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Functional characterization of the turkey macrophage migration inhibitory factor

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Highlights

- Turkey MIF was identified and its biological functions explored, including migration inhibitory activities of macrophages, cell proliferative activity, and induction of pro-inflammatory as well as Th1/Th2/Th17 cytokines in activated immune cells.
- Turkey and chicken MIFs share high sequence homology, functional similarities and cross-reactivity.

Abstract

Macrophage migration inhibitory factor (MIF) is a soluble protein that inhibits the random migration of macrophages and plays a pivotal immunoregulatory function in innate and adaptive immunity. The aim of this study was to clone the turkey MIF (TkMIF) gene, express the active protein, and characterize its basic function. The full-length TkMIF gene was amplified from total RNA extracted from turkey spleen, followed by cloning into a prokaryotic (pET11a) expression vector. Sequence analysis revealed that TkMIF consists of 115 amino acids with 12.5 kDa molecular weight. Multiple sequence alignment revealed 100%, 65%, 95% and 92% identity with chicken, duck, eagle and zebra finch MIFs, respectively. Recombinant TkMIF (rTkMIF) was expressed in *E. coli* and purified through HPLC and endotoxin removal. SDS-PAGE analysis revealed an approximately 13.5 kDa of rTkMIF monomer containing T7 tag in soluble form. Western blot analysis showed that anti-chicken MIF (ChMIF) polyclonal antisera detected a monomer form of TkMIF at approximately 13.5 kDa size. Further functional analysis revealed that rTkMIF inhibits migration of both mononuclear cells and splenocytes in a dose-dependent manner, but was abolished by the addition of anti-ChMIF polyclonal antisera. qRT-PCR analysis revealed elevated transcripts of pro-inflammatory cytokines by rTkMIF in LPS-stimulated monocytes. rTkMIF also led to increased levels of IFN-γ and IL-17F transcripts in Con A-activated splenocytes, while IL-10 and IL-13 transcripts were decreased. Overall, the sequences of both the turkey and chicken MIF have high similarity and comparable biological functions with respect to migration inhibitory activities of macrophages and enhancement of pro-inflammatory cytokine expression, suggesting that turkey and chicken MIFs would be biologically cross-reactive.

Keywords: Macrophage migration inhibitory factor; turkey; chemotaxis; cytokines

1. Introduction

Macrophage migration inhibitory factor (MIF), an evolutionarily conserved multi-functional protein, was originally identified as activated T cell-derived factor inhibiting random migration of macrophages (David et al., 1966). Following determination of complementary DNA sequence of human MIF (Weiser et al., 1989), a variety of biological properties has been reported and defined MIF as a cytokine, enzyme, and chemokine-like function (CLF) chemokine. MIF is constitutively expressed in a wide range of tissues and cells, and rapidly released after stimulation with Gram-negative bacteria, bacterial endotoxin (LPS), pro-inflammatory mediators (Calandra et al., 1994), and low concentration of glucocorticoids (Calandra et al., 1995). Due to the absence of N-terminal consensus leader sequence, MIF is swiftly secreted through non-classical pathway that requires the activation of the Golgi-associate protein p115a (Flieger et al., 2003).

As a pleiotropic inflammatory cytokine, MIF modulates both innate and adaptive immune responses through the activation of macrophages and T cells (Calandra et al., 2003). MIF upregulates the expression of TLR4 in response to stimuli and prompts induction of pro-inflammatory cytokines and chemokine (TNF-α, IFN-γ, IL-1β, IL-2, IL-6, IL-8), nitric oxide (NO) (Calandra et al., 1994; 1995; Bacher et al., 1996), and macrophage inflammatory protein 2 (MIP2) (Makita et al., 1998). In adaptive immunity, MIF inhibits $CD8^+$ T lymphocytes (CTL) cytotoxicity and regulates T cell trafficking (Abe et al., 2001). MIF reverses the anti-inflammatory and immunosuppressive activities of glucocorticoids, and sustains inflammatory response against them (Calandra et al., 1995). A high-affinity interaction of MIF with CD74 is responsible to induce cell proliferation by activation ERK ½ family of mitogen-activated protein in growth-promoting signaling pathway (Leng et al., 2003). Induction of cyclooxygenase-2

(COX-2) and products of the arachidonic acid pathway (PGE_2) by MIF is required to suppress apoptotic-inducing function of the tumor suppress protein (p53), which promotes cell survival (Mitchell et al., 2002). Structural analysis revealed MIF exists as a homotrimer form, and two adjacent sites between monomers possess enzymatic activities (Lubetsky et al., 1999), such as a $_D$-dopachrome tautomerase (Rosengren et al., 1996), a phenylpyruvate tautomerase (Rosengren et al., 1997), and a thiol-protein oxidoreductase (Kleemann et al., 1998). Moreover, MIF is classified into CLF chemokine based on the structural and functional similarities with chemokines. Comparison of crystal structure revealed that MIF monomer resembles the dimer form of CXCL8 (Weber et al., 2008). The non-cognate interaction of MIF with chemokine receptors, CXCR2, CXCR4 and CXCR7, promotes chemotactic migration and leukocytes arrest (Bernhagen et al., 2007; Tarnowski et al., 2010).

In birds, chicken MIF was identified as a marker for cellular differentiation in developing chicken eye lens (Wistow et al., 1993) and upregulated MIF transcript was observed in *Eimeria*-infected chickens, thus supporting involvement of MIF in intestinal immune responses (Hong et al., 2006). Molecular function of chicken MIF was characterized by analysis of cell migration, transcription of Th1/Th2-associated and pro-inflammatory cytokines, and cell proliferation after LPS stimulation (Kim et al., 2010). Recently, it was verified that ChMIF binds to macrophages via the surface receptor CD74p41 (Kim et al., 2014).

Compatative analyses of the turkey and chicken genomes revealed high similarity between the two sequences being relatively conservative and stable despite 40 million years of species divergence (Dalloul et al., 2010). However, these two species showed lower similarity (83%) at the protein level than at the genome level (90%) (Arsenault et al., 2014). To elucidate these distinctions at the protein level, further biological characterization is required. To date,

89 several cytokines have been biologically characterized in turkeys, and also describing the cross-
90 reactivities of avian cytokines including IFN-γ, IL-2, IL-10, IL-13, and IL-18 (Lawson et al.,
91 2000; 2001; Kaiser et al., 2002; Powell et al., 2012).

92 Given that these cytokines are functionally cross-reactive between two closely related
93 Galliformes (turkey and chicken), MIF may also have a similar role in both species. To describe
94 the biological function of MIF in turkeys that may have cross-reactivity with chicken MIF, we
95 cloned the full-length turkey MIF (TkMIF) gene, and explored its biological functions including
96 inhibitory effect of random cell migration, proliferative effect of splenic lymphocytes, and
97 expression of pro-inflammatory and Th1/Th2/Th17 cytokines by activated immune cells.
98

99 **2. Materials and Methods**

100 2.1. Turkey, RNA sources for cloning

101 Tissue samples, including heart, liver, brain, thymus, spleen, small intestine sections
102 (duodenum, jejunum, ileum), proventriculus, cecal tonsil and bursa, were collected from 21-day-
103 old male and female commercial turkey (*Meleagris gallopavo*). A total of 30 mg tissue samples
104 was excised and homogenized in lysis buffer containing β-mercaptoethanol (β-Me) with stainless
105 steel beads using TissueLyser II (Qiagen, CA) for 5 min at 25 Hz. Total RNA was isolated from
106 homogenized tissues using the RNeasy Mini kit (Qiagen) according to the manufacturer's
107 instructions.
108

109 2.2. Sequence analyses

110 Nucleotide and deduced amino acid sequences of TkMIF were compared with other
111 sequences reported in NCBI's GenBank using Clustal Omega program (Sievers and Higgins,

2014). The phylogenetic tree was constructed from the aligned sequences by the neighbor-joining (NJ) method and evaluated with 1000 bootstrap replicates using MEGA4 (Tamura et al., 2007). The molecular weight (MW) and theoretical isoelectric point (pI) of MIF were computed using the Translate software. The presence of signal peptide and potential N-glycosylation sites were predicted using SignalP3.0 and NetNGlyc 1.0, respectively. The protein secondary structure of MIF was determined using SSpro 5.1 (Magnan and Baldi, 2014).

