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The Macrophage Migration Inhibitory Factor Homolog of *Entamoeba histolytica* Binds to and Immunomodulates Host Macrophages

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The host inflammatory response contributes to the tissue damage that occurs during amebic colitis, with tumor necrosis factor alpha (TNF-α) being a key mediator of the gut inflammation observed. Mammalian macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that plays an important role in the exacerbation of a wide range of inflammatory diseases, including colitis. We identified a MIF gene homolog in the *Entamoeba histolytica* genome, raising the question of whether *E. histolytica* MIF (*Eh*MIF) has proinflammatory activity similar to that of mammalian MIF. In this report, we describe the first functional characterization of *Eh*MIF. Antibodies were prepared against recombinantly expressed *Eh*MIF and used to demonstrate that *Eh*MIF is expressed as a 12-kDa protein localized to the cytoplasm of trophozoites. In a manner similar to that of mammalian MIF, *Eh*MIF interacted with the MIF receptor CD74 and bound to macrophages. *Eh*MIF induced interleukin-6 (IL-6) production. In addition, *Eh*MIF enhanced TNF-α secretion by amplifying TNF-α production by lipopolysaccharide (LPS)-stimulated macrophages and by inhibiting the glucocorticoid-mediated suppression of TNF-α secretion. *Eh*MIF was expressed during human infection, as evidenced by the presence of anti-*Eh*MIF antibodies in the sera of children living in an area where *E. histolytica* infection is endemic. Anti-*Eh*MIF antibodies did not cross-react with human MIF. The ability of *Eh*MIF to modulate host macrophage function may promote an exaggerated proinflammatory immune response and contribute to the tissue damage seen in amebic colitis.

Entamoeba histolytica is the protozoan parasite that causes amebic colitis. Diarrheal disease is second only to pneumonia as a leading cause of death globally in children under the age of 5 years, and intestinal amebiasis is one of the leading causes of severe diarrhea in the developing world (1, 2). For example, in a cohort of Bangladeshi infants, *E. histolytica* was present in 20% of all diarrheal episodes (3). Infection with *E. histolytica* trophozoites results in marked mucosal inflammation. Along with *E. histolytica* cytolytic factors, the host inflammatory response contributes to the tissue destruction seen in amebic colitis (4). Tumor necrosis factor alpha (TNF-α) is a proinflammatory cytokine that plays a central role in intestinal inflammation, including the gut inflammation seen in amebic colitis. More TNF-α production was shown to correlate with *E. histolytica* diarrhea in children, and blocking of TNF-α with monoclonal antibodies reduced inflammation and intestinal damage from amebic infections in mice (5, 6). TNF-α was also recently shown to mediate the tissue destruction seen in amebic liver abscesses (7).

Mammalian macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine with mitogenic and proinflammatory functions (8). Proinflammatory functions include the following: (i) MIF induces the secretion of inflammatory mediators, such as interleukin-6 (IL-6); (ii) MIF enhances TNF-α production by lipopolysaccharide (LPS)-stimulated immune cells; and (iii) MIF can counterregulate the anti-inflammatory activities of glucocorticoids (9–17). Many of the inflammatory effects of MIF are initiated via direct binding to its cell surface receptor, CD74 (18). Several studies have implicated a key role for MIF in colitis. In one study of patients with inflammatory bowel disease (IBD), a condition characterized by inflammation of the colon, patients with IBD were found to have significantly higher concentrations of MIF in plasma (17). Furthermore, there is genetic evidence to support the role of MIF in colitis. The MIF −173 G/C single nucleotide polymorphism is associated with elevated MIF expression and, in turn, increased susceptibility to IBD (19, 20). Additionally, MIF knockout mice are protected from dextran-induced colitis, while antibodies targeting MIF prevent experimental colitis (21, 22).

MIF homologs from several pathogenic protozoans have been characterized (23–28). We identified a MIF homolog in the *E. histolytica* genome (EHI_092370). The *E. histolytica* MIF (*Eh*MIF) gene has a coding sequence consisting of 342 bp, with no intron and a GC content of 31%. The gene encodes a protein with a predicted molecular mass of 12 kDa. Microarray studies showed that there is at least an 8-fold increase in *Eh*MIF expression from the dormant cyst stage to the invasive and proliferative trophozoite stage (29, 30). Similar to other protozoan MIF homologs, *Eh*MIF has a relatively low identity (28%) to human MIF but appears to be structurally conserved (Fig. 1). Protozoan MIF homologs have demonstrated biological activity similar to that of mammalian MIF. For example, *Plasmodium* MIF was shown to enhance TNF-α secretion by immune cells after stimulation with LPS (11, 16). The presence of a MIF homolog in *E. histolytica* implies a potential mechanism that may contribute to the proinflammatory host response that occurs during infection.

