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Characterization of *Neospora Caninum* macrophage migration inhibitory factor

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Abstract

The present study is the first characterization of *Neospora caninum* macrophage migration inhibitory factor (NcMIF). BLAST-N analysis of NcMIF revealed high similarity (87%) to the *Toxoplasma gondii* MIF. NcMIF was cloned and expressed in *Escherichia coli* in 3 forms, NcMIF (mature protein), NcMIFm (mutation of proline -2 to glycine), and NcMIFhis (addition of a polyhistidine tag at the N-terminus). None of these recombinant NcMIFs (rNcMIF) had tautomerase, oxidoreductase, or immunologic regulatory activities. rNcMIF was unable to compete with recombinant human MIF for a MIF receptor (CD74), suggesting that NcMIF does not bind to this MIF receptor. The glycine substitution for proline-2 of NcMIF resulted in increased retention time on SEC-HPLC and decreased formation of dimers and trimers. The addition of N-terminal HIS-tag led to increased formation of trimers. Immunofluorescence staining demonstrated that NcMIF was localized to the apical end of *N. caninum* tachyzoites. Immunoelectron microscopy further revealed that NcMIF was present in the micronemes, rhoptries, dense granules, and nuclei. NcMIF was abundant in the tachyzoite lysate and present in excretory and secretory antigen (ESA_g) preparations. Total and secretory NcMIF was more ($P < 0.05$) abundant in a non-pathologic clone, Ncts-8, than in the wild type isolate (NC1). Furthermore, NcMIF release by the both isolates was increased in the presence of calcium ionophore. This differential production of NcMIF by the pathologic and non-pathologic isolates of *N. caninum* may suggest a critical role of this molecule in the infectious pathogenesis of this parasite.

Keywords

macrophage migration inhibitory factor; MIF; NcMIF; tautomerase; CD74; *Neospora caninum*

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Instruction

Macrophage migration inhibitory factor (MIF) was one of the first cytokines identified nearly four decades ago (Bloom et al., 1969). MIF has been described as a cytokine with two potentially physiologically relevant catalytic activities (tautomerase and oxidoreductase) and is involved in both innate and adaptive immune responses (Sun et al., 1996; Suzuki et al., 1996a). The most important role of MIF is the regulation of macrophage (Calandra et al., 1994; Onodera et al., 1997) and lymphocyte response and endocrine functions (Abe et al., 2001; Bacher et al., 1996). MIF counter-regulates the anti-inflammatory effect of glucocorticoids, upregulates the Toll-like receptor 4 (TLR4) expression by immune cells in response to the microbial products, and suppresses activation-induced p53-dependent apoptosis (Calandra et al., 1995; Hudson et al., 1999; Roger et al., 2001). MIF also induces expression of proinflammatory molecules such as TNF, IL-1, IL-2, IL-6, IL-8 and IFN- γ (Bacher et al., 1996; Calandra et al., 2003; Donnelly et al., 1999) and promotes production of nitric oxide (Donnelly et al., 1997). MIF upregulation of TNF production further promotes the expression of MIF, which leads to further TNF release by macrophages (Calandra et al., 1994). In addition, MIF is involved in tumor progression and angiogenesis (Chesney et al., 1999; Onodera et al., 1999). It has clearly shown that MIF is a natural ligand for CD74 (Leng et al., 2003) and a noncognate ligand of CXC chemokine receptors (Bernhagen et al., 2007).

MIF does not have a N-terminal leader sequence or an apparent internal signal sequence. However, it is effectively secreted from the immune and non-immune cells by a non-conventional leaderless pathway (cite: Merk M, Baugh J, Zierow S, Leng L, Pal U, Lee SJ, Ebert AD, Mizue Y, Trent JO, Mitchell R, Nickel W, Kavathas PB, Bernhagen J, Bucala R. 2009. The Golgi-associated protein p115 mediates the secretion of macrophage migration inhibitory factor. *J Immunol*. 182:6896–6906. Chemical crosslinking studies suggest that the mouse MIF forms a homotrimer under native solution conditions (Bendrat et al., 1997), while human MIF tends to form a mixture of monomers, dimers and trimers under the same conditions, which has been verified by equilibrium sedimentation velocity studies (cite: El-Turk F, Cascella M, Ouertatani-Sakouhi H, Narayanan RL, Leng L, Bucala R, Zweckstetter M, Rothlisberger U, Lashuel HA. *Biochemistry*. 2008 47:10740–56). X-ray crystallographic studies also support the conclusion that human and mouse MIFs exist as homotrimers (Sugimoto et al., 1996; Sun et al., 1996) and moreover, that they have significant structural homology with the bacterial enzymes 4-Oxalocrotonate tautomerase and 5-carboxymethyl-2-hydroxymuconate isomerase (Sugimoto et al., 1999). The N-terminal proline at position 2, which becomes the N-terminus after proteolytic processing of the initiating methionine, is the critical residue for the enzymatic activity of both bacterial enzymes and MIF (Bendrat et al., 1997; Stamps et al., 1998). A correlation between the tautomerase activity and immunological activities of MIF has been suggested with the results of a genetic knockin mouse of a catalytically inactive MIF indicating that the structural integrity of this site mediates protein-protein interactions critical for MIF bioactivity (Fingerle-Rowson G, Kaleswarapu DR, Schlander C, Kabgani N, Brocks T, Reinart N, Busch R, Schütz A, Lue H, Du X, Liu A, Xiong H, Chen Y, Nemajero A, Hallek M, Bernhagen J, Leng L, Bucala R. 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).

