The Macrophage Is an Important and Previously Unrecognized Source of Macrophage Migration Inhibitory Factor

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Summary

For over 25 years, the cytokine known as macrophage migration inhibitory factor (MIF) has been considered to be a product of activated T lymphocytes. We recently identified the murine homolog of human MIF as a protein secreted by the pituitary in response to endotoxin administration. In the course of these studies, we also detected MIF in acute sera obtained from endotoxin-treated, T cell-deficient (nude), and hypophysectomized mice, suggesting that still more cell types produce MIF. Here, we report that cells of the monocyte/macrophage lineage are an important source of MIF in vitro and in vivo. We observed high levels of both preformed MIF protein and MIF mRNA in resting, nonstimulated cells. In the murine macrophage cell line RAW 264.7, MIF secretion was induced by as little as 10 pg/ml of lipopolysaccharide (LPS), peaked at 1 ng/ml, and was undetectable at LPS concentrations >1 μg/ml. A similar stimulation profile was observed in LPS-treated peritoneal macrophages; however, higher LPS concentrations were necessary to induce peak MIF production unless cells had been preincubated with interferon γ (IFN-γ). In RAW 264.7 macrophages, MIF secretion also was induced by tumor necrosis factor α (TNF-α) and IFN-γ, but not by interleukins 1 β or 6. Of note, MIF-stimulated macrophages were observed to secrete bioactive TNF-α. Although previously overlooked, the macrophage is both an important source and an important target of MIF in vivo. The activation of both central (pituitary) and peripheral (macrophage) sources of MIF production by inflammatory stimuli provides further evidence for the critical role of this cytokine in the systemic response to tissue invasion.

N umerous observations over the years indicate that the hypothalamic-pituitary-adrenal axis is essential in the normal host response to infection and tissue invasion (1-4). In the course of investigating the role of the pituitary in systemic inflammatory responses, we identified the murine homolog of human macrophage migration inhibitory factor (MIF)[1] as a protein secreted by the anterior pituitary in response to LPS stimulation (5). Pituitary MIF was found to contribute to the MIF present in plasma in the postacute phase (>2 h) of endotoxemia. Recombinant murine MIF markedly increased lethality when coinjected with LPS, and anti-MIF antibody conferred full protection against lethal endotoxemia, suggesting that MIF is an important and possibly critical mediator of endotoxic shock.

Historically, MIF has been considered a product of activated T lymphocytes and appears to exhibit a number of macrophage-activating properties (6-12). While studying MIF production in experimental endotoxemia, however, we detected MIF in acute sera obtained from LPS-injected, T cell-deficient (nude), and hypophysectomized mice, suggesting that yet additional cell types may produce MIF in vivo. Since the macrophage is a major source of the cytokines that appear in response to LPS administration (13), we examined the possibility that MIF also is produced by cells of the monocyte/macrophage lineage.

In this study, we report that monocytes/macrophages synthesize and release MIF after stimulation with LPS, TNF-α, and IFN-γ. In contrast to previously studied macrophage-derived cytokines, both MIF protein and mRNA are present in substantial, preformed amounts in these cells. Moreover, stimulation of macrophages with recombinant MIF induced the secretion of significant amounts of TNF-α, suggesting that these two cytokines may act together in a proinflammatory loop. Although previously overlooked, we conclude that the macrophage is not only a cellular target of T cell or pituitary MIF but also is an important source of MIF production in vivo.

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[1] *Abbreviations used in this paper:* FBS, fetal bovine serum; MIF, macrophage migration inhibitory factor.

Material and Methods

Reagents. *Escherichia coli* O111:B4 LPS, polymyxin B, carbenicillin, PMSF, and Tween-20 were obtained from Sigma Chemical Co. (St. Louis, MO). LPS was resuspended in pyrogen-free water, vortexed, sonicated, aliquoted (5 mg/ml), and stored at −20°C. Serial dilutions of LPS were prepared in pyrogen-free water by sonication (model 3210; Branson Ultrasonics Corp., Danbury, CT). Gentamicin was from GIBCO BRL (Gaithersburg, MD). Thioglycollate broth (Difco, Detroit, MI) was prepared according to the manufacturer's recommendation, autoclaved, and stored protected from light at room temperature. Horseradish peroxidase–conjugated goat anti–rabbit antibody was purchased from Pierce (Rockford, IL) and 4-chloro-1-naphthol and stabilized 3,3′,5,5′-tetramethylbenzidene (TMB) substrate for horseradish peroxidase were from Promega Corp. (Madison, WI). Polyclonal anti-MIF serum was generated by immunizing New Zealand White rabbits (Hare Marland, Hewitt, NJ) with purified recombinant murine MIF (rMIF). At weeks 1 and 2, rabbits were inoculated intradermally with 100 μg of rMIF diluted in CFA, and with 50 μg of rMIF diluted in IFA on week 4. Immune serum was collected 1 wk after the last inoculation.

