Distinct immunoregulatory properties of macrophage migration inhibitory factors encoded by *Eimeria* parasites and their chicken host

Seung I. Jang a,1, Hyun S. Lillehoj a,b, Sung Hyen Lee a, Duk Kyung Kim a, Marc Pagès a,2, Yeong Ho Hong b, Wongi Min c, Erik P. Lillehoj d

a Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705, United States
b Department of Animal Science and Technology, Chung-Ang University, Anseong, Gyeonggi-Do 456-756, South Korea
c College of Veterinary Medicine & Research Institute of Life Science, Kyungpook National University, Jinju 660-701, South Korea
d Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD 21201, United States

**A R T I C L E   I N F O**

Article history:
Received 19 May 2011
Received in revised form 22 August 2011
Accepted 10 September 2011
Available online 21 September 2011

**Keywords:**
MIF
Chicken
Coccidiosis
Eimeria
Immunomodulation

**A B S T R A C T**

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that plays an important role in host defense against a variety of microorganisms including protozoan parasites. Interestingly, some microbial pathogens also express a MIF-like protein, although its role in disease pathogenesis is not well understood. The aim of this study was to compare an *Eimeria*-encoded MIF (E.MIF) protein with chicken MIF (C.MIF) on the basis of their structural, immunological, and biological properties. E.MIF and C.MIF proteins, each with a glutathione S-transferase epitope tag, were expressed in *Escherichia coli* or COS-7 cells and purified by glutathione affinity chromatography. Rabbit antiserum against the purified proteins demonstrated their mutual immunological cross-reactivity on Western blots, and immunolocalized intracellular native E.MIF to the *Eimeria* schizont, merozoite, and oocyst life cycle stages. HD11 chicken macrophages treated in vitro with C.MIF recombinant protein expressed increased levels of transcripts encoding interleukin-6 (IL-6), IL-17, and tumor necrosis factor superfamily member 15 (TNFSF15), but decreased levels of IL-8 transcripts, compared with cells treated with the PBS control; similar treatment with E.MIF only down-regulated IL-8 transcripts. Unlike recombinant E.MIF, C.MIF exhibited in vitro chemotactic activity for HD11 cells. Conversely, E.MIF, but not C.MIF, enhanced protection against experimental *Eimeria* infection, compared with the PBS control. These studies provide evidence for overlapping structural and antigenic properties, but distinct immunoregulatory roles, of E.MIF and C.MIF.

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1. Introduction

Macrophage migration inhibitory factor (MIF) is a conserved 12.5 kDa proinflammatory cytokine that is expressed in invertebrates and vertebrates [1]. MIF was originally identified as a soluble factor from sensitized guinea pig peritoneal lymphoid cells which inhibited the *in vitro* migration of normal peritoneal exudate macrophages [2]. In mammals, MIF is critically involved in the pathogenesis of immune and inflammatory diseases, including septic shock, rheumatoid arthritis, inflammatory bowel disease, and tumor metastasis [3–5]. It has been speculated that MIF may play a role in the regulation of macrophage function during host defense against infectious pathogens through suppression of the anti-inflammatory effects of glucocorticoids [6,7]. However, the biological mechanism and significance of MIF in immunity remain to be elucidated.

Recently, our laboratory cloned an MIF-like gene from *Eimeria acervulina* (E.MIF), one of the seven species of *Eimeria* protozoa responsible for avian coccidiosis [8]. This full-length clone encoded a 116-amino acid protein that shares between 35% and 38% amino acid identity with MIFs of vertebrates, including chicken MIF (C.MIF). By comparison there is 71% amino acid identity between human and mouse MIF [9]. Gene expression analysis suggested that the E.MIF transcript was expressed primarily during the merozoite developmental stage. Interestingly, high levels of C.MIF transcripts were detected in the gut of *Eimeria tenella*-infected chickens, suggesting co-expression of these biologically related molecules during avian coccidiosis. However, comparisons of the biological, immunological, and functional activities of E.MIF and C.MIF have not been reported. Therefore, in this study, we expressed and purified recombinant forms of both MIF polypeptides and evaluated...
2. Materials and methods