MIF homologues have been reported in a number of protozoan parasites including *Eimeria* spp, *Plasmodium* spp, and *Leishmania* spp and these MIF-like proteins were similar in structure and function to the mammalian MIFs and have been suggested to play a role in immunoregulation and immune evasion (Alam et al., 2011; Cordery et al., 2007; Han et al.,

2010; Jang et al., 2011; Miller et al., 2012; Miska et al., 2007; Shao et al., 2010; Wang et al., 2012). The present study describes the first cloning and expression of NcMIF in *E. coli* and the characterization of its structures and possible functions.

Materials and Methods

NcMIF Gene Cloning and Sequencing

Total RNA was isolated from *N. caninum* tachyzoites (NC1 isolate) using the RNeasy Mini Kit (Qiagen, MD). Based on the NC1 McMIF mRNA sequence (FR823385), specific primers were designed to amplify the NcMIF gene by polymerase chain reaction (PCR). The forward primer sequence is 5'-ATACATATGCCAAAGTGCATGATCTAC-3' with a Nde I restriction site at the 5' end and the reverse primer sequence is 5'-TAAAGCTTTTAGCCAAAGGTGCGGTC-3' with a Hind III restriction site at the 5' end. A forward primer was designed to produce a mutated NcMIF (NcMIFm) at the second amino acid position from proline (P) to glycine (G) by PCR method using this primer 5'-ATACATATGGGCAAGTGCATGATCTAC-3'. A forward primer with a BamH I restriction site at the 5' end (5'-ATAGGATCCATGCCAAAGTGCATGATCTAC-3') was designed to produce NcMIF with a polyhistidine tag (NcMIFhis) at the N-terminus. NcMIFm and NcMIFhis share the same reverse primer with NcMIF for PCR amplification. The cDNA (1 µg) was synthesized from total RNA using the M-MuLV reverse transcription enzyme (NEB, Ipswich, MA) with the reverse primer. The PCR reaction was performed as follows: 95°C for 10 min followed by 35 cycles at 95°C for 40s, 55°C for 30s, 72°C for 40s with a final extension of 72°C for 10 min. The PCR product was purified by the PCR purification kit (Promega, Madsion, WI) and ligated into the pGEM-T vector (Promega, Madsion, WI), followed by transformation into *E. coli* DH5⁺. The recombinant plasmid was extracted by the Wizard[®] Plus SV Minipreps kits (Promega, Madsion, WI) from the overnight liquid culture of LB medium containing ampicillin (50 µg/ml) and sequenced (Functional Biosciences, Inc., Madison, WI).

Expression, endotoxin removal, purification and refolding of recombinant NcMIF and antisera preparation

Three forms of recombinant NcMIFs (rNcMIFs) were produced, including rNcMIF without any modification, rNcMIFhis and rNcMIFm. NcMIF and NcMIF mutant (NcMIFm) genes were subcloned from the pGEM-T to pET26b(+) vector and the NcMIFhis was inserted into the pET28a vector (EMD Millipore, San Diego, CA). rNcMIFs were expressed in *E. coli* BL21 (DE3) (Novagen, Madison, WI, USA) and induced at 30°C for 3 hours in the presence of 1 mM isopropyl thiogalactopyranoside (IPTG). The cultures were harvested by centrifugation at 4,000×g for 20 min at 4°C, followed by resuspending the pellet in lysis buffer consisting of 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl with (for Ni-NTA column purification) or without (SEC-HPLC purification) 10 mM imidazole. The cells were lysed by 5 freeze-thaw cycles followed by digestion with 10 µg/ml DNase/RNase for 30 min at 37°C. The soluble fraction of the bacterial lysate was obtained by centrifugation at 20,000×g for 20 min at 4°C.

Triton X-114 (Sigma, Louis, MO) was used to remove endotoxin prior to purification (Aida and Pabst, 1990). Triton X-114 was added to the soluble lysate to a final concentration of 1%. The mixture was vortexed for 10s and incubated on ice for 5 min before it was vortexed again and then incubated at 37°C for 10 min. The mixture was centrifuged at 20,000 g for 2 min at 38°C and the upper aqueous phase containing rNcMIF was collected. This procedure was repeated 7 times for complete removal of endotoxin. rNcMIF and rNcMIFm were purified from the extract by size exclusion high performance liquid chromatography (SEC-HPLC) with a size exclusion column (7.7 mm × 300 mm, Biosuite 5 µm HR; Waters, MA).

