Interaction between parasite-encoded JAB1/CSN5 and macrophage migration inhibitory factor proteins attenuates its proinflammatory function

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Multiple protozoans produce homologs of the cytokine MIF which play a role in immune evasion, invasion and pathogenesis. However, how parasite-encoded MIF activity is controlled remains poorly understood. Cytokine activity can be inhibited by intracellular binding partners that are released in the extracellular space during cell death. We investigated the presence of an endogenous parasite protein that was capable of interacting and interfering with MIF activity. A screen for protein-protein interaction was performed using immunoaffinity purification of amebic cell lysate with specific anti-Entamoeba histolytica MIF (EhMIF) antibody followed by mass spectrometry analysis, which revealed an E. histolytica-produced JAB1 protein (EhJAB1) as a potential binding partner. JAB1 was found to be highly conserved in protozoans. Direct interaction between the EhMIF and EhJAB1 was confirmed by several independent approaches with GST pull-down, co-immunoprecipitation, and Biolayer interferometry (BLI) assays. Furthermore, the C-terminal region outside the functional JAMM deneddylase motif was required for EhMIF binding, which was consistent with the top in silico predictions. In addition, EhJAB1 binding blocked EhMIF-induced IL-8 production by human epithelial cells. We report the initial characterization of a parasite-encoded JAB1 and uncover a new binding partner for a protozoan-produced MIF protein, acting as a possible negative regulator of EhMIF.

Protozoan parasites represent a major threat to health and contribute significantly to morbidity and mortality worldwide. For example, Entamoeba histolytica is a protozoan parasite that causes colitis. Severe forms of amebic colitis are associated with high case fatality rates ranging from 40% to 89%. There is neither an effective vaccine nor have there been advancements in therapies for amebic colitis for over fifty years. Therefore, there remains an ongoing need to find new drug and vaccine targets through a better understanding of parasite biology.

Macrophage migration inhibitory factor (MIF), one of the first cytokines to be discovered, is a pleiotropic inflammatory cytokine and a critical upstream mediator of innate immunity. Many of the inflammatory effects of MIF are mediated through direct binding to the CD74 cell surface receptor, causing the secretion of proinflammatory cytokines such as IL-8. An increase in MIF expression contributes to excessive inflammation and immunopathology. Hence, MIF has been reported to have a role in the pathogenesis of several inflammatory diseases such as inflammatory bowel disease and rheumatoid arthritis. MIF proinflammatory properties also make it a crucial mediator in the immune response against a wide range of pathogens.

Counterintuitively, MIF homologs have been characterized in several pathogenic protozoans including Entamoeba, Plasmodium, Toxoplasma, and Leishmania. These protozoan MIF homologs have demonstrated similar proinflammatory activities to that of human MIF, and play a role in immune evasion, invasion and pathogenesis. Despite the growing literature on protozoan-encoded MIF proinflammatory activity, very little is known about how it is regulated. Here, we uncover a parasite-encoded JAB1 (c-Jun activation domain binding protein 1),

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which is highly conserved throughout protozoan parasites, as a novel binding partner and potential regulator of the MIF homolog of Entamoeba histolytica.

**Results**

**Identification and characterization of a parasite-produced JAB1.** Co-immunoprecipitation (co-IP) of proteins followed by mass spectrometric identification is a standard approach for identifying novel protein-protein interactions. Parasite cell lysates were incubated with a specific antibody against Entamoeba histolytica MIF (EhMIF) or IgG control antibody. The immunoprecipitates were subjected to mass spectrometry analysis. An Entamoeba histolytica-encoded JAB1 (EhJAB1) amino acid sequence. Peptides unique to the EhJAB1 protein identified by mass spectrometry are highlighted (blue). JAMM motif (EXnHXHX10D) shown in red box. (C) Structural homology between Human JAB1 (HuJAB1) and EhJAB1. HuJAB1 (orange) was superimposed with the predicted structure of EhJAB1 (blue). (D) Multiple sequence alignment of the conserved JAMM motif of JAB1 from pathogenic parasites. Identical (green), conserved (blue), semi-conserved (pink), and non-conserved residues (red).