2.3. Tissue distribution of TkMIF

In order to analyze TkMIF expression in various tissues of male and female turkeys, qRT-PCR was performed using 7500 Fast Real-Time PCR system (Applied Biosystems, CA). Specific primer sets were designed using Primer Express (Ver 3.0; Applied Biosystems) (Table 1). First-strand cDNA was synthesized with 2 µg of total RNA from turkey tissues using High-capacity cDNA Reverse Transcription kit (Applied Biosystems). Synthesized cDNA was diluted to 1:25 with nuclease-free water and 1 µl of diluted cDNA was used as template with 0.1 µM primers and 5 µl of 2× Fast SYBR Green Master Mix (Applied Biosystems) in 10 µl volume of final qRT-PCR reaction. The PCR reaction was performed as follows: samples were initially denatured at 95°C for 20 sec, followed by 40 cycles of denaturation at 95°C for 3 sec and annealing/extension at 57°C for 30 sec. Reactions were prepared in triplicate and GAPDH was used as reference gene. TkMIF expression was normalized to GAPDH and calculated relative to that of the heart by the $2^{-\Delta\Delta Ct}$ comparative method.

2.4. Construction of recombinant TkMIF (rTkMIF) expression plasmid

The full-length TkMIF gene was amplified from total RNA extracted from turkey spleen using primers designed by Kim et al. (2010) as follows: initial denaturation at 92°C for 2 min, followed by 35 cycles of denaturation at 92°C for 15 sec, annealing at 57°C for 15 sec, and extension at 72°C for 30 sec with a final extension at 72°C for 7 min. The amplified PCR product was purified using Wizard SV Gel and PCR Clean-up system (Promega, WI), ligated into pGEM-T vector, and followed by transformation into *E. coli* Top10. Transformants containing the target gene were selected by combination of colony PCR screening and endonuclease digestion with *EcoR* I (New England Biolabs, MA), confirmed by sequencing (Virginia Bioinformatics Institute at VT, VA). For sub-cloning into a prokaryotic expression vector, TkMIF was digested with restriction endonucleases *Nde* I and *Nhe* I (New England Biolabs), and sub-cloned into the pET11a vector. The recombinant plasmid was transformed into *E. coli* Top10 and positive clones including TkMIF were selected and confirmed by sequencing.

2.5. Expression and purification of rTkMIF by SEC-HPLC

The TkMIF in pET11a plasmid was transformed into *E. coli* BL21 (DE3) and cultured at 30°C overnight and the production of recombinant TkMIF was induced by shake-incubating for 5 hr in the presence of 1 mM IPTG. The cells were harvested and lysed by rapid sonication-freeze-thaw cycles in 20 mM NaH_2PO_4, 500 mM NaCl (pH 7.8), followed by treatment of RNase A (10 µg/ml) and DNase I (10 µg/ml) on ice for 15 min. By centrifugation, the supernatant including rTkMIF was collected. For endotoxin removal prior to purification, TX-114 (Sigma, MO) was added to the bacterial lysate containing rTkMIF to a final concentration of 1%. The mixture was shortly vortexed and incubated at 41°C for 5 min, followed by centrifugation to

collect the upper aqueous phase containing rTkMIF. This procedure was repeated three times. Subsequently, size exclusion high performance liquid chromatography (SEC-HPLC) was used to purify rTkMIF. In SEC-HPLC, a mobile phase containing 50 mM K_2HPO_4, 150 mM NaCl (pH 6.8) was passed through two size exclusion columns (7.7mm×300mm, Biosuite 5 µm HR; Waters, MA) at a rate of 0.5 ml/min and the absorbance was monitored with a photo diode array detector (Model 997; Waters, MA) at 214 nm and 280 nm. Following injection of lysates, fractions were collected, analyzed by SDS-PAGE, and the concentration of proteins determined by BCA assay (Thermo Scientific, IL). The level of endotoxin in purified protein sample was measured using Limulus Amebocyte Lysate (LAL) chromogenic endotoxin quantitation kit (Rockfold, IL).

2.6. Western blot analysis

Western blotting was performed to examine whether a rabbit anti-ChMIF polyclonal antisera (Kim et al., 2010) would recognize TkMIF as it shares high identity with ChMIF. Briefly, 1 ng of purified TkMIF was resolved on SDS-PAGE gel under reduced conditions, transferred to a PVDF membrane (Millipore, MA) and incubated with anti-ChMIF polyclonal antisera in a 1:1000 dilution as the primary antibody. Goat anti-rabbit IgG conjugated with HRP (Thermo Scientific, IL) was applied as the secondary antibody and the blot was incubated in the SuperSignal® West Pico Chemiluminescent Substrate (Rockford, IL), and exposed to X-ray film (Genesee Scientific, CA).

2.7. Isolation of peripheral blood mononuclear cells (PBMCs) and splenocytes

In order to perform cellular assay, turkey PBMCs were isolated from freshly drawn blood by density-gradient centrifugation. Briefly, 20 ml of blood were collected from the heart (immediately following euthanasia) and diluted with equal volume of Hank's Buffered Salt Solution without magnesium and calcium (HBSS; HyClone, UT). Following centrifugation at 50 x *g* for 10 min, the supernatant and buffy coat were collected and then carefully overlaid on Histopaque-1077 (Sigma, MO). After centrifugation at 400 x *g* for 30 min at room temperature, mononuclear cells from the interphase were collected by Pasteur pipette and mixed with PBS for washing. After centrifugation at 250 x *g* for 10 min, the collected cells were washed with Dulbecco's Modified Eagle Medium (DMEM; Mediatech, VA), counted using a hemocytometer and cultured at 1.0×10^6 cells/well in a 24-well plate for 3 hr at 39°C with 5% CO_2 humidified air. By gently washing with DMEM, non-adherent cells were removed leaving adherent monocytes/macrophages on the plate.

For turkey splenocytes isolation, spleens were cut into small pieces and smashed through a 0.22 μm cell strainer (BD, CA). Cell suspension was washed three times with HBSS to remove cell debris and overlaid onto Histopaque-1077, followed by isolation of splenocytes as described above. Isolated splenocytes were resuspended with RPMI-1640 (Mediatech, VA) supplemented with 20% fetal calf serum (FCS) and 1% penicillin/streptomycin and cultured for 24 hr at 39°C with 5% CO_2 humidified air. After overnight incubation, non-adherent cells were collected and adjusted to a cell density of 2×10^6 cells/ml.

2.8. Chemotaxis assay

To measure the ability of TkMIF in inhibiting the random migration of immune cells, serially diluted rTkMIF (0.01, 0.1, 1.0 μg/ml) with DMEM supplemented with 10% FCS and 1%

penicillin/streptomycin were freshly prepared. Diluted rTkMIF (25 μl) was loaded to the bottom wells of the Boyden chemotaxis chamber in absence or presence of anti-rChMIF polyclonal antisera along with the medium supplemented with 10% FCS and serum-free medium as positive and negative controls, respectively. Polycarbonate filter membrane (Neuro Probe, MD) was placed with forceps and then 50 μl of prepared PBMCs or splenocytes (1.0×10^5 cells/ml) were loaded on the top well above the membrane. After incubation at 39°C with 5% CO_2 for 4 hr, cells that migrated to the bottom side of the membrane were fixed, stained using Diff-Quick Staining (Fisher Scientific, NJ) and counted. The percentage of migration inhibition was calculated as previously described (Kotkes et al., 1979).

