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A macrophage migration inhibitory factor-like tautomerase from *Teladorsagia circumcincta* (Nematoda: Strongylida)

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Summary

A macrophage migration inhibitory factor (MIF)-like molecule, Tci-MIF-1, was characterised in *Teladorsagia circumcincta*. A cDNA representing *Tci-mif-1* possessed a 348 bp open reading frame (ORF) and the closest homologue of Tci-MIF-1 in public databases was a MIF from the hookworm *Ancylostoma ceylanicum* with 83% amino acid identity and 91% similarity across all 115 residues. Messenger RNA (mRNA) representing the Tci-MIF-1 was present in eggs, L3 and adult stages of *T. circumcincta*. The transcript was also present, but to a much lesser extent in the L4 stage and the protein expression profile of Tci-MIF-1 reflected these transcript levels. In sections of L3 worms, Tci-MIF-1 localised to gut tissue but also, in a more diffuse manner, to material between the cuticle and gut wall. Sections of L4 larvae showed a similar pattern of localisation with more intense staining along the gut wall and localisation in adults was almost exclusively to gut tissues. A recombinant version of Tci-MIF-1 and somatic extract of L3 *T. circumcincta* both possessed the dopachrome tautomerase activity associated with active MIF molecules, however neither native Tci-MIF-1, nor the recombinant molecule dramatically influenced sheep monocyte migration in *in vitro* analyses of MIF activity.

Keywords

macrophage migration inhibitory factor (MIF); cytokine; parasitic nematode; immunoregulation

Introduction

Parasitic helminths produce a variety of host-immunomodulatory factors, on their cuticular surfaces and in their excretory and secretory (ES) material, which regulate the outcomes of

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infection and permit their survival in the host (reviewed by Maizels and Yazdanbakhsh, 2003)(add Johnston et al 2009 Parasitology 136:125-141?). Several studies have shown the production of macrophage migration inhibitory factor (MIF) orthologues by parasitic nematodes (reviewed in Vermeire et al., 2008). Mammalian MIF, a cytokine which is synthesized by a variety of cells including T lymphocytes, inhibits random migration of monocytes, influences cytokine production and acts as an important pro-inflammatory cytokine in innate immunity (Bernhagen et al., 1994; Calandra and Roger, 2003).

The relationships between macrophages and parasitic nematodes during infection may be complex: While macrophages are not thought to possess nematode antigen-specific receptors, they do co-localise with lymphocytes responding to nematode antigens during infection. In addition, nematode-elicited macrophages, a type of alternatively activated macrophage, are generated in the presence of *Bm*-MIF-1, a nematode-derived MIF of the filarioid *Brugia malayi*, resulting in an induced eosinophilia (Falcone et al., 2001). *Bm*-MIF-1, and a related homologue *Bm*-MIF-2, also promote the IL-4-dependent alternative activation of functionally suppressive macrophages in vitro (Prieto-Lafuente et al 2009 J Leuk Biol 85:844-854). Because these alternatively activated macrophages drive the differentiation of naïve T-cells into T_H2 cells in a mouse model, they represent an important contribution to the development of the T_H2-polarised host immune response to helminth infection (Allen et al., 1996). Both mammalian MIF and *Bm*-MIFs can act as pro-inflammatory cytokines (Zang et al., 2002), however, the continuous release of *Bm*-MIF by adult worms in the host is thought to activate an anti-inflammatory pathway which would be more in keeping with the anti-inflammatory phenotype of the infection (Kleeman et al., 2000; Maizels and Yazdanbakhsh, 2003).

Ovine parasitic gastroenteritis (PGE) in temperate regions is primarily due to infection with the nematode *Teladorsagia circumcincta* and, through a process of proteomic and genomic analysis, we have identified a number of vaccine candidates for Teladorsagiosis (Redmond et al., 2006; Nisbet et al., 2008,2009; Smith et al., 2009). In addition to these antigens, MIFs of parasitic nematodes have also been described as “ideal targets” for vaccine based strategies (Vermeire et al., 2008). Given this potential, and the rationale that a better understanding of parasite:host interplay is likely to assist in the rational selection of drug targets [against parasite-derived immunomodulatory molecules for example (Maizels, 2009)], we have investigated MIF from *T. circumcincta* (Tci-MIF-1). We have previously demonstrated the presence of a transcript encoding MIF in a cDNA library which had been enriched for genes differentially expressed in the infective larval stage (xL3) of *T. circumcincta* and showed, by microarray, that this transcript was expressed at a higher level in xL3 than in the L4, early parasitic stage, of the worm (Nisbet et al., 2008). Here we describe the stage- and tissue-specific expression of Tci-MIF-1 and investigate both its biochemical properties and potential as a cytokine in host immune modulation.