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Figure 1. Characterization of EhJAB1. (A) Schematic of procedure for identifying novel protein-protein interaction with co-immunoprecipitation followed by mass spectrometric analysis. (B) Entamoeba histolytica JAB1 (EhJAB1) amino acid sequence. Peptides unique to the EhJAB1 protein identified by mass spectrometry are highlighted (blue). JAMM motif (EXnHXHX10D) shown in red box. (C) Structural homology between Human JAB1 (HuJAB1) and EhJAB1. HuJAB1 (orange) was superimposed with the predicted structure of EhJAB1 (blue). (D) Multiple sequence alignment of the conserved JAMM motif of JAB1 from pathogenic parasites. Identical (green), conserved (blue), semi-conserved (pink), and non-conserved residues (red).
with the IgG antibody control (Fig. 2D–F). Together, these findings suggest a direct binding between EhMIF and EhJAB1 proteins.

High-affinity binding between EhMIF and EhJAB1 proteins. Biolayer interferometry (BLI) is a useful technique for measuring interactions between proteins in real time\(^\text{26}\). We determined the equilibrium dissociation constant for EhMIF binding to EhJAB1 using BLI. GST-tagged EhJAB1 was coupled to the surface of anti-GST antibody-coated BLI sensors, followed by binding measurements in different concentrations of EhMIF. Analysis revealed a dissociation constant of K\(_D\) of 3.86 × 10\(^{-8}\) M (Fig. 3A). GST alone coupled to anti-GST antibody-coated BLI sensors was used as control. BLI measurements demonstrate that EhMIF did not bind to the GST control, K\(_D\) not applicable (Fig. 3B). These findings suggest EhMIF binds to EhJAB1 within the range considered biologically relevant\(^\text{27,28}\).

EhJAB1 interacts with EhMIF via its C-terminal domain. We investigated the binding domain of EhJAB1 responsible for interacting with EhMIF. First, an in silico approach using pyDockWEB was applied to assess the binding of EhJAB1 to EhMIF. pyDockWEB allows the best rigid-body docking orientations generated by pyDock scoring function, which consists of electrostatics, desolvation energy and limited van der Waals contribution\(^\text{25}\). The C-terminal domain of EhJAB1 was revealed to be the EhMIF binding region in all top ten predictions (Figs 4A and S6). Next, we further examine the region of EhJAB1 that is responsible for the formation of the complex with EhMIF using deletion mutant analysis. GST-tagged full-length EhJAB1 and various GST-tagged deletion mutants of EhJAB1, with either N-terminal, MPN, JAMM or C-terminal domain deletion (Fig. 4B,C), were immobilized on magnetic beads and incubated with EhMIF protein. EhMIF specifically interacted with GST–EhJAB1, except for the mutant lacking the C-terminal domain. Interaction was regained when the 187–246 amino acid sequence of the C-terminal was restored (Fig. 4D). These results indicate binding of EhJAB1 to EhMIF is mediated through the C-terminal region outside of the MPN domain.

EhJAB1 inhibits EhMIF interaction with the CD74 receptor and EhMIF-induced cytokine production. CD74 is a cell surface receptor for human MIF, which mediates many of its inflammatory effects\(^\text{39}\). Several parasite MIF homologs, including EhMIF, were previously shown to interact with the CD74 receptor\(^\text{14,17,29–31}\). Here, we were able to reproduce this finding and found that EhMIF–CD74 interaction was blocked by preincubating EhMIF with EhJAB1 (Fig. 5A). The epithelial surfaces of the skin, nasal, intestinal, respiratory, and genitourinary tracts are the first points of contact for many protozoans. Epithelial cells express CD74 and are a rich source of IL-8\(^\text{32}\). Stimulation of IL-8 production has been the most reproducible activity of protozoan MIF homologs. EhMIF was recently shown to stimulate IL-8 secretion from human intestinal epithelial cells\(^\text{18}\). IL-8 is a potent neutrophil chemotacticant that contributes to inflammation in various infectious and inflammatory diseases. We proceeded to examine if EhJAB1 binding to EhMIF attenuates its proinflammatory function using two different colonic epithelial cell lines. EhJAB1 blocked EhMIF-induced IL-8 secretion by HCT116 and Caco2 colonic cells (Fig. 5B,C). These data indicate that when bound to EhJAB1, EhMIF is no longer capable of carrying out its inflammatory functions.
Discussion

Parasites can exploit the host inflammatory response to promote tissue invasion\(^1\). *EhMIF*-stimulated human intestinal epithelial cells secrete IL-8, a potent promoter of inflammation\(^19\). One of the downstream effects of *E. histolytica* MIF-induced inflammation is an increase in matrix metalloproteinases (MMPs) production, which was shown recently to promote *E. histolytica* tissue invasion in human colon\(^19,33\). That said, parasites are likely to have developed mechanisms to regulate their MIF actions especially in situations where they fail to evade the host inflammatory response induced by producing such a molecule.