2.9. Cell proliferation assay

Cell proliferation was determined with CellTiter 96® Non-Radioactive Cell Proliferation Assay Kit (Promega, WI). For this assay, isolated splenocytes (1.0×10^5 cells/ml) were treated with medium alone, Concanavalin A (Con A) alone (10 μg/ml), rTkMIF (0.01 and 0.1 μg/ml) or rTkMIF (0.01 and 0.1μg/ml) with Con A (10 μg/ml) in the presence or absence of anti-ChMIF polyclonal antibody at 39°C with 5% CO_2 for 24 hr. After incubation, the Dye solution was added and the mixture incubated at 39°C with 5% CO_2 for 4 hr. The Solubilization solution/Stop mix were added followed by incubation at 39°C with 5% CO_2 for 1 hr, after which absorbance was measured at 595 nm and 630 nm using a microplate reader. The results were analyzed after subtraction of the 630 nm value as a background.

2.10. Cell stimulation assay and cytokine transcripts analysis

PBMCs were cultured at 2.0×10^6 cells/well in a 24-well plate and treated with medium alone, LPS alone (5 μg/ml), rTkMIF (0.01 and 0.1 μg/ml) or rTKMIF (0.01 and 0.1 μg/ml) with LPS (5 μg/ml) at 39°C with 5% CO_2 for 6 hr. The supernatants were collected for NO assay using Griess Reagent System (Promega, WI). The cells were lysed with Buffer RLT (Qiagen) containing β-Me followed by RNA extraction using RNeasy Mini Kit (Qiagen). After cDNA synthesis using 1 μg of RNA, expression levels of pro-inflammatory cytokines were analyzed.

Isolated splenocytes were cultured at 2.0×10^6 cells/well in a 24-well plate and treated with medium alone, Con A alone (10 μg/ml), rTkMIF (0.01 and 0.1μg/ml) or rTKMIF (0.01 and 0.1 μg/ml) with Con A (10 μg/ml) at 39°C with 5% CO_2 for 6, 12, or 24 hr. After incubation, NO assay was performed using the supernatant and total RNA was extracted. After cDNA synthesis, the transcripts of Th1/Th2/Th17 cytokines were analyzed.

2.11. Statistical analysis

All data were analyzed by Student's *t*-test or one-way analysis of variance (ANOVA) using JMP software (Ver 11) and significant differences between groups were considered significant by Tukey-Kramer multiple comparison test at $P < 0.05$.

3. Results

3.1. Sequence and phylogenetic analyses of TkMIF

The full-length TkMIF amplified from turkey spleen contained 348 bp nucleotides encoding a 115-amino acid protein, which had 97% nucleotide and 100% amino acid identities with Chicken MIF (Figure S1 and Figure S2A). Multiple sequence alignment and phylogenetic analysis revealed that TkMIF shares 71% identity with human and mouse MIFs, and over 61%

identity among bird species with the highest identity with eagle (95%) and zebra finch (92%) MIFs (Figure S2). The phylogenetic tree shows that turkey MIF is closest to the chicken MIF as well as clusters together with eagle and zebra finch MIFs. Similar to mammalian MIF and ChMIF, TkMIF retained conserved amino acid residues, Pro^{2}, Lys^{33}, Ile^{65}, Tyr^{96}, Asn^{98}, which are essential for enzymatic activities. The putative TkMIF showed a calculated MW of 12.5 kDa and theoretical isoelectric point of 7.82. Computational analysis revealed two possible N-glycosylations (^{73}Asn-Lys-Thr^{75}, ^{110}Asn-Gly-Ser^{112}) and four cysteine residues (Cys^{11}, Cys^{57}, Cys^{60}, Cys^{81}) in the amino acid sequence. Cys^{11} is only conserved among the chicken, eagle, and zebra finch MIFs that are highly similar to TkMIF, and Cys^{57} and Cys^{60} formed conserved Cys-X-X-Cys motif mediated by enzymatic oxidoreductase activity. Secondary structure of TkMIF exhibits two α-helices and six β-strands (Figure S1), similar to that of human MIF monomer.

3.2. TkMIF expression in tissues

The expression patterns of TkMIF gene was measured in various tissues of male and female turkeys including heart, liver, brain, thymus, spleen, proventriculus, cecal tonsils, bursa, and intestinal sections using qRT-PCR (Figure 1). The expression level was normalized to GAPDH expression as an endogenous reference gene and then fold changes were calculated relative to the lowest expression level of heart. The results demonstrated that TkMIF is ubiquitously expressed in all tested tissues, with the lowest level in the heart and relatively highest levels in the spleen and thymus in both males and females. Slightly different expression levels were observed between males and females with high levels in ileum of females, but not in that of males.

3.3. Expression and Western analysis of TkMIF

rTkMIF was expressed in *E. coli* BL21 (DE3) as a soluble form and 20% of protein from bacterial lysates was detected in predicted MW of rTkMIF on a gel after endotoxin removal by TX-114 extraction. rTkMIF was purified and collected from fractions 19 and 20 by SEC-HPLC with 80% purity. Purified rTkMIF was observed around 13.5 kDa by SDS-PAGE (Figure 2B, left), which is slightly higher molecular weight than that of only rTkMIF, 12.5 kDa due to the presence of T7 tag (approximately 1.3 kDa) in the recombinant protein that was encoded by the plasmid vector. Endotoxin concentration of purified rTkMIF was 0.04 EU (endotoxin units) per μg protein. Since turkey and chicken MIFs showed high identify, we examined whether anti-ChMIF polyclonal antisera (Kim et al., 2010) can bind rTkMIF molecule (Figure 2B). The anti-ChMIF polyclonal antisera recognized 13.5 kDa of rTkMIF along with rChMIF, which was used as a positive control. Based on the Western blot results, anti-ChMIF polyclonal antisera were used to neutralize rTkMIF in further assays.

3.4. Chemotactic activity of rTkMIF

In order to evaluate the regulation of PBMCs and splenocytes migration by rTkMIF, chemotaxis assay was performed. Migration of PBMCs was inhibited by rTkMIF in a dose-dependent manner, with 90% and 60% migration inhibition at high (1 μg/ml) and low (0.01 μg/ml) concentration of rTkMIF, respectively (Figure 3A). Although the inhibition level of cell migration is slightly lower than in PBMCs, rTkMIF also inhibited migration by approximately 80% (1 μg/ml) and 10% (0.01 μg/ml) of splenic lymphocytes (Figure 3B). The results show that rTkMIF has appreciable inhibition activity of migration on PBMCs as well as on splenocytes, revealing different inhibitory pattern between these two cell types. Since 0.01 and 0.1 μg/ml of

293 rTkMIF showed noticeable reduction of both PBMCs and splenocytes migration, these two
294 concentrations were used in subsequent assays.

295 To substantiate its biological specificity, rTkMIF was neutralized using anti-ChMIF
296 polyclonal antisera to examine whether MIF-induced inhibition of cell migration can be
297 abolished. Pre-incubation of rTkMIF (0.1 μg/ml) with anti-ChMIF polyclonal antisera blocked
298 approximately 70% and 30% migration inhibition of PBMCs and splenocytes, respectively
299 (Figure 4). Anti-ChMIF antisera alone had no effect on migration of both PBMCs and
300 splenocytes.

301

302 3.5. The effect of rTkMIF on proliferation of splenic lymphocytes

303 To determine the effect of rTkMIF on cell proliferation, isolated splenocytes were
304 cultured with rTkMIF in the presence or absence of Con A (Figure 5). We did not observe any
305 significant changes in cell proliferation when treating with 0.01 μg/ml rTkMIF both in the
306 presence and absence of Con A. However, treatment with 0.1 μg/ml of rTkMIF slightly induced
307 splenocytes proliferation. Additionally, 0.1 μg/ml of rTkMIF enhanced proliferation of Con A
308 co-stimulated splenocytes. As percentages, 15% and 12% splenocyte proliferation were induced
309 by 0.1 μg/ml of rTkMIF both in the presence and absence of Con A, respectively. This rTkMIF-
310 induced splenocyte proliferation was abolished by pre-incubation with anti-ChMIF antisera,
311 further substantiated its biological activity on cell proliferation.