The PBS consisting of 50mM Na₂HPO₄, 200 mM NaCl (pH 7.2) was used as the mobile phase and a flow rate of 0.5 ml/min was used. The lysate (10 mg/ml) was injected in a 100 µl volume and fractions (0.5ml/fraction) were collected and analyzed by SDS-PAGE and BCA assay (Thermo Scientific, Rockford, IL). rNcMIFhis was purified by a nickel-nitrilotriacetic acid (Ni-NTA) column under native conditions according manufacturers' instructions (QIAGEN Inc., Valencia, CA).

Proteins refolding was carried out as previously described (Lubetsky et al., 2002). In brief, lyophilized rNcMIFs were dissolved in 350 µl PBS with 8 M urea and incubated for 1 h at room temperature, followed by addition of 4 µl of 1 M dithiothreitol (DTT) with additional incubation for 1 h. DTT (350 µl at 10 mM) then was added, mixed, and incubated at room temperature for 30 min, this step was repeated for 3 consecutive times followed by a final incubation at 4°C for 30 min. The mixture was centrifuged at 14,000 rpm for 10 min at 4°C and the supernatant dialyzed overnight at 4°C against 10 mM DTT in Tris-buffered saline, pH 7.4 using a dialysis cassette MWCO7K (Pierce, Rockford, IL). Additional dialysis against Tris-buffered saline alone continued for 3 days. Dialyzed samples were collected and centrifuged at 4°C for 10 min at 20,000 g and stored until analyzed.

Purified rNcMIF was used to immunize a sheep to produce anti-NcMIF antiserum. Rabbit anti-rNcMIF antisera were produced by the Pacific immunology Corp. (Romona, CA) by immunizing 2 rabbits with purified rNcMIF. Animal care and use was approved by Beltsville Agricultural Research Center (BARC) Animal Care and Use Committee.

NcMIF Cross-linking

An NcMIF crosslinking experiment was performed as described previously (Mischke et al., 1998). In brief, rNcMIF at 2 µg/ml in 50 mM potassium phosphate buffer, pH 6.5 was incubated in the presence of 1% glutaraldehyde (Sigma, Louis, MO) for 3 h at room temperature. The cross-linked products were stabilized by addition of NaBH₄ (2M stock solution in 0.1 M NaOH) (Sigma, Louis, MO) to a final concentration of 50 mM. Sodium deoxycholate (Sigma, Louis, MO) was added at a final concentration of 0.01% 20 min later. The protein was then precipitated by the trichloroacetic acid method and analyzed by SDS-PAGE under reducing conditions. Proteins were visualized by silver staining using a staining kit (Sigma, Louis, MO).

Preparation of excretory and secretory antigen (ESAg) and whole parasite soluble antigen (NcAg) from the NC1 and NcTs-8 isolates

N. caninum tachyzoites were harvested from cell culture as described previously (Tuo et al., 2005; Yin et al., 2012). Briefly, purified tachyzoites were suspended to 10⁸ parasites/ml in RPMI 1640 (25 mM Glutamine, 50 µg/ml gentamicin) and cultured at 37°C for NC1 isolate and 32°C for NcTs-8 isolate and 4°C for both isolates with or without the treatment by 5 µM calcium ionophore (Sigma, Louis, MO) for 1 hour. The supernatant was collected by centrifugation at 20,000 g for 20 min at 4°C and stored at -70°C until use. The sample was heated for 10 min at 70 °C prior to analysis by SDS-PAGE. The NcAg was prepared by sonication of the tachyzoites with three 15s pulses at maximal frequency followed by centrifugation at 20,000 g at 4 °C for 20 min and the supernatant was collected and stored at 70°C until use.

Western Blot analysis of NcMIF in whole parasite lysate and ESAg

Western blotting was performed as described previously (Tuo et al., 2005). Briefly, samples were separated on a 15% SDS-PAGE gel under reducing conditions. After electrophoresis, protein was transferred to a PVDF membrane (Millipore, Bedford, MA). The blot was incubated in blocking solution of 3% skim milk in PBS for 1 h followed by incubation in

sheep anti-NcMIF antibody (1:500) in blocking solution for 1 h at room temperature. Following washing, the blot was incubated for 1 h with rabbit anti-sheep IgG-HRP (1:5000). The blot was then washed and developed with a chemiluminescence substrate. The image was captured using the α imaging system (describe softwares and give reference). To determine the relative levels of secreted NcMIF, ESAG was concentrated 20-fold by the trichloroacetic acid method. Briefly, TCA was added to 0.8 ml ESAG to a final concentration of 1%, and incubated on ice for 15 min followed by centrifugation at 14,000 g for 10 min. The pellet was washed twice with 100 μ l acetone, air-dried, and dissolved in 50 μ l of 2x SDS sample buffer. Concentrated ESAG in the amount of 2×10^7 tachyzoites equivalent was used in gel analysis. The integrated optical density (IOD) of NcMIF specific band was determined by the imaging system. The IOD for NcMIF released by tachyzoites at 4°C was subtracted from those for NcMIF secreted at 37°C prior to data analysis.