Cytokines. rMIF was expressed in *E. coli* BL21/DE3 (Novagen, Madison, WI) (5) and purified to homogeneity by anion exchange (Mono Q; Pharmacia, Piscataway, NJ) and reverse phase chromatography (C8-SepPak; Millipore Corp., Milford, MA), lyophilized, and reconstituted in sodium phosphate buffer (20 mM, pH 7.2) following procedures to be reported elsewhere (Bernhagen, J., R. A. Mitchell, T. Calandra, W. Voelter, A. Cerami, and R. Bucala, manuscript submitted for publication). MIF bioactivity was established by measuring dose-dependent, MIF-induced augmentation of *Leishmania major* killing by macrophages and by neutralization of this activity with anti-MIF antibody (Gessner, A., personal communication). rMIF contained 9 pg of endotoxin per microgram of protein as determined by the chromogenic *Limulus* amoebocyte assay (BioWhittaker, Inc., Walkersville, MD). Recombinant murine TNF-α was a generous gift from Dr. Michael Paladino (Genentech, San Francisco, CA). Recombinant murine IL-1β and IL-6 (5 μg/ml after reconstitution) were obtained from R&D Systems, Inc. (Minneapolis, MN) and recombinant murine IFN-γ (10⁵ IU/ml) was from Boehringer Mannheim (Indianapolis, IN). Cytokines were reconstituted in pyrogen-free water containing 0.1% of very low endotoxin BSA (Miles, Inc., Kankakee, IL) and stored at −80°C. The endotoxin content of the reconstituted cytokines was 0.5 ng/μg of TNF-α, 2 ng/μg of IL-1β, 1.1 ng/μg of IL-6, and 0.6 pg/U of IFN-γ as determined by the chromogenic *Limulus* amoebocyte assay.

Animals and Experimental Design. 8–10-wk-old (19–21 g) female BALB/c (control), T cell–deficient BALB/c^nu/nu^ (nude), and hypophysectomized BALB/c mice were purchased from Charles River Labs. (Kingston, NY). Animals were housed in groups of 5 or 10 mice per cage with free access to food and water (supplemented with 5% glucose for hypophysectomized mice) and were acclimatized for 5 d before experimentation. Mice were injected intraperitoneally with nonlethal doses of LPS (2.25 mg/kg for BALB/c and T cell–deficient mice and 50 μg/kg for hypophysectomized mice), and killed 2, 8, and 20 h after LPS challenge to collect serum. The LPS dose was adjusted to provide comparable lethality in the control BALB/c group as among the hypophysectomized mice, which are hypersensitive to endotoxin (4). 5 μl of serum was analyzed by Western blotting and visualized with anti-MIF antibody as described below.

Organ Distribution of Murine MIF Protein. The organ distribution of MIF protein was examined in a 8–10-wk-old female BALB/c

mouse killed by CO_2 asphyxiation and necropsied under aseptic conditions. Organs (brain, liver, spleen, kidneys, adrenals, and lungs) were excised, sectioned, and homogenized at 4°C with Tris-buffered saline (50 mM Tris-Base, 150 mM NaCl, pH 7.5) containing 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 2 mM EDTA, and 1 mM PMSF. Cellular debris was pelleted and aliquots of organ lysate supernatants, adjusted for protein concentration, were diluted with an equal volume of reducing SDS-PAGE sample buffer. Samples (equivalent to 60 μg of protein) were electrophoresed through 18% polyacrylamide gels and transferred to nitrocellulose membranes for Western blot analysis using polyclonal anti-MIF serum.

Cells. RAW 264.7 murine macrophages, THP-1 human monocytes, ASL-1 murine and Jurkat human T lymphocyte cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 medium (Gibco BRL) containing 2 mM glutamine, 10% heat-inactivated fetal bovine serum (FBS) (HyClone Labs., Logan, UT), and 50 μg/ml of carbenicillin and gentamicin. Medium for ASL-1 cells was supplemented with 1 mM sodium pyruvate. Thioglycollate-elicited peritoneal macrophages were obtained from BALB/c mice that were injected intraperitoneally 3–4 d previously with 2 ml of sterile thioglycollate broth. Cells were harvested under strict aseptic conditions by lavage of the peritoneal cavity with 5 ml of an ice-chilled 11.6% sucrose solution. After centrifugation for 10 min at 800 g, cells were resuspended in RPMI/10% FBS, enumerated, and plated at a density of 2 × 10⁶ or 4 × 10⁶ cells/well. Human PMN were isolated from peripheral blood by density gradient centrifugation as previously described (14, 15).

MIF Content of Macrophage and T Lymphocyte Lysates. Aliquots of 10⁶ cells of each type were lysed with Tris-buffered saline containing 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS and 2 mM EDTA. Cellular debris was pelleted and the supernatants were diluted with an equal volume of reducing SDS-PAGE sample buffer. 10 μl of lysate (equivalent to 5 × 10³ cells) was electrophoresed through 18% polyacrylamide gels and transferred to nitrocellulose membranes for Western blot analysis using polyclonal rabbit anti-MIF serum.