2.1. Cloning and purification of recombinant E.MIF and C.MIF proteins

The full-length E.MIF gene (GenBank accession number DQ332351), originally cloned in the pET28(a) vector [8], was subcloned into the pGEX vector (GE Healthcare Biosciences, Piscataway, NJ) with a glutathione S-transferase (GST) epitope tag. The full-length C.MIF gene with a GST epitope tag was amplified by PCR from total RNA using oligonucleotide primers based on the published sequence [10] (GenBank accession number M95776). Both recombinant proteins were expressed in Escherichia coli BL21 in TY broth containing 100 μg/ml ampicillin. The bacteria were grown to an optical density at 600 nm of 0.5, induced with 1.0 mM isopropyl-

2.4. Immunofluorescence microscopy

Three-week-old chickens were orally infected with 2.0 × 10⁴ E. acervulina sporulated oocysts as described [11] using protocols approved by the Beltsville Area Institutional Animal Care and Use Committee. At 5 and 7 days post-infection, the intestinal duodenum was removed, snap-frozen in liquid nitrogen, and 5 μm sections on glass slides were air-dried and fixed in acetone for 10 min (Sakura, Torrance, CA). Tissue sections were embedded in OCT compound and incubated overnight at 4 °C with anti-E.MIF or anti-C.MIF antibodies (1:50 in 1.0% horse serum). The slides were washed, incubated at room temperature for 2 h with AlexaFluor 488-conjugated goat anti-rabbit IgG secondary antibody (1:500) (Invitrogen), washed, counterstained with 0.01 mg/ml propidium iodide, and examined using a confocal microscope (LSM 510 META, Carl Zeiss, Thornwood, NY) with 488 nm and 543 nm laser excitation lines. In other experiments, merozoites were collected from E. acervulina-infected chickens as described [8], spread onto poly-L-lysine-coated glass slides (Sigma) by cytocentrifugation (Shandon Southern Products, Cheshire, England), and immunostained with anti-E.MIF antibody.

2.5. Macrophage migration inhibition assay

HD11 chicken macrophages (5.0 × 10⁶ cell/ml) were added to the upper chambers of Transwell tissue culture inserts containing 5.0 μm pore membranes (Costar, Lowell, MA). One hundred microgram of purified recombinant E.MIF or C.MIF proteins expressed in COS-7 cells and E.MIF or C.MIF proteins pre-incubated with each antibody were added to the lower chambers and incubated at 41 °C for 24 h in 5% CO₂. As a negative control, an equivalent volume of culture medium was added to the lower chamber. Following incubation, the Transwells were removed and the number of cells in the lower chambers was enumerated by light microscopy.

2.6. Quantitative RT-PCR

Confluent cultures of HD11 macrophages in 24-well plates were incubated at 37 °C for 48 h with 100 μg of purified recombinant E.MIF or C.MIF expressed in COS-7 cells. Total RNA was extracted using TRIzol (Invitrogen) and 1.0 μg was incubated at room temperature for 15 min with 1.0 U of DNase I and 1.0 μl of 10× reaction buffer (Sigma), followed by 1.0 μl of stop solution and heating at 70 °C for 10 min. RNA was reverse transcribed using the Affinity Script Multiple Temperature cDNA Synthesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s recommendations. Quantitative RT-PCR oligonucleotide primers for chicken interleukin-1β (IL-1β), IL-6, IL-8, IL-17, and tumor necrosis factor superfamily member 15 (TNFSF15) are listed in Table 1. Amplification and detection were carried out using equivalent amounts of total RNA using the Mx3000P system and Brilliant SYBR Green PCR master mix (Stratagene) as described [12]. Standard curves for each transcript were generated using log₁₀-diluted standard RNA and levels of individual transcripts were normalized to those of GAPDH analyzed by the Q-gene program [13]. Each analysis was performed in triplicate. To normalize RNA levels between samples within an experiment, the mean threshold cycle (Cₜ) values for the amplification products were calculated by pooling values from all samples in that experiment.