Immunolocalization of NcMIF in *N. caninum* tachyzoites

N. caninum tachyzoites (10ul) at 10^6 parasites/ml in PBS were pipetted onto individual wells of multi-well glass slides (Erie Scientific Co., Portsmouth, NH) and allowed to air-dry. After drying, the slides were immersed for 5 min in cold methanol and then blocked by 2% skim milk in PBS at room temperature for 30 min. After washing, the slides were incubated for 2 h at room temperature with a 1:100 dilution of rabbit anti-NcMIF or pre-immune serum. Following 5 washes with PBS, FITC-labeled anti-rabbit IgG (Sigma, Louis, MO) was added and incubated for 1 h at room temperature. After washing with PBS, the slides were overlaid with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and cover-slipped. The image was taken using a fluorescence microscope (Carl Zeiss MicroImaging, Inc, Thornwood, NY).

Immunogold electron microscopy (IEM) staining was performed as reported elsewhere (Jenkins et al., 2009). In brief, *N. caninum* tachyzoites were pelleted by centrifugation for 2 min at 5000 g and resuspended in 100 μ l fixative consisting of 3% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M cacodylate buffer. Following a 5 min fixation, tachyzoites were collected by centrifugation for 5 min at 5000 g, and washed with cacodylate buffer, and then mixed to form a dispersed pellet. The tachyzoites were then dehydrated in a graded ethanol series, infiltrated overnight with LR White hard grade acrylic resin (London Resin Company, London, UK), and cured at 55°C for 24 h. Sections (90 nm thickness) were obtained using a Diatome diamond knife on a Reichert/AO Ultracut microtome and collected on 200-mesh Formvar-coated nickel grids. These grids were floated on drops of PBS containing 0.1M glycine and 1% bovine serum albumin and on drops containing PBS-T-NFDM. Grids were incubated for 2 h at room temperature on drops of PBS containing a 1:100 dilution of sheep anti-NcMIF serum, 1:100 dilution of rabbit anti-NcMIF, or pre-immune sera. The grids were rinsed with PBS and incubated for 1 h at room temperature on PBS containing a 1:100 dilution of gold-labeled anti-sheep IgG or anti-rabbit IgG (KPL, Gaithersburg, MD). Following sequential washes with PBS, PBS and H₂O, grids were air-dried, stained with 5% uranyl acetate for 30 min, and examined with a HT 7700 electron microscope.

Tautomerase and oxidoreductase assays

The tautomerase activity of the NcMIF was determined as reported previously (Pennock et al., 1998). In brief, 10 mM stock solutions of L-3,4-dihydroxyphenylalanine methyl ester (Sigma, Louis, MO) and 20 mM stock sodium periodate (Sigma, Louis, MO) were prepared in water. Seven hundred twenty two microliter of the 50 mM sodium phosphate, pH 6.2, 1 mM EDTA, was mixed with 48 μ l of L-3,4-dihydroxyphenylalanine methyl ester and 32 μ l of periodate to generate L-dopachrome methyl ester in a 1 ml polystyrene disposable cuvette. The spontaneous decay of the substrate at A₄₇₅ due to decarboxylation was also

monitored. The refolded or non-refolded rNcMIF, rNcMIFm or rNcMIFhis at a final concentration of 10 µg/ml were added to the cuvette and read for 20 min using a spectrophotometer (Beckman, Atlanta GA). The rate of tautomerization is expressed as uM/min/mg and calculated by the initial change of absorbance based on the extinction coefficient of the substrate dopachrome.

The oxidoreductase activity of NcMIF and NcMIFm were determined by the insulin reduction assay (Chandler and Varandani, 1975). Briefly, the reaction was initiated by adding 49 µl rNcMIF or mMIF and 6 µl 200 mM reduced glutathione (GSH) (Sigma, Louis, MO) to 195 µl of ice-cold 1 mg/ml insulin (Sigma, Louis, MO) in a buffer containing 100 mM potassium phosphate buffer (pH 7.2) and 2 mM EDTA. The MIF-catalyzed insulin reduction was measured against the control solution which contained GSH only. The absorbance was measured at 650 nm for 96 min at 23°C using a spectrophotometer (Beckman, Atlanta, GA).