312

313 3.6. Expression of pro-inflammatory cytokines and chemokine by TkMIF in PBMCs

314 The administration of rTkMIF alone did not affect cytokine expression (data not shown),
315 but overall treatment of rTkMIF with LPS enhanced mRNA level of pro-inflammatory cytokines

316 (IL-1β, IL-6) and chemokine (IL-8) compared to those of LPS alone-treated cells (Figure 7).
317 Transcripts of IL-1β and iNOS were enhanced approximately 14-fold and 19-fold, respectively,
318 by incubation with 0.01 μg/ml rTkMIF but not with 0.1 μg/ml. IL-1β transcript was enhanced by
319 13-fold following 12 hr incubation (data not shown). The addition of rTkMIF induced mRNA
320 levels of IL-6 and IL-8 regardless of concentration, and markedly enhanced IL-8 transcript
321 shown for 6 hr as well as 12 hr incubation. LPS-stimulated PBMCs exhibited no significant
322 induction of IL-12β (p40) and had reduced IL-18 transcript after 6 hr incubation with rTkMIF.
323 However, enhanced IL-12β and IL-18 transcripts by 2-fold were shown at 12 hr incubation with
324 0.01 μg/ml rTkMIF (data not shown). In addition, the production of NO by PBMCs was
325 observed after rTkMIF (0.01μg/ml) stimulation in combination with LPS, but not after treatment
326 with rTkMIF alone (Figure 6).
327
328 3.7. Expression of Th1/Th2/Th17 cytokines by TkMIF in splenocytes
329 Splenocytes were treated with rTkMIF (0.01 and 0.1 μg/ml) in the presence of Con A for
330 6, 12, and 24 hr (Figure 8). Transcript of IFN-γ, a Th1 cytokine, was induced by Con A
331 treatment, but no effect was observed by rTkMIF at 6 hr point; however, rTkMIF enhanced IFN-
332 γ transcript in a dose-dependent manner at 12 hr post-stimulation. No difference in mRNA level
333 was observed by Con A and/or rTkMIF at 24 hr post-stimulation. On the other hand, rTkMIF
334 reduced transcripts of Th2 cytokines (IL-10 and IL-13) after 24 hr stimulation; however, IL-10
335 transcript was enhanced when the Con A-activated splenocytes were incubated with 0.1 μg/ml of
336 rTkMIF at 6 hr. rTkMIF significantly enhanced mRNA level of IL-17F over the tested
337 incubation periods, especially 24 hr post-stimulation. MIF transcript was not changed with
338 either Con A treatment alone or Con A and rTkMIF treatment.

4. Discussion

Previously, the molecular cloning and biological characterization of ChMIF have been described (Kim et al., 2010). Interestingly, MIF homologue from turkey has high sequence identity with the corresponding gene of its sister Galliformes bird, the domesticated chicken. This finding led us to characterize the biological activities of TkMIF in order to compare this cytokine functions between two closely related Galliformes species (Kim et al., 2010). In this study, we cloned the full-length MIF from domesticated turkey spleen and characterized its biological functions ex vivo. Sequence analysis revealed that TkMIF contains conserved residues including CXXC motif mediating enzymatic activity, similar to human and mouse. In addition, secondary structure analysis revealed that TkMIF possesses two α-helices and six β-strands in the same order as mammalian MIF (Sun et al., 1996; Suzuki et al., 1996), implying a similar tertiary structure and function between turkey and mammalian MIFs. The conserved sequences mediating enzymatic activities suggest the potential similar activities of TkMIF. However, catalytic activities were not exhibited in TkMIF in contrast to mammalian MIFs (Sugimoto et al., 1999). Interestingly, a lack of catalytic properties also was exhibited in chicken MIF (Kim et al., 2010). Also, TkMIF shares high homology with variant-1 of zebra finch among its two isoforms. MIF is highly conserved among birds and mammals, indicating this molecule is evolutionary conserved across species and hence implying its significant function.

MIF is ubiquitously expressed not only by immune cells as macrophages and activated T lymphocytes, but also by non-immune cells such as endothelial, epithelial and parenchymal cells (Lue et al., 2002; Calandra et al., 2003). Consistent with the distribution patterns of MIF in various species, TkMIF was ubiquitously expressed in all tissues examined, and highly expressed

362 in the primary and secondary lymphoid tissues (thymus and spleen), in contrast to abundant
363 ChMIF transcript in stomach (Kim et al., 2010). Of note, only subtle differences were observed
364 in TkMIF expression between male and female tissues. Mammalian MIF is more expressed by
365 monocytes and T lymphocytes, and up-regulated by stimulation with bacterial LPS and certain
366 cytokines like IFN-γ and TNF-α (Calandra et al., 1994). Although TkMIF is constitutively
367 expressed, it is not significantly induced by stimulated monocytes and lymphocytes, similar to
368 ChMIF (Kim et al., 2010).

369 Like mammalian MIFs, TkMIF lacks an N-terminal signal sequence, indicating it is
370 easily released from its intracellular stores as a soluble form via a non-conventional mechanism
371 (Weiser et al., 1989). As expected from high sequence identity between turkey and chicken
372 MIFs, the ability of anti-ChMIF antisera to bind TkMIF was substantiated by performing
373 Western blotting, which shows the cross reactivity of chicken MIF antibody against TkMIF.

374 In the mouse, MIF regulated the recruitment of monocytes, T lymphocytes, and
375 neutrophils like a CLF chemokine (Bernhagen et al., 2007). The migration inhibitory properties
376 of MIF on monocytes and lymphocytes were examined in the fish and further confirmed by
377 neutralizing antibody (Qiu et al., 2013). Consistent with previous reports, rTkMIF inhibited
378 random migration of both monocytes and splenic lymphocytes in a dose-dependent manner.
379 This inhibitory effect was abolished in the presence of anti-ChMIF polyclonal antisera,
380 demonstrating that the observed inhibitory effect on the migration of immune cells was
381 specifically associated with rTkMIF. The rTkMIF exhibited similar pattern of chemotactic
382 activity with ChMIF, suggesting that chemokine-like properties of MIF is conserved in both
383 mammalian and avian species.

Based on the finding that MIF was abundantly expressed in the epithelial cells of chicken embryonic lens (Wistow et al., 1993), MIF has been considered an important factor for cell growth and differentiation. Mammalian MIF induced a survival cascade via interaction with CD74, resulting in B cell proliferation and survival (Starlets et al., 2006). Immuno-neutralization of MIF indicated its proliferative effect on splenocytes and T lymphocytes (Bacher et al., 1996; Calandra et al., 1998). Additionally, MIF is secreted by murine dendritic cells (DCs) and neural stem/progenitor cells (NSPCs) that can support the proliferation and survival of NSPCs (Ohta et al., 2012). In chickens, MIF induced proliferation of lymphocytes primed by Con A, although MIF alone did not impact cell proliferation. In the present study, enhancement of cell proliferation was detected by addition of TkMIF on splenic lymphocytes both in the presence and absence of Con A stimulation. The proliferative effect of TkMIF was suppressed by anti-ChMIF antisera. These small but statistically significant effects support its ability to promote cell proliferation.