2.2. Western blot analysis

Purified recombinant E.MIF and C.MIF proteins expressed in E. coli were heated at 94 °C for 4 min in 0.125 M Tris–HCl, pH 6.8, 4.0% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.004% bromophenol blue, and resolved on 10% SDS-polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose (Immobilon-P, Millipore, Billerica, MA), and the membranes were treated with SuperBlock buffer (Fisher, Pittsburgh, PA) at 4 °C for 2 h and incubated with rabbit antisera commercially prepared against recombinant E.MIF or C.MIF proteins (Pacific Immunology, Ramona, CA) diluted 1:50 in PBS at 22 °C for 1 h. Membranes were washed 3 times with PBS and incubated at 22 °C for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Sigma). The membrane was washed 3 times with PBS, 3 times with distilled water, and developed with 4-chloro-1-naphthol substrate (Sigma).

2.3. Parasites

The wild type strains of E. tenella and E. acervulina were originally developed and maintained at the Animal Parasitic Disease Laboratory of the Animal and Natural Resources Institute (Beltsville, MD). Oocysts were cleaned by floatation on 2.5% sodium hypochlorite, washed 3 times with PBS, and counted using a hemocytometer.

their abilities to modify body weight gain and fecal oocyst shedding in an in vivo experimental model of avian coccidiosis, and to modulate the migration and expression of proinflammatory cytokine and chemokine genes by chicken macrophages cultured in vitro.
The predicted 116 amino acid open reading frames of E.MIF and C.MIF share 70.7% (82/116) sequence homology, 36.2% (42/116) sequence identity, and 55.6% (10/18) sequence identity within the 18 residues thought to contribute to the catalytic site of the parasite [8] (Fig. 1). Therefore, it was of interest to assess the immunological cross-reactivities of these homologous, but distinct, polypeptides. Both proteins were expressed with GST epitope tags in COS-7 cells and purified from spent cell culture media by glutathione-agarose affinity chromatography. As previously reported [9], a single Coomassie blue-stained band was detected (data not shown). As shown in Fig. 2A, purified E.MIF and C.MIF proteins were equally reactive on Western blots with a rabbit antiserum prepared against purified recombinant E.MIF. Similarly, a separate rabbit antiserum against recombinant C.MIF recognized both proteins equally well (Fig. 2B). Pre-immune sera from both rabbits showed no reactivity with either protein (data not shown).

### 3. Results

#### 3.1. Homology of recombinant E.MIF and C.MIF proteins

Our prior study suggested developmental regulation of E.MIF gene expression in various life cycle stages of the parasite, and immunofluorescence staining of *E. acervulina* merozoites indicated that native E.MIF protein was distributed throughout the cytosol of the parasite [8]. Therefore, we asked whether a corresponding change in E.MIF protein levels were apparent in the different stages of the microorganism. Intestinal duodenum

#### 3.2. Developmental expression of native E.MIF protein

Table 1

<table>
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2.7. In ovo immunization, *E. tenella* infection, and measurements of body weight gains and fecal oocyst shedding

Eggs of commercial broiler chickens (Moyer’s Chicks Inc., Quakertown, PA) were randomly distributed into six groups (20 eggs/group) and injected with 100 μl of sterile PBS, pH 7.4, or 50 or 100 μg of recombinant E.MIF or C.MIF proteins expressed in COS-7 cells at day 18 of embryonic development using a 17.5 cm 18-gauge needle with the Intellitect system (AviTech, Easton, MD) as described [14]. At day 11 post-hatch, the chickens were orally challenged with 1.0 × 10⁵ sporulated oocysts of *E. tenella*. Body weight gains were determined between days 0 and 10 post-infection. Fecal samples were collected daily between days 5 and 10 post-infection and oocysts were enumerated using a McMaster counting chamber as described [14].

2.8. Statistical analyses

All data were expressed as mean ± S.D. values and subjected to one-way analysis of variance using SPSS software (SPSS 15.0 for Windows, Chicago, IL). The Duncan’s multiple range test was used to analyze differences between mean values. Differences were considered statistically significant at *P* < 0.05.