Immunoregulatory activity of NcMIF

Glucocorticoid counter-regulatory and nitric oxide (NO) and TNF- inducing activities of NcMIF was assessed as described previously (Calandra et al., 1995; Calandra and Bucala, 1995). Briefly, murine RAW264.7 macrophages were plated in 48-well plates at 3×10^5 cells/ml in 500 µl DMEM containing 10% FBS, 25 mM glutamine, and 50 µg/ml gentamicin and cultured for 20–24 h at 37°C in the presence of 5% CO₂. To determine the glucocorticoid counter-regulatory activity of NcMIFs, cells were pre-incubated with or without dexamethasone (DEX, 10nM) (Sigma, Louis, MO) alone or in the presence of increasing concentrations of rNcMIF, rNcMIFm or rNcMIFhis for 1 h prior to addition of LPS (*E. coli* O111:B4, Sigma-Aldridge, Louis, MO) at a final concentration of 10ng/ml. Following additional 16 h of incubation, cell culture supernatants were collected and centrifuged at 20,000 g at 4°C for 20 min. To assess the immunoregulatory function of NcMIF on TNF- and NO production, cells were treated with increasing concentrations of rNcMIF, rNcMIFm or rNcMIFhis in the presence or absence of 10 nM LPS for 16 h and supernatants were collected and stored at –80°C until assayed. TNF- and NO in supernatants were determined by ELISA kit (eBioscience Inc, San Diego, CA) and by Griess reaction assay, respectively. Griess reaction assay was carried out as described elsewhere (Straccia et al., 2011). In brief, 150 µl of the sample was added into wells of 96-well plates, followed by addition of 20 µl of 1µM nicotinamide adenine dinucleotide phosphate (Sigma, Louis, MO) and 30 µl of the master mix consisting of glucose-6-phosphate (Sigma, Louis, MO), glucose-6-phosphate dehydrogenase (Sigma, Louis, MO) and Nitrate reductase (Roche Diagnostics, Indianapolis, IN). The plate was mixed for half hour and then 20 µl 1% sulfanilamide and 20 µl 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride (Sigma, Louis, MO) were added. After 5 min incubation, the plate was read at 550nm and 650nm by a microplate reader (Molecular Devices SpectraMax Plus384, Ramsey, MN). Nitrite concentration was determined from a sodium nitrite standard curve.

Analysis of binding of rNcMIF to human CD74

rNcMIFs were tested for binding to the human MIF receptor, CD74, as described previously (Leng et al., 2003). In brief, 96-well plates were coated with recombinant, soluble CD74 ectodomain (sCD74^{73–232}). Biotinylated recombinant human MIF (rhMIF, 2 ng/µl) (Roche Applied Sciences) was added together in triplicate wells with increasing concentrations of purified (non-biotinylated) rNcMIF or rNcMIFm. The bound biotinylated rhMIF was detected by adding streptavidin-conjugated alkaline phosphatase (R&D), followed by detection of the alkaline phosphatase with p-nitrophenyl phosphate (Sigma). Plates were read at 405 nm and plotted as percent A405 relative to wells containing biotinylated rhMIF alone.

RESULTS

NcMIF sequence and phylogenetic analyses

The NcMIF gene was cloned from the NC1 tachyzoite mRNA and showed 100% identity to NcMIF cDNA from the *N. caninum* Liverpool (FR823385). This gene was predicted to encode a 116-amino acid polypeptide which appeared to be similar to MIFs of other protozoan parasites. It has a single amino acid insertion at the 12th position when compared to the mammalian MIFs (Fig. 1A). The calculated NcMIF molecular mass was 12.2kDa. A sequence alignment and phylogenetic analysis (CLUSTALW, <http://www.ebi.ac.uk/Tools/msa/clustalw2/>) of MIF aa sequences from protozoan parasites and mammals revealed that NcMIF shares the highest homology (81.9%) with *T. gondii* MIF, but only shares moderate homology with human and mouse MIFs (24.3–27.0%) or other protozoan parasite MIFs (31.0–36.5%) (Fig. 1A and B). NcMIF does not appear to have a conventional signal sequence (SignalP 4.0, <http://www.cbs.dtu.dk/services/SignalP/>). It has been predicted to have 6 conserved amino acid residues in mammalian MIFs that are essential for the tautomerase activity (Bendrat et al., 1997), however, NcMIF only retained Pro2 (*i.e.* Pro1 after proteolytic processing of Met1), which is the catalytic residue for substrate tautomerization, Lys33, Ile65, and Tyr96, but not Asn98 and Val107 (Fig. 1A, marked by asterisks). The Cys3 and Cys4 present in *Plasmodium* spp. was predicted to be responsible for its chemokine function, however, NcMIF and TgMIF only have the Cys4 but not the Cys3 (Fig. 1A, marked by open triangles). The Cys57-X-X-Cys60 motif identified in mammalian MIFs which is predicted to be responsible for mediating the catalytic oxidoreductase activity (Nguyen et al., 2003) is absent from NcMIF and TgMIF by substitution of the Cys 60/59 by Ile59 (Fig. 2A, identified by filled triangles).

Expression and purification of rNcMIFs

rNcMIF, rNcMIFhis and rNcMIFm were expressed in *E.coli* BL21 (DE3) as soluble proteins. SDS-PAGE analysis showed that both rNcMIF and rNcMIFm had a molecular mass of 12kDa and rNcMIFhis a molecular mass of 15kDa (Fig. 2, insert). Mutation of Pro2 to Gly2 did not alter the migration pattern of rNcMIF on SDS-PAGE (Fig. 2, insert). Both rNcMIF and rNcMIFm were purified by SEC-HPLC and exhibited a single absorbance peak at 280 nm. However, the mutation of Pro2 to Gly2 in rNcMIFm resulted in a prolonged retention time (35–45 min) as compared to that (18–19 min) of rNcMIF on SEC-HPLC (Fig. 2), although these two proteins had a similar molecular mass as analyzed by SDS-PAGE (Fig. 2, insert). rNcMIFhis with a polyhistidine tag at the N-terminus was purified on Ni-NTA column. The purified rNcMIFhis was further analyzed by SEC-HPLC and showed that its retention time was similar to that for rNcMIF, but not to rNcMIFm (data not shown).