Furthermore, MIF activated macrophages and induced significant production of pro-inflammatory cytokines and NO in stimulated macrophages/monocytes (Bernhagen et al., 1994; Calandra et al., 1995). In chickens, upregulated expression of pro-inflammatory cytokines and iNOS was shown in response to 0.01 µg/ml of rChMIF by LPS-primed monocytes/macrophages. The current findings that addition of TkMIF significantly augmented pro-inflammatory cytokines and chemokine (IL-1β, IL-6, IL-8) transcription and NO release by LPS-stimulated monocytes are consistent with previous reports. TkMIF stimulation induced IL-12β and IL-18 at later time-points when compared with other pro-inflammatory cytokines and chemokine. These inductions may consequently result in synergistic action of IL-12β and IL-18 that would lead to IFN-γ production and stimulation of a Th1 response (Takeda et al., 1998). Taken together, these data

support the pro-inflammatory roles of avian MIFs in stimulated immune cells. Given that avian MIF promotes pro-inflammatory responses of innate immune cells, these findings suggest its potential role in host innate immune defenses of infected birds.

In regards to MIF involvement in T cell immunity, murine MIF promoted Th1 cytokine production, typically IL-2 and IFN-γ, in activated T cells (Bacher et al., 1996). In chickens, the production of Th1 and Th2 cytokines was regulated by MIF levels in Con A-stimulated lymphocytes (Kim et al., 2010). In the present study, the addition of rTkMIF induced the expression of IFN-γ at 12 hr, and reduced transcripts of the Th2 cytokines IL-10 and IL-13 at 24 hr. The expression of IL-10 was briefly reduced and elevated after stimulation with low (0.01 μg/ml) and high (0.1 μg/ml) concentrations of TkMIF at 6 hr, and then gradually decreased over 24 hr. Given the ability of avian IL-10 to inhibit IFN-γ expression by stimulated splenocytes (Rothwell et al., 2004; Powell et al., 2012), late enhancement of IFN-γ may be caused by gradual decline in IL-10 transcript combined with synergistic activity of IL-12β and IL-18. As to the expression patterns of Th1 and Th2 cytokines in MIF-stimulated lymphocytes, TkMIF promoted Th1 transcript whereas suppressed Th2 transcripts, contrast to ChMIF that enhanced the transcript of both Th1 and Th2 cytokines. These findings indicate different expression profiles of Th1 and Th2 between two species that may mediate the different susceptibilities to host-specific pathogens; turkeys were extremely susceptible to *Histomonas meleagridis* exhibiting high mortality, while chickens were resistant to the parasite (Powell et al., 2009). In this regard, it would be interesting to investigate whether MIF is associated with the susceptibility of turkeys to protozoan pathogens. The stimulatory effect of MIF on IL-17 production was observed in activated murine lymphocytes (Stojanovic et al., 2009). Similarly, rTkMIF continuously stimulated IL-17F production over the 24 hr incubation period, suggesting the possibility that

avian MIF might be involved in differentiation of Th17 cells. Abundant MIF transcript by stimulation with PMA/ION was observed in mice (Bacher et al., 1996), whereas TkMIF was not significantly induced by LPS-stimulated splenic lymphocytes as well as monocytes from turkeys similar to chicken MIF, indicating that avian MIFs are constitutively expressed in immune cells regardless of stimulation. These findings indicate the unique expression pattern of avian MIF contrast to most cytokines and chemokines that are expressed by activated cells. TkMIF alone is not sufficient to induce cytokine expression in splenic lymphocytes as well as in monocytes, similar to results by ChMIF alone. Overall, these data suggest that MIF can be directly involved in the modulation of Th1/Th2/Th17 cytokines in turkeys, further revealing different innate immune responses in stimulated cells between turkeys and chickens.

In summary, Turkey MIF was cloned and its biological functions characterized including migration inhibitory effect, proliferative effect, and the ability to modulate production of pro-inflammatory mediators as well as Th1/Th2/Th17 cytokines. These results help us to better understand the biological roles of evolutionarily conserved avian MIFs in the birds' immune system, and predict functional cross-reactivity between turkey and chicken MIFs.

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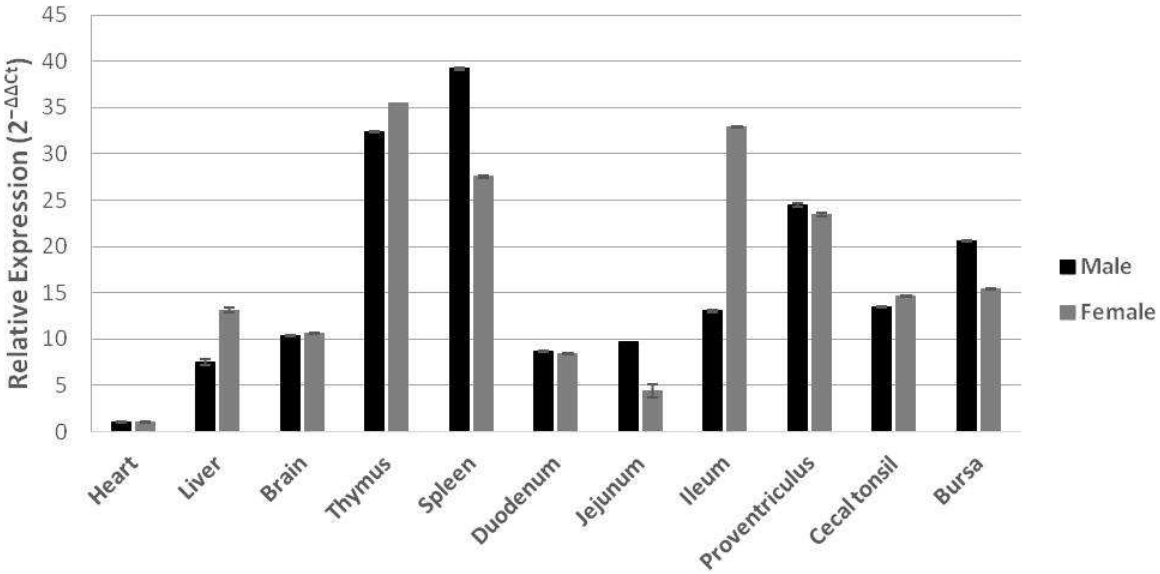
578 Acad Sci U S A. 90, 1272-1275.

579 **Table 1.** Primers sequences used for gene cloning and qRT-PCR analysis.
580

Name	Sequence (5'→3')	GenBank accession No.	Application
TkMIF_F	GATCATATGAGATCTATGCCCATGTTCACCATCCACACC	From turkey genome	Gene cloning
TkMIF_R	GATGCTAGCCTATGCAAAGGTGGAACCGTTCCA		
MIF_F	CGGATCCCTGCGCTCTCT	XM_425824	qRT-PCR
MIF_R	TGTTCTGCTGCCCTCCGATT		
IFN- γ_F	CAAAGCCGCACATCAAACAC	AJ000725.1	
IFN- γ_R	GCCATCAGGAAGGTTGTTTTTC		
IL-1b_F	CCGACACGCAGGGACTTT	DQ393271.1	
IL-1b_R	GAAGGTGACGGGCTCAAAAA		
IL-2_F	GAGCATCGCTATCACCAGAAAA	AF209705.1	
IL-2_R	TTGTTCTTGCTTTCTTCCAGTATTTCTA		
IL-6_F	ACTCAGCCACCCAGAAATCC	XM_003207130.1	
IL-6_R	TCTCTATCCACGCCTTATCTGACT		
IL-8_F	GGTTTCAGCAGCTCTGTCACA	DQ393276.1	
IL-8_R	TGGCACCGCAGCTCGTT		
IL-10_F	CCAGCCACCAGGAGAGCAT	AM493432.1	
IL-10_R	GCGCTTCATTGTCATCTTCAG		
IL-12B_F	ACTACTGTCCATTTGCCGAAGA	XM_003210283.1	
IL-12B_R	CATCAATGACCTCCAGGAACA		
IL-13_F	CGAGCTCCATGCCCAAGAT	AM493431.1	
IL-13_R	TGTTGAGCTGCTGGATGCTT		
IL-17F_F	GTCTCCAATCCCTTGTTCTCCTT	XM_003204633.1	
IL-17F_R	GACAGCACGGCCAGCAA		
IL-18_F	TGCCCGTCGCATTCAG	AJ312000.1	
IL-18_R	CCATGCTCTTTCTCACAACACA		
iNOS_F	TTGGGTGGAAGCCGAAAT	XM_003211871.1	
iNOS_R	TTGCTTGGAGAATGAGTGGAACT		
GAPDH_F	GCTGAGAATGGGAAACTTGTGAT	NM_001303179.1	
GAPDH_R	GGGTTACGCTCCTGGAAGATAG		

581



582
583 **Figure 1.** Tissue-specific mRNA expression of TkMIF. The relative TkMIF transcription in
584 each tissue of male and female turkeys was calculated by the $2^{-\Delta\Delta Ct}$ methods using GAPDH as a
585 reference gene, and the relative expression level was compared with the expression level in heart
586 (arbitrarily set at 1.0). Error bars represent SEM.