![Fig. 1. Amino acid sequence alignment of E.MIF and C.MIF proteins. Predicted amino acid sequences of E.MIF (GenBank accession number DQ332516) and C.MIF (GenBank accession number M95776) were aligned using ClustalW. The 18 residues in C.MIF that are believed to be involved in the formation of the catalytic site are underlined [14]. ++, identical residues; --, conserved substitutions; --, semi-conserved substitutions; --, gaps to maximize alignment.](image)

![Fig. 2. Western blot analysis of recombinant E.MIF and C.MIF proteins. Purified recombinant C.MIF (lane 1) and E.MIF (lane 2) proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with rabbit anti-E.MIF antibody (A) or rabbit anti-C.MIF antibody (B) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody and 4-chloro-1-naphthol substrate. M, molecular weight markers indicated on the right in kiloDaltons (kDa).](image)
tissue sections taken from chickens at 5 or 7 days post-infection with *E. acervulina* contained parasites that were immunoreactive with the antiserum prepared against the recombinant E.MIF protein. At day 5 post-infection, schizonts were visualized in the tissues that were stained with the anti-E.MIF antibody (Fig. 3A). At day 7 post-infection, oocyst immunostaining was apparent (Fig. 3B). Identical results were observed using the cross-reactive anti-C.MIF antibody (Fig. 3C and D, respectively). Host C.MIF outside of the parasites was not seen in any of these tissue sections. Next, *E. acervulina* merozoites were harvested from the duodenal loop of chickens at day 4 post-infection, purified using an established method [15], and subjected to immunofluorescence staining using the anti-MIF antiserum. The anti-E.MIF antiserum stained the outer membrane, apical end, and nucleus of the merozoites (Fig. 4A), whereas the cross-reactive anti-C.MIF antiserum prominently stained the entire cytosol of the merozoites (Fig. 4B). No immunostaining of any parasite developmental stage was observed using pre-immune sera (data not shown).

### 3.3. Chemotactic activity of recombinant C.MIF protein

Although MIF was originally discovered as an inhibitor of random macrophage migration [2], recent evidence indicates that this soluble immune mediator also possess chemoattractant properties [16]. However, the ability of E.MIF or C.MIF to induce leukocyte chemotaxis has not been reported. Therefore, both proteins were evaluated at 24 h in a standard chemotaxis assay using a modified Boyden chamber. In preliminary experiments, no difference in chemotaxis was seen between 24 and 48 h treatment times (data not shown). Purified recombinant C.MIF, but not E.MIF, in the lower chamber of Transwell cell cul-

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**Fig. 3.** Immunofluorescence staining of native E.MIF and C.MIF proteins in tissue sections. Chickens were infected with *E. acervulina*. At day 5 post-infection, parasite schizonts in intestinal duodenum tissue sections were stained with rabbit anti-E.MIF antibody (A) or rabbit anti-C.MIF antibody (C) plus AlexaFluor488-conjugated goat anti-rabbit IgG secondary antibody (green) and examined by confocal immunofluorescence microscopy. Red staining represents nuclei of intestinal epithelial cells that were counter-stained with propidium iodide. At day 7 post-infection, parasite oocysts in duodenum tissue sections were stained with rabbit anti-E.MIF antibody (B) or rabbit anti-C.MIF antibody (D) and processed as above. Scale bar, 20 μm. Arrows indicate positive staining parasites. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

**Fig. 4.** Immunofluorescence staining of native E.MIF and C.MIF proteins in *E. acervulina* merozoites. Merozoites were purified from the intestine of parasite-infected chickens, air-dried on glass slides, and fixed with acetone. The parasites were stained with rabbit anti-E.MIF antibody (A) or rabbit anti-C.MIF antibody (B) plus AlexaFluor488-conjugated goat anti-rabbit IgG secondary antibody (green) and examined by confocal immunofluorescence microscopy. In A, nuclei were counter-stained with propidium iodide (red). Scale bar, 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
ture inserts increased the migratory response of HD11 chicken macrophages from the upper to lower chamber, compared with the medium control (Fig. 5). Pre-incubation of C.MIF with rabbit anti-C.MIF antiserum inhibited its ability to augment macrophage migration.

3.4. Chicken macrophage cytokine levels in response to E.MIF or C.MIF

Previous studies in mammalian systems have demonstrated that MIF not only regulates macrophage migration, but also modulates the expression of proinflammatory cytokines and chemokines elicited by these leukocytes [17]. Therefore, we compared the ability of E.MIF and C.MIF to up- or down-regulate the expression of a subset of chicken cytokines and chemokines that have been shown to play key roles in the avian immune response. Treatment of HD11 macrophages with C.MIF increased the levels of transcripts encoding IL-6, IL-17 and TNFSF15, and decreased the levels of transcripts encoding IL-8 (CCLii2), compared with cells treated with the medium control ($P<0.05$; Fig. 6). In the case of E.MIF, only IL-8 levels were decreased in response to agonist treatment. Neither MIF protein affected the levels of IL-1B mRNA.

