The *D*-dopachrome tautomerase (*DDT*) gene product is a cytokine and functional homolog of macrophage migration inhibitory factor (MIF)

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Edited* by Peter Cresswell, Yale University School of Medicine, New Haven, CT, and approved July 12, 2011 (received for review February 21, 2011)

Macrophage migration inhibitory factor (MIF) is a pivotal regulator of the immune response. Neutralization or genetic deletion of MIF does not completely abrogate activation responses, however, and deletion of the MIF receptor, CD74, produces a more pronounced phenotype than MIF deficiency. We hypothesized that these observations may be explained by a second MIF-like ligand, and we considered a probable candidate to be the protein encoded by the homologous, *D*-dopachrome tautomerase (D-DT) gene. We show that recombinant D-DT protein binds CD74 with high affinity, leading to activation of ERK1/2 MAP kinase and downstream proinflammatory pathways. Circulating D-DT levels correlate with disease severity in sepsis or malignancy, and the specific immunoneutralization of D-DT protects mice from lethal endotoxemia by reducing the expression of downstream effector cytokines. These data indicate that D-DT is a MIF-like cytokine with an overlapping spectrum of activities that are important for our understanding of MIF-dependent physiology and pathology.

inflammation | lipopolysaccharide | septic shock

Macrophage migration inhibitory factor (MIF) is the first cytokine activity described and a key regulatory mediator that is released upon activation of different cell types (1–3). MIF increases macrophage antimicrobial responses and it is expressed upstream of cytokines such as tumor necrosis factor (TNF)-α, IFN-γ, and IL-1β (4). MIF activates immune cells by binding to CD74, leading to the recruitment of CD44 into a signaling complex, the stimulation of nonreceptor tyrosine kinases, and initiation of the ERK1/2 MAP kinase pathway (5, 6). The chemokine receptors CXCR2 and CXCR4 also become activated by MIF via non-cognate interactions that are reinforced in the presence of CD74 (7). Among mesenchymal cell types, MIF binding to cardiomyocyte CD74 stimulates the AMP-activated kinase (AMPK) cascade to mediate protection from ischemic injury (8, 9).

Although MIF receptor knockout mice ($CD74^{-/-}$) phenocopy features of MIF deficiency (10–12), recent observations have led to the hypothesis that there may be a second ligand for CD74. MIF-deficient B cells, for example, are more sensitive to apoptosis than wild-type B cells, but the magnitude of this defect is twofold more pronounced in CD74-deficient cells (13). Intravital microscopy studies also have shown a more pronounced effect of antagonism of CD74 than MIF in monocyte arrest (7). Finally, anti-MIF antibodies, although highly effective in experimental studies, do not completely inhibit CD74-dependent cellular activation responses (14).

We hypothesized that these observations may be explained by a second MIF-like ligand, and we considered a likely candidate to be the protein encoded by the *DDT* gene, *D*-dopachrome tautomerase (D-DT). *DDT* and *MIF* show a conserved intron–exon structure and their coding regions are highly homologous. The genes for MIF and D-DT are in close apposition to each other and to two theta-class glutathione S-transferases, suggesting that these gene clusters arose by an ancestral duplication event (Fig. 1).

D-DT was named for its ability to tautomerize the non-naturally occurring, *D*-stereoisomer of dopachrome, which is a catalytic property shared with MIF. This activity has been hypothesized to be a vestigial function that reflects MIF's ancestral origin in the invertebrate melanotic encapsulation response (15). A crystal structure of D-DT has verified its 3D similarity with MIF (16). With the exception of recent studies indicating an interaction between the *MIF* and *DDT* genes in the expression of proangiogenic factors and COX-2 in adenocarcinoma cell lines (17, 18), there have been no studies of the biologic functions of D-DT. In this report, we describe the functional characterization of human and mouse D-DT. We produced recombinant D-DT and demonstrate that it binds with high affinity to CD74, activates the ERK1/2 MAP kinase signaling cascade, and recapitulates many of the inflammatory functions of MIF, including modulation of macrophage migration and glucocorticoid-induced immunosuppression. The administration of an anti-DDT antibody, like anti-MIF, protects mice from lethal endotoxic shock by reducing the circulating levels of proinflammatory cytokines (TNF-α, IFN-γ, IL-12p70, and IL-1β) and increasing the circulating concentration of the anti-inflammatory cytokine, IL-10. An analysis of clinical samples from patients with sepsis or cancer also revealed that D-DT is systemically expressed and that circulating levels correlate with MIF and with the severity of inflammatory disease and malignancy.

Results

Purification and Characterization of the D-DT Protein. The genes for MIF and D-DT lie within 0.1 kb of each other in both the mouse and human genomes and have a similar organizational relationship with nearby genes for matrix metalloproteinase 11 and two theta class GSTs (Fig. 1). The amino acid sequences show 34% identity between human MIF and human D-DT and 27% identity between murine MIF and murine D-DT. The D-DT proteins share with MIF a canonical N-terminal proline (formed after posttranslational excision of the initiating methionine), which catalyzes substrate tautomerization (19), but they lack two of the three conserved cysteines (Cys^{59} and Cys^{80}) that appear in

Author contributions: M.M. and R.B. designed research; M.M., S.Z., L.L., R.D., X.D., W.S., J.F., H.L., Y.C., and H.X. performed research; F.C., J.B., E.L., G.M., and O.L. contributed new reagents/analytic tools; M.M., L.L., R.D., Y.C., H.X., and J.B. analyzed data; and M.M. and R.B. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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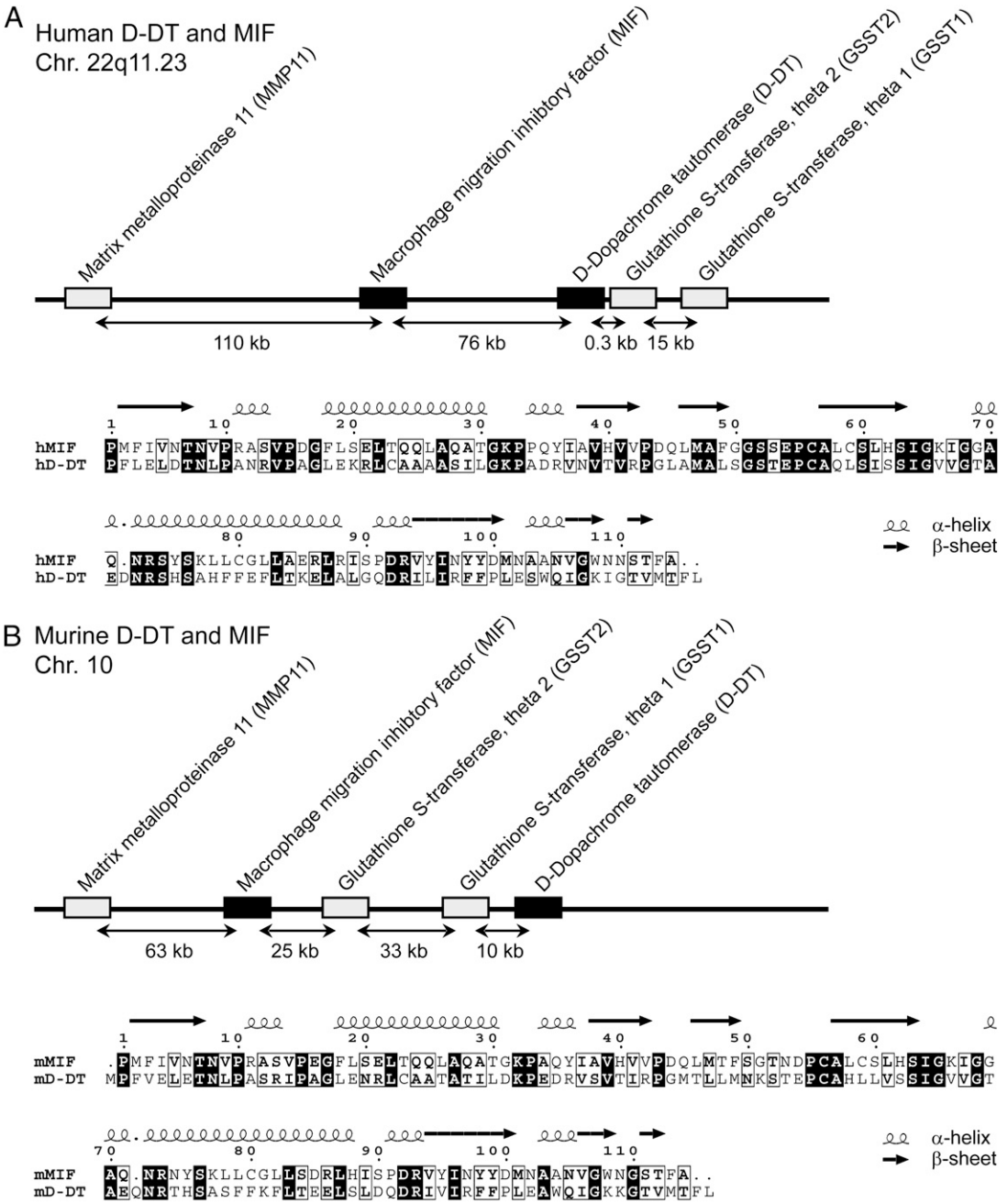