The role of the *Eh*MIF homolog in *E. histolytica*-induced gut

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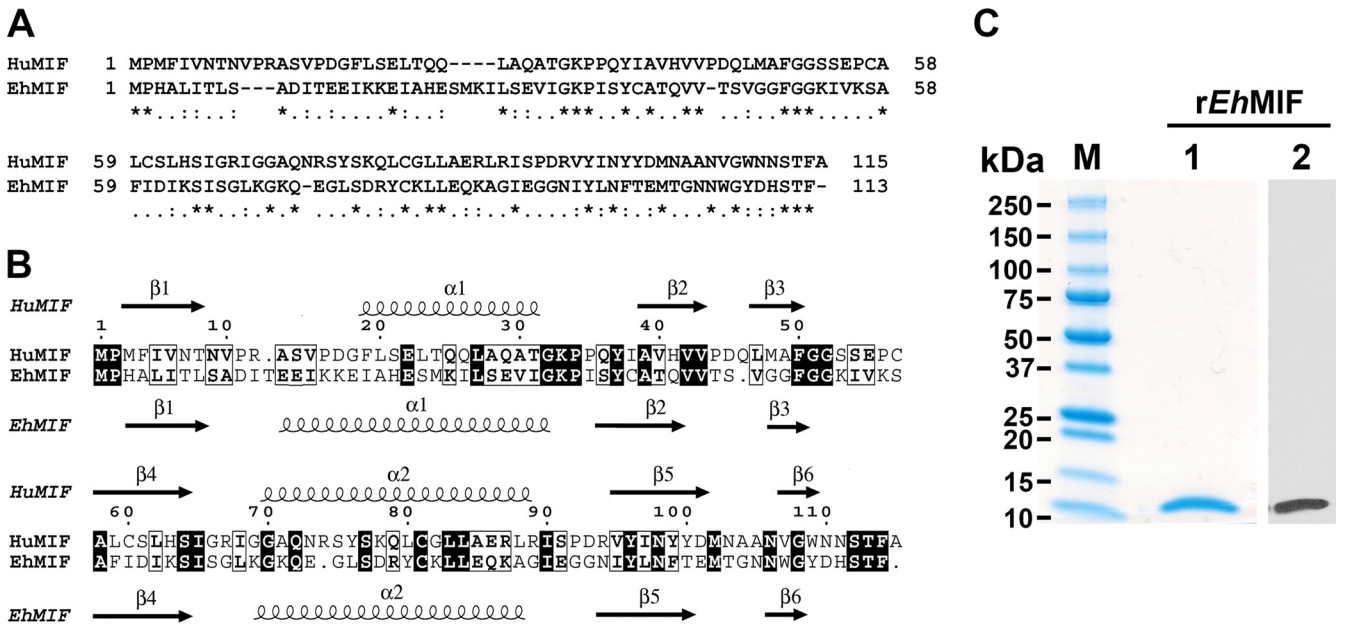

FIG 1 Characterization of *Eh*MIF. (A) Pairwise amino acid sequence alignment of human MIF (*Hu*MIF) and *Entamoeba histolytica* MIF (*Eh*MIF). Sequence alignment was performed using Needle (EMBOSS). Symbols: asterisks, identical residues; colons, conserved residues; periods, semiconserved residues. (B) Secondary structure homology between *Hu*MIF and *Eh*MIF. The secondary structure elements of *Hu*MIF and *Eh*MIF are shown above and below the alignment, respectively. The figure was generated with ClustalW2 and ESPript. (C) Purified recombinant *Eh*MIF (2 μg) in an SDS-PAGE gel stained with Coomassie Blue (lane 1), with an estimated molecular mass of 12 kDa, and immunoblot analysis of purified recombinant *Eh*MIF (25 ng), using anti-penta-His–HRP conjugate (lane 2).

inflammation has not yet been characterized. We generated recombinant *Eh*MIF and examined its ability to modulate host macrophage function. We also investigated the cellular localization of *Eh*MIF and whether anti-*Eh*MIF antibodies were present in the sera of children living in an area where *E. histolytica* infection is endemic.

MATERIALS AND METHODS

Expression and purification of recombinant *Eh*MIF. The *Eh*MIF gene was codon optimized for *Escherichia coli* expression and cloned into the pJexpress414 vector (DNA2.0). The expression plasmid was transformed into *E. coli* BL21(DE3) competent cells. Expression of *Eh*MIF with a C-terminal polyhistidine tag was induced at an optical density at 600 nm (OD_{600}) of 0.6 by use of isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C. The pelleted cells were sonicated, and the bacterial extract was purified by Ni-nitrilotriacetic acid (Ni-NTA) chromatography (Qiagen) and dialyzed into phosphate-buffered saline (PBS). Protein concentrations were determined by Bradford assay (Thermo Scientific). Sample purity was analyzed by SDS-PAGE. Protein expression was also confirmed by immunoblotting, using an anti-penta-His–horseradish peroxidase (HRP) conjugate (Qiagen). Triton X-114 and polymyxin B-agarose (Sigma) were used during wash and purification procedures for removal of endotoxin. The resulting proteins contained <1 pg LPS/μg protein as quantified by the *Limulus* amebocyte lysate (LAL) assay (Thermo Scientific). His-tagged mouse MIF (*M*MIF) and glutathione *S*-transferase (GST) were purified, and endotoxin removed, under the same conditions as those for recombinant *Eh*MIF.