Stimulation of Macrophages by LPS and Cytokines. RAW 264.7 macrophages were washed with fresh medium, harvested by gentle scraping, resuspended in RPMI/10% FBS, and incubated at 2 × 10⁶ or 4 × 10⁶ cells/well in 3.5-cm tissue culture plates (Linbro®; Flow Laboratories, McLean, VA). After 3 h of incubation at 37°C in a humidified atmosphere with 5% CO_2, nonadherent cells were removed and wells were washed twice with RPMI/1% FBS. Cells then were incubated for 12 h with LPS (at concentrations ranging from 1 pg/ml to 10 μg/ml) or with cytokines (1 or 10 ng/ml for TNF-α, IL-1β, and IL-6, and 10, 100, or 1,000 IU/ml for IFN-γ) diluted in RPMI/1% FBS. For time course experiments, conditioned media of parallel cultures were removed at 3-, 6-, 9-, 12-, and 15-h intervals after LPS stimulation. Thioglycollate-elicited peritoneal macrophages (10⁷ cells/well) were harvested as described and conditioned similarly to RAW 264.7 cells. Cells were incubated for 12 h with either LPS or IFN-γ plus LPS. When costimulated with IFN-γ and LPS, cells were first incubated for 1 h with IFN-γ (100 IU/ml) before addition of LPS at the indicated concentrations. At the end of each experiment, cell culture media were collected, centrifuged for 10 min at 800 g, and supplemented with PMSF (1 mM). Supernatants then were concentrated 10-fold by membrane filtration (10 kD cut-off) (Centricon-10; Amicon, Beverly, MA). Cellular RNA was extracted from adherent cells as described below.

RNA Extraction and Isolation of MIF mRNA by Reverse Transcription

and Polymerase Chain Reaction (RT-PCR). Total cellular RNA was extracted from macrophages with RNAzol-B (Tel-Test Inc., Friendswood, TX) following the manufacturer's protocol. 1 μg of RNA was reversed transcribed using oligo (dT)$_{12-18}$ and M-MLV reverse transcriptase (GIBCO BRL) in a 50 μl reaction. 5 μl of cDNA was amplified by PCR in a thermal cycler (model 9600; Perkin-Elmer/Cetus, Norwalk, CT) (denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and elongation for 1 min at 72°C) using murine MIF primers (32 cycles), TNF primers (22 cycles), or β-actin primers (25 cycles). Murine intron-spanning MIF and β-actin primers have been reported elsewhere (5). Intron-spanning TNF primers were 5'-GCGGAGTCCGGGCAGGTCTA-3' and 3'-GGG-GGGCTGGCTCTGTGAGG-5'. DNA amplification products were analyzed on 2% agarose gels and gel loading was normalized to β-actin PCR products. Quantification of MIF mRNA from LPS-stimulated RAW 264.7 macrophages was done by competitive PCR as described (5, 16). The 273-bp competitive cDNA template was added at the indicated concentrations and coamplified with MIF cDNA using MIF primers.

Western Blots. For Western blotting, samples were resolved on 18% SDS polyacrylamide gels under reducing conditions and transferred onto nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH) at 50 V and 150 mA for 16 h. Membranes were blocked with 500 mM NaCl, 50 mM Tris-Base, pH 7.5, buffer containing 5% nonfat dry milk and 0.05% Tween-20 and incubated first with polyclonal rabbit anti-rMIF serum and then with horseradish peroxidase–conjugated goat anti–rabbit IgG antibody (each diluted 1:1,000 in 500 mM NaCl, 50 mM Tris-Base, pH 7.5 buffer with 1% BSA and 0.05% Tween-20). Polyclonal rabbit anti–murine MIF serum was demonstrated to react with both natural and recombinant murine and human MIF. Incubations were for 1 h each. MIF was visualized by development with chloronaphthol/H_2O_2 or with TMB substrate. Serum MIF bands were quantified by laser densitometry (model CS-9000U; Shimadzu Corp., Braintree, MA) (17). Faint densitometric signals in the MIF region were detected in most sera before LPS administration (0 h). Hence, scanning integrals are presented as relative serum MIF content (i.e., fold increase) normalized to the respective 0 h zone (i.e., background or baseline staining) on each membrane. Each plotted point is the mean ± SEM of individual sera from two to five animals. To further quantify the amount of circulating MIF after LPS administration in vivo, serum MIF bands were compared and quantitated relative to rMIF standards electrophoresed on the same SDS gels. Calculated estimates of serum MIF concentrations were expressed as molar concentrations.

MIF-induced Secretion of TNF by RAW 264.7 Macrophages. Cells (4 × 10⁶/well) were processed as described above and stimulated for 12 h with rMIF at specified concentrations ranging from 100 pg/ml to 1 μg/ml. At 1 μg/ml of rMIF, LPS contamination was 9 pg/ml as assessed by *Limulus* assay. To neutralize this minute amount of LPS, rMIF was preincubated for 1 h at 37°C with polymyxin B (1 μg/ml). MIF-induced TNF activity in cell culture supernatant was quantitated by L929 cell cytotoxicity as described previously (18). MIF-induced TNF activity was blocked completely by anti-TNF antibodies. rMIF did not contribute to TNF activity, as recombinant murine TNF (dose range, 5 pg/ml–1 μg/ml) cytotoxicity remained unchanged when rMIF (10 pg/ml–10 μg/ml) or anti-MIF polyclonal serum was added to L929 cells together with rTNF.