Fig. 1. Genomic organization and protein homology of MIF and D-DT. (*A*) Schematic diagrams showing the relationship between human *MIF* and *DDT* with matrix metalloprotease 11 and GST genes (*Upper*) and the amino acid sequence and secondary structure homologies of the two proteins (*Lower*). (*B*) Mouse *Mif*, *Ddt*, and adjacent genes (*Upper*) and the corresponding amino acid sequence and secondary structure homologies of the two proteins (*Lower*). Gene structure was compiled from www.ensembl.org and sequence alignment performed using *ClustalX* and http://espript.ibcp.fr/ESPript/ESPript/.

all known mammalian MIF proteins. Murine and human D-DT also lack the *pseudo(E)LR* (Arg^{11}, Asp^{44}) motif that mediates MIF's noncanonical interactions with the CXCR2 chemokine receptor (20). The mRNA for D-DT does not encode either an N-terminal or an internal secretory signal sequence, suggesting that like MIF, D-DT is secreted by a specialized, nonclassical export pathway (21).

The cDNAs for human and mouse D-DT were prepared from monocytes and cloned into a bacterial expression vector for recombinant protein production. We considered it critical to work with native sequence proteins because of structure-function studies that have shown that modifications of the N or C termini interfere with trimer formation, the functional unit of MIF (19, 22, 23). Recombinant D-DT protein was purified by anion exchange chromatography followed by high performance liquid chromatography (HPLC) (Fig. 2*A*). Mass spectroscopy of purified mouse D-DT protein gave an *m/z* of 12,947, which lies within 0.02% of the calculated mass for D-DT (Fig. 2*B*). A minor peak of 13,079 Da also was detected; this peak corresponds to the molecular mass of D-DT with an uncleaved N-terminal methionine (expected *m/z* = 13,077).

MIF tautomerizes model substrates such as *D*-dopachrome and *p*-hydoxyphenylpyruvate (HPP) (whether a physiological substrate exists is unknown) (15), and D-DT purified from liver

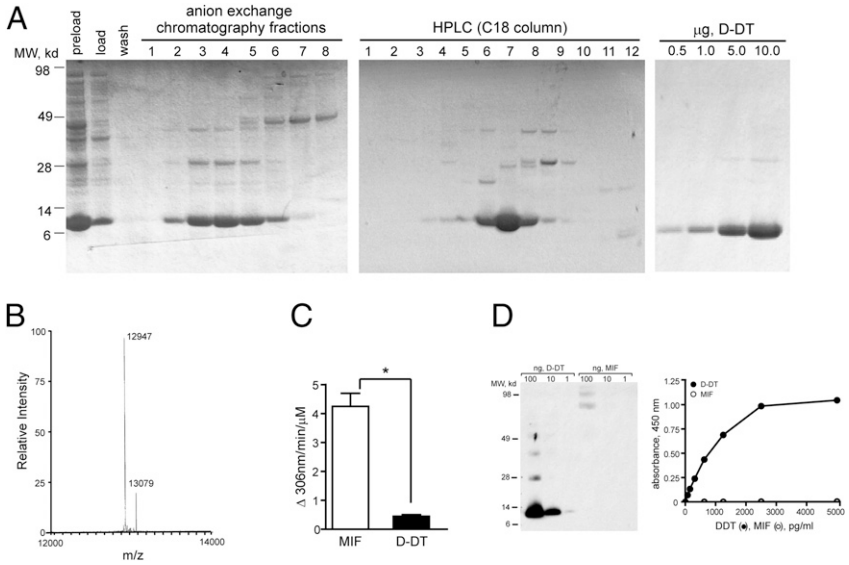

Fig. 2. Characterization of the D-DT protein. (*A*) SDS/PAGE and Coomassie analysis of sequential purification steps of recombinant D-DT protein. The examples shown are for mouse D-DT but qualitatively identical results were obtained for human D-DT. (*B*) Electrospray ionization mass spectrometry of mouse D-DT showing a molecular mass (*m/z*) that is within 0.02% accuracy of the predicted *m/z* (12,946 Da). (*C*) Tautomerization activity of human MIF and D-DT measured with the substrate, *p*-hydoxyphenylpyruvate. Results are expressed as mean ± SD of duplicate measurements and are representative of three experiments. (*D*) Anti-DDT antibody specifically recognizes D-DT. (*Left*) Anti-DDT antibody recognizes recombinant murine D-DT protein in Western blotting (1–100 ng/lane), but does not detect recombinant mouse MIF. (*Right*) D-DT ELISA quantifies concentrations in the picogram range and shows no cross-reactivity to MIF. Results are expressed as mean ± SD of duplicate measurements and are representative for two independent experiments.

has tautomerization activity (24, 25). We verified that recombinant D-DT tautomerizes HPP with a specific activity that is ∼10 times lower than that measured for MIF (D-DT = 0.5 ± 0.1 $\Delta 306 \cdot min^{-1} \cdot \mu M^{-1}$, and MIF = 4.3 ± 1.1 $\Delta 306 \cdot min^{-1} \cdot \mu M^{-1}$, $P < 0.001$) (Fig. 2*C*). A possible explanation for the discrepancy in activity might be structural differences in the active site regions of D-DT and MIF, resulting in a reduced affinity of D-DT to its substrate (16, 22). A polyclonal anti-DDT antibody was prepared to establish an ELISA. This antibody recognized both murine and human D-DT but did not cross-react with MIF in its denatured form (assessed by Western blot; Fig. 2*D*, *Left*) or in its native form (assessed by ELISA; Fig. 2*D*, *Right*).

D-DT Binds the MIF Receptor, CD74, and the Intracellular Protein, JAB1. MIF activates ERK1/2 phosphorylation by engaging CD74, and a high-affinity binding interaction between MIF and the CD74 ectodomain ($CD74^{73–232}$ or sCD74) has been demonstrated by surface plasmon resonance (6). We first studied the interaction between D-DT and sCD74 in a competition binding assay. D-DT reduced MIF binding to the CD74 ectodomain in a dose-dependent manner, with a maximal effect of ∼50% compared with MIF (Fig. 3*A*). Measurement of the equilibrium dissociation constants between human D-DT or MIF and sCD74 by surface plasmon resonance (BIAcore analysis) revealed a high-affinity binding interaction between D-DT and the MIF receptor (K_D of 5.42×10^{-9} M) (Fig. 3*B*), albeit 60% lower than for MIF ($K_D = 1.40 \times 10^{-9}$ M). Detailed analysis revealed a k_a of 1.2×10^5 $M^{-1} \cdot s^{-1}$ for D-DT and only 4.3×10^4 $M^{-1} \cdot s^{-1}$ for MIF, whereas the dissociation rate (k_d) was 11-fold lower for MIF than for D-DT ($6 \times 10^{-5} \cdot s^{-1}$ and $6.6 \times 10^{-4} \cdot s^{-1}$, respectively). These measurements demonstrate that D-DT has an ∼3-fold higher binding rate to the receptor CD74, but also dissociates much faster than MIF.