GST pulldown assay. GST pulldown assays were carried out as previously described (31). Briefly, the mouse CD74 ectodomain cDNA was cloned into the pGEX-6P-1 GST expression vector (GE Healthcare). The empty pGEX-6P-1 vector (GST-only control) and the GST-CD74 expression plasmid were transformed into BL21(DE3) competent cells. Protein expression was induced at an OD_{600} of 0.6 by the addition of IPTG. Approximately 0.25 μg of GST or GST-CD74 in bacterial lysate was added to glutathione Sepharose beads (GE Healthcare). After washing with PBS, recombinant *Eh*MIF was added to a final concentration of 1 μM in 500 μl binding buffer (50 mM $K_xH_yPO_4$ [pH 7.2], 50 mM NaCl, 5% glycerol, 0.01% Tween). Mixed fractions were incubated at 4°C for 1 h with rotation. The beads were washed three times with binding buffer and then subjected to SDS-PAGE. *Eh*MIF was detected by immunoblotting with anti-penta-His–HRP conjugate (Qiagen).

Cell binding analyses by flow cytometry. The cell-binding properties of *Eh*MIF were determined based on a previously described method (32). *Eh*MIF was biotinylated using an EZ-Link sulfo-NHS-LC biotinylation kit (Thermo Scientific) per the manufacturer's instructions. Biotinylated *Eh*MIF (1 μg) was incubated with 2×10^5 murine RAW-Blue macrophages (InvivoGen) for 45 min at 4°C to prevent internalization. In selected experiments, macrophages were incubated with mouse anti-CD74 antibody (Santa Cruz Biotechnology) for 30 min before exposure to *Eh*MIF, or *Eh*MIF was incubated with *M*MIF at a final dilution of 1:20. After washing with PBS, samples were incubated with streptavidin-Alexa Fluor 647 (Invitrogen) for 30 min. Samples incubated with streptavidin-Alexa Fluor 647 only were used as negative controls. The cells were washed and analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton, Dickinson). Flow cytometry data were analyzed with FlowJo software.

Effects of *Eh*MIF on cytokine production. RAW-Blue macrophages (10^6 cells/well in 6-well plates) were stimulated with increasing concentrations of *Eh*MIF (0 to 100 ng/ml) for 8 h. Macrophages were also treated with 25 μg/ml anti-CD74 antibody or control IgG. IL-6 levels in supernatants were measured by enzyme-linked immunosorbent assay (ELISA) (eBioscience) according to the manufacturer's instructions. The effect of *Eh*MIF on TNF-α production was evaluated using a modification of a previously described method (9). RAW-Blue macrophages were incubated for 8 h with *Eh*MIF or *Eh*MIF and LPS (from *E. coli* O111:B4; Sigma) at 100 ng/ml. TNF-α levels in supernatants were measured by ELISA

(eBioscience) according to the manufacturer's instructions. Cytokines secreted by macrophages into culture supernatants were also measured after stimulation with *Eh*MIF preincubated with purified rabbit polyclonal anti-*Eh*MIF IgG or control IgG.

Glucocorticoid override assay. RAW-Blue macrophages were preincubated for 1 h with 100 nM dexamethasone (Sigma) or with dexamethasone plus *Eh*MIF (100 ng/ml) before the addition of LPS (100 ng/ml). Supernatants from macrophage cultures were collected after 4 h. TNF-α levels in supernatants were measured by ELISA (eBioscience).

Immunoblotting and immunofluorescence assay (IFA). *E. histolytica* HM1:IMSS trophozoites in TYI-S33 medium were harvested by centrifugation. The pellet was washed with PBS and sonicated. The lysate was centrifuged at 18,000 × *g* for 30 min at 4°C, and the supernatant was then subjected to SDS-PAGE. Protein extracts were transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were incubated overnight at 4°C with anti-*Eh*MIF rabbit serum or preimmune rabbit serum followed by peroxidase-conjugated anti-rabbit IgG (Sigma). Peroxidase activity was detected by enhanced chemiluminescence assay (Thermo Scientific).

The localization of *Eh*MIF within trophozoites was determined using fluorescence microscopy. Trophozoites were fixed in 4% formalin for 30 min and then spotted on polylysine-coated slides. The cells were permeabilized with PBS containing 0.2% Triton X-100 for 5 min and blocked with 3% bovine serum albumin (BSA) plus 0.05% Tween 20 in PBS for 1 h at 37°C. IFA was performed using sera containing rabbit polyclonal anti-*Eh*MIF antibodies. Fluorescence staining was achieved with Alexa Fluor 488-conjugated secondary antibodies specific to rabbit IgG (Invitrogen). Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole). The slides were then mounted with Vectashield mounting medium (Vector Laboratories). Images were captured using an Olympus BX51 fluorescence microscope equipped with an Olympus DP70 digital camera.

