Targeting Parasite-Produced Macrophage Migration Inhibitory Factor as an Antivirulence Strategy With Antibiotic–Antibody Combination to Reduce Tissue Damage

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Targeting virulence factors represents a promising alternative approach to antimicrobial therapy, through the inhibition of pathogenic pathways that result in host tissue damage. Yet, virulence inhibition remains an understudied area in parasitology. Several medically important protozoan parasites such as Plasmodium, Entamoeba, Toxoplasma, and Leishmania secrete an inflammatory macrophage migration inhibitory factor (MIF) cytokine homolog, a virulence factor linked to severe disease. The aim of this study was to investigate the effectiveness of targeting parasite-produced MIF as combination therapy with standard antibiotics to reduce disease severity. Here, we used Entamoeba histolytica as the model MIF-secreting protozoan, and a mouse model that mirrors severe human infection. We found that intestinal inflammation and tissue damage were significantly reduced in mice treated with metronidazole when combined with anti–E. histolytica MIF antibodies, compared to metronidazole alone. Thus, this preclinical study provides proof-of-concept that combining antiparasite MIF-blocking antibodies with current standard-of-care antibiotics might improve outcomes in severe protozoan infections.

Keywords. virulence; MIF; protozoan; antibody; antibiotic; parasite–host interactions; tissue damage; immunopathology; immunotherapeutic; antivirulence.

Protozoan parasites represent a major threat to global public health, causing >1 million deaths yearly [1]. No effective vaccines have been developed to prevent disease from any of the protozoan parasites to date. Therefore, the management of infected patients continues to rely on treatment with antibiotics and supportive care. New strategies to fight such infections are urgently needed due to the emergence of multidrug-resistant pathogens that limit available treatment options. In addition, poor clinical outcomes are associated with severe infections, even when appropriate therapy is administered [2]. For example, Entamoeba histolytica is a protozoan parasite that causes inflammatory diarrhea, termed amebic colitis, which is characterized by colonic inflammation and tissue damage. Entamoeba histolytica infects millions of people annually, making amebic colitis a leading cause of severe diarrhea worldwide, estimated to kill approximately 50 000–100 000 people each year [3, 4]. Severe forms of amebic colitis carry high fatality exceeding 50%, even despite treatment with the nitroimidazole antibiotics, such as metronidazole, which are the treatment choice. New therapeutic strategies are needed as metronidazole alone is sometimes not enough, and even drastic measures such as the surgical resection of the inflamed portion of colon may not prevent death [4–7].

Virulence factors are molecules or proteins produced by pathogens that promote disease by damaging host tissue. Targeting virulence factors by inhibiting specific mechanisms that promote tissue damage and disease symptoms is a promising alternative strategy to new antimicrobial development. Also, removing pathogens of their virulence properties without harming their survival hopefully will reduce the potential of antimicrobial selection pressure and development of drug-resistant mutations [2, 8, 9]. While substantial progress in antivirulence approaches have been made in the field of bacteriology, virulence factor inhibition in parasitology remains significantly understudied.
Macrophage migration inhibitory factor (MIF) is an inflammatory cytokine that is a critical upstream mediator of inflammation. Secreted MIF binds to its receptor, CD74, on immune and epithelial cells and stimulates expression of various cytokines, for example, interleukin 8 (IL-8) and tumor necrosis factor alpha (TNF-α) [10, 11]. Pathogenic protozoan parasites, such as *Plasmodium*, *Entamoeba*, *Toxoplasma*, and *Leishmania*, secrete homologs of the cytokine MIF. Avoiding immune clearance allows these protozoa to persist in their host, which exacerbates the damage caused by the lingering inflammatory response to invading parasites [12], compounded by the fact that these parasites secrete MIF cytokine that can directly drive inflammation [13]. The inflammatory properties of protozoan-produced MIF contribute to immunopathology, damaging the host, and are linked to more severe disease [14–22]. However, a critical unanswered question is whether antibodies to protozoan MIF can reduce disease severity. The aim of this preclinical study was to investigate the benefit of neutralizing antiprotozoan MIF antibodies as an add-on therapy to antibiotics in severe disease using *E. histolytica* as the model organism. We found that blocking the virulence factor *E. histolytica* MIF (Eh-MIF) with neutralizing antibodies combined with antibiotics resulted in improved inflammatory outcomes and less host damage in severe infection.