The intracellular transcriptional regulator and COP9 signalosome component JAB1 is a well characterized binding partner of MIF that has been implicated in MIF-dependent control of cell proliferation (26). D-DT binds to JAB1 as demonstrated by coimmunoprecipation (Fig. 3*C*), and the interaction affinity between JAB1 and D-DT appears comparable to that observed between JAB1 and MIF.

Differential Expression of D-DT and MIF. We assessed the possibility that D-DT may be differentially expressed in tissue compared with MIF. Esumi et al. have published Northern blotting data for *Ddt* expression that suggest enhanced expression in the murine brain compared with *Mif* (27). We analyzed eight different mouse organs by Western blotting for D-DT, MIF, and CD74. D-DT and MIF were present in constitutive and appreciable levels in all tissues examined, with the greatest difference observed in the testis, where D-DT appeared in severalfold higher concentrations compared with MIF (Fig. 3*D*). Immunostaining of murine tissue confirmed these results and showed that D-DT, like MIF (28), is detected in virtually all cells, with prominent staining in the epithelia of the kidney, the lung, the bowel, hepatocytes, and the follicular area of the spleen (Fig. 3*E*).

D-DT Initiates ERK-1/2 Phosphorylation in a MIF Receptor-Complex–Dependent Manner, Mediates Macrophage Migration Arrest, and Counterregulates Glucocorticoid-Induced Immunosuppression. MIF binding to CD74 leads to the recruitment of CD44 and the intracellular phosphorylation of these proteins, resulting in the activation of SRC family nonreceptor tyrosine kinases and the initiation of the ERK1/2 MAP kinase cascade (5, 6). Stimulation of cultured macrophages with increasing concentrations of recombinant D-DT produced a dose-dependent phosphorylation of ERK1/2, with an activating effect that was both sustained (2 h) (29) and comparable to that observed for MIF (Fig. 4*A*, *Top*). Costimulation with D-DT and MIF revealed an additive effect of the two proteins in the ERK1/2 MAP kinase pathway (Fig. 4*A*, *Middle*). D-DT–induced ERK1/2 phosphorylation was strictly dependent on the expression of both CD74 and CD44, as previously reported for MIF (5) (Fig. 4*A*, *Bottom*).

We analyzed the biologic activity of recombinant D-DT by first assaying for MIF's effect on macrophage chemotaxis (30). D-DT inhibited chemotaxis induced by CCL2/monocyte chemotactic protein (MCP)-1, but with a less steep dose response and re-

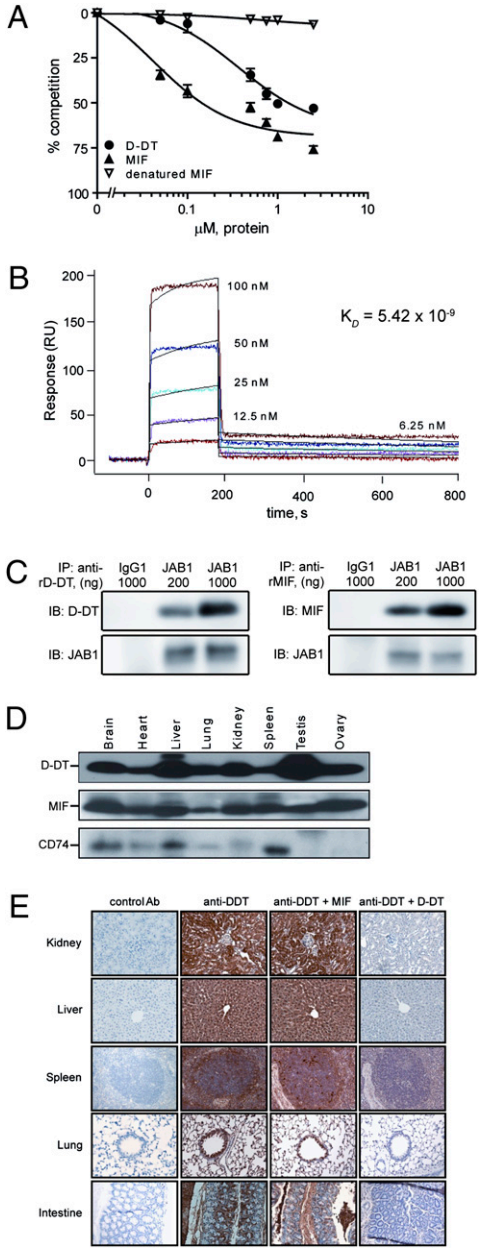

Fig. 3. D-DT binds with high affinity to the MIF receptor, CD74. (*A*) Concentration-dependent binding of D-DT and MIF to the MIF receptor sCD74 using biotinylated human MIF as competitor. Heat-denatured MIF served as a negative control. (*B*) Real-time surface plasmon resonance analysis (BIAcore) of the interaction between D-DT and sCD74. (*C*) Coimmunoprecipitation of D-DT/JAB1 and MIF/JAB1. (*Left*) Cells were lysed and recombinant D-DT was added. JAB1/D-DT–containing protein complexes were coprecipitated by pull-down of JAB1, and D-DT was detected by Western blot. (*Right*) Coimmunoprecipitation between JAB1 and MIF following the same protocol. (*D*) D-DT is differentially expressed in mouse tissue. Protein lysates (75 μg) were separated by SDS/PAGE and analyzed by Western blot for D-DT, MIF, and CD74 (n = 2 mice studied). (*E*) D-DT protein expression analyzed by immunostaining of five representative organs from a C57BL/6 mouse (n = 3 mice studied).

duced inhibitory action at 1 μg/mL compared with MIF (Fig. 4*B*). This observation may be explained by the reduced binding affinity of D-DT for the MIF receptor or different rates of ligand association/dissociation and a consequent reduction in the downstream events necessary for CCL2 desensitization.

MIF is unique among immune mediators in its ability to counterregulate the immunosuppressive action of glucocorticoids, which occurs via intracellular pathways that involve cytoplasmic phospholipase A2, IκB1, and MKP-1 (31–33). Using a standardized assay (31), we further observed that D-DT, like MIF, counterregulated the inhibitory effect of glucocorticoids on TNF production from lipopolysaccharide (LPS)-stimulated macrophages (Fig. 4*C*). Similar to observations in the migration assay, D-DT shows a decreased counterregulatory potential at low concentrations compared with MIF, which may be attributed to lower binding affinity to the MIF receptor.

D-DT Is Produced in Response to LPS and Mediates Lethal Endotoxemia. Whereas macrophages have been considered historically to be a main target of MIF action, these cells also are a major source of MIF production in response to microbial products and tissue invasion in vivo (34). Cultured macrophages were stimulated with 1 μg/mL of *Escherichia coli* LPS and the secretion of D-DT and MIF was quantified by specific ELISA. LPS-stimulated macrophages released D-DT into conditioned medium with kinetics that were very similar to those of MIF (Fig. 5*A*). Peak levels were detectable at 16 h and decreased thereafter. Unstimulated cells also slowly released these proteins into supernatants, which in the case of MIF has been attributed to a low level of constitutive secretion (21). We addressed whether there may be reciprocal regulation of D-DT and MIF expression in macrophages. We depleted D-DT or MIF in immortalized macrophages by siRNA-mediated knockdown but detected no effect on the expression level of the reciprocal protein in response to LPS stimulation (Fig. 5*B*).