ELISA and immunoblot analyses of children's sera. A total serum IgG ELISA was performed on sera from children. Sera were taken from 160 preschool children (2 to 5 years old) in the area of Mirpur, within Dhaka, Bangladesh, an area where *Entamoeba histolytica* infection is endemic. The collection of blood samples for research purposes was reviewed and approved by the Institutional Review Board at the University of Virginia and the Ethical Review Committee of the International Center for Diarrheal Disease Research, Bangladesh, Dhaka, Bangladesh. A codon-optimized MIF open reading frame (DNA2.0) was PCR amplified and cloned into the expression vector pGEX-6p-1 (GE Healthcare), using BamHI and SalI sites. Competent *E. coli* BL21(DE3) cells were transformed with vector alone or with pGEX-6p-1-MIF, and the cells were induced with 1 mM IPTG for 3 h at 37°C. GST alone or GST-MIF was purified using glutathione Sepharose beads (GE Healthcare) per the manufacturer's instructions. Ninety-six-well ELISA plates (Maxisorp; Nunc) were coated overnight with 0.5 μg GST alone or GST-MIF per well. Children's sera, diluted 1:250, followed by peroxidase-conjugated anti-human IgG (1:10,000), were added to the ELISA plates. The optical density (OD) was read at 450 nm. A sample was considered to be positive for anti-*Eh*MIF antibody if the OD reading was >2 standard deviations (SD) above the mean for the GST-only control (33, 34).

Immunoblot analysis was also performed to confirm the presence of anti-*Eh*MIF antibody in children's sera and to evaluate whether anti-*Eh*MIF antibody cross-reacted with recombinant human MIF (*Hu*MIF). Recombinant *Eh*MIF and *Hu*MIF, at 100 ng and 50 ng, were separated by SDS-PAGE. An immunoblot was performed as described above, using 1:100 dilutions of anti-*Eh*MIF antibody-positive and -negative sera.

Data analysis. All data were analyzed using IBM SPSS 20 and GraphPad Prism 6 software. Results are presented as means ± SD or as individual data points. Comparisons between groups were determined using the independent-sample *t* test or the paired-sample *t* test. *P* values of <0.05 (two-tailed) were considered statistically significant.

RESULTS

***Eh*MIF interacts with the cell surface receptor CD74 and binds to host macrophages.** Mammalian MIF achieves some of its inflammatory effects by binding to the MIF receptor (CD74) through interaction with the CD74 ectodomain (18). This interaction has been shown to be conserved in MIF homologs of *Leishmania* and *Plasmodium* spp. (16, 27, 31). We examined the ability of purified recombinant *Eh*MIF (Fig. 1C) to interact with CD74 by performing GST pulldown assays. We expressed and purified the mouse CD74 ectodomain (CD74ec) fused to GST (Fig. 2A and B). As shown in Fig. 2C, CD74ec fused to GST readily pulled down recombinant *Eh*MIF relative to the GST control, demonstrating that *Eh*MIF interacted with the MIF receptor.

CD74 is expressed on the surfaces of a variety of mammalian cells, including macrophages. The binding of *Eh*MIF to host macrophages was investigated by flow cytometry. Macrophages were incubated with biotinylated *Eh*MIF, and binding of *Eh*MIF to macrophages was observed (Fig. 2D). Macrophages pretreated with anti-CD74 antibodies resulted in a reduction of *Eh*MIF binding. Anti-CD74 antibodies caused only a partial inhibition of *Eh*MIF binding. This was not surprising, as MIF homologs have been shown to bind with other cell surface receptors, such as the chemokine receptors CXCR2 and CXCR4 (35). The receptor-binding similarities between *Eh*MIF and mammalian MIF were further supported when incubation with a 20-fold excess of mouse MIF (*M*MIF) resulted in a pronounced reduction of *Eh*MIF binding to macrophages (Fig. 2D).

***Eh*MIF immunomodulates host macrophages.** The binding of *Eh*MIF to host macrophages suggested that *Eh*MIF might be capable of interfering with or modulating macrophage activity. MIF homologs have been shown to promote inflammation by enhancing proinflammatory cytokine production (9, 11, 16, 17). For cytokine assays, we used endotoxin-free recombinant *Eh*MIF (<1 pg LPS/μg protein). Cytokines (IL-6 and TNF-α) secreted into cell culture supernatants were collected after 8 h of stimulation with *Eh*MIF. *Eh*MIF induced IL-6 production in a dose-dependent manner (Fig. 3A). Treatment aimed at blocking CD74 or *Eh*MIF (treatment with anti-*Eh*MIF or anti-CD74 neutralizing antibodies) inhibited *Eh*MIF-induced IL-6 production (Fig. 3B). Macrophages incubated with endotoxin-free *Eh*MIF only did not show any significant increase in TNF-α secretion (Fig. 3C), in agreement with a previous study done with mammalian MIF (10).

There are two well-characterized proinflammatory effects of MIF. First, MIF enhances TNF-α production by LPS-stimulated immune cells. Second, MIF can override the anti-inflammatory activities of glucocorticoids (9–16). We tested the effect of *Eh*MIF on TNF-α secretion by host macrophages with LPS stimulation. As shown in Fig. 3C, *Eh*MIF was capable of augmenting TNF-α production by LPS-stimulated macrophages. To assess the effect of *Eh*MIF on glucocorticoid-mediated immunosuppression, we pretreated macrophages with dexamethasone, with or without *Eh*MIF, followed by stimulation with LPS. Dexamethasone inhibited TNF-α production by macrophages stimulated with LPS. Treatment of macrophages with *Eh*MIF overcame dexamethasone inhibition of TNF secretion (Fig. 3D).