Identifying a binding partner that inhibits MIF function provides insight into how MIF’s actions are regulated\(^27\). In this study, a proteomics approach was used to identify parasite-encoded protein interaction partners of a homolog of MIF from *Entameoba histolytica*. We found that *EhJAB1* protein interacts with *EhMIF*. This interaction was validated by multiple independent approaches with GST-pulldowns, co-immunoprecipitation, in silico experiments and Biolayer interferometry.

**JAB1** function is not limited to CSN dependent deneddylation. JAB1 is also stable and functional in a free, monomeric, CSN independent form\(^46-48\). JAB1 monomers are catalytically inactive, but bind to certain proteins and alter their activity\(^34,48\). We showed that the JAMM catalytic motif of *EhJAB1* was not required for its interaction with *EhMIF*, which is consistent with the previous report on mammalian proteins\(^35\). The dissociation.

Figure 3. Characterization of *EhMIF* binding to *EhJAB1* using the Biolayer interferometry. (A) Representative binding and dissociation curves for *EhMIF* binding to *EhJAB1*. Anti-GST antibody-coated biosensors were loaded with GST-*EhJAB1*. Sensors were placed into solutions with *EhMIF*, concentrations range from 5 to 80 nM (association analysis: 0 to 600 secs). Subsequently, the sensors were transferred to buffer without *EhMIF* for dissociation analysis (from 600 to 1200 secs). Analysis revealed a dissociation constant of \(K_D\) of \(3.86 \times 10^{-8}\) M. (B) Biosensors loaded with GST only were used as controls.
constant for \( \text{EhJAB1-} \text{EhMIF} \) complex was well within the range considered physiologically relevant, suggesting a biologically significant interaction. Intracellular molecules released from damaged cells can have profound effects on the immune response. There is growing evidence that not all of these immunomodulatory molecules are pro-inflammatory, and interestingly, some have immunosuppressive or regulatory effects. These anti-inflammatory actions occur either directly or indirectly. For example, in the case of human \( \text{MIF} \), an endogenous binding partner that is released in the extracellular space during cell death interacts and negatively interferes with \( \text{MIF} \) activity. \( \text{JAB1} \) is an intracellular protein, which is consistent with our mass spectrometric analysis of the parasite cytosolic fraction; while \( \text{MIF} \) homologs, such as \( \text{EhMIF} \) are secreted proteins. \( \text{E. histolytica} \) parasite has developed a number of mechanisms to evade the host immune response. However, amebic parasites could become damaged if they fail to evade the inflammatory response triggered by \( \text{EhMIF} \). It would be reasonable to speculate that the free monomeric \( \text{EhMIF} \) released from injured cells into the extracellular environment would then form complexes with \( \text{EhJAB1} \), preventing interaction with the host receptor CD74 and reducing \( \text{EhMIF} \)-induced inflammation, as a negative feedback mechanism. In addition, given the structural and functional similarity between human and \( \text{E. histolytica} \) \( \text{MIF} \) and \( \text{JAB1} \) proteins, we do postulate that \( \text{EhJAB1} \) could also interact with human \( \text{MIF} \). It would be interesting to determine whether an \( \text{EhJAB1} \) is a therapeutic target against this devastating parasitic disease.

**Methods**

**Plasmids, cloning and PCR.** The \( \text{EhJAB1} \) gene, codon optimized for expression in \( \text{E.coli} \) BL21(DE3) cells, was cloned within \text{pDEST15} vector. Deletion constructs were prepared by inverse PCR on the \text{pDEST15-full length JAB1} clone using the primers listed in the Supp. Fig. S4. A schematic of the mutation strategy is provided in the Supp. Fig. S5. Clones were screened by PCR across the gene boundaries within the vector followed by confirmation with sequencing. The \( \text{EhJAB1} \) gene was amplified from the \text{pDEST15} vector with primers carrying \( \text{5’ BamHI} \) and \( \text{3’ XhoI} \) sites and sub-cloned within \text{BamHI} and \text{XhoI} sites of \text{pGEX-4T1} vector to utilize the thrombin site for cleaving off the \text{GST} tag from the \text{GST- \text{EhJAB1}} protein. Also, the previously described codon optimized \text{EhMIF} gene cloned within \text{pJexpress414} vector (\text{DNA2.0}) was used in this study.