Cross-linking of rNcMIFs

To demonstrate the oligomerization state of rNcMIFs, all 3 forms of rNcMIFs were crosslinked as described previously and analyzed under reducing conditions (Mischke et al., 1998). Crosslinking experiment showed that all 3 rNcMIFs without crosslinking were predominantly in the monomer form, but the dimer was also evident (Fig. 3A–C). rNcMIF existed in equal amount as monomer, dimer and trimer, and low levels of higher oligomerization were apparent (Fig. 3A). rNcMIFm was primarily present as a monomer with minor species of both dimer and trimer (Fig. 3B). Intriguingly, rNcMIFhis existed primarily as a trimer with low levels of monomer and dimer together with higher oligomerization forms (Fig. 3C).

Analysis of NcMIF in whole tachyzoite lysate and ESAG prepared from NC1 and Ncts8 isolates

The level of NcMIF in equal amount of ESAG (2×10^7 equivalent) from NC1 or Ncts8 tachyzoites prepared under various conditions was analyzed by Western blotting. Low levels of NcMIF were detected in ESAG prepared at 4°C (Fig. 4A, lanes 7 and 8). The release of NcMIF by NC1 tachyzoites was not increased at 37°C when compared to that at 4°C (Fig. 4A, Lane 4 and Fig. 4B), however, NC1 NcMIF release was dramatically increased (by approximately 85 fold) when co-cultured with calcium ionophore (Fig. 4B, Lanes 4 vs. Lane 6) ($P < 0.05$). The basal level of NcMIF release by Ncts8 tachyzoites was higher ($P < 0.05$) than that of NC1 tachyzoites, but treatment with calcium ionophore was only able to enhance NcMIF release by approximately 6 folds ($p < 0.05$) above the basal release. Overall, higher ($P < 0.05$) levels of NcMIF was secreted at either 4°C or 32°C by Ncts8 when compared to those of NC1 tachyzoites. In addition, NcMIF in whole parasite lysate was higher ($p < 0.05$) in Ncts8 than in NC1 tachyzoites.

Immunolocalization of *N. caninum* tachyzoites

No apparent difference in NcMIF staining was seen in NC1 and Ncts8 phenotypes (data not shown). NcMIF appeared to be localized to the apical end of the tachyzoites of NC1 isolate (Fig. 5A panel f). The similar staining pattern was not observed when PBS alone, goat anti-rabbit IgG-FITC alone, pre-immune sera, or a irrelevant immune serum was used (Fig. 5A, a, b, c, and d). Rabbit anti-Neospora profilin (Jenkins et al., 2010) was used as positive control and its localization was more diffuse than that of NcMIF which was intensely localized to the tip of the apical end (Fig. 4A, e and f). In immunoelectron microscopy, NcMIF was specifically localized to the rhoptries and micronemes (Fig. 5b, c, and d), dense granules (Fig. 5e) and nuclei (Fig. 5f).

rNcMIF does not possess tautomerase and oxidoreductase activities

Tautomerase activity is the signature property for MIF although little is known about its physiological relevance. To determine this activity in rNcMIF with or without refolding, the dopachrome substrate was used in the tautomerase assay. The mouse MIF used as a positive control had tautomerase activity detectable at 0.6 µg/ml at 315 µM/min/mg, as reported previously (Fig. 6A). However, rNcMIF or rNcMIFm at 10 µg/ml had no detectable tautomerase activity before or after the recombinant proteins were subjected to refolding (Fig. 6B). rNcMIFhis had very low but detectable tautomerase activity at high concentrations (10 µg/ml); this activity was attributable to imidazole present in the elution buffer used during protein purification (data not shown). rNcMIF also showed no detectable oxidoreductase activity (data not shown).

rNcMIF does not have detectable immunoregulatory activities and does not bind to the MIF receptor, human CD74

rNcMIF, rNcMIFm, or rNcMIFhis with a purity of 98% or greater and undetectable levels of endotoxin, with or without a refolding, had no effect to counter-regulate glucocorticoid activity in macrophages or to regulate TNF and nitric oxide production by macrophages (data not shown). In addition, both rNcMIF and rNcMIFm failed to compete with the rhMIF for binding to the human MIF receptor ectodomain CD74^{73–232} (Fig. 7).

DISCUSSION

A MIF homologue from *N. caninum* characterized in the present study was highly similar (81.9%) to the MIF of its close family member, *T. gondii*. As anticipated, it only shared moderate sequence homology with the mammalian MIFs or those of other parasites. Despite

limited homology, the NcMIF has most of the aa residues characteristic of the mammalian MIF. This was the basis of our rationale that the NcMIF should also share similar biological activities similar to the mammalian MIFs.

Surprisingly, the NcMIFm (NcMIF mutated at the second proline to glycine) produced for the comparison of tautomerase activity was shown structurally different from its wild type. This was demonstrated by increased retention time on SEC-HPLC and changes in oligomer ratios in crosslinking experiments. The NcMIF exhibited a similar pattern of oligomer formation in human MIF (Mischke et al., 1998). Strikingly, the single aa mutation impaired the formation of both dimers and trimers. Furthermore, addition of a HIS-tag (a total of 34 aa) at the N-terminus increased the formation of trimers. These results suggest that alteration or chemical modification of aa sequence at the N-terminus of MIF can significantly change the structure of this protein, which may or may not be essential for functions. Previous reports indicate the presence of prolines in an aa sequence is critical for protein conformation and configuration (ref). However, the role of proline in NcMIF structure remains to be elucidated.