Results

Serum MIF Levels in LPS-injected Mice. We recently reported that pituitary MIF contributes significantly to the MIF that appears in serum during endotoxemia. Serum MIF reached peak levels in normal mice at 20 h but was undetectable in hypophysectomized mice at this time, suggesting that the pituitary is a major source of the MIF that appears in serum after LPS injection (5).

The central role played by MIF in endotoxic shock, together with the known hypersensitivity of hypophysectomized mice to LPS, led us to examine more closely the kinetics of MIF appearance in the circulation during the early, acute phase (i.e., 2–8 h) of endotoxemic shock. For purposes of comparison, we also examined serum MIF kinetics in nude mice, which lack a T cell source of MIF. Control, hypophysectomized, and nude mice were injected with LPS and serum prepared at intervals and quantified for MIF content by Western blotting and laser scanning densitometry (Fig. 1). Whereas serum MIF concentrations increased gradually over 20 h in control mice, hypophysectomizeed mice exhibited a rapid rise and then prompt fall in MIF that peaked 2 h after LPS injection. In nude mice, the rise in serum MIF was delayed by 8–12 h, but increased in a manner that was similar to that of the control mice. Peak serum MIF concentrations were compared and quantitated relative to rMIF standards electrophoresed and transferred on the same nitrocellulose membrane. Estimates of peak serum MIF in the different experimental groups were calculated to be in the range of 30–60 nM.

Given the lack of a pituitary source of MIF in the hypophysectomized mice, we considered that the MIF present in the circulation at 2 h must reflect the release of MIF by an additional LPS-sensitive cell population. Such serum MIF kinetics were reminiscent of a macrophage TNF response (19–21) and we next examined the possibility that MIF is produced by cells of the monocyte/macrophage lineage.
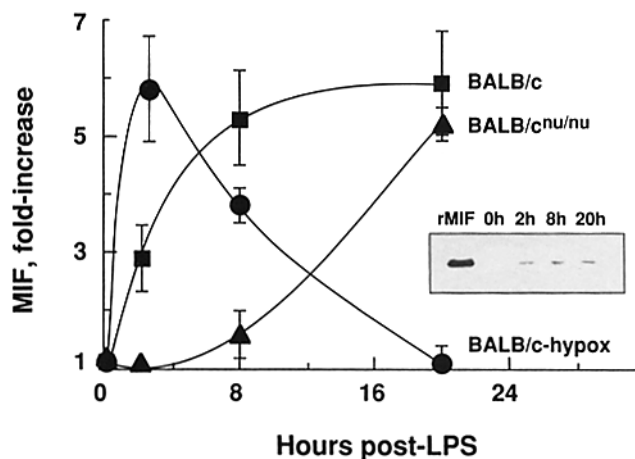

Figure 1. Western blotting and densitometric analysis of serum MIF in BALB/c, BALB/c$^{nu/nu}$, and hypophysectomized BALB/c mice. Mice were injected intraperitoneally with LPS and blood sampled at intervals for Western blotting analysis as described in Materials and Methods. The content of MIF in serum aliquots (5 μl) was quantified by laser densitometry with reference to electrophoresed rMIF standards. (*Inset*) MIF blot for the BALB/c mice, demonstrating the time-dependent increase in serum MIF after LPS administration. Each plotted point is the mean ± SEM of individual sera from two to five animals.

Expression of MIF in Resting Macrophages and Mouse Tissues. Since both a pituitary cell line (AtT-20) and the whole pituitary in vivo contain substantial amounts of preformed MIF (5), we first analyzed the intracellular MIF content of various inflammatory cells and cell lines. Significant amounts of preformed MIF protein were found to be present in cell lysates obtained from resting, nonstimulated murine RAW 264.7 cells, murine peritoneal macrophages, and human THP-1 monocytes (Fig. 2 *A*). We estimate that on average there is 0.1–1 pg of immunoreactive, preformed MIF protein per macrophage. The MIF content of murine or human monocytes/macrophages was similar to that of two T cell lines, the human Jurkat and the murine ASL-1. In contrast, cell lysates obtained from purified PMN exhibited no detectable MIF protein (Fig. 2 *A*).

We then examined the organ distribution of MIF protein in normal mice. Mouse tissues were homogenized and aliquots of total protein electrophoresed and analyzed by Western blotting. As shown in Fig. 2 *B*, preformed MIF was detectable in mouse liver, spleen, kidney, and brain. These organs contain significant amounts of macrophages, suggesting that the MIF protein present in these tissues is at least partly macrophage associated.