Fig. 5. Chemotaxis of HD11 macrophages (MΦ) in response to C.MIF. HD11 cells were incubated in the upper chamber of Transwell cell culture inserts and their migration into the lower chamber was measured at 24 h in response to medium control, or to purified recombinant E.MIF (rE.MIF) or recombinant C.MIF (rC.MIF) proteins added to the lower chamber at the indicated concentrations. In other experiments, E.MIF or C.MIF proteins were pre-incubated with rabbit anti-E.MIF or rabbit anti-C.MIF antibodies, respectively prior to the migration assay. Each bar represents the mean ± S.D. value ($n=3$). Bars with different letters are significantly different according to the Duncan’s multiple range test ($P<0.05$). The results are representative of 3 independent experiments.

Fig. 6. Cytokine mRNA levels in HD11 cells in response to E.MIF or C.MIF proteins. HD11 macrophages (MΦ) were treated at 41°C for 48 h with purified rE.MIF or rC.MIF proteins. Total RNA was isolated and used as template for amplification of transcripts encoding IL-1β, IL-6, IL-8, IL-17, or TNFSF15 by quantitative RT-PCR, and normalized to GAPDH transcript levels. Each bar represents the mean ± S.D. value ($n=3$). Bars with different letters are significantly different according to the Duncan’s multiple range test ($P<0.05$). NS, not significant.
3.5. Effects of in ovo treatment with E.MIF or C.MIF on experimental Eimeria infection

On the basis of the published studies demonstrating the ability of MIF to regulate host immunity to infectious pathogens [3], we next assessed protection against experimental E. tenella infection following treatment of chicken embryos with E.MIF or C.MIF. In ovo injection of embryos was chosen because it is currently used in commercial poultry production facilities and offers the advantages of inducing early and heightened immunity compared with post-hatch immunization methods [14,18–20]. Infected birds treated with 50 μg of E.MIF had significantly increased body weight gain compared with animals given the PBS control (P < 0.05; Fig. 7). By contrast, weight gains in the 100 μg dose of E.MIF or either dose of C.MIF were identical to the PBS control group. Similarly, fecal oocyst shedding was significantly reduced in chickens treated with E.MIF, but not with C.MIF, compared with the PBS control (P < 0.05; Fig. 8). Again, 50 μg of E.MIF was superior to the higher dose in reducing parasite excretion.

4. Discussion

This study was conducted to compare the structural, immunological, and biological properties of E.MIF and C.MIF. The major findings are that (1) native E.MIF protein was detected in E. acervulina schizonts, merozoites, and oocysts using antisera prepared against the purified recombinant proteins, (2) HD11 chicken macrophages treated in vitro with either of the recombinant proteins expressed increased levels of transcripts encoding IL-6, IL-17, and/or TNFSF15, but decreased levels of IL-8 transcripts, compared with cells treated with the PBS control, (3) C.MIF, but not E.MIF, displayed in vitro chemotactic activity for HD11 cells, and (4) E.MIF, but not C.MIF, enhanced protection against experimental E. tenella infection following in ovo treatment of chicken embryos, compared with embryos treated with the PBS control. Thus, although E.MIF and C.MIF proteins share structural and antigenic similarities, their biological properties are quite distinct.

MIF is a pleiotropic cytokine produced not only by leukocytes, including monocytes, macrophages, and T cells, but also by non-lymphoid cells such as those of the anterior pituitary gland [21–23]. MIF induces the expression of proinflammatory mediators, including IL-1β, IL-6, IL-8, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) [3,6,7,24]. Bacher et al. [22] reported that MIF plays a critical regulatory role in the activation of T cells through the elaboration of these, and other, cytokines and chemokines. Moreover, macrophages themselves secrete MIF in response to IFN-γ and TNF-α [3]. Murine models of Leishmania major and Trypanosoma cruzi infections revealed that MIF-induced proinflammatory responses were protective [25,26]. Flores et al. [27] reported that MIF increased the resistance against acute Toxoplasma gondii infection in mice through multiple mechanisms, i.e., by enhancing macrophage microbicidal activity, by increasing early proinflammatory cytokine production, and by up-regulating cytokine receptor expression. These studies have been extended to humans where it has been demonstrated that MIF secreted by activated macrophages plays an important role in innate immune defense against Mycobacterium tuberculosis [28].

