant systolic and diastolic dysfunction. This dysfunction, however, was not present when the anti-MIF antibody III.D.9 was given because no significant differences from sham mice were identified in this treatment group.

**DISCUSSION**

In the present study we describe for the first time the pathophysiological role of MIF in a model of burn injury and its associated cardiac dysfunction. In this model, cardiac MIF is released from tissues, including heart, and this release is accompanied by a peak in MIF serum concentration. More importantly, when MIF was neutralized by monoclonal antibodies, cardiac function improved significantly by 12 h and returned to baseline by 24 h, confirming that MIF is an important mediator of cardiac dysfunction after burn injury. These results confirm and extend our recently published finding indicating that MIF is a cardiac-derived myocardial depressant protein after endotoxin challenge in mice (26).

In the mouse model of burn injury described in the present study, the molecular pathogenesis of cardiac dysfunction involves a tlr-4-mediated pathway (52, 53). Presumably, endotoxin is responsible for tlr-4 activation, but another tlr-4 ligand cannot yet be excluded. The source of LPS is believed to be gut derived owing to several potential insults associated with burn injury. These insults include intestinal ischemia (4), bacterial translocation (14, 16), and increased intestinal permeability (15). It is thought that the production and release of inflammatory factors become systemic through gut-associated lymphoid tissues (45).

Most cytokines are not expressed constitutively and have tightly regulated expression after stimulation. MIF, however, exists preformed in substantial amounts in the cytosol and nucleus of multiple cells, and its elaboration relies not only on de novo protein synthesis but on secretion from preformed stores (7, 20, 22, 38, 41). This is consistent with the present study, which identified constitutive MIF in whole cell cardiac

![Fig. 6. Cardiac function determination by Langendorff preparation 18 h after burn injury as a function of increasing Ca2+ concentrations. Cardiac function is expressed as means ± SE of 30 independent Langendorff experiments [11 sham, 9 burn injury, 5 burn injury after anti-MIF III.D.9 pretreatment, 5 burn injury after isotype control (IgG1) pretreatment]. Separate analyses were performed for each LV pressure, maximum positive and negative first derivative of LV pressure, respectively (+dp/dt max and −dp/dt max), as a function of treatment group and perfusate Ca2+ concentrations using a repeated-measures ANOVA and a multiple comparison procedure employing the Bonferroni method to determine significant differences between groups (*P < 0.05 compared with burn injury mice (with or without pretreatment with isotype control antibody).](http://ajpheart.physiology.org/)
lysates by Western immunoblot and in ventricular cardiomyocytes by immunohistochemistry. Intracellular MIF decreases significantly 8 h after burn injury, suggesting the possibility that MIF is released in response to burn injury. Oxidative stress in cardiomyocytes promotes MIF release (23). This is significant in relation to the present study because major burn injury is associated with increased oxidative stress as shown by increased malondialdehyde levels in plasma and cardiac tissue (13). Moreover, antioxidant therapy has been shown to decrease inflammatory cytokine release after burn injury (29). Therefore, the cardiac release of preformed MIF demonstrated in this study may have been initiated by increases in oxidative stress that signaled the release of MIF.

Decreases in spleen and kidney MIF expression were observed by immunohistochemistry in the present study, whereas liver MIF expression did not differ significantly from baseline (data not shown) after burn injury. Previous studies have identified the presence of MIF in the heart, liver, spleen, and kidney (3, 26). After LPS challenge, decreased MIF expression by immunohistochemistry has been identified in the heart, liver, and spleen (26). This additional evidence suggests that tissue MIF modulation after burn injury differs from LPS challenge specifically by modulating kidney MIF and not affecting liver MIF.

Because previous studies have demonstrated that burn injury involves skin necrosis in our model (56), and because MIF has been identified in the skin and localized to the basal layers of the epidermis (48), MIF may be directly released from necrotic cells of the skin. In addition to the necrotic release of preformed MIF, epidermal damage has been shown to increase the expression of MIF (1, 47, 49). Systemic levels of MIF may have increased after burn injury in the present study more quickly and dramatically (2.2-fold by 4 h) compared with the endotoxosis model (1.5-fold increase by 8 h in the endotoxosis model) owing to MIF released from skin sources.

Numerous studies have demonstrated that MIF secretion results from a variety of stimuli such as endotoxin (LPS), TNF-α (9), angiotensin II (43), corticosteroids (8), and ACTH (38). Most cytokines have a tightly regulated expression of mRNA that increases after stimulation and is followed by increases in protein production and release. However, substantial amounts of MIF protein are found constitutively in many cell types (3, 26, 34). In experimental endotoxemia, MIF mRNA significantly increases by 24–48 h in both rat and mouse models (3, 26). Therefore, a distinct discordance between stimulus, protein release, and MIF mRNA upregulation has previously been identified and was identified after burn injury in the heart in the present study. Other preformed cytokines such as pro-IL-1β/IL-1β demonstrate a similar delayed upregulation of both protein and mRNA after inflammatory stimulus (LPS) as identified in the present study for MIF (30).