MIF Secretion by LPS-stimulated Macrophages. MIF was observed to be secreted by macrophages into culture medium after LPS stimulation (Fig. 3). In RAW 264.7 macrophages,
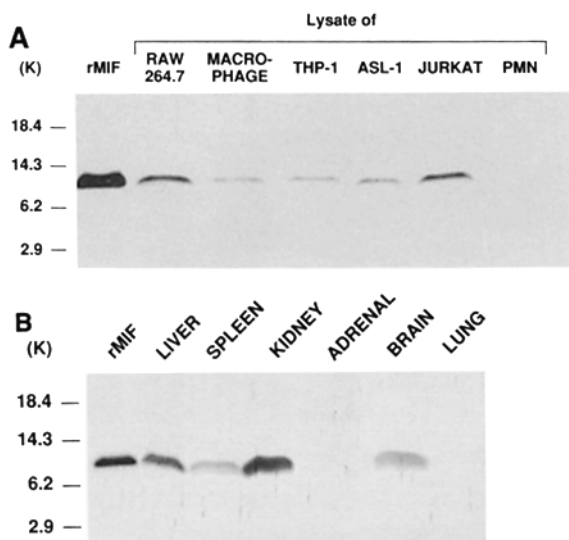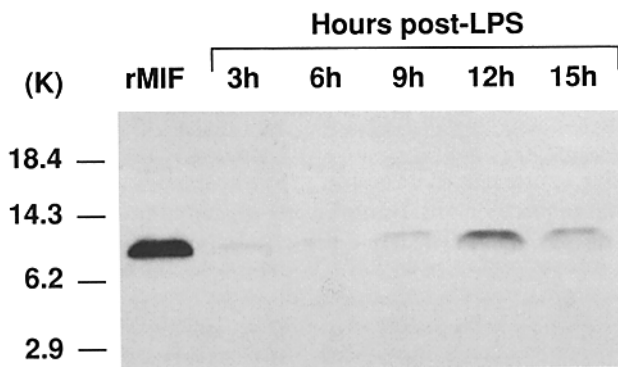
Figure 2. (*A*) Western blot analysis of the MIF content of nonstimulated monocytes/macrophages, T-lymphocytes, and PMN. 10 μl of cell lysate (equivalent to 5 × 10³ cells) were electrophoresed through 18% gels, transferred to nitrocellulose membrane, and analyzed with anti-MIF polyclonal antibody. 30 ng of rMIF was electrophoresed and transferred as a standard. (*B*) Western blot analysis of the organ distribution of murine MIF protein. Organs were obtained from an untreated BALB/c mouse and prepared as described in Materials and Methods. Aliquots (60 μg of protein) of liver, spleen, kidney, and adrenal, brain, and lung lysates were electrophoresed through 18% gels, transferred to nitrocellulose membrane, and analyzed with anti-MIF polyclonal antibody. 20 ng of rMIF was electrophoresed and transferred as a standard. Although not visible, a MIF band of much lower intensity also was detected in the adrenals and lungs.
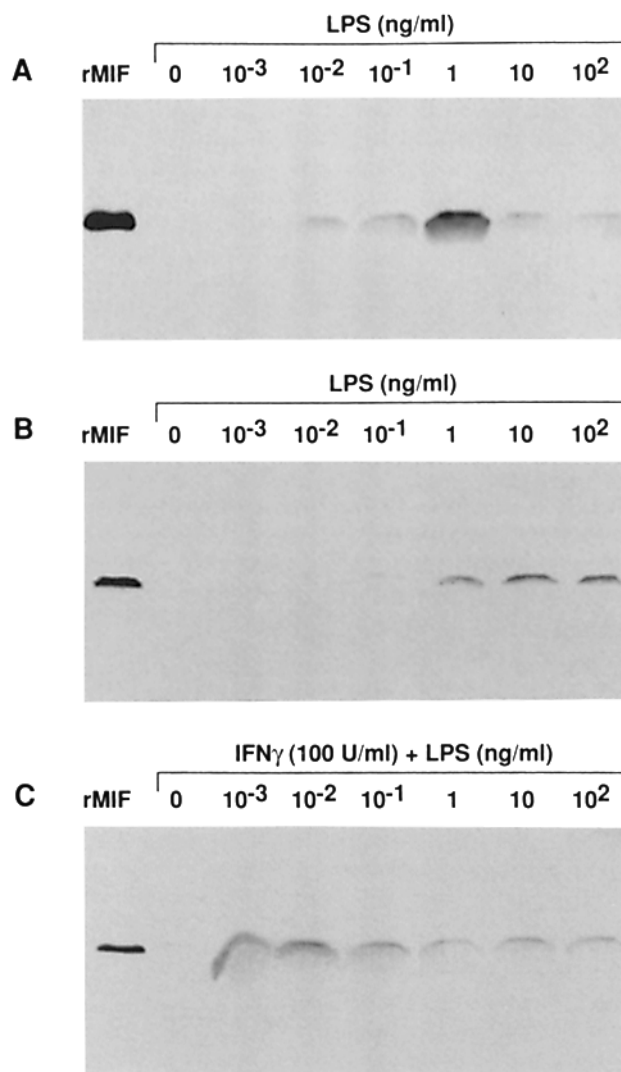
Figure 3. Western blot analysis of MIF secretion by RAW 264.7 cells (*A*) and by thioglycollate-elicited peritoneal macrophages stimulated with LPS (*B*) or with IFN-γ plus LPS (*C*). Macrophages (4 × 10⁶ RAW 264.7 cells or 10⁷ peritoneal macrophages) were incubated for 12 h with LPS or IFN-γ plus LPS at the indicated concentrations. The content of MIF secreted into the medium was analyzed by Western blotting as described in Materials and Methods. rMIF (30 ng in *A* and 20 ng in *B* and *C*) was electrophoresed and transferred as a standard.