Materials and Methods

Cloning and sequence determination for *Tci-mif-1*

Examination of a putative MIF-encoding sequence from a *T. circumcincta* xL3-enriched EST dataset (Nisbet et al, 2008; Accession number CB043804) suggested that the entire

coding sequence of *Tci-mif-1* was contained within that EST but with a potentially spurious, premature, termination codon. Gene-specific oligonucleotide primers were designed to incorporate the initiation and putative correct termination codons (primer sequences are available from the authors on request) and the open reading frame (ORF) of *Tci-mif-1* was amplified by reverse transcriptase polymerase chain reaction (PCR). The PCR was conducted using cDNA synthesized from xL3 *T. circumcincta* RNA [prepared as described in (Redmond et al., 2006)] as a template, employing Advantage™ 2 polymerase (Clontech) according to the manufacturer's instructions. Cycling parameters were 95 °C 1 min (1 cycle), 95 °C 30 sec, 60 °C 30 sec, 68 °C 1 min (30 cycles) with a final extension cycle of 68 °C for 1 min. The resultant amplicon was column-purified (QIAquick® PCR purification kit, Qiagen) and ligated into the vector pGEM®-T (Promega). The constructs were transformed into *Escherichia coli* JM109 (Promega) and cultured in Luria Bertani medium (LB) prior to selection on LB agar containing ampicillin (100 µg ml⁻¹), 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 80 µg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). Colonies with plasmids containing *Tci-mif-1* were isolated and plasmids extracted, using Wizard® Plus SV Minipreps (Promega), after overnight liquid culture in LB medium containing ampicillin (100 µg ml⁻¹). Automated sequencing (eurofins MWG operon) confirmed the sequence of each of four plasmids isolated in this manner. Nucleotide sequences were compared with those in public databases including the GenBank non-redundant database, using the Basic Local Alignment Search Tool (BLASTn and BLASTx) programme from the National Center for Biotechnology Information and the Parasite Genomes WU-Blast2 Nematoda database (BLASTn and BLASTx), administered by the European Bioinformatics Institute.

The predicted amino acid sequence of the full length Tci-MIF-1 was aligned with putative orthologues from other species of invertebrate organisms using the Clustal X algorithm (Jeanmougin et al., 1998) and a neighbour-joining tree drawn according to relationships inferred by the multiple sequence alignment. The neighbour joining tree was bootstrapped 1000 times using Clustal X (Jeanmougin et al., 1998) and the resulting tree viewed with TreeView (Page, 1996).

Expression of recombinant *Tci-mif-1*

Purified plasmid containing the full-length ORF was used as a template in the PCR amplification of, and accompanying addition of *Bam*H-I, *Hind*-III, *Nde*-I and/or *Xho*-I restriction sites to, *Tci-mif-1*. PCR conditions were as described above and the PCR products were sub-cloned into the expression vector pET-22b(+) (Novagen) with (*Bam*H-I and *Hind*-III restricted product) or without (*Nde*-I and *Xho*-I restricted product) a periplasmic secretion signal and containing a C-terminal His-tag, cultivated in *E. coli* JM109 competent cells and plasmid was extracted as above. After sequencing to confirm that the constructs were in frame, the plasmids were used to transform *E. coli* BL21-CodonPlus® (DE3)-RIL competent cells (Stratagene) and recombinant protein expression was induced using 1 mM IPTG. Soluble r*Tci-mif-1* was purified from cell lysates by nickel column affinity chromatography using HisTrap™ HP columns (GE Healthcare) according to the manufacturer's instructions. Purified r*Tci-mif-1* was used for electrophoresis on a NuPAGE® Bis-Tris 4-12% gel under reducing conditions and stained with SimplyBlue™

according to the manufacturer's instructions (Invitrogen). To confirm the identity of r*Tci-mif-1*, the single visible band was excised, destained and reductively alkylated using DTT and iodoacetamide. The gel pieces were digested overnight with trypsin at 37 °C. Digests were analysed on an Ultraflex II MALDI-ToF-ToF mass spectrometer (Bruker Daltonics), scanning the 600 to 5000 Dalton region in reflectron mode producing monoisotopic resolution. The spectra generated were mass calibrated using known standards and the peaks deisotoped. Masses obtained were used for database searching with the MASCOT search engine using the Swiss-Prot and local databases with a 50 ppm mass tolerance window. Significant matches from the Peptide Mass Fingerprint data were confirmed by MS/MS analysis using the search criteria detailed above and an MS/MS tolerance window of 0.5 Da.