The secretion of MIF has been shown to be a result of not only enhanced MIF expression and de novo protein synthesis, but also an induction of preexisting MIF stores (22). MIF has recently been identified as a protein that is secreted by “non-classical” pathways; that is, it does not traffic through the endoplasmic reticulum because it does not contain an NH₂-terminal signal sequence (22). The prototype of leaderless cytokine secretion is IL-1β, which accumulates as pro-IL-1β in the cytosol (preformed) in secretory lysosomes that are exocytozed when induced by exogenous ATP (2, 44). Other leaderless cytokines include HMGB1, which, like MIF, is found in the cytosol and exhibits a delayed release in response to inflammatory stimuli (25). Interestingly, IL-1β and HMGB1 are found in distinct lysosomal compartments that are released differentially (IL-1β early, HMGB1 late) (25).

Cardiac dysfunction was identified as early as 4 h after burn injury, and this early dysfunction was not changed by anti-MIF treatment. We speculate that this early cardiac depression may be due to cytokines that are released early after burn injury (such as TNF-α and IL-1β), which have been reported to be released from cardiomyocytes in as little as 2 h after burn injury (35). These cytokines have additionally been shown to synergize with IL-6 in mediating cardiac dysfunction experimentally, whereby complex interactions may account for the observed cardiac dysfunction (35). Although circulating TNF-α and IL-1β were not detected in the serum in the present study, it is thought that elevated cardiac cytokine levels may be present without elevated circulating levels, resulting in auto-crine/paracrine cardiodepressive effects (40). Immediately after this nadir in function, however, neutralization of MIF resulted in cardioprotection, reaching statistical significance at 12 h. We interpret these data to indicate that MIF is a critical mediator of late and prolonged cardiac dysfunction in this model. Of the 10 cytokines investigated in addition to MIF in the serum, only systemic IL-6 and IL-12 levels were modulated after burn injury. Increases in IL-6 after burn injury have previously been reported, and IL-6 has been implicated in directly mediating cardiac dysfunction (35, 56). To determine whether MIF plays a role in modulating IL-6 in the present study, we investigated the effects of anti-MIF on burn injury-induced IL-6 release (data not shown). Although anti-MIF pretreatment did not result in significant decreases in systemic IL-6 levels in the present study, it is still possible that IL-6 may be blocked locally in the heart, preventing cardiodynamic dysfunction in this manner. Interestingly, systemic IL-12 levels were significantly decreased during the 12 h after burn injury, which parallel decreases in IL-12 reported in postsurgical sepsis patients (21). Decreases in IL-12 have been postulated to be involved in sepsis-induced immune energy in animal models and humans (39, 46, 54).

Our initial study observed that MIF mediated late cardiac depressive effects in a model of sublethal endotoxemia in mice (4 mg/kg 0111:B4 LPS) (26). There are several differences between the burn injury model described in the present study and these initial observations made in a model of endotoxemia. First, in mice given burn injury, serum MIF is released at an earlier time point (4 h) than the serum MIF release seen after LPS challenge (8 h). Moreover, the serum MIF levels are significantly higher (2.2-fold baseline) in the burn injury model compared with the endotoxemia model (1.5-fold baseline). Second, cardiac release of MIF occurred by 8 h after burn injury, which did not occur until 12 h post-LPS challenge in the endotoxemia model. Third, the degree of cardiac dysfunction (measured by echocardiography) in the burn injury model at 4 h after burn injury was not as great (38% decrease in FS% from baseline) as that seen in the endotoxemia model 4 h after LPS challenge (53.7% decrease in FS% from baseline). Fourth, cardiac MIF mRNA levels were significantly increased 8 h after burn injury, which was not seen until 48 h after LPS challenge in the endotoxemia model. Lastly, systemic changes
in IL-6 and IL-12 were identified in the burn injury model, whereas changes in TNF-α, IL-1β, IL-12, IL-6, IL-10, IFN-γ, and GM-CSF were increased in the endotoxemia model (unpublished data). Although MIF mediates a late cardiac dysfunction in each of these models, the underlying pathogenesis is considerably different. Moreover, these findings suggest that burn injury-induced changes in MIF are not solely due to LPS.

The cytokine MIF plays a significant role in the late cardiac dysfunction associated with burn injury. Although we have previously identified that MIF itself has cardiodepressive effects ex vivo (26), it is not clear whether this direct effect accounts for any of the observed cardiac dysfunction after burn injury. MIF may indirectly induce cardiac dysfunction by stimulating the release of downstream cytokines and/or act synergistically with other cytokines (i.e., IL-6) to mediate late burn injury-associated cardiac dysfunction. Because MIF release is delayed after burn injury, the development of small molecule inhibitors (i.e., tautomerase inhibitors) that appear to have in vitro anti-MIF activity (18) may make MIF a good potential therapeutic target.

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