MIF secretion was induced by as little as 10 pg/ml of LPS, peaked at 1 ng/ml, and was not detectable at LPS concentrations >1 μg/ml (Fig. 3 *A*). In elicited peritoneal macrophages, 1 ng/ml of LPS was required to induce MIF secretion, and a maximal response was observed with 10–100 ng/ml of LPS (Fig. 3 *B*). Of significance, costimulation of cells with IFN-γ (100 IU/ml, given 1 h before LPS) plus LPS resulted in a >1,000-fold increase in LPS responsiveness (Fig. 3 *C*).

We next examined the time course of MIF secretion in parallel cultures of RAW 264.7 macrophages maximally stimulated with 1 ng/ml of LPS. As assessed by Western blotting, MIF was first detected 6–9 h after LPS stimulation. Maximum amounts of MIF appeared in medium between 9 and 12 h after LPS stimulation (Fig. 4). MIF levels then decreased,
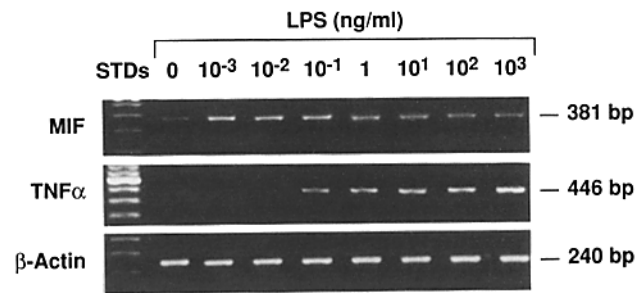
Hours post-LPS

| (K) | rMIF | 3h | 6h | 9h | 12h | 15h |

18.4 —
14.3 —
6.2 —
2.9 —

Figure 4. Time course of MIF secretion by LPS-stimulated RAW 264.7 macrophages. Parallel cultures (4 × 10⁶ cells) were incubated with 1 ng/ml of LPS and the conditioned medium removed at 3-h intervals for Western blot analysis of MIF content. 20 ng of rMIF was electrophoresed and transferred as a standard.

LPS (ng/ml)

STDs 0 10⁻³ 10⁻² 10⁻¹ 1 10¹ 10² 10³

MIF — 381 bp
TNFα — 446 bp
β-Actin — 240 bp

Figure 5. RT-PCR analysis of MIF, TNF-α, and β-actin expression by LPS-stimulated RAW 264.7 macrophages. Cells (4 × 10⁶) were incubated for 12 h in medium containing RPMI/1% FBS and LPS at the indicated concentrations. Total cellular RNA was extracted, cDNA prepared, and gene-specific PCR products analyzed by agarose gel electrophoresis as described in Materials and Methods.

indicating that after 12 h, MIF is degraded at a rate that exceeds that of synthesis and release by macrophages.

Expression of MIF mRNA by LPS-stimulated Macrophages. The expression of MIF mRNA by LPS-stimulated RAW 264.7 macrophages was investigated by RT-PCR. Parallel cultures were incubated for 12 h with medium or increasing amounts of LPS and analyzed for the expression of MIF, TNF-α, and β-actin. MIF mRNA was expressed constitutively in non-stimulated murine RAW 264.7 (Fig. 5) and in elicited peritoneal macrophages (data not shown). As expected, the expression of TNF-α increased over the range of LPS concentrations (1 pg/ml–1 μg/ml). In contrast, MIF mRNA levels correlated inversely with LPS concentration. MIF mRNA level was highest in cells induced with 1 pg/ml of LPS and lowest in those induced by 1 μg/ml of LPS.

To assess more quantitatively the induction of MIF mRNA, cDNA was prepared from control and LPS-stimulated cultures of RAW 264.7 macrophages and analyzed by competitive PCR. The amount of competitive template that was required to obtain equivalent levels of MIF and competitor DNA amplification products was 1.5 pg for nonstimulated (control) macrophages and 3 pg for LPS-induced (100 pg/ml) RAW 264.7 macrophages (Fig. 6). Therefore, macrophage MIF mRNA increased approximately twofold after LPS induction, a level comparable to that observed in pituitary cells in vivo (threefold after induction with 45 μg of LPS injected intraperitoneally) (5). We next examined the time course of MIF mRNA in RAW 264.7 macrophages incubated with 1 ng/ml of LPS, the LPS concentration that induced max-

imum MIF secretion in these cells (see Fig. 3). MIF mRNA levels increased 6 h after LPS induction and remained elevated for up to 12 h after LPS induction (data not shown).

Interaction between MIF and Proinflammatory Cytokines. The detection of elevated levels of MIF in blood after LPS administration (Fig. 1) together with the finding of an important role for MIF in experimental endotoxemia (5) led us to investigate the interaction between MIF and other proinflammatory cytokines. We first studied MIF secretion by RAW 264.7 macrophages stimulated with recombinant murine TNF-α, IL-1β, IL-6, or IFN-γ. By Western blotting, TNF-α and IFN-γ were found to induce MIF secretion in a dose-dependent fashion (Fig. 7). The lowest concentration of cytokine that was effective under these experimental conditions was 1 ng/ml of TNF-α and 10 IU/ml of IFN-γ. The TNF-α and IFN-γ effects could not be accounted for by LPS contamination of recombinant cytokine preparations, which were found to be 0.5 pg/ng of TNF-α and 60 fg/U of IFN-γ. LPS and IFN-γ had an additive/synergistic effect on MIF secretion, whereas TNF-α and IL-1β did not (data not shown). Finally, MIF production was not induced by either IL-1β or IL-6 at 1 or 10 ng/ml.