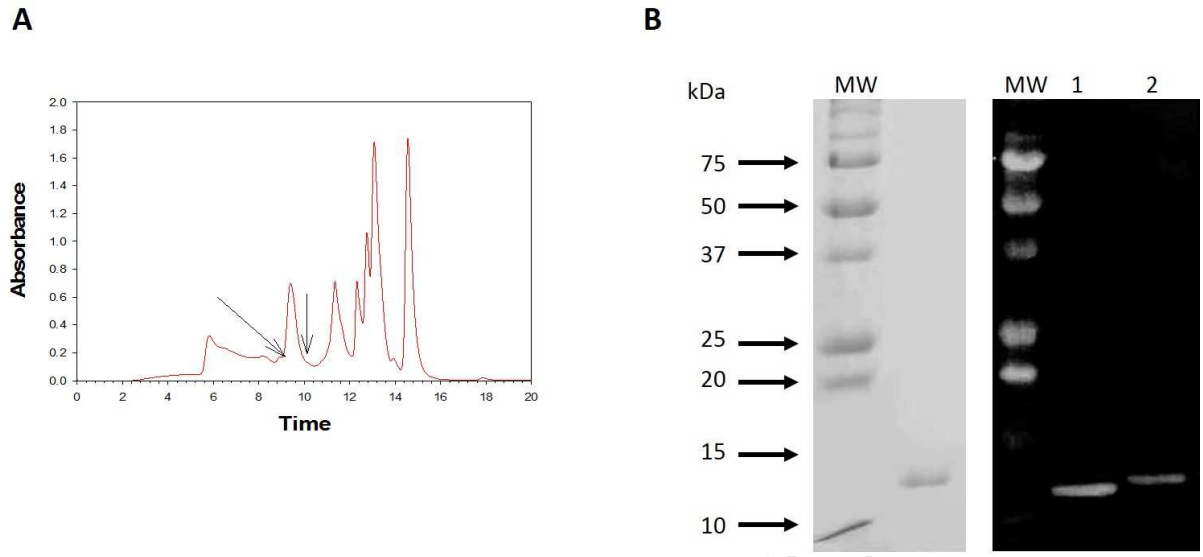
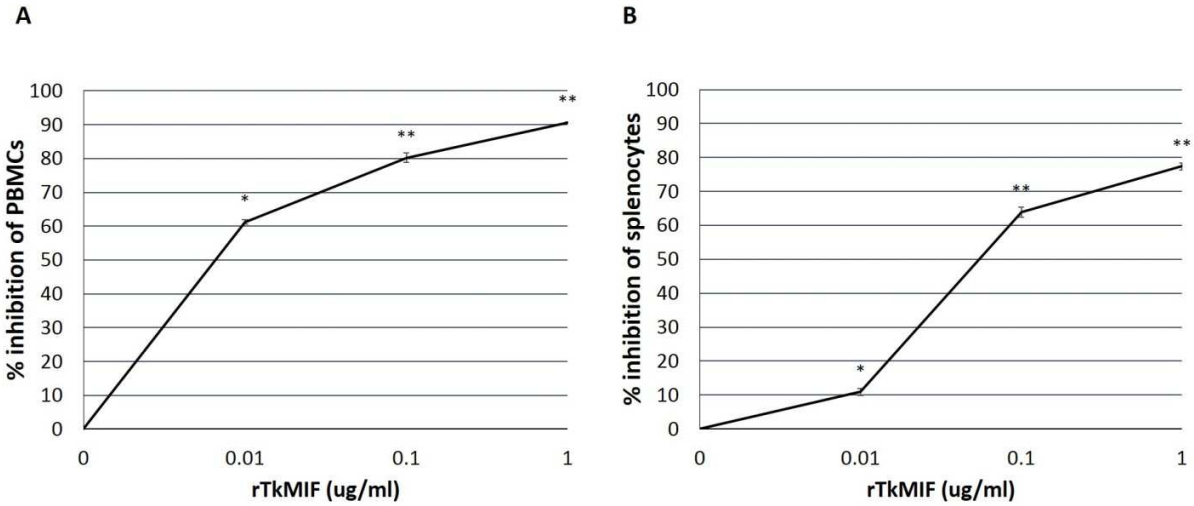


Figure 2. Purification and Western blot analysis of rTkMIF. (A) rTkMIF was purified from bacterial lysate by SEC-HPLC and scanned the gel. The fractions containing rTkMIF proteins are indicated by arrows. (B) Purified rTkMIF was analyzed by SDS-PAGE analysis (left). Western blot analysis of rTkMIF was performed with anti-chicken MIF polyclonal antisera (1:1000) (right), MW, protein molecular weight marker; lane 1, rChMIF (1 ng); lane 2, rTkMIF (1 ng).



595
596

597 **Figure 3.** Inhibition the random migration of PBMCs and splenocytes by rTkMIF. Migration of
598 turkey PBMCs-derived monocytes (A) and splenic lymphocytes (B) was observed in the
599 presence of serially diluted rTkMIF (0.01, 0.1, and 1.0 µg/ml). Experimental wells were set up
600 in triplicate and values represent mean of two independent experiments. Error bars represent
601 SEM. Asterisks (*) indicate statistically significant differences (*, ** = $P < 0.05$, 0.01,
602 respectively).

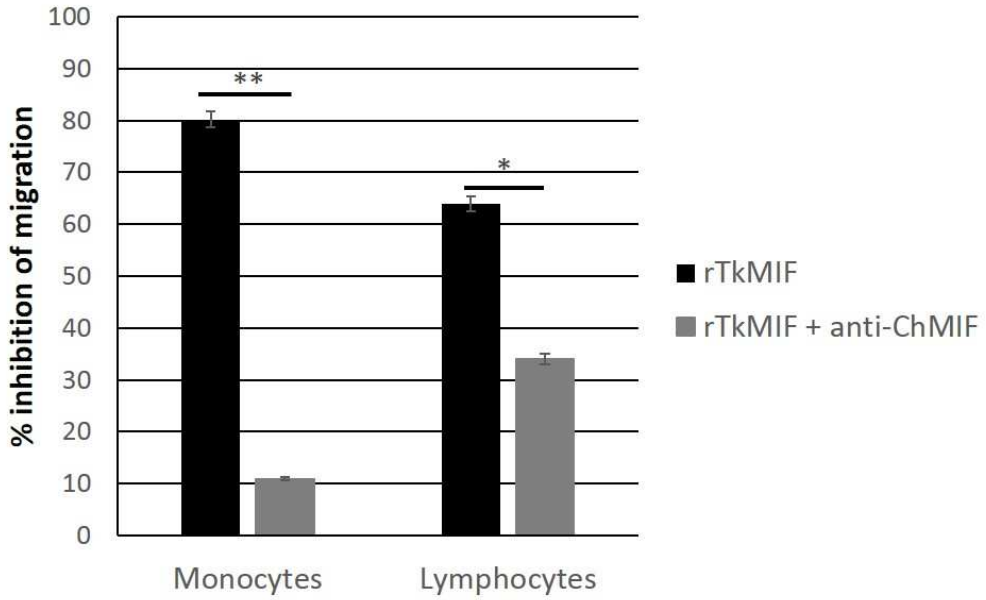


Figure 4. Blocking of MIF-induced inhibition of cell migration. Migration of PBMC-derived monocytes and splenic lymphocytes were examined in the combination of rTkMIF (0.1 µg/ml) in the absence or presence of anti-ChMIF antisera (1:1000 dilution). The experiment was set up in triplicate and data represent mean of two independent experiments. Error bars represent SEM. Statistically significant differences indicated by asterisks (*, ** = $P < 0.05$, 0.01, respectively).