The administration of LPS to mice resulted in a time-dependent increase in plasma D-DT concentrations (6.0 ± 4.3 ng/mL to 26 ± 12 ng/mL at 24 h), and this increase mimicked the rise observed for MIF (1.0 ± 0.9 ng/mL to 43 ± 28 ng/mL at 24 h) (Fig. 5*C*). D-DT also is detectable in plasma under basal conditions and at comparable levels to MIF (D-DT = 6.0 ± 4.3 ng/mL and MIF = 1.0 ± 0.9 ng/mL) (31). It is noteworthy that whereas plasma MIF and D-DT circulate in similar concentrations under basal or stimulated conditions, LPS-stimulated macrophages produce 20-fold more MIF than D-DT (Fig. 5*A*). This observation suggests that nonmacrophage sources of D-DT contribute significantly to plasma D-DT expression in vivo.

Immunoneutralization of D-DT Protects from Lethal Endotoxemia. Immunoneutralization or genetic deletion of MIF protects mice from endotoxic shock (35, 36) and subsequent studies demonstrated that this protective effect is due to a reduction in the expression of tissue-damaging, effector cytokines such as TNF and IL-1 (37, 38). The administration of a specific anti-DDT antibody before the injection of an LD_{80} dose of LPS increased survival from 20 to 79% (Fig. 5*D*), which is a level of protection comparable to that of anti-MIF (35). An analysis of plasma cytokine expression showed that D-DT neutralization was associated with a significant reduction in the circulating concentration of several proinflammatory cytokines (TNF-α, IL-1β, IFN-γ, and IL-12p70) implicated in shock pathogenesis (Fig. 5*E*). In contrast, the concentration of the anti-inflammatory cytokine IL-10 was increased in the anti–DDT-treated group compared with controls.

D-DT Expression Is Up-Regulated in Patients with Sepsis or Invasive Cancer. To determine whether D-DT is up-regulated in human disease and to assess potential relationships between D-DT and MIF production in vivo, we analyzed the serum concentrations of these mediators in individuals with sepsis or with ovarian cancer, which are two conditions characterized by high levels of MIF in plasma (38, 39). There was a statistically significant increase in circulating D-DT protein in patients with sepsis compared with

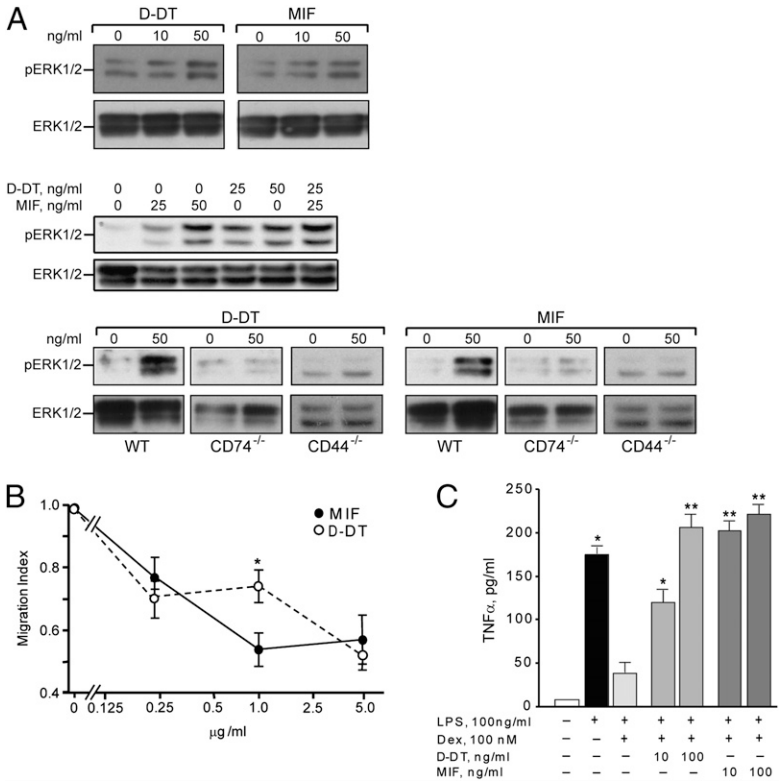

Fig. 4. Functional comparison of D-DT and MIF. (*A*) D-DT activates the sustained ERK1/2 MAP kinase pathway in a MIF receptor complex (CD74/CD44)-dependent manner. (*Top*) Macrophages (1×10^6/mL) were treated with 0, 10, or 50 ng/mL of D-DT or MIF for 2 h. Cell lysates were analyzed for phosphorylation of ERK1/2. (*Middle*) Macrophages were treated with the indicated concentrations of D-DT, MIF, or D-DT plus MIF. Lysates were analyzed for the phosphorylation status of ERK1/2. (*Bottom*) Wild-type and MIF receptor knockout ($CD74^{-/-}$ or $CD44^{-/-}$) macrophages were treated with 50 ng/mL of D-DT or MIF for 2 h, and cell lysates were analyzed by Western blot. Results are representative of at least two independent experiments. (*B*) Increasing concentrations of D-DT or MIF inhibit the chemotaxis of human peripheral blood monocytes to MCP-1. Data shown are mean ± SD of quadruplicate assays and statistical significance for the comparison of MIF vs. D-DT was analyzed by an unpaired Student's *t* test; $*P < 0.01$. (*C*) D-DT or MIF inhibits glucocorticoid-mediated suppression of TNF production. Macrophages were preincubated for 1 h with or without dexamethasone (Dex, 100 nM), MIF, or D-DT (100 ng/mL) and then stimulated with LPS (100 ng/mL). Supernatants were collected after 4 h and TNF was quantified by ELISA. Data shown are mean ± SD of triplicate samples from one experiment and are representative of four independent experiments. $*P < 0.005$, $**P < 0.001$ vs. LPS + Dex condition by an unpaired Student's *t* test.

healthy controls (sepsis patients, 55.5 ± 61.3 ng/mL; control group, 5.9 ± 3.9 ng/mL; $P < 0.0001$) (Fig. 6*A*). MIF levels also were elevated, as expected from prior work (38, 40, 41) (sepsis patients,111.0 ± 69.0 ng/mL; control group, 6.3 ± 6.2 ng/mL; $P < 0.0001$). Receiver operator characteristic (ROC) analysis revealed an area under the curve of 0.99 for MIF or D-DT, indicating that both proteins show excellent sensitivity and specificity for the diagnosis of sepsis. These measurements further revealed that serum levels of D-DT, like MIF (40, 42), correlate with disease severity as determined by APACHE II clinical severity scores (Fig. 6*B*). Both D-DT and MIF show a significant association with outcome parameters; however, a more precise assessment of the prognostic value of these proteins may be obtained by serial measurements. Serum D-DT and MIF concentrations also correlate in healthy individuals, and the correlation coefficient increases from $R = 0.32$ to $R = 0.5$ for the analysis of these mediators in patients with sepsis (Fig. 6*C*).

We also found circulating D-DT concentrations to be elevated in patients with ovarian cancer (cancer patients, 15.2 ± 13.8 ng/mL; control group, 5.9 ± 3.9 ng/mL; $P < 0.0001$) (Fig. 6*D*). ROC analysis revealed an area under the curve of 0.8, which is comparable to that observed for MIF (0.7). The correlation between MIF and D-DT serum concentrations was stronger and showed greater statistical significance than that observed for septic patients ($R = 0.9$, $P = 0.0001$).