**Protein expression and purification.** Protein expression of the recombinant \text{EhMIF} and \text{EhJAB1} was done following the previously described protocol except that the induction with isopropyl \( \beta \)-D – thiogalactoside (\text{IPTG}) was done for 18 hours at 15 °C. Cells were pelleted and lysed in CelllyticTM B Cell Lysis Reagent (Sigma) at room temperature for 15 minutes and lysate was collected following 30 min spin at maximum speed at 4 °C.
The purification of GST-fusion protein and His-tagged fusion protein was done as previously described using glutathione-sepharose (GE-Healthcare) and Ni-NTA agarose beads (Qiagen) respectively. Polymyxin B (Sigma) was used in the purification procedures for the removal of endotoxin. On-column cleavage of the GST-EhJAB1 protein was done with the Thrombin Cleavage Capture Kit (Millipore) that utilizes biotinylated thrombin for its streptavidin agarose based removal.

**GST-Pulldown Assay.** GST pull down assays were done using the MagneGST™ Protein Purification System kit (Promega). Bacterial lysate expressing GST fused- full length or deletion mutant EhJAB1 protein, diluted in MagneGST binding/wash buffer to a concentration 500 ng/ml in 500 µl, was mixed with 25 µl magnetic beads and was incubated overnight at 4 °C with rotation. Beads were washed 3 times with 500 µl binding/wash buffer followed by incubation with 1 µg purified EhMIF at 4 °C for overnight. The GST-CD74 pulldown assays were performed as previously described. Briefly, EhMIF protein was pre-incubated with or without 1X, 2X or 5X concentration of EhJAB1 protein for 30 minutes in 500 µl binding/wash buffer prior to incubation with the GST-CD74 bound magnetic beads at 4 °C for overnight. The bound complexes were eluted in 40 µl elution buffer after washing the beads 5 times with 500 µl binding/wash buffer. In a control experiment equivalent amount of GST protein was used in place of GST-EhJAB1 or GST-CD74 proteins. Twenty five percent of the eluted samples were assayed by immunoblots.

**Figure 5.** Effect of EhJAB1 binding on EhMIF activity. (A) EhJAB1 inhibits EhMIF interaction with the human MIF receptor CD74 by GST pull-down competition assay. Input (lane 1) and GST control (lane 2). EhMIF was preincubated with or without EhJAB1 at increasing doses before mixing with GST-CD74 (lanes 3–6). (B,C) EhJAB1 inhibits EhMIF-induced IL-8 production in a dose-dependent manner. Human colonic epithelial cells HCT116 and Caco2 were incubated with EhMIF with or without EhJAB1 at increasing doses. Culture supernatants were collected after 8 h and IL-8 was quantified by ELISA. Data represent mean and SD of triplicates from 1 experiment and are representative of 3 independent experiments. *P < 0.01; **P < 0.001. ns, not significant.
Immunoprecipitation. Approximately 1.25 × 10^7 trophozoites of E. histolytica, grown at 37 °C in TY1-S-33 medium, were harvested in 3 ml non-denaturing lysis buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 1% TritonX-100, 2 mM EDTA, 1x Protease inhibitor freshly added). Lysis was done with sonication followed by centrifugation at high speed for 10 minutes at 4 °C. Immunoprecipitations were performed with anti-EhMIF rabbit serum and rabbit IgG (Santa Cruz Biotechnology). For each, 100 µl Dynabeads™ Protein A (Invitrogen) was chemically conjugated with 10 µg antibodies using 5 mM Bis[Sulfo succinimidyl] carbonate (Thermo Scientific) following the manufacturer’s manual. Antibody conjugated beads were added to 500 µl amebic lysate and incubated overnight at 4 °C with rotation. Following binding, beads were washed 5 times with 300 µl wash buffer (10 mM Tris HCl pH 7.4, 150 mM NaCl, 1% TritonX-100, 1 mM EDTA, 1x Protease inhibitor freshly added). The bound complexes were eluted in 40 µl 1x Laemmli sample buffer (Bio-Rad) by boiling. Co-precipitated endogenous proteins were analyzed by Mass-Spectrometry. For direct binding experiment, tag free EhJAB1 protein was biotinylated using the EZ-Link® Sulfo-NHS-LC-Biotinylation kit (Thermo Scientific). Immunoprecipitation was performed with 50 µl dynabeads bound to 10 µg anti-EhMIF rabbit serum or rabbit IgG. For each set, 1.5 µg of EhMIF mixed with 500 ng biotinylated EhJAB1 protein was incubated with the antibody coated beads in 300 µl binding buffer (as described above) for overnight at 4 °C with rotation. Wash and elution of the bound complexes were done the same way as above. The immunoprecipitation was repeated in the presence of 5 µg and 10 µg non-biotinylated tag free EhJAB1 protein. Twenty-five percent of the eluted samples were assayed by immunoblots.