*Eh*MIF demonstrated immunomodulatory activity comparable to that of *M*MIF. The immunomodulatory effects of *Eh*MIF on macrophages were inhibited by a neutralizing anti-*Eh*MIF antibody. We used recombinant GST, expressed, purified, and cleared

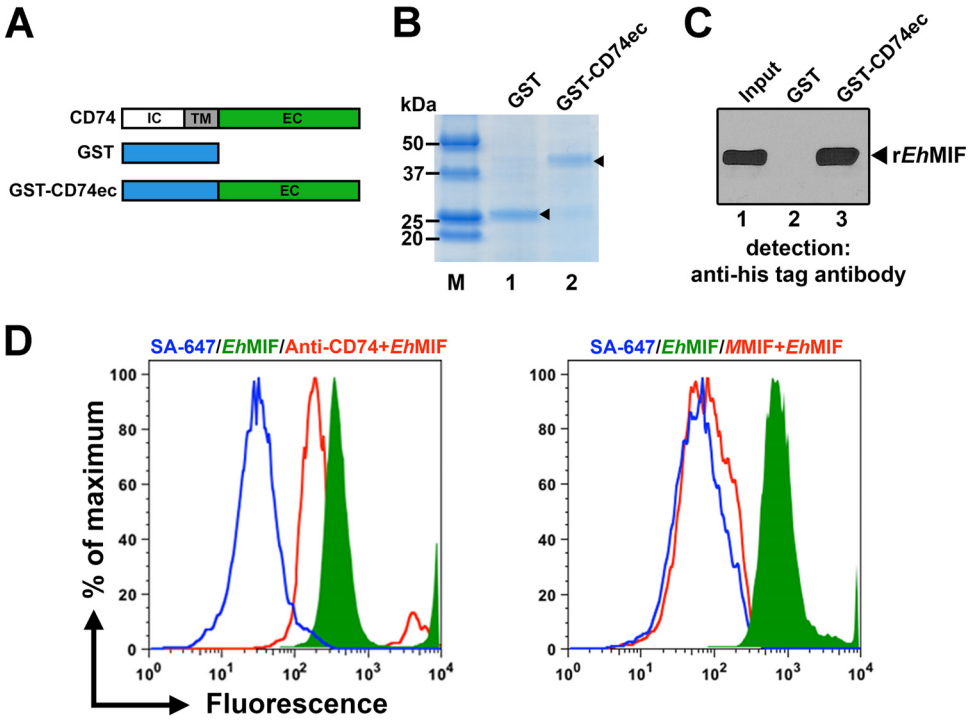

FIG 2 *Eh*MIF interacts with the MIF receptor (CD74) and binds to host macrophages. (A) Schematic representation of the MIF receptor (CD74). IC, TM, and EC, intracellular, transmembrane, and extracellular domains, respectively. Also represented are GST and GST fused to the extracellular domain of CD74 (CD74ec). (B) Purified recombinant proteins used in the GST pulldown assay (arrowheads). Approximately 0.25-μg samples of GST (lane 1) and GST-CD74ec (lane 2) were separated by SDS-PAGE and stained with Coomassie blue. (C) Interaction between *Eh*MIF and CD74ec by GST pulldown assay. GST and GST-CD74ec were mixed with His-tagged *Eh*MIF. One percent of the input (lane 1) and the pull-down material (lanes 2 and 3) were separated by SDS-PAGE, and His-tagged *Eh*MIF was detected by immunoblot analysis using anti-His antibody. (D) Binding of *Eh*MIF to macrophages as determined by flow cytometry analysis. A total of 2×10^5 murine RAW-Blue macrophages were incubated at 4°C for 45 min with biotinylated *Eh*MIF followed by streptavidin-Alexa Fluor 647 or with a streptavidin-Alexa Fluor 647-only control (SA-647). A red line represents the binding of biotinylated *Eh*MIF to macrophages pretreated with anti-CD74 antibody (left) or binding of biotinylated *Eh*MIF to macrophages in the presence of a 20-fold excess of mouse MIF (*M*MIF) (right). Data are representative of two independent experiments.

of endotoxin under the same conditions as those for *Eh*MIF, as a negative control. GST did not have any effect on cytokine secretion. Mouse and rabbit IgG controls had no effect on *Eh*MIF activity (Fig. 3A to D).