Discussion

We report herein the functional characterization of the eukaryotic MIF homolog, D-DT. MIF and D-DT are highly conserved in their overall genomic structure, amino acid sequence similarity, and MIF receptor activation properties. Both mouse and human D-DT proteins show conservation in their catalytic, N-terminal proline but they lack MIF's –CXXC- and *pseudo(E)LR* motifs, the latter of which mediates noncognate interactions with CXCR2 and CXCR4 (7, 20). We produced recombinant, endotoxin-free D-DT protein and demonstrated that D-DT, like MIF, tautomerizes the model substrate, *p*-hydroxyphenylpyruvate, albeit the measured velocity is ~10 times lower than that analyzed for MIF. D-DT is constitutively expressed in different tissues and it is up-regulated by proinflammatory activation.

MIF binds to the type II transmembrane protein, CD74, leading to its intracellular phosphorylation, the recruitment of the coreceptor CD44, and the activation of SRC family kinases and the ERK1/2 MAPK pathway (5, 6). Although the interaction between D-DT and sCD74 was reduced in comparison with MIF when measured by both competition binding and BIAcore studies, the ERK1/2 activation potential of D-DT and MIF appeared comparable by Western blotting. The K_D of sCD74 for D-DT was 60% of that measured for MIF; D-DT was found to have an ~3-fold higher association rate (k_a) to sCD74 but to dissociate (k_d) ~11-fold faster than MIF. These differences in association and dissociation values may differentiate the signaling action of the

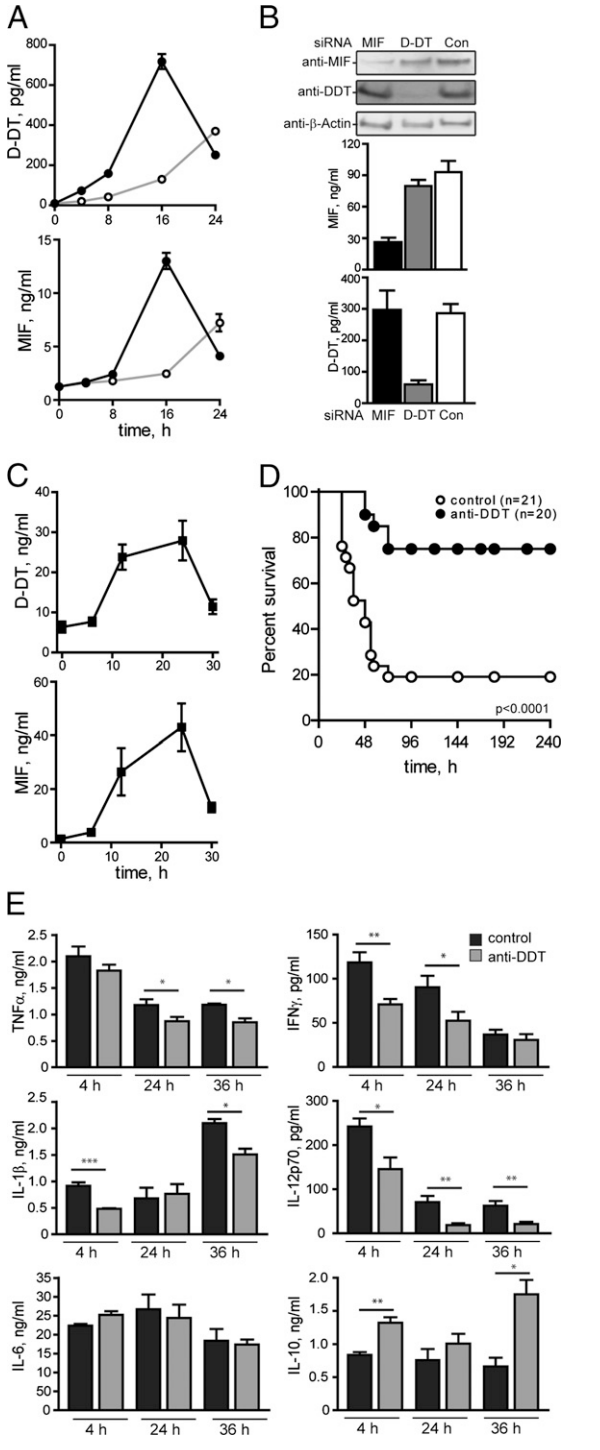

Fig. 5. Neutralization of D-DT protects from lethal endotoxic shock. (*A*) D-DT is released from macrophages after LPS stimulation. Peritoneal macrophages (1×10^6/mL) were stimulated with LPS or PBS (control) and supernatants were analyzed by ELISA for D-DT and MIF content. Results are expressed as mean ± SD of duplicate assays and are representative of at least three independent experiments. (*B*) Reciprocal regulation of MIF and D-DT. Macrophages were transfected with MIF, D-DT, or control siRNA, respectively, and cultivated for 4 d. (*Upper*) Cells were lysed and a Western blot was performed. (*Lower*) Macrophages were stimulated with LPS (1 ng/mL) and after 6 h the supernatants were collected for ELISA. (*C*) LPS challenge leads to increased D-DT concentrations in serum. BALB/c mice (8 wk, female) were challenged with 12.5 mg/kg of LPS, and blood was drawn 0, 6, 12, and 24 h after i.p. LPS administration. Serum was analyzed by ELISA for D-DT and MIF content. The results are expressed as mean values ± SD of two independent experiments ($n = 10$), and statistical significance was by Student's *t* test, $^{**}P < 0.01$, $^{***}P < 0.001$. (*D*) Neutralization of D-DT protects from lethal endotoxemia. BALB/c mice were injected i.p. with anti-DDT antibody or nonimmune antibody (control) 2 h before LPS administration (20 mg/kg). Data points are from three independent experiments. Survival was 75% (15 of 20) in mice treated with anti-DDT antibody and 19% (4 of 21) in mice treated with control antibody. $P < 0.0001$, Kaplan–Meier test. (*E*) Neutralization of D-DT influences serum cytokine concentrations. Mice were treated with LPS as in *C* and blood was drawn for cytokine analysis by Luminex. The results are expressed as mean values ± SD of three independent experiments (Student's *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$).

two proteins; e.g., D-DT binding to the receptor might not always trigger a signaling cascade but might result in its internalization. MIF showed a steeper dose response than D-DT in measurements of macrophage migration inhibition and glucocorticoid overriding, but the resolution of these cell-based assays may be too limited to make precise, quantitative conclusions. These results may support the notion that D-DT is a less potent cytokine that might be associated with the down-regulation of inflammation.

We produced anti-DDT antibodies that do not cross-react with MIF; these antibodies allowed for measurements of this protein in biologic specimens. D-DT was expressed in the serum of endotoxemic mice at levels that were ~60% those of MIF. MIF is a high, upstream mediator of septic shock, and immunoneutralization, genetic deletion, or pharmacologic inhibition of MIF protects from lethal shock induced by different microbial pathogens, even when administered many hours after microbial invasion (35, 36, 43–45). Immunoneutralization of D-DT conferred protection from lethal endotoxemia and this effect was accompanied by a broad reduction in the systemic expression of TNF-α, IL-1β, IL-12p70, and IFN-γ and an increase in the circulating levels of the anti-inflammatory mediator, IL-10. Given current development of MIF inhibitors for the treatment of the inflammatory diseases (44, 46, 47), it would be of interest to determine whether the combined neutralization of MIF and D-DT has a synergistic effect.

MIF levels are elevated in patients with systemic inflammatory disease or with neoplasia, and in many instances a correlation between disease severity and MIF levels has been observed (48). Like MIF, D-DT circulates at baseline levels in healthy individuals and there is a correlation in the plasma concentrations of the two mediators. Circulating D-DT levels increase significantly in patients with sepsis or ovarian cancer and show a strong correlation with MIF, suggesting that the two mediators may be coordinately expressed in response to similar activating stimuli.