We then examined the secretion of TNF-α and IL-1β by RAW 264.7 macrophages stimulated with rMIF. MIF samples were preincubated for 1 h with polymyxin B at 1 μg/ml to neutralize small amounts of contaminating LPS (9 pg/μg of MIF by the chromogenic *Limulus* assay). rMIF at concentrations ≥100 ng/ml was found to induce the secretion of bioactive TNF-α as determined by the L929 cytotoxicity assay (Fig. 8). The MIF-induced TNF bioactivity was blocked completely by anti-TNF antibodies and rMIF did not contribute

MIF Competitor DNA (pg)

15 10.5 7 6 4.5 3 2 1 0.25

LPS (ng/ml)
0
0.1

— 381 bp
— 273 bp
— 381 bp
— 273 bp
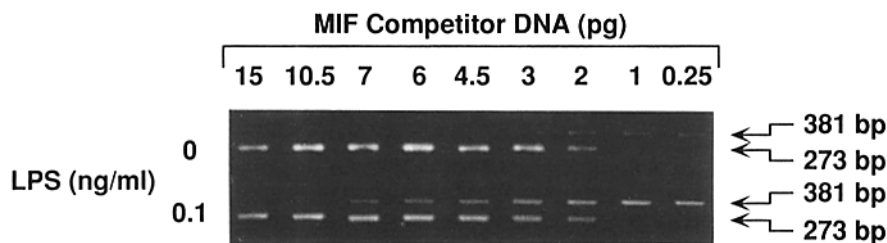
Figure 6. Competitive PCR analysis of macrophage MIF mRNA after LPS stimulation. RAW 264.7 cells (4 × 10⁶) were incubated for 12 h in RPMI/1% FBS with or without LPS (100 pg/ml). RNA was extracted and MIF mRNA levels analyzed as described in Materials and Methods. The amount of MIF competitor template DNA ranged from 0.25 to 15 pg per reaction as shown.
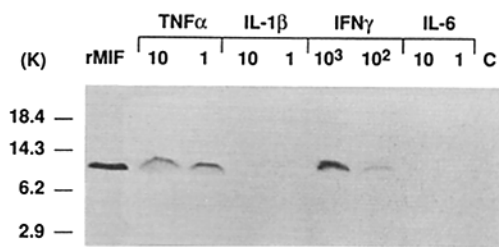
Figure 7. Western blot analysis of cytokine-induced MIF secretion by RAW 264.7 macrophages. Cells (4 × 10⁶) were incubated for 12 h with TNF-α, IL-1β, IL-6 (10 or 1 ng/ml), or IFN-γ (1,000 or 100 IU/ml). 20 ng of rMIF was electrophoresed and transferred as a standard.

to TNF cytotoxicity, as recombinant murine TNF cytotoxicity was unchanged when rMIF or anti-MIF polyclonal serum was added to L929 cells together with rTNF (data not shown). IL-1β secretion, in contrast, was not detectable by Western blotting over the concentration of rMIF tested.

Discussion

MIF was described originally as a T lymphocyte–associated activity that inhibited the random migration of guinea pig peritoneal macrophages (6, 7). Over the years, MIF activity was reported to be associated with delayed-type hypersensitivity (6, 7), to be produced by lectin-activated T cells, and to enhance macrophage adherence, phagocytosis (8, 9), and tumoricidal activity (10). Nevertheless, the biological activi-
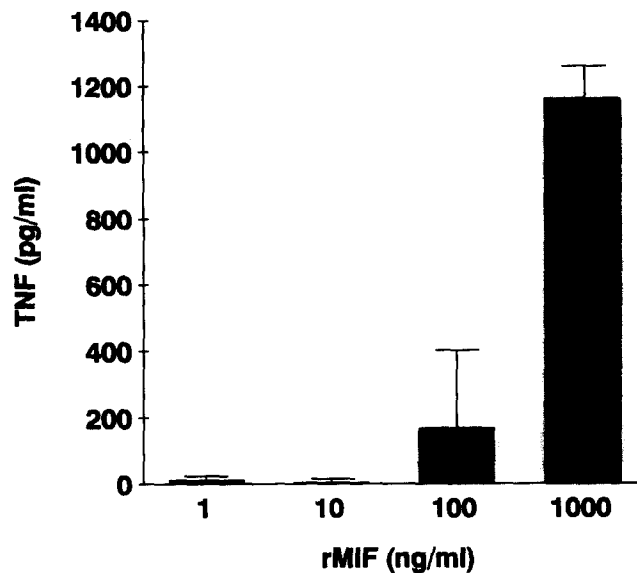
Figure 8. Concentration of TNF-α in cell culture supernatants of RAW 264.7 macrophages stimulated with rMIF. Cells (4 × 10⁶) were incubated for 12 h with the indicated concentrations of rMIF or with medium alone (control). Culture medium then was removed and the TNF-α content quantified by L929 cell cytotoxicity. TNF-α concentration is expressed as the difference between the level produced by rMIF-stimulated cells and by control (nonstimulated) cells. Data are expressed as the mean ± SD of three separate experiments.

ties attributed to MIF remained ambiguous, as these studies were performed with lymphocyte culture supernatants which were shown subsequently to contain other cytokines with MIF activity, such as IFN-γ and IL-4 (22–24). More recent experiments conducted with rMIF suggest that MIF in fact activates monocytes/macrophage to kill *Leishmania donovani* (Gessner, A., personal communication) and tumor cells (12).