**MATERIALS AND METHODS**

**Coculture of Human Cells With *E. histolytica* Parasites**

Human intestinal epithelial cells (HCT-116) and human macrophages (differentiated THP-1 cells) were cultured with *E. histolytica* strain HM1:IMSS trophozoites at a ratio of 10:1 human cells to parasite in M199 medium [17, 23]. IL-8 and TNF-α in cell culture supernatant were measured by enzyme-linked immunosorbent assay (ELISA; eBioscience).

**Mice and Amebic Colitis**

*Entamoeba histolytica* strains capable of evading immune clearance were generated by passing trophozoites through mice intestine. *Entamoeba histolytica* trophozoites that persisted in an inflamed intestine for at least 5 days were used for severe colitis experiments. Wild-type CBA/J mice were obtained from the Jackson Laboratory. Male mice were used at 10 weeks of age. Mice were treated with granulocyte colony-stimulating factor (G-CSF) 125 μg/kg subcutaneously twice per day for 3 days [24]. On day 4, animals were anesthetized, laparotomized, and intracally infected with 10⁶ *E. histolytica* trophozoites [25]. Treatment began 24 hours after infection [6] and continued for a total of 3 days. One group received metronidazole (10 mg/kg per day) [26] plus 1 mg mouse anti–*Eh*-MIF blocking antibodies given by parenteral (intraperitoneal) injection. The control group received equivalent amounts of metronidazole plus control antibody. At the end of the treatment course mice were killed, and the cecal tissue and luminal contents were obtained for further analysis.

**ELISA**

Intestinal tissue was prepared for ELISA as described previously [27]. Intestinal tissue lysates and luminal contents were evaluated by ELISA for CXCL1 (R&D Systems), TNF-α (eBioscience), myeloperoxidase (MPO; R&D Systems) [28], and albumin (Bethyl Laboratories) according to the manufacturers’ instructions. Total protein concentration was measured using the Pierce BCA Protein Assays Kit (Thermo Scientific).

**Histopathological Examination**

Mouse tissue was fixed in Bouin solution (Sigma) and stored in 70% ethanol. Tissue staining with hematoxylin and eosin was performed by the University of Virginia Research Histology Core [17]. Histological scoring was performed by 2 independent blinded scorers as previously described [29].

**Structure Analysis and Bioinformatics**

The coordinates of the *Eh*-MIF protein structure have been deposited in the Protein Data Bank under the accession code 6CUQ. Structural comparison between the *Eh*-MIF and human MIF proteins were done using the University of California, San Francisco Chimera software version 1.10.2. Amino acid sequences of MIF proteins from human and *E. histolytica* were aligned by Multiple Sequence Comparison by Log Expectation (MUSCLE) software [30].

**Protein Expression, Purification, and Biotinylation**

The CD74 ectodomain cDNA was subcloned from pGEX-6P-1 CD74 plasmid (previously described in [16]) into pET28-MBP-TEV vector (Addgene plasmid number 69929) within 5’BamH1 and 3′XhoI sites followed by transformation into *Escherichia coli* BL21 (DE3) cells for expression and purification of the recombinant MBP-CD74 protein. Both MBP and MBP-CD74 proteins were expressed by induction with 1 mM isopropyl β–d–thiogalactoside for 18 hours at 15°C. Purification of these proteins was done as previously described [31]. In brief, proteins were affinity purified with amylose resin (New England Biotechnologies) and eluted with 10 mM maltose. The expression and purification of *Eh*-MIF and human MIF recombinant proteins were done as previously described [16]. Purified proteins were concentrated and buffer exchanged into 1× phosphate-buffered saline using Amicon Ultra-15 centrifugal filter units (Millipore Sigma). Biotinylation of MBP and MBP-CD74 proteins were done by the EZ-Link Sulfo-NHS-LC-Biotinylation kit (Thermo Scientific).

**Binding Kinetics Using Biolayer Interferometry Assay**

The binding affinity of MBP-CD74 for *Eh*-MIF and human MIF was determined using the Blitz System (Octet Red 96 system, ForteBio). In brief, Streptavidin Dip and Read
Biosensors (ForteBio) were hydrated for 10 minutes followed by baseline stabilization for 5 minutes in the sample dilution buffer (1× Dulbecco’s PBS, 0.02% Tween 20), and 25 µg biotinylated MBP-CD74 or MBP was loaded onto the biosensors for 5 minutes. After washing the loaded biosensors in sample dilution buffer for 5 minutes, they were exposed for 10 minutes to five 2-fold dilution series of Eh-MIF or human MIF protein starting at 800 nM concentration. Postbinding dissociation was done for 10 minutes in the sample dilution buffer. Binding affinities (K_D) were calculated using the Blitz system software (ForteBio).