NcMIF possesses the key proline at position 2 and the other three associated residues (Lys33, Ile65, Tyr96) out of the five residues essential for tautomerase (Bendrat et al., 1997). One of the intriguing findings of the study was that both NcMIF and NcMIFm lacked the tautomerase activity, in contrast to other protozoan MIFs which possess this signature function (Augustijn et al., 2007; Richardson et al., 2009). The lack of this activity may not be due to a folding issue because we attempted to refold the proteins and the refolded proteins were still inactive in catalyzing the substrate. We have detected low levels of tautomerase activities in the NcMIFhis preparations, but this was later confirmed due to the presence of low levels of imidazole which was used for protein purification (data now shown). We speculate that the proline may be important for the tautomerase activity of all MIF homologues, but the other aa residues/sequence that involved in the formation of the tautomerase active site may also be critical for this function. For NcMIF, the tautomerase active site may not be functional due to the lack of other aa residues or sequences. Similarly, the oxidoreductase activity was also lacking in both NcMIF and NcMIFm, which may be explained that the one of the two cysteines critical for this function was missing in NcMIF (Alam et al., 2011). The lack of MIF-like activities may be further supported by the lack NcMIF binding to the MIF receptor, CD74 (Leng et al., 2003). Although there is no evidence that *N. caninum* may infect humans, this parasite could practically infect cells *in vitro* from all species tested. In addition, bovine CD74 shares high aa sequence homology with human CD74, and the fragment of human CD74 used for the binding experiment was 77% similar to the corresponding sequence of bovine CD74 (Cho et al., 2007; Leng et al., 2003), suggesting structural similarity between human and bovine CD74 molecules.

NcMIF was primarily localized to the apical end of the tachyzoite. Immunoelectron microscopy further revealed that NcMIF was localized in structures likely associated with the rhoptries, micronemes, dense granules, and nuclei. Of particular note is that NcMIF was clearly present in the nuclei. There were reports describing the localization of MIF in nuclei (Kamimura et al., 2000; Vedder et al., 2000), but these observations were not reported in later studies. NcMIF, as shown in the present study, as well as MIFs from other species does not contain a signal sequence or a nuclear localization signal (Arnoys and Wang, 2007), but the localization in the cytoplasm and nucleic as well as the extracellular compartments was apparent (Vedder et al., 2000). The present study clearly demonstrated that NcMIF was abundant in tachyzoite lysate and was also present in the ESAG preparation, suggesting that NcMIF is secretory which makes it possible for NcMIF to function as a cross-regulatory immune modulator of the host immune responses (Kleemann et al., 2000). It is interesting that the Ncts-8 clone produced more NcNIF than the NC1 isolate; however, the magnitude

of increase in NcMIF secretion by the NC1 isolate was dramatically more than that of the Ncts-8 clone when stimulated by the calcium ionophore. This may be due to the fact that the NC1 isolate had a lower basal secretion of NcMIF and may further suggest that NcMIF is released by the micronemes in a similar fashion of the microneme protein release (Lovett et al., 2002). Most strikingly, it was shown that increased production of MIF by the transgenic *P. yoelii* resulted in attenuation of this parasite, leading to reduced pathogenesis (Thorat et al., 2010). In patients infected *Plasmodium* spp, the parasite MIF is readily detectable in circulation and the level correlates with patient temperature, parasitemia, and levels of cytokines and circulating human MIF (Han et al., 2010; Shao et al., 2008).

Both *Leishmania* and *Plasmodium* MIFs have been shown to be biologically active. However, the present study found that NcMIF lacks the tautomerase activity and the bioactivity in modulating host immune cell responses. The differential production of NcMIF by pathogenic and non-pathogenic isolates of *N. caninum* may suggest that NcMIF is a key molecule mediating parasite-host interactions and possesses unconventional functions. Alternatively it remains possible that the function of NcMIF is limited to the life cycle of *N. caninum* and not to modulation of the host-parasite interaction. Nonetheless, the role of NcMIF in *N. caninum* pathogenesis remains to be investigated. Additional comparative research may be done to elucidate the mechanisms by which structural changes in MIF lead to corresponding functional changes.

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Fig. 1. Sequence alignment (A) and phylogenetic analysis (B) of representative MIF orthologues from Apicomplexan parasites and mammals. The sequences include those encoding the *Homo sapiens* MIF (NP_002406), *Mus musculus* MIF (NP_034928), *P. falciparum* MIF (AAN36370), *E. tenella* MIF (ABC73371), *N. caninum* MIF (CBZ51173), *T. gondii* MIF (ABC69140), and *L. major* MIF (XP_001685935). The six amino acid residues in mammalian MIF previously predicted to interact with the tautomerase substrate are marked by asterisks, the chemokine motif of CXXC present in mammalian MIF is marked by filled triangles and the adjacent Cys2 and Cys3 present in *P. falciparum* MIF are labeled with open triangles.