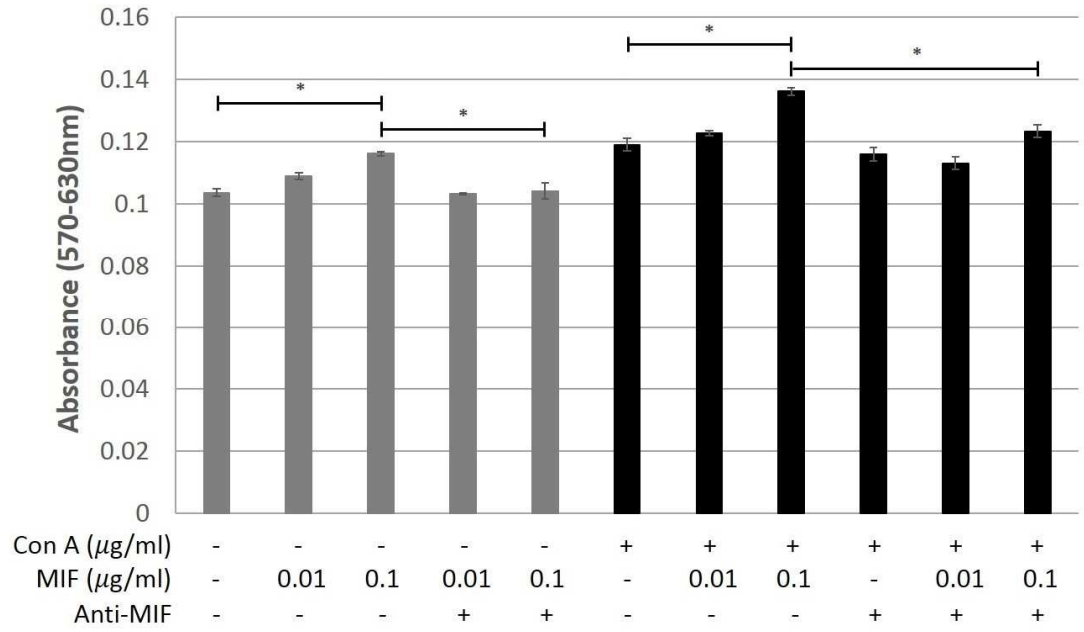


Figure 5. The proliferative effect of rTkMIF on splenic lymphocytes. $1x10^5$ cells were treated with media alone, Con A (10 µg/ml) alone, rTkMIF (0.01 and 0.1 µg/ml) alone, Con A with rTkMIF (0.01 and 0.1 µg/ml), rTkMIF (0.01, 0.1 µg/ml) with anti-rChMIF polyclonal antibody (1:1000 dilution) and Con A with anti-rChMIF polyclonal antisera in the absence or presence of rTkMIF (0.01 and 0.1 µg/ml) for 24 hr. The cell proliferation assay was performed in triplicate per manufacturer's instruction. Data represent the mean of two independent experiments and significant differences are indicated by asterisks ($P < 0.05$). Error bars represent SEM.

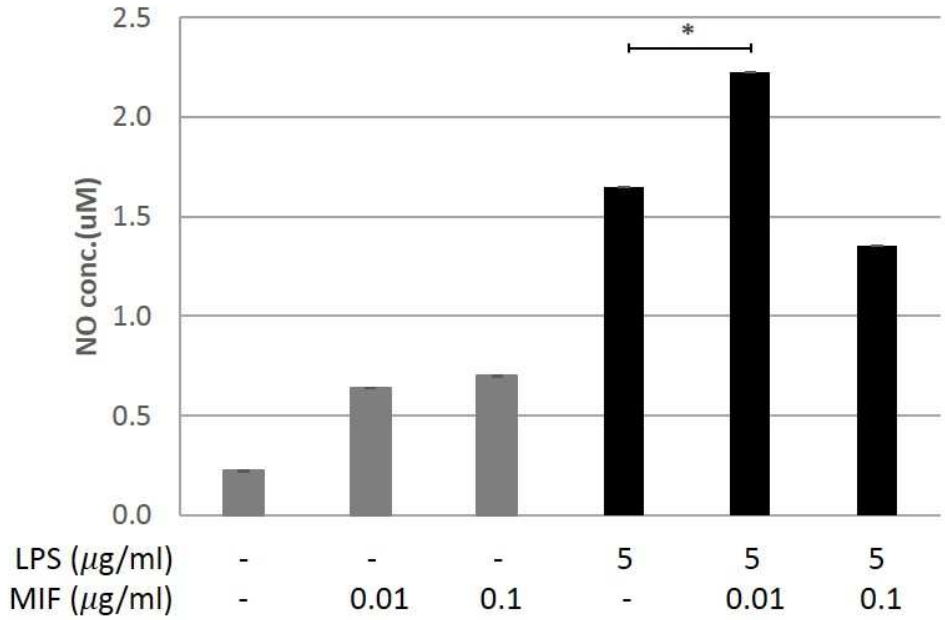


Figure 6. Nitric oxide release of rTkMIF-treated PBMC derived monocytes. Monocytes ($1x10^6$ cells/well) were treated with media alone, rTkMIF (0.01 and 0.1 µg/ml) alone, LPS (5 µg/ml) alone, rTkMIF (0.01 and 0.1 µg/ml) with LPS (5 µg/ml) for 6 hr. NO assay was performed in triplicate following the manufacturer's instructions. Data represent the mean of three independent experiments with significant differences indicated by asterisks ($P < 0.05$). Error bars represent SEM.