**Immunoblotting**

Recombinant Eh-MIF and human MIF were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by transfer onto polyvinylidene difluoride membranes (Millipore). The membranes were incubated overnight with affinity purified mouse anti–Eh-MIF antibody at 4°C followed by antimouse immunoglobulin G (IgG) horseradish peroxidase conjugate (Sigma) secondary antibody. Enhanced chemiluminescence (Thermo Scientific)–based substrates were used to detect antibody conjugated peroxidase activity.

**MIF Homologs and IL-8 Secretion Assay**

IL-8 secretion assays were carried out as previously described [17, 18]. In brief, 10^6 cells/mL human Caco2 colonic epithelial cells were treated with 0.5 µg/mL human or E. histolytica MIF in the presence of 5, 20, and 50 µg/mL anti–E. histolytica antibodies for 8 hours. IL-8 in cell culture supernatant was measured by ELISA (eBioscience).

**Antibody Purification**

Antibodies used in cytokine secretion assays and passive immunization were purified using the Melon Gel IgG Purification Kit (Thermo Scientific) for purification of IgG from Eh-MIF immunized or control mice as previously described [32]. This allows both groups to have the same antibody profile except for neutralizing antibodies against the Eh-MIF protein.

**Statistical Analysis**

Statistical differences between 2 groups were determined using Student t test and Mann–Whitney U test. Pearson correlation was used for correlation analysis. A P value <.05 was considered statistically significant.

**Study Approval**

All animal procedures were approved by the University of Virginia Institutional Animal Care and Use Committee (IACUC). All animal studies were performed in compliance with the federal regulations set forth in the Animal Welfare Act, the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the guidelines of the University of Virginia IACUC.

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**RESULTS**

**E. histolytica MIF Protein is a Bona Fide Homolog of Human MIF**

Given that structural similarity between proteins is strong predictor of functional similarity [33], we determined the crystal structure of the Eh-MIF protein and investigated whether it is an authentic homolog of human MIF. The X-ray crystal structure of Eh-MIF was solved at a resolution of 2.45 Å (Protein Data Bank identifier: 6CUQ). Similar to the human protein, Eh-MIF formed a stable trimer (Figure 1A). Structural homology between Eh-MIF and human MIF was measured by the root mean square deviation (RMSD) [34, 35]. Superimposition of all the 113 α-carbon atoms of the backbone resulted in a RMSD value of 1.678 Å, which falls well within the range of RMSD for homologous proteins, which is <3 Å [36]. Since RMSD calculation is inherently biased by further apart atoms [35], we also found that the RMSD value of 100 residues best aligned with the human protein was 1.076 Å. Therefore, our findings indicate that Eh-MIF protein is structurally homologous to human MIF.

Next, we compared the binding affinity of E. histolytica and human MIF proteins to the MIF receptor CD74. Biolayer interferometry (BLI) is a useful technique for measuring inter-action affinity between proteins in real time [37]. Biotinylated MBP-CD74 fusion protein was loaded onto the surface of streptavidin BLI sensors, followed by binding measurements in different concentrations of E. histolytica or human MIF proteins. Analysis revealed a dissociation constant (K_D) of 1.12 × 10⁻⁸ M for Eh-MIF (Figure 1B), similar to that for the human MIF, which was 1.82 × 10⁻⁸ M (Figure 1B). Biotinylated MBP alone coupled to the streptavidin BLI sensors was used as control. BLI measurements demonstrated that E. histolytica and human MIF proteins did not bind to the MBP-only control, K_D not applicable (Figure 1B). Therefore, the MIF receptor CD74 has similar high binding affinity for both E. histolytica and human MIF proteins.

Collectively, these structural and receptor binding data along with previously described proinflammatory properties of Eh-MIF [16–18], supports Eh-MIF as a bona fide homolog of the human proinflammatory cytokine MIF.

**Antibodies Against E. histolytica MIF Do Not Cross-React With Human MIF**

While human and Eh-MIF homologs share structural and functional similarities, their sequence homology is low; they share only 28% sequence identity (Figure 2A). Antibody cross-reactivity depends on the extent of protein sequence similarity. Therefore, the low sequence homology facilitates generating antibodies against Eh-MIF that do not cross-react with human MIF. In support of this, we found that anti–Eh-MIF antibodies were highly specific to the parasite protein and did not cross-react with the host protein by immunoblot analysis (Figure 2B). This is consistent with our earlier data that human and animal sera containing anti–Eh-MIF antibodies did not cross react with the recombinant host MIF [16, 17].
to immunoblot assay, we evaluated cross-reactivity with functional analysis of neutralizing antibodies against *Eh*-MIF. Both human and *Eh*-MIF stimulate IL-8 production from Caco-2 intestinal epithelial cells [17]. Using this assay, we found that these neutralizing antibodies inhibited *Eh*-MIF activity but had no effect on human MIF–induced IL-8 production (Figure 2C). These results, which include sequence identity, immunoblot, and functional analysis, support the lack of cross-reactivity of anti–*Eh*-MIF antibodies with human MIF.