We recently isolated and cloned the murine homolog of human MIF after identifying its release by the pituitary in response to LPS administration. Pituitary MIF was found to contribute to circulating MIF levels after endotoxemia and exogenous MIF increased markedly the lethality of experimental endotoxemia in mice (5). In this study, we sought to identify additional cellular sources of MIF. MIF was detected in the blood of LPS-challenged, T cell–deficient, and hypophysectomized mice suggesting that, in addition to the T cell and the pituicyte, other cell types contribute to MIF production in vivo. The extremely rapid rise in serum MIF which occurred in hypophysectomized mice further suggested to us a response that could be attributed to an LPS-sensitive immune cell such as the monocyte/macrophage.

Although previously unsuspected, we found that macrophages are an important source of MIF production in vivo. Resting, nonstimulated RAW 264.7 macrophages and thioglycollate-elicited peritoneal macrophages contain abundant quantities of preformed MIF. MIF protein was detected in normal mouse liver, spleen, kidney, and brain. Much lower amounts of MIF also were found in the adrenals and lungs. These data are consistent with recent studies reporting the presence of MIF mRNA in various tumor cells and tissues (25, 26). We detected MIF protein in macrophage-rich organs such as liver, spleen, brain, or kidney, suggesting that the MIF present in various tissues may be at least partly macrophage associated.

Various proinflammatory stimuli, such as LPS, TNF-α, and IFN-γ were observed to be potent inducers of macrophage MIF secretion. Secretion of significant amounts of MIF occurred at relatively low LPS concentrations (10–100 pg/ml), suggesting that macrophage-derived MIF is likely to be released in patients with gram-negative bacterial infections (27–29). Surprisingly, MIF secretion by RAW 264.7 macrophages, and to a lesser extent by peritoneal macrophages, decreased at high concentration of LPS (1–10 μg/ml). Analysis of the expression of MIF mRNA yielded similar results, as MIF mRNA levels decreased markedly with increasing LPS concentrations (>0.1 ng/ml of LPS). This contrasts with the macrophage TNF response where expression of both TNF protein and mRNA increased in a dose-dependent fashion. Possible mechanisms for the apparent downregulation of MIF at high LPS concentration are not immediately evident. Nevertheless, the relatively low levels of LPS which initiate this downregulation suggests that a similar downregulatory response is likely to occur in vivo as well.

The net concentration of MIF in supernatants of macrophage cultures peaked about 12 h after LPS stimulation. In vivo, however, elevated serum levels of MIF were detected as early as 2 h after LPS administration and increased in a

time-dependent manner. MIF levels were nearly maximal 8 h after LPS administration and plateaued thereafter. In hypophysectomized mice, serum MIF levels peaked 2 h after LPS injection. The mechanism responsible for this rapid rise in serum MIF is unknown, but may be related to the well-described hypersensitivity of hypophysectomized animals to LPS (4). In contrast, the appearance of serum MIF was delayed in T cell–deficient mice, suggesting that T cells either contribute directly to the early rise of serum MIF, or act indirectly by releasing cytokines such as TNF-α or IFN-γ that then promote the release of macrophage MIF.

Indeed, TNF-α and IFN-γ but not IL-1β and IL-6 were found to be potent inducers of MIF secretion by macrophages in vitro. Of importance, rMIF also was found to induce TNF secretion by macrophages, suggesting that MIF and TNF may act together to augment acute, proinflammatory responses. In the situation of a severe infection however, the proinflammatory effects of MIF may be detrimental as indicated by our recent findings that MIF markedly increased lethality when coinjected with LPS, whereas anti-MIF antibody prevented LPS lethality. These data also suggest that in extreme situations such as septic shock, MIF, like TNF, may be overproduced, and neutralization of either cytokine can be shown to fully protect against the toxicity of endotoxin administration (5, 30–33).

The production of MIF by LPS-stimulated macrophages indicates that this cytokine may serve an important role in acute inflammatory responses. Taken together with the finding that MIF is a major protein constituent of the pituitary that is released during endotoxemia, this study supports the concept that MIF plays a systemic, regulatory function in host immunity. Macrophage MIF, once released by a proinflammatory stimulus, may act in concert with TNF-α and other cytokines to coordinate host defenses against infection or tissue invasion. Pituitary MIF, on the other hand, may serve to prime systemic immune responses once a localized inflammatory site fails to contain an invasive agent, or else act as a central nervous system–derived stress signal to activate the immune system in anticipation of an impending, invasive stimulus. Studies are in progress to delineate the role of pituitary, macrophage, and T cell MIF in acute and chronic inflammatory conditions.

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