Our main findings that D-DT is a close structural and functional homolog of MIF, that it binds and activates the MIF receptor, and that targeting D-DT, similar to MIF, protects mice from lethal endotoxemia raise the question: What are the distinctive functional features of D-DT, if any, compared with MIF? Like MIF (28), D-DT is widely expressed in tissues. We did find that macrophages produce 20-fold more MIF than D-DT in response to LPS, suggesting that there may be cell-specific release of these mediators and that in contrast to MIF, nonmacrophage sources of D-DT contribute more importantly to the systemic expression of D-DT than MIF. Computational inspection of the D-DT and MIF promoter regions shows similarities in the presence of serum-responsive, SP-1 and AP-1 binding elements (http://alggen.lsi.upc.es/). Of note, the human *DDT* gene lacks the polymorphic, $CATT_{5-8}$ microsatellite repeat that exists in the *MIF* promoter (*rs*5844572) and that regulates *MIF* expression and is associated with the severity of autoimmune inflammatory diseases (48). Conceivably, one difference between *MIF* and *DDT* may lie in the allele-specific regulation of *MIF*

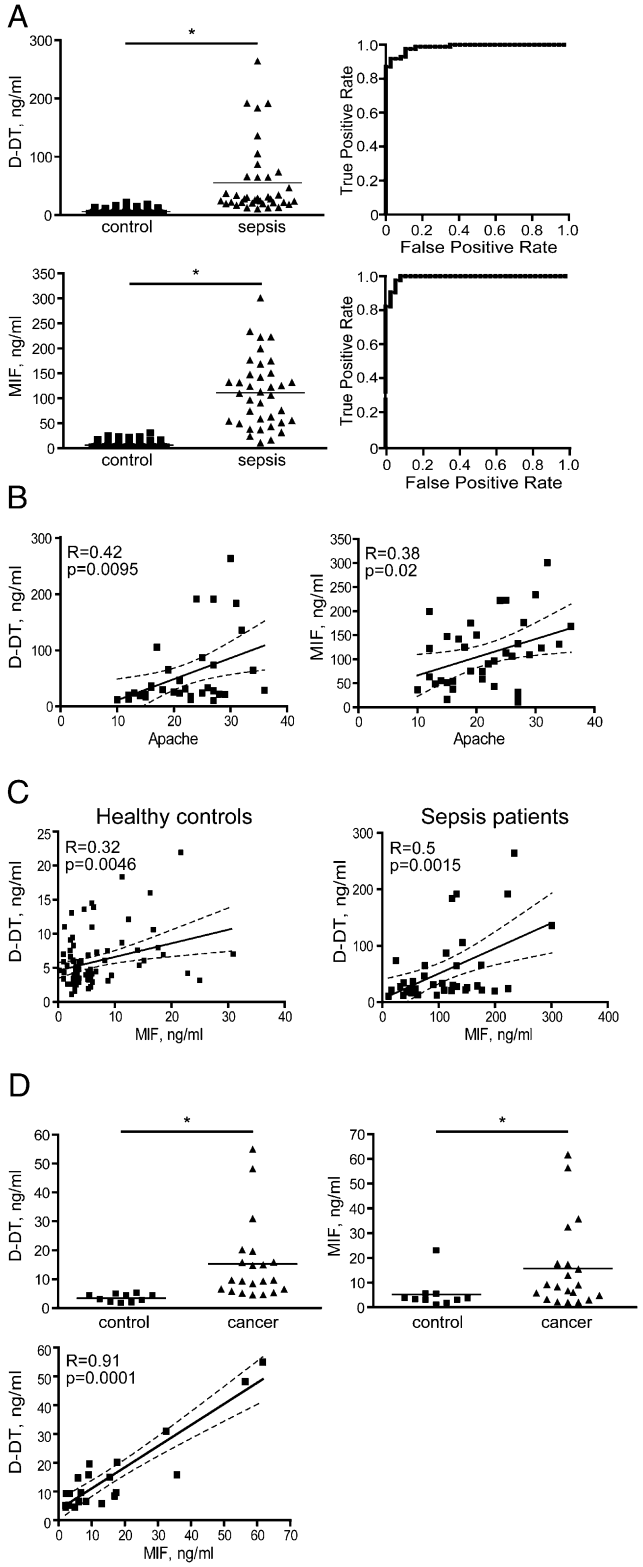

Fig. 6. Human serum concentrations of D-DT correlate with MIF, sepsis severity, and the presence of ovarian cancer. (*A*) D-DT and MIF are elevated in the serum of patients with sepsis. Median concentrations of D-DT and MIF in healthy controls were 6.9 ng/mL and 6.3 ng/mL, respectively. In patients with sepsis, the median concentrations were 56 ng/mL for D-DT and 111 ng/mL for MIF (*$P < 0.0001$ by nonparametric *t* test). ROC analysis revealed an area under the curve of 0.99 for both proteins. (*B*) Positive correlation between the APACHE II (sepsis severity) scores and the levels of either D-DT or MIF. (*C*) D-DT and MIF show a significant correlation both in the serum of healthy individuals and in the serum of patients with severe sepsis. (*D*) D-DT and MIF are elevated in the serum of patients with ovarian cancer and show a positive correlation. Statistical significance between sera from healthy and diseased individuals was determined by nonparametric *t* test, *$P < 0.001$, and the significance of correlation was by Pearson calculation.

expression but not D-DT, although this conclusion will require closer investigation of potential *DDT* polymorphisms.

At the protein level, D-DT lacks MIF's *pseudo(E)LR* domain, which is necessary for activation of CXCR2. Bernhagen and colleagues have reported that MIF initiates coordinated receptor interactions between CD74 and CXCR2/CXCR4 (7, 20). D-DT's high binding affinity to CD74 also may facilitate direct chemotactic effects, perhaps in concert with MIF or IL-8, which is expressed upon CD74 signaling (18, 49). D-DT binds the intracellular protein, JAB1, indicating that it might affect the regulation of cell cycle control and signalosome function, similarly to MIF (26). Like MIF, D-DT lacks an N-terminal signal sequence and it may be produced in sufficient intracytoplasmic concentrations to influence these regulatory pathways.

In conclusion, these data identify D-DT to be a cytokine and a close functional homolog of MIF. We propose that D-DT be designated "MIF-2", which would be in accordance with the nomenclature for nematode MIF-like genes (50) and a prior suggestion that the *DDT* gene product be renamed to more appropriately reflect its function (51). Better definition of D-DT (MIF-2) versus MIF (MIF-1) functions may be uncovered by the development of a *Ddt*-KO mouse, which is underway. Finally, we note that the current emphasis on the development MIF-directed therapies, which have shown strong efficacy in different preclinical models of disease, may be enhanced by the simultaneous targeting of D-DT.

Materials and Methods

Cloning and Purification of D-DT Protein. The cDNAs for the human and mouse D-DT proteins were prepared by amplification of mRNA from human or murine monocytes and subcloned into the pET22b expression vector. For native protein expression, a stop codon was engineered. Bacterial extract was purified using a Q Sepharose column (Amersham) and subsequent HPLC using a C18 column (Amersham). D-DT was refolded using the protocol established for MIF renaturation (52). Protein purity was verified by Coomassie and fidelity confirmed by mass spectroscopy. The resulting proteins contained <1 pg LPS/μg protein as quantified by the PyroGene Recombinant Factor C assay (Cambrex). MIF proteins were produced as described earlier (52). *D*-dopachrome tautomerase activity was assessed using the substrate HPP, measuring the change in absorbance at 306 nm for 180 s (53).

Anti-DDT Antibody and ELISA. Polyclonal antibodies against recombinant mouse or human D-DT were produced in rabbits. IgG antibody fractions were isolated by Protein A agarose affinity chromatography (Pierce) and sterile filtered. Microtiter plates (Nunc) were coated with 15 μg/mL of polyclonal anti-DDT, washed, and blocked in 1% BSA and 1% sucrose. Samples were added and incubated for 2 h, followed by biotinylated anti-DDT antibody and a streptavidin-HRP conjugate. The D-DT concentrations were calculated by extrapolation from a sigmoidal quadratic standard curve using native D-DT protein (dynamic range, 0–625 pg/mL). For mouse or human D-DT, the detection limit was 15 pg/mL.