We further tested if the antibody could neutralize the effects of the parasite-secreted MIF on human epithelial and immune cells using coculturing assays [17, 23]. We found that neutralizing antibodies blocked *E. histolytica*-stimulated IL-8 and TNF-α production by human intestinal epithelial cells (HCT116) and macrophages (differentiated THP-1), respectively (Figure 2D). Together, these data further support the concept that *Eh*-MIF can be specifically targeted in *E. histolytica*-induced inflammation.

**Combination Therapy With Neutralizing Anti–*E. histolytica* MIF Antibodies Is Superior to Metronidazole Alone**

Since elevated parasite MIF levels positively correlate with inflammation and disease severity [13, 14, 17, 38], we hypothesize that blocking *Eh*-MIF activity would be most beneficial in the setting of severe infections. To test this hypothesis, we used a mouse model that simulates severe human amebic colitis. That is, in patients with amebic colitis, excess intestinal tract neutrophil infiltration is associated with more tissue destruction and severe disease [39–42]. First we serially infected mice, then obtained *E. histolytica* strains that were capable of evading the immune clearance by persisting in the inflamed intestine for at least 5 days. These strains were used for future studies. G-CSF is a potent stimulator of white blood cell production, and in particular, neutrophil production [24]. MPO, a major component of neutrophils, is a widely used marker of neutrophil influx and intestinal inflammation [43]. A healthy intestine with an intact epithelial and endothelial barrier prevents the spilling of albumin into the gut lumen [44]. Intestinal tissue damage caused by *E. histolytica* infection results in loss of the intestinal permeability barrier and can be quantified by measuring the flux of albumin from the serum into the intestinal lumen [45]. Infected mice pretreated with G-CSF had increased neutrophil infiltration as measured by tissue MPO levels. As expected, increased neutrophil infiltration correlated with intestinal damage as evidenced by more severe histopathology and elevated luminal albumin, modeling severe human amebic colitis (Figure 3).
Next, we use this model to evaluate the benefit of add-on therapy with neutralizing anti-\(Eh\)-MIF antibodies in severe infection. Both treatment groups received metronidazole and were able to clear the parasites within 72 hours. Adding anti-\(Eh\)-MIF antibodies to the treatment regimen as opposed to metronidazole only, significantly reduced parasite-induced gut inflammation, as measured by CXCL1 (IL-8 homolog), TNF-\(\alpha\), and MPO levels, and the \(E.\ histolytica\)-induced tissue damage, as analyzed by histological score and by mucosal barrier integrity (Figure 4). These data provide evidence that antibodies to the amebic virulence factor MIF can provide additional benefits over antibiotics alone in severe amebic colitis.

**DISCUSSION**

During an infection, host tissue destruction occurs by direct damage by the pathogen and inflammatory-mediated damage (immunopathology) [46]. Extensive tissue destruction correlates with poor clinical outcomes, even when appropriate antibiotics are administered promptly for treatment [5, 39, 46]. This implies that antibiotics alone are not always sufficient to disrupt the effects arising from severe parasitic infection. Disarming the
parasite of important virulence factors offers an attractive targeted approach that can be used as an adjunct to protozoacidal antibiotics. Neutralizing secreted virulence factors, as well as virulence factors released during parasite death and cell lysis, may help to limit ongoing damage and inflammation. We have used *E. histolytica* as a prototype to show a novel therapeutic strategy for reducing tissue damage by directing therapy against the parasitic MIF virulence factor.

The targeting of microbial virulence factors offers several putative therapeutic advantages. As the pathogen itself is typically not destroyed, antivirulence treatments should not cause selection pressure of drug-resistant mutants, which has been a major challenge with traditional antibiotics [2]. Another benefit of antivirulence strategies may be lack of direct effects on beneficial host commensals, which are unlikely to harbor virulence factors [2]. Metronidazole, for example, in addition to having antiprotozoal activity, can cause undesired disturbance to a wide range of enteric anaerobes. Additionally, antivirulence may be able to more quickly inactivate targets than antibiotics, which act by inhibition of growth and replication [2]. Hence anti–*Eh*-MIF holds potential as a rapid-onset strategy for decreasing disease severity in amebic colitis without anticipated selection of resistance or disturbance of host microbiomes.