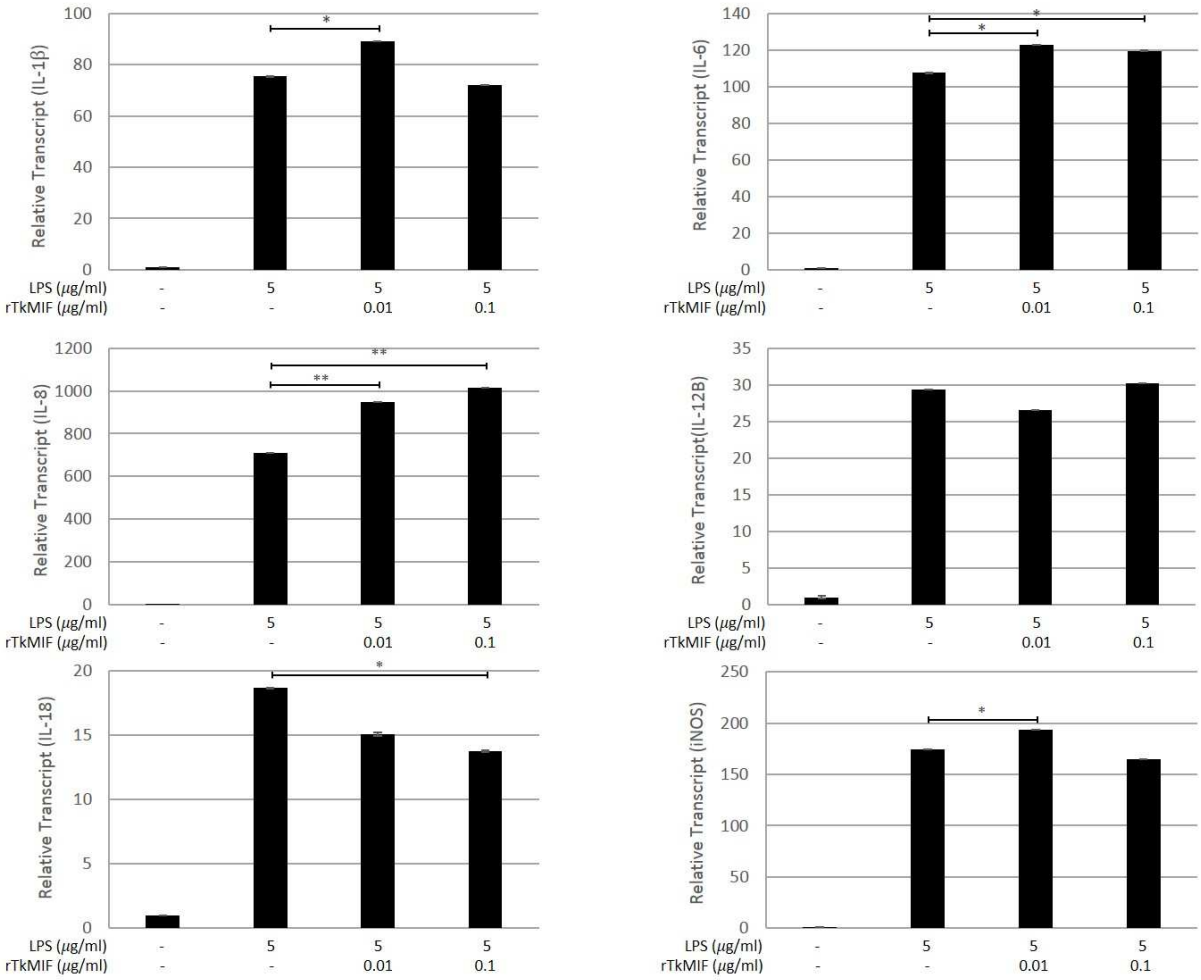


Figure 7. mRNA expression of pro-inflammatory cytokines and chemokine on rTkMIF treated monocytes. PBMCs derived monocytes ($1x10^6$ cells/well) were treated with media alone, LPS (5 µg/ml) alone, rTkMIF (0.01 and 0.1 µg/ml) with LPS (5 µg/ml) for 6 hr and the expression of pro-inflammatory cytokines was examined by qRT-PCR. Transcript levels were standardized to GAPDH and compared to media alone. Data shown represent the mean of three different experiments with significant differences indicated by asterisks (*, ** = $P < 0.05$, 0.01, respectively). Error bars represent SEM.

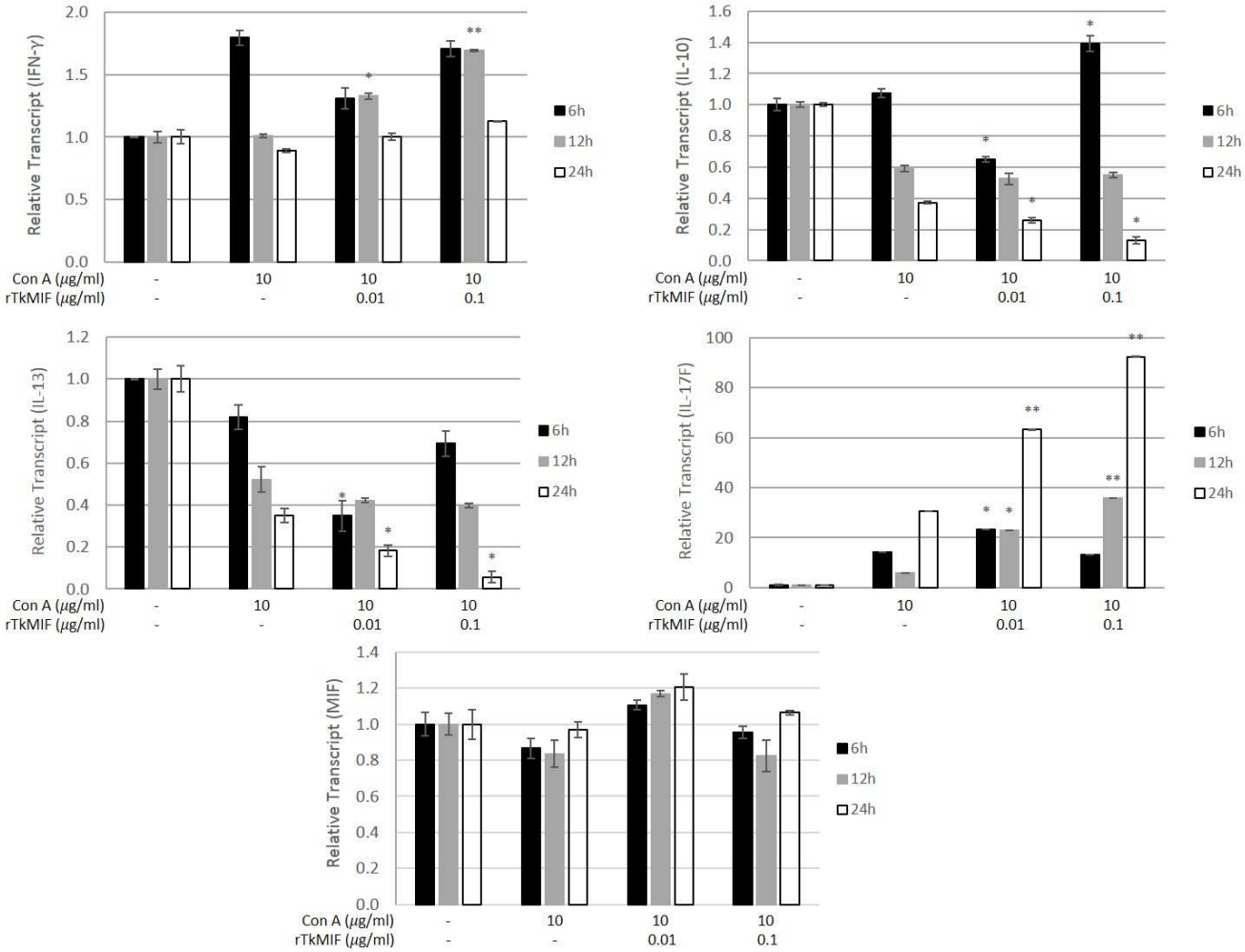


Figure 8. mRNA expression of Th1/Th2/Th17 cytokines on rTkMIF treated splenocytes. Splenic lymphocytes ($1x10^6$ cells/well) were treated with media alone, Con A (10 µg/ml) alone, rTkMIF (0.01and 0.1 µg/ml) with Con A (10 µg/ml) for 6, 12, 24 hr and the expression of cytokines was examined by qRT-PCR. Transcript levels were standardized to GAPDH and compared to media alone. Data shown represent the mean of two independent experiments with significant difference of transcription compared to that of Con A alone indicated by asterisks (*, ** = $P < 0.05$, 0.01, respectively). Error bars represent SEM.

Functional characterization of the turkey macrophage migration inhibitory factor

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Highlights

- Turkey MIF was identified and its biological functions explored, including migration inhibitory activities of macrophages, cell proliferative activity, and induction of pro-inflammatory as well as Th1/Th2/Th17 cytokines in activated immune cells.
- Turkey and chicken MIFs share high sequence homology, functional similarities and cross-reactivity.