MIF Receptor Binding Studies. Binding of D-DT to the MIF receptor, CD74, was studied by competition binding assay as described previously (6). Real-time binding interaction of MIF or D-DT with $CD74^{73–232}$ was measured by surface plasmon resonance using a BIAcore 2000 optical biosensor (54). The MIF receptor sCD74 was immobilized to the chip, and binding of the ligands D-DT and MIF was measured in five serial dilutions, three times for each dilution sample. Sensorgram response data were analyzed in the BIA evaluation kinetics package and the equilibrium binding constants calculated in the same experiment.

D-DT Protein Expression in Murine Tissues. Tissues were isolated from C57BL/6 mice, and proteins were analyzed by Western blot (28). For immunohistochemistry, tissue sections from C57BL/6 mice (10) were deparaffinized and antigen retrieval was performed using the Target Retrieval Solution (Dako). The specificity of anti-DDT antibody staining was established by preabsorbing an aliquot of the antibody with a 1,000-fold molar excess of either D-DT or MIF. Slides were incubated with rabbit anti-DDT or IgG control antibody (1:50) overnight and visualized with the Liquid DAB + Substrate Chromogen System (Dako). To allow a semiquantitative comparison of different tissues, all slides were developed for 10 min.

siRNA-Mediated Knockdown of D-DT and MIF. Immortalized murine macrophages (55) were transfected with 50 nM siRNA using HiPerFect (Qiagen). Sequences used were 5′-TCAACTATTACGACATGAA-3′ for MIF and 5′-GCATGACCCTGTTGATGAA-3′ for D-DT.

Signal Transduction Studies. Mouse peritoneal macrophages (1×10^6/well) were rendered quiescent by incubation in 0.1% FBS before stimulation with D-DT or MIF for 2 h (29). Cells were lysed in RIPA buffer and lysates were run on a 4–12% Bis-Tris NuPage gel (Invitrogen). Immunoblotting was conducted with Abs directed against total ERK1/2, and phospho-ERK-1/2 (Cell Signaling).

Migration and Glucocorticoid Overriding Assays. Migration assays were performed as described previously (30). Briefly, human monocytes were incubated for 20 min with MIF or D-DT. Media with or without 25 ng/mL MCP-1 was added in the lower compartment of migration chambers and monocytes was added to the transwell (0.5-μm pore size) for 90 min. Cells from the lower migration chamber were lysed, and DNA was fluorescently labeled and enumerated at 480/520 nm. Following the original glucocorticoid overriding methodology of Calandra et al. (31), macrophages were preincubated for 1 h with 100 nM dexamethasone (Sigma) and MIF or D-DT before adding 100 ng/mL LPS (Sigma). TNF levels in supernatants were measured by ELISA (eBioscience).

MIF/JAB1 Coimmunoprecipitation. *Mif* $^{-/-}$ MEFs were lysed in ice-cold buffer and incubated with MIF or D-DT, respectively (26). Two micrograms of anti-JAB1 (2A10.8; Gene Tex/Biozol) or IgG1 control was added and the protein complexes were pulled down with magnetic protein G beads (Invitrogen). Blotted proteins were visualized using an anti-MIF or anti-DDT antibodies and then reprobed with anti-JAB1 antibody.

Endotoxemia Model. Endotoxemia was induced in female BALB/c mice (8 wk old) by i.p. administration of *E. coli* LPS 0111:B4 (Sigma) at a dose of 12.5 mg/kg for serum cytokine measurement and 20 mg/kg for intervention experiments (LD_{80}). For D-DT neutralization studies, mice were injected i.p. with 200 μL of rabbit anti-D-DT antiserum or nonimmune serum 2 h before administration of LPS. Mice were monitored every 4 h within the first 72 h and then twice daily until death or until 14 d. Cytokine levels were obtained by bleeding mice 4, 24, and 36 h after LPS challenge, and serum cytokines were analyzed by Luminex (Bio-Rad).

Patient Samples. Serum concentrations of D-DT and MIF were measured in 85 healthy individuals and in 37 septic patients hospitalized in the medical intensive care unit (56). The median APACHE II score at the time of intensive care unit admission was 22 points (range: 10–36 points). The mortality rate was 27%. The etiologic agents of sepsis were Gram-negative bacteria (43%) and Gram-positive bacteria (49%). Two patients had an infection with Gram-positive and Gram-negative bacteria and one with fungi. Sera from women with biopsy-proven ovarian cancer (n = 21) were from Yale–New Haven Hospital.

ACKNOWLEDGMENTS. We thank Robert Mitchell, PhD, Gunter Fingerle-Rowson, MD, PhD, and Stefan Endres, MD for thoughtful discussions. Immortalized macrophages (C57BL/6 background) were a kind gift from E. Latz. We thank Adriana Blakaj for assistance with the initial cloning of mouse D-DT. Studies were supported by the National Institutes of Health and by Deutsche Forschungsgemeinschaft Grant BE 1977/4-2 (to J.B.).