The concept of using antibodies to neutralize virulence factors is well described for several bacterial infections, and has been used for more than a century in the treatment of children with life-threatening diphtheria who are given both antibiotics and horse antiserum derived against diphtheria toxin, for example [2]. To our knowledge, this strategy for parasitic infections has been understudied [2], and we believe that anti–*Eh*-MIF offers a suitable and rationale prime candidate. Anti–*Eh*-MIF is a naturally occurring antibody in humans, produced as part of the adaptive immune response to *E. histolytica* infection. *Eh*-MIF is structurally identical to human MIF, but exhibits relatively low identity sequence homology. Hence, *Eh*-MIF antibodies can be generated with high affinity and specificity, but without significant cross-reactivity with human MIF, as we have shown here and in prior human and animal studies [16, 17]. Taken together, these data further support the safety of anti–*Eh*-MIF as an antivirulence candidate that can effectively neutralize parasite MIF, without anticipated adverse off-target effects on human MIF.

Limitations of this approach include the following: Anti–*Eh*-MIF does not impair amebic growth or have amebicidal activity, meaning that as antibody levels wane with time, if the parasite has not been killed, there could be a risk of recurrence.

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**Figure 3.** Tissue destruction caused by *Entamoeba histolytica* parasite. **A**, Increased tissue myeloperoxidase (MPO) and luminal albumin levels in infected mice pretreated with granulocyte colony-stimulating factor (G-CSF) compared with infected mice without G-CSF pretreatment and uninfected controls. **B** and **C**, Representative hematoxylin and eosin–stained images and histology scores. Scale bars, 50 μm. Data represent mean and standard deviation (n = 5 mice per group). **P** < .01.
of disease. To overcome this, we suggest that at this time anti-
*Eh*-MIF be developed as a strategy to minimize inflammation-
induced tissue damage in combination with antibiotic therapy,
rather than as monotherapy. Second, an early microbiologic di-
agnosis is required to deploy antivirulence therapy in an effec-
tive manner, as these strategies are too specific for broad-range
empiric use. The increasing availability of rapid molecular diag-
nostics such as multiplex enteric pathogen panels, however, will
facilitate the ability to carry this out in a timely manner in the
future [4].

We can further improve upon this work by pursuing
pathways for polyclonal antibody development, such as
standardization and supply of anti-*Eh*-MIF, in order to
study clinical applications. Polyclonal antibodies offer the
advantage of binding to multiple epitopes and hence offer
higher affinity with less vulnerability to minor antigenic
changes, but large-scale production can be challenging. One
solution may be to engineer a monoclonal antibody candi-
date, through the use of recombinant DNA or other tech-
nology, which can be made more readily in larger quantities.
Previously such technologies required large investments in
time and technical skills, but the increasing demand for
monoclonal antibodies in the treatment of cancer, autoim-
mune conditions, and other infections is paving the way for
faster production capacity and improved manufacturing
processes, making this a viable and perhaps even affordable
option with time [47, 48].

In summary, we have demonstrated that anti-*E. histolytica*
MIF offers a promising candidate for adjunctive treatment of
severe amebic colitis, which otherwise carries a high fatality,
even when treated with appropriate antibiotics. We also more
broadly demonstrate the concept that antiparasite virulence
factors can be blocked to treat disease, providing a preclin-
ical basis for the development of innovative strategies to save
and protect lives from these devastating neglected tropical
diseases.

Notes

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Figure 4. Anti-*Entamoeba histolytica* macrophage migration inhibitory factor (MIF) antibody adjunctive therapy reduces tissue damage. A–C, Reduced tissue CXCL1,
tumor necrosis factor–α, and myeloperoxidase, luminal albumin levels, and histology scores in infected mice treated with anti-*E. histolytica* MIF antibodies combined with
metronidazole compared to metronidazole alone (control). Scale bars, 50 μm. D, Schematic diagram of the possible mechanism by which combination therapy reduces tissue
damage. Data represent mean and standard deviation (n = 7 mice per group). *P < .05; **P < .01; ***P < .001. Abbreviations: Ab, antibody; *Eh*, *Entamoeba histolytica*; IL-8,
interleukin-8; MIF, macrophage migration inhibitory factor; MPO, myeloperoxidase; TNF-α, tumor necrosis factor alpha.
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