T lymphocytes are the major effector cells against Eimeria infection [29]. Our previous studies showed that multiple pro- and anti-inflammatory cytokines are produced by intestinal T cells following gut infection of chickens with Eimeria parasites, including IL-1β, IL-6, IL-8, IL-10, IL-17, IFN-γ, TNF-α, and TNFSF15 [12,30]. In addition, C.MIF transcripts were detected in T lymphocytes following E. tenella or E. maxima infection, but interestingly enough, only following primary infection and not after secondary parasite infection. It is possible that early C.MIF production following Eimeria infection leads to the subsequent elaboration of a larger pool of immune cytokines and chemokines that drive and amplify the avian immune response to the parasite. Given this scenario, one may question the relative role being played by Eimeria-derived MIF in the context of C.MIF production. Although E.MIF failed to mobilize HD11 cells in the chemotaxis assay and did not affect IL-1β, IL-6, IL-17, or TNFSF15 mRNA levels in these cells, unlike C.MIF, it did enhance protection against E. tenella infection, as assessed by increased weight gain and reduced oocyst shedding. It is possible that the latter effects may be mediated by alternative immune mechanisms not investigated in this study.

It is also unclear why MIF is expressed by Eimeria, since it would seem to be counter-intuitive for a pathogen to produce a proinflammatory mediator that is detrimental to its survival. Part of the answer may lie in the fact that while MIF has proinflammatory properties at low doses, higher amounts of the mediator produce an anti-inflammatory effect [31]. Support for this possibility comes from an earlier study showing that the enteric protozoan parasite Entamoeba histolytica induced host anti-inflammatory factors that inhibited the migration of leukocytes to the focus of infection...
Indeed, the peculiar MIF dose–response relationships on body weight gain and oocyst shedding observed in this study, where the 50 µg dose was more efficacious than the 100 µg amount, may be relevant here. It might be hypothesized that these opposing effects are utilized by the host to efficiently regulate inflammation against microbial pathogens simply by controlling the expression of a single molecule. In other words, a proinflammatory state would be favored early in the course of infection when MIF expression is relatively low; later, as the pathogen infection was cleared from the host and levels of the mediator had accumulated, inflammation would be dampened to prevent bystander tissue damage. Nevertheless, it is clear that production of an MIF-like protein is not particular to Eimeria, and the discovery of MIF orthologues in other parasites indicates that it also has a survival advantage for these microorganisms [33–36]. We speculate that Eimeria protozoa, as well as other pathogens, may have evolved an MIF-like molecule in a manner such that it can be expressed at relatively high levels vis-à-vis its host counterpart early during infection, thus allowing the invading pathogen to preempt the developing inflammatory response. Further studies to elucidate the role of this interesting molecule in molecular and cellular parasitology should shed new light on these questions.

In summary, this report documents that E.MIF and C.MIF mediate distinct in vitro and in vivo immunoregulatory roles in chickens while sharing common structural and antigenic properties. Given its ability to increase protection against experimental Eimeria infection, recombinant E.MIF may be a potential vaccine candidate as an in ovo-delivered, post-hatch immunomodulator to control avian coccidiosis in the commercial poultry industry. Alternatively, recombinant C.MIF may be applicable to other poultry diseases based on its ability to affect leukocyte migration and cytokine responses. Future studies to elucidate the underlying immune mechanisms mediated by E.MIF and C.MIF in various avian clinical conditions will provide important milestones in the quest to obtain an effective control strategy against avian diseases of commercial importance.

Acknowledgements

This work was supported by ARS CRIS 1265-32000-086 and the World Class University Program (R33-10013) of the Ministry of Education, Science and Technology of South Korea. The authors thank Ms. Margie Nichols for technical assistance.

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