Cytoplasmic localization of *Eh*MIF. Host immune cells secrete MIF by a nonclassical pathway involving an ABC transporter. Once secreted, MIF then acts in an autocrine/paracrine fashion. Similar to mammalian MIF, *Eh*MIF lacks a characteristic signal peptide (Fig. 1A), and putative ABC transporter genes can be found in the *E. histolytica* genome (29, 36). Cells constitutively express MIF protein, and high levels accumulate in the cytoplasm (37). We hypothesized that *Eh*MIF was also localized to the parasite cytoplasm. Rabbit sera containing anti-*Eh*MIF antibodies were generated. First, we determined if *Eh*MIF was present in the soluble extract of trophozoites. Trophozoites were sonicated and centrifuged at 18,000 × *g*, and the supernatant (soluble cell extract) was then subjected to immunoblot analysis. Anti-*Eh*MIF serum recognized a protein of 12 kDa, the predicted molecular mass of *Eh*MIF, while no bands were seen with the preimmune serum (Fig. 4A). We next investigated the cellular localization of *Eh*MIF. Trophozoites were fixed and permeabilized on polylysine-coated slides, and cellular localization of *Eh*MIF was examined by immunofluorescence analysis using sera containing rabbit polyclonal anti-*Eh*MIF antibodies. As shown in Fig. 4B, *Eh*MIF was localized to the cytoplasm of trophozoites. Fluorescence was not seen in parasites treated with preimmune serum.

Anti-*Eh*MIF antibodies are present in children's sera. Previous studies have demonstrated the presence of antibodies against parasite MIF homologs, including those of the protozoan *Plasmodium* and the intestinal helminth *Strongyloides*, in the sera of individuals living in areas of endemicity (24, 32). We hypothesized that children infected with *E. histolytica* are capable of generating anti-*Eh*MIF antibodies. To test this hypothesis, we first developed an ELISA to screen for anti-*Eh*MIF antibodies in the sera of children living in an area where *Entamoeba histolytica* infection is endemic. We coated ELISA plates with a GST-*Eh*MIF fusion protein, and GST-only plates were used as a control. Children's sera and anti-human IgG were added to the ELISA plates. The ELISA OD values were significantly higher in the presence of *Eh*MIF ($P < 0.0001$), indicating the presence of anti-*Eh*MIF IgG antibody in children's sera (Fig. 5A). Next, we used immunoblot analysis to confirm the presence of anti-*Eh*MIF antibody in children's sera. As shown in Fig. 5B, children's sera positive for anti-*Eh*MIF antibody reacted with recombinant *Eh*MIF, and no reaction was seen with sera negative for anti-*Eh*MIF antibody. We then evaluated whether anti-*Eh*MIF antibody would cross-react with recombinant human MIF. Children's sera positive for anti-*Eh*MIF antibody did not cross-react with human MIF (Fig. 5B).