Fig. 2.

Purification of rNcMIF and rNcMIFm by size exclusion chromatography on HPLC and analysis by SDS-PAGE. The chromatograph for both purified rNcMIF (first peak in solid line) and rNcMIFm (second peak in broken line) obtained at the same conditions is shown. The coomassie blue stained SDS-PAGE gel for the analysis of purified rNcMIFs is shown in the insert. M, molecular weight ladder; Lane 1, purified rNcMIF; Lane 2, purified rNcMIFm.

Fig. 3.

Chemical crosslinking of rNcMIF (A), rNcMIFm, and rNcMIFhis (C) and analysis by silver-stained 4–12% NuPAGE. Recombinant protein (2ug) was chemically crosslinked, precipitated by the TCA method prior to separation on NuPAGE. Lane 1, protein fixed by NaBH₄ without 1% glutaraldehyde; Lane 2, protein treated with 1% glutaraldehyde without NaBH₄ fixation; Lane 3, protein treated with 1% glutaraldehyde followed by NaBH₄ fixation; and Lane 4, protein without any treatment. M, monomer; D, dimer; T, trimer. Molecular weight ladder is shown to the left. The results represent 3 experiments.

Fig. 4.

Analysis of the presence and relative levels of NcMIF in whole tachyzoite lysate (NcAg) and secretory products (ESAg) prepared from the NC1 and Ncts-8 isolates. ESAG was prepared from purified tachyzoites and concentrated 20-fold using the TCA method and concentrated ESAG in the amount of 2×10^7 tachyzoites equivalent was subjected to analysis by Western blotting using a sheep anti-rNcMIF serum at 1:500 dilution. A, Western blotting of *N. caninum* ESAG; and B, integrated optical density analysis of the NcMIF band (Lanes 4 through 7) in NcESAG from NC1 and Ncts8 in the absence or presence of calcium ionosphere. Lane 1, rNcMIFhis (25 ng/lane); Lane 2, rNcMIF (25 ng/lane); Lane 3, rNcMIFm (25 ng/lane); Lane 4, NC1 EsAg at 37°C; Lane 5, Ts-8 EsAg at 32°C; Lane 6, NC1 EsAg in the presence of 5 μM calcium ionophore at 37°C; Lane 7, Ts-8 EsAg in the presence of 5 μM calcium ionophore at 32°C; Lane 8, Nc1 EsAg at 4°C; Lane 9, Ts-8 EsAg at 4°C; and Lane 10, NcAg of Nc1 (5 μg/lane). C, Western blotting of NcAg and Ncts-8Ag; and D, integrated optical density analysis of the NcMIF band in NcAg from NC1 and Ncts8 isolates. Lane 1, NcAg (10 μg/ml); Lane 2, Ncts-8Ag (10 μg/ml). The level of NcMIF was assessed by integrated optical density using an imaging system and data represent the mean $\pm$ standard error of 3 independent experiments.

Fig. 5. Immunolocalization of NcMIF in *Neospora* tachyzoites by indirect fluorescence assay (IFA) (A) and immunoelectron microscopy (B). A, IFA using a rabbit anti-NcMIF serum as first antibody and goat anti-rabbit IgG-FITC as secondary reagent. A: Localization of NcMIF by IFA. a, PBS alone without application of antibodies; b, second antibody (1:100) alone; c, pre-immune rabbit serum (1:100 dilution); d, rabbit antisera to a non-*N. caninum* recombinant protein (beta-giardin of *Giardia lamblia*) (1:100 dilution) as an irrelevant antibody control; e, rabbit antisera to recombinant *Neospora* profilin (1:100 dilution) as a positive control; f, rabbit antisera against rNcMIF (1:100). B, localization of NcMIF by immunoelectron microscopy using sheep antiNcMIF as first antibody and rabbit anti-sheep IgG-gold as secondary antibody. R, rhoptry; M, microneme; A, apical; N, nucleus; DG, dense granule. a, 10,000x; b-f, 50,000x.

Fig. 6.

Dopachrome tautomerase activity of mMIF (A) and rNcMIFs (B). mMIF and rNcMIF tautomerase activities were assayed using the dopachrome substrate by a spectrophotometer using a wavelength of 574 nm. The reaction was monitored at a 30s interval for a total of 20 min. mMIF was used as a positive control in the dopachrome tautomerase activity at a final concentration of 0.5 μ g/ml (A). rNcMIF and rNcMIFm at a final concentration of 10 μ g/ml were assayed for the dopachrome tautomerase activity (B). An equivalent volume of PBS was used in place of MIF to determine the spontaneous substrate decay. Data represent 3 independent experiments.

Fig. 7. Comparison of binding by rNcMIF, and rNcMIFm, rhMIF to the human MIF receptor ectodomain (sCD74) in an *in vitro* capture assay using biotinylated-rhMIF as competitor. The data represent three independent experiments. Each data point represents mean $\pm$ SEM.