1. Bloom BR, Bennett B (1966) Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. *Science* 153:80–82.
2. David JR (1966) Delayed hypersensitivity in vitro: Its mediation by cell-free substances formed by lymphoid cell-antigen interaction. *Proc Natl Acad Sci USA* 56:72–77.
3. Weiser WY, et al. (1989) Molecular cloning of a cDNA encoding a human macrophage migration inhibitory factor. *Proc Natl Acad Sci USA* 86:7522–7526.
4. Calandra T, Roger T (2003) Macrophage migration inhibitory factor: A regulator of innate immunity. *Nat Rev Immunol* 3:791–800.
5. Shi X, et al. (2006) CD44 is the signaling component of the macrophage migration inhibitory factor-CD74 receptor complex. *Immunity* 25:595–606.
6. Leng L, et al. (2003) MIF signal transduction initiated by binding to CD74. *J Exp Med* 197:1467–1476.
7. Bernhagen J, et al. (2007) MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. *Nat Med* 13:587–596.
8. Miller EJ, et al. (2008) Macrophage migration inhibitory factor stimulates AMP-activated protein kinase in the ischaemic heart. *Nature* 451:578–582.
9. Qi D, et al. (2009) Cardiac macrophage migration inhibitory factor inhibits JNK pathway activation and injury during ischemia/reperfusion. *J Clin Invest* 119: 3807–3816.
10. Mizue Y, et al. (2005) Role for macrophage migration inhibitory factor in asthma. *Proc Natl Acad Sci USA* 102:14410–14415.
11. Meyer-Siegler KL, Iczkowski KA, Leng L, Bucala R, Vera PL (2006) Inhibition of macrophage migration inhibitory factor or its receptor (CD74) attenuates growth and invasion of DU-145 prostate cancer cells. *J Immunol* 177:8730–8739.
12. Topilski I, Harmelin A, Flavell RA, Levo Y, Shachar I (2002) Preferential Th1 immune response in invariant chain-deficient mice. *J Immunol* 168:1610–1617.
13. Gore Y, et al. (2008) Macrophage migration inhibitory factor induces B cell survival by activation of a CD74-CD44 receptor complex. *J Biol Chem* 283:2784–2792.
14. Chagnon F, Metz CN, Bucala R, Lesur O (2005) Endotoxin-induced myocardial dysfunction: Effects of macrophage migration inhibitory factor neutralization. *Circ Res* 96:1095–1102.
15. Fingerle-Rowson G, et al. (2009) A tautomerase-null macrophage migration-inhibitory factor (MIF) gene knock-in mouse model reveals that protein interactions and not enzymatic activity mediate MIF-dependent growth regulation. *Mol Cell Biol* 29:1922–1932.
16. Sugimoto H, et al. (1999) Crystal structure of human D-dopachrome tautomerase, a homologue of macrophage migration inhibitory factor, at 1.54 A resolution. *Biochemistry* 38:3268–3279.
17. Xin D, et al. (2010) The MIF homologue D-dopachrome tautomerase promotes COX-2 expression through β-catenin-dependent and -independent mechanisms. *Mol Cancer Res* 8:1601–1609.
18. Coleman AM, et al. (2008) Cooperative regulation of non-small cell lung carcinoma angiogenic potential by macrophage migration inhibitory factor and its homolog, D-dopachrome tautomerase. *J Immunol* 181:2330–2337.
19. Bendrat K, et al. (1997) Biochemical and mutational investigations of the enzymatic activity of macrophage migration inhibitory factor. *Biochemistry* 36:15356–15362.
20. Weber C, et al. (2008) Structural determinants of MIF functions in CXCR2-mediated inflammatory and atherogenic leukocyte recruitment. *Proc Natl Acad Sci USA* 105: 16278–16283.
21. Merk M, et al. (2009) The Golgi-associated protein p115 mediates the secretion of macrophage migration inhibitory factor. *J Immunol* 182:6896–6906.
22. Sun HW, Bernhagen J, Bucala R, Lolis E (1996) Crystal structure at 2.6-A resolution of human macrophage migration inhibitory factor. *Proc Natl Acad Sci USA* 93: 5191–5196.
23. El-Turk F, et al. (2008) The conformational flexibility of the carboxy terminal residues 105-114 is a key modulator of the catalytic activity and stability of macrophage migration inhibitory factor. *Biochemistry* 47:10740–10756.
24. Rosengren E, et al. (1996) The immunoregulatory mediator macrophage migration inhibitory factor (MIF) catalyzes a tautomerization reaction. *Mol Med* 2:143–149.
25. Odh G, Hindemith A, Rosengren AM, Rosengren E, Rorsman H (1993) Isolation of a new tautomerase monitored by the conversion of D-dopachrome to 5,6-dihydroxyindole. *Biochem Biophys Res Commun* 197:619–624.
26. Kleemann R, et al. (2000) Intracellular action of the cytokine MIF to modulate AP-1 activity and the cell cycle through Jab1. *Nature* 408:211–216.
27. Esumi N, et al. (1998) Conserved gene structure and genomic linkage for D-dopachrome tautomerase (DDT) and MIF. *Mamm Genome* 9:753–757.
28. Bacher M, et al. (1997) Migration inhibitory factor expression in experimentally induced endotoxemia. *Am J Pathol* 150:235–246.
29. Mitchell RA, Metz CN, Peng T, Bucala R (1999) Sustained mitogen-activated protein kinase (MAPK) and cytoplasmic phospholipase A2 activation by macrophage migration inhibitory factor (MIF). Regulatory role in cell proliferation and glucocorticoid action. *J Biol Chem* 274:18100–18106.
30. Hermanowski-Vosatka A, et al. (1999) Enzymatically inactive macrophage migration inhibitory factor inhibits monocyte chemotaxis and random migration. *Biochemistry* 38:12841–12849.
31. Calandra T, et al. (1995) MIF as a glucocorticoid-induced modulator of cytokine production. *Nature* 377:68–71.
32. Flaster H, Bernhagen J, Calandra T, Bucala R (2007) The macrophage migration inhibitory factor-glucocorticoid dyad: Regulation of inflammation and immunity. *Mol Endocrinol* 21:1267–1280.
33. Roger T, Chanson AL, Knaup-Reymond M, Calandra T (2005) Macrophage migration inhibitory factor promotes innate immune responses by suppressing glucocorticoid-induced expression of mitogen-activated protein kinase phosphatase-1. *Eur J Immunol* 35:3405–3413.
34. Calandra T, Bernhagen J, Mitchell RA, Bucala R (1994) The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. *J Exp Med* 179:1895–1902.

35. Bernhagen J, et al. (1993) MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. *Nature* 365:756–759.
36. Bozza M, et al. (1999) Targeted disruption of migration inhibitory factor gene reveals its critical role in sepsis. *J Exp Med* 189:341–346.
37. Mitchell RA, et al. (2002) Macrophage migration inhibitory factor (MIF) sustains macrophage proinflammatory function by inhibiting p53: Regulatory role in the innate immune response. *Proc Natl Acad Sci USA* 99:345–350.
38. Calandra T, et al. (2000) Protection from septic shock by neutralization of macrophage migration inhibitory factor. *Nat Med* 6:164–170.
39. Visintin I, et al. (2008) Diagnostic markers for early detection of ovarian cancer. *Clin Cancer Res* 14:1065–1072.
40. Bozza FA, et al. (2004) Macrophage migration inhibitory factor levels correlate with fatal outcome in sepsis. *Shock* 22:309–313.
41. Lehmann LE, et al. (2001) Plasma levels of macrophage migration inhibitory factor are elevated in patients with severe sepsis. *Intensive Care Med* 27:1412–1415.
42. Emonts M, et al. (2007) Association between high levels of blood macrophage migration inhibitory factor, inappropriate adrenal response, and early death in patients with severe sepsis. *Clin Infect Dis* 44:1321–1328.
43. Calandra T, Spiegel LA, Metz CN, Bucala R (1998) Macrophage migration inhibitory factor is a critical mediator of the activation of immune cells by exotoxins of Gram-positive bacteria. *Proc Natl Acad Sci USA* 95:11383–11388.
44. Arjona A, et al. (2007) Abrogation of macrophage migration inhibitory factor decreases West Nile virus lethality by limiting viral neuroinvasion. *J Clin Invest* 117: 3059–3066.
45. McDevitt MA, et al. (2006) A critical role for the host mediator macrophage migration inhibitory factor in the pathogenesis of malarial anemia. *J Exp Med* 203:1185–1196.
46. Lolis E, Bucala R (2003) Therapeutic approaches to innate immunity: Severe sepsis and septic shock. *Nat Rev Drug Discov* 2:635–645.
47. Cournia Z, et al. (2009) Discovery of human macrophage migration inhibitory factor (MIF)-CD74 antagonists via virtual screening. *J Med Chem* 52:416–424.
48. Grieb G, Merk M, Bernhagen J, Bucala R (2010) Macrophage migration inhibitory factor (MIF): A promising biomarker. *Drug News Perspect* 23:257–264.
49. Binsky I, et al. (2007) IL-8 secreted in a macrophage migration-inhibitory factor- and CD74-dependent manner regulates B cell chronic lymphocytic leukemia survival. *Proc Natl Acad Sci USA* 104:13408–13413.
50. Vermeire JJ, Cho Y, Lolis E, Bucala R, Cappello M (2008) Orthologs of macrophage migration inhibitory factor from parasitic nematodes. *Trends Parasitol* 24:355–363.
51. Coggan M, Whitbread L, Whittington A, Board P (1998) Structure and organization of the human theta-class glutathione S-transferase and D-dopachrome tautomerase gene complex. *Biochem J* 334:617–623.
52. Bernhagen J, et al. (1994) Purification, bioactivity, and secondary structure analysis of mouse and human macrophage migration inhibitory factor (MIF). *Biochemistry* 33: 14144–14155.
53. Stamps SL, Taylor AB, Wang SC, Hackert ML, Whitman CP (2000) Mechanism of the phenylpyruvate tautomerase activity of macrophage migration inhibitory factor: Properties of the P1G, P1A, Y95F, and N97A mutants. *Biochemistry* 39:9671–9678.
54. Kamir D, et al. (2008) A Leishmania ortholog of macrophage migration inhibitory factor modulates host macrophage responses. *J Immunol* 180:8250–8261.
55. Duewell P, et al. (2010) NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature* 464:1357–1361.
56. Lesur O, et al. (2010) Proven infection-related sepsis induces a differential stress response early after ICU admission. *Crit Care* 14:R131.