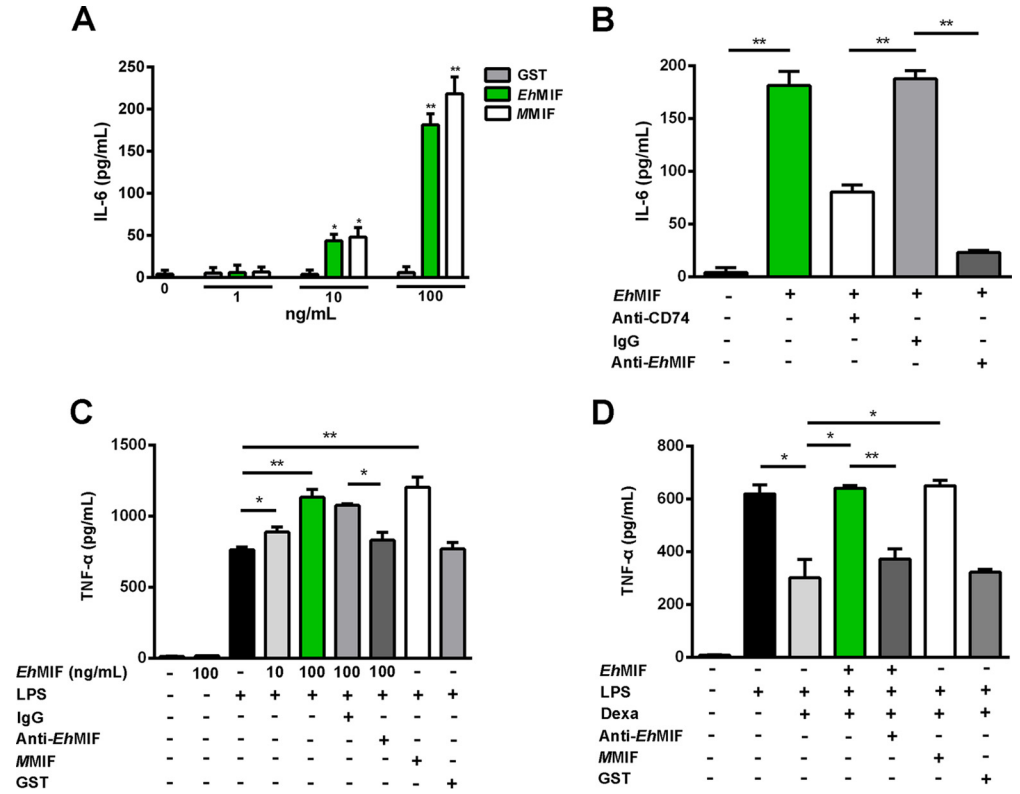

FIG 3 *Eh*MIF immunomodulates macrophages. (A) *Eh*MIF stimulates IL-6 production in a dose-dependent manner. RAW-Blue macrophages were stimulated with GST, *Eh*MIF, or *M*MIF (0 to 100 ng/ml) for 8 h. (B) Inhibition of *Eh*MIF-induced IL-6 production by neutralizing anti-CD74 and anti-*Eh*MIF antibodies relative to that with the IgG control. Macrophages treated with anti-CD74 antibody or the IgG control were incubated with *Eh*MIF (100 ng/ml) for 8 h. The amount of IL-6 secreted by macrophages into culture supernatants was measured after 8 h of stimulation with *Eh*MIF preincubated with anti-*Eh*MIF antibody or the IgG control. (C) *Eh*MIF enhances TNF-α production of LPS-stimulated macrophages. Macrophages were incubated with *Eh*MIF or *Eh*MIF plus LPS (100 ng/ml). TNF-α secreted into culture supernatants was collected after 8 h and measured by ELISA. (D) *Eh*MIF inhibits glucocorticoid-mediated suppression of TNF-α production. Macrophages were preincubated with dexamethasone (Dexa; 100 nM), with or without *Eh*MIF (100 ng/ml), for 1 h and then stimulated with LPS (100 ng/ml). Culture supernatants were collected after 4 h, and TNF was quantified by ELISA. Data shown are means ± SD for triplicates from one experiment and are representative of three independent experiments. *, $P < 0.005$; **, $P < 0.001$.

DISCUSSION

The host immune response and the proinflammatory cytokine TNF-α, in particular, play a major role in the intestinal pathology induced by *E. histolytica* infection (5, 6, 38). Understanding the mechanisms by which *E. histolytica* drives gut inflammation is critical for the development of improved preventive and therapeutic strategies. In this report, we describe the first functional characterization of the *E. histolytica* homolog of the cytokine MIF.

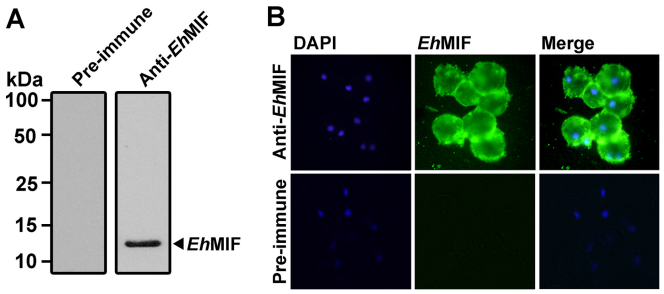

FIG 4 Cytoplasmic localization of *Eh*MIF. (A) Immunoblot analysis of soluble *E. histolytica* trophozoite extract, using serum containing anti-*Eh*MIF antibodies or preimmune serum. Anti-*Eh*MIF antibodies recognized a protein of 12 kDa, the predicted molecular mass of *Eh*MIF. (B) Immunofluorescence detection of *Eh*MIF in the cytoplasm of *E. histolytica* trophozoites. Trophozoites were permeabilized with 0.2% Triton X-100, and IFA was performed using serum containing rabbit anti-*Eh*MIF antibodies or preimmune serum. Fluorescence staining was achieved with Alexa Fluor 488-conjugated secondary antibodies. Nuclei were stained with DAPI.

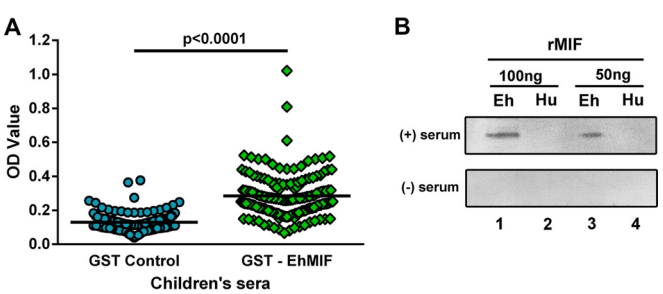

FIG 5 Anti-*Eh*MIF antibodies are present in children's sera. (A) ELISA with sera from children exposed to *E. histolytica*. Children's sera were added to ELISA plates coated with GST-*Eh*MIF or GST only. There was a statistically significant difference between the GST-*Eh*MIF assay (mean = 0.28; SD = 0.13) and the GST control assay (mean = 0.13; SD = 0.05) ($P < 0.0001$; paired-sample *t* test). (B) Serum positive for anti-*Eh*MIF antibody recognized *Eh*MIF and did not cross-react with human MIF. Human (Hu) and *E. histolytica* (Eh) recombinant MIF (rMIF) proteins were analyzed at 100 ng and 50 ng by immunoblotting with anti-*Eh*MIF antibody-positive and -negative sera.

We produced recombinant *Eh*MIF purified to remove endotoxin and showed that in a manner similar to that for mammalian MIF, *Eh*MIF was capable of binding to host macrophages, at least in part through interaction with the MIF receptor, CD74. We went on to show that *Eh*MIF induced IL-6 production, amplified the secretion of TNF-α by LPS-stimulated macrophages, and reversed the glucocorticoid inhibition of TNF-α secretion.