Mass Spectrometry. The entire eluted fraction from anti-EhMIF and rabbit IgG immunoprecipitations were subjected to electrophoresis on SDS polyacrylamide gel. Proteins were separated via SDS-PAGE for a length of 1 cm^3. About 1 cm × 1 cm section of the gel, spanning all of the resolved proteins in each well, were excised. The gel samples were submitted to the W. M. Keck Biomedical Mass Spectrometry Laboratory for mass spectrometry analysis.

Immunoblotting. Protein samples obtained from GST-pulldown and immunoprecipitation experiments were resolved by SDS-PAGE followed by transfer onto polyvinylidene difluoride membranes (Millipore). The membranes were incubated overnight with primary antibodies at 4 °C. For EhMIF protein detection, rabbit anti-EhMIF antibody was used followed by anti-rabbit IgG HRP conjugate (Sigma) secondary antibody. For biotinylated EhJAB1, goat anti-biotin HRP conjugated antibody (Cell Signaling Technology) was used. Enhanced chemiluminescence (Thermo scientific) based substrates were used to detect antibody conjugated peroxidase activity.

IL8 secretion assay by ELISA. The human colonic epithelial cells (HCT116 & Caco2, American Type Culture Collection) with densities 10^6 cells/ml were cultured in 48 well plate (Corning) with 100 µL complete media (Dulbecco's Modified Eagle Medium, Gibco) for 12 hours followed by gentle washing and incubation with 100 µl serum free media for 12 hours. After washing the plates with 200 µl media, cells were treated with 0.5 µg/ml EhMIF in presence or absence of 0.5, 1 and 2.5 µg/ml EhJAB1 protein for 8 hours. IL-8 in cell culture supernatant was measured by enzyme linked immunosorbent assay (ELISA, ebioscience).

Binding kinetics using BLI assay. The binding affinities between GST-EhJAB1 and EhMIF proteins were measured using the Blitz System (Octet® Red 96 system, ForteBio). Briefly, Anti-GST Dip and Red™ Biosensors (ForteBio) were hydrated for 10 minutes in the sample dilution buffer (1x DPBS, 0.1% BSA, 0.02% Tween 20) followed by 3 cycles of priming and neutralization, 20 seconds each, in regeneration buffer (10 mM Glycine, pH 1.7) and the sample dilution buffer, respectively. Next, baseline stabilization of the primed biosensors was done in the dilution buffer for 5 minutes. Then, 10 µg GST-JAB1 or GST proteins diluted in sample dilution buffer 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 5, 2-fold dilution series of EhMIF protein starting at 80 nM concentration. Post-binding dissociation of EhMIF was done for 10 minutes in the sample dilution buffer. Binding affinities (K_d) were calculated using the Blitz system software (ForteBio).

Bioinformatics. Orthologues of JAMM/MPN^4 motifs of the EhJAB1 protein from different protozoan parasites were aligned by Multiple Sequence Comparison by Log Expectation (MUSCLE) software^24. 3D structure of EhJAB1 was constructed by Protein Homology/Analogy Recognition Engine v 2.0 (PHYRE)^25. The predicted EhJAB1 structure was then compared with that of the Human JAB1 protein using the UCSF Chimera software v. 1.10.2. The protein-protein interaction between EhJAB1 and EhMIF was examined and potential docking interfaces were predicted using pyDockWeb that applies a rigid body protein-protein docking prediction model by electrostatic and desolvation scoring^26. The predictions were viewed using the UCSF Chimera software v. 1.10.2.

Statistical Tests. Statistical differences were determined using ANOVA followed by Dunnnett's post-hoc test. A p value less than 0.05 was considered statistically significant.

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S.G., L.A.L., L.F., A.B. and S.M. conducted experiments. S.G. and S.M. wrote the paper.

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