Previously, coculture of human cells with *E. histolytica* trophozoites was shown to increase expression and secretion of proinflammatory cytokines, including IL-6 (39). Our finding that *Eh*MIF induced IL-6 secretion is consistent with previous studies of MIF homologs (16, 17, 25, 40–42). IL-6 is a proinflammatory cytokine, and its main sources in the intestine are macrophages. IL-6 plays a crucial role in the uncontrolled inflammation of the intestinal mucosa that is characteristic of IBD (43, 44). These findings support the concept that *Eh*MIF might contribute to the immunopathology of intestinal amebiasis.

Blocking of CD74 decreased *Eh*MIF-induced IL-6 secretion. Naturally resistant B6 mice become highly susceptible to amebic colitis after disruption of the anti-inflammatory cytokine IL-10 gene. It is interesting that CD74 is upregulated in these IL-10-deficient C57/B6 mice and also in inflammatory bowel disease (45, 46). One could postulate that host CD74 overexpression would enhance *Eh*MIF activity, which would partly explain the increased susceptibility of IL-$10^{-/-}$ mice.

LPS is a major component of the Gram-negative bacterial outer membrane. It is possible that during amebic colitis, LPS may enter the host lamina propria through at least two paths. First, *E. histolytica* infection results in disruption of the mucosal barrier, which might lead to infiltration of bacteria into the lamina propria. Second, trophozoites feed on gut flora; hence, invading parasites could carry bacterial products, including LPS (46, 47). As reported previously, *Eh*MIF in combination with LPS increased TNF-α production by macrophages *in vitro*. The concentration of TNF-α produced plays a pivotal role in gut inflammation, and an excess of TNF-α can result in damage to intestinal tissue (48). Roger et al. demonstrated that MIF enhances the host response to LPS by upregulating Toll-like receptor 4 (TLR4), the receptor for LPS (9).

The gut mucosa contains the largest number of immune cells in the body, and tight regulation of the mucosal immune response is required to maintain intestinal homeostasis. Glucocorticoids are locally synthesized in the intestine and play an important role in downregulating the inflammatory immune response and mediating repair of a damaged mucosa (49–55). Mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1) is induced by glucocorticoids to dephosphorylate and therefore deactivate MAPK activity in response to proinflammatory stimuli (e.g., LPS). MIF inhibits glucocorticoid-induced MKP-1 expression and inhibition of TNF-α production by LPS-stimulated immune cells (15, 54, 56). It is reasonable to deduce that the glucocorticoid-overriding activity of *Eh*MIF disrupts the tightly regulated immune response, creating a proinflammatory state in the gut during *E. histolytica* infection.

It appears counterintuitive that parasite genomes would encode a proinflammatory cytokine, but *Eh*MIF may play several important roles during the parasite life cycle. It has been shown that TNF-α has a chemotactic effect on *E. histolytica*; hence, it is possible that by enhancing TNF-α, *Eh*MIF promotes parasite invasion (57, 58). Also, TNF-α promotes adherence of *E. histolytica* to host cells (59). It was recently shown that the *Plasmodium* MIF cytokine interferes with the development of immunological memory by inducing the development of short-lived effector cells rather than memory cells (16). *Eh*MIF might therefore perform a function similar to that of *Plasmodium* MIF, rendering the host susceptible to reinfection by the parasite. Furthermore, *E. histolytica* has developed an impressive number of mechanisms to evade the host immune response (4). For example, the amebic surface protein peroxiredoxin possesses antioxidant activity, which protects the parasite from immune cell reactive oxygen species (60). In addition to cytokine functions, mammalian MIF, through interaction with the JAB-1 protein, coordinates cell cycle checkpoints promoting DNA repair (61, 62). Since a putative JAB-1 gene homolog can be found in the *Entamoeba* genome (29), *Eh*MIF might have a physiological function involving cell cycle regulation in amebae. It would be interesting in future studies to determine whether an *Eh*MIF–JAB-1 functional interaction exists in replicating trophozoites.

The present study demonstrated that an anti-*Eh*MIF IgG antibody present in the sera of children recognized *Eh*MIF but did not cross-react with human MIF. Although *Eh*MIF and human MIF share similar structures and biological functions, their relatively low amino acid sequence identity (28%) allows for different immunoreactive epitopes. This finding supports the possibility of developing immunotherapy against *Eh*MIF without affecting normal human MIF activity. Immunized rats possessing antibodies against the MIF homolog of the intestinal parasite *Strongyloides* were previously shown to have a reduced worm burden compared to that in nonimmunized rats, suggesting a potential protective role of antibodies against parasitic MIF homologs (32). However, it is not known whether anti-*Eh*MIF antibodies protect against amebiasis.

In conclusion, our study suggests that the interactions between the *E. histolytica* homolog of the cytokine MIF and host immune cells leads to an exaggerated inflammatory response and may contribute to disease. This study also generates the hypothesis that neutralization of *Eh*MIF may serve as potential immunotherapy against this devastating disease. We plan to examine whether antibodies against *Eh*MIF can prevent and/or attenuate disease in an amebic mouse model and whether anti-*Eh*MIF antibodies present in children's sera are associated with protection.

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