Analysis of transcription pattern of *Tci-mif-1*

Single stranded cDNA (ss-cDNA) was synthesized from mRNA isolated from *T. circumcincta* eggs, infective L3, L4 (harvested at 7 days p.i.) and adults (harvested at 28 days p.i.) as described previously (Redmond et al., 2006). Approximately 5 ng ss-cDNA were used as template in semi-quantitative PCR reactions using primers specific for *Tci-mif-1* (sequences available on request). The following cycling conditions were used: 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 1 min with a final 10 min extension at 72°C. Equal loading and integrity of each of the ss-cDNA preparations was verified by PCR using primers designed to amplify a 320 bp fragment of the *T. circumcincta* β -tubulin gene (Accession number Z69258; Elard et al., 1996). Negative controls were performed where ss-cDNA was omitted from the reactions. Amplification products were separated on a 1.5 % (w/v) agarose gel and visualized by staining with GelRed™ (Biotium).

Detection of Tci-MIF-1 in parasite extracts

T. circumcincta homogenates from L3, L4 and adults (12 µg total protein each) prepared as described previously (Nisbet et al., 2009) and pure rTci-MIF-1 were electrophoresed on NuPAGE® Bis-Tris 4-12% gels under reducing conditions employing NuPAGE® MES SDS running buffer (Invitrogen). Proteins were transferred to a nitrocellulose membrane according to the manufacturer's instructions (Invitrogen). After transfer, the membrane was washed briefly in TNTT (10 mM Tris, 0.5 M NaCl, 0.05% Tween 20, 0.01% thiomersal pH 7.4) then incubated in TNTT overnight at 4 °C to block non-specific protein adsorption. To detect Tci-MIF-1, the proteins were incubated with serum raised in rabbits against rTci-MIF-1. Blots were incubated with rabbit sera (pre-immune and anti-rTci-MIF-1) diluted (1:50 in TNTT) for 1 h at RT with constant rocking. The blots were washed (10 min/wash) three times in TNTT, before being incubated in goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (DakoCytomation) diluted 1:1000 in TNTT. Following incubation at RT for 1 h, the blots were washed a further three times in TNTT and peroxidase activity was revealed using 3,3'-Diaminobenzidine (DAB, Sigma) as substrate.

Immunolocalisation of Tci-MIF-1 in *T. circumcincta*

Live *T. circumcincta* [L3,L4 (day 8 p.i.) and adult (day 28 p.i.)] were fixed in 10% formal saline for 1 h, washed (x 2) in PBS, then resuspended in 5% gelatin in PBS. Once set, the pellet was re-fixed overnight and processed into paraffin wax prior to cutting 5 µm-thick

sections. Sections were dried at 40 °C, dewaxed in xylene and rehydrated. Sections were treated with 0.01% sodium periodate for 5 min followed by sodium borohydride (0.1 mg ml⁻¹) for 2 min to remove endogenous peroxidase. Following washing in ddH₂O, sections were incubated in TNTT for 30 min at RT before incubation for 1.5 h at RT with pre-immunisation serum or serum raised in rabbits against rTci-MIF-1 (1:25 dilution in TNTT). Goat anti-rabbit IgG-HRP conjugate (1:1000 dilution in TNTT, DakoCytomation) followed by DAB substrate (Sigma) were used to detect binding. Negative controls were prepared by omitting the primary antibody.

Dopachrome tautomerase activity of Tci-MIF-1

T. circumcincta homogenate from L3 was prepared by grinding snap-frozen L3 to a powder under liquid nitrogen and re-constituting the powder in sodium phosphate buffer (20 mM sodium phosphate pH 7.0, 2 mM EDTA, 0.15 M NaCl, 0.1 mM dithiothreitol). The resulting suspension was centrifuged (15,000g at 4°C for 20 min) and the supernatant stored at -80 °C until use. Tci-MIF-1 was enriched from L3 extracts by phenyl-agarose chromatography using HiTrap™ Phenyl HP columns (GE Healthcare) as described previously (Pennock et al., 1998b). The tautomerisation of dopachrome to 5,6-dihydroxyindole-2 carboxylic acid (DHICA), catalysed by Tci-MIF-1 or rTci-MIF-1 (expressed with or without the periplasmic secretion signal of pET22b, purified and dialysed against 20 mM sodium phosphate pH7.4, 0.15M NaCl), was measured using L-dopachrome methyl ester, generated from 3,4-dihydroxy-L-phenylalanine (L-DOPA) methyl ester and periodate, as described previously (Pennock et al., 1998a). Recombinant MIFs of *Brugia malayi* [*Bm*-MIF-1 (enzymically active) and *Bm*-MIF-1G (inactive) (Zang et al., 2002)] were used as positive and negative controls in these tautomerase assays. All assays were performed in triplicate (as a minimum) and repeated three times. Spontaneous decay of L-dopachrome methyl ester as a result of decarboxylation of the substrate was measured by omitting enzyme, L3 extract, or its chromatographic fractions, from the assay. Values for dopachrome tautomerase activity of the extracts were calculated after correction for this spontaneous decay.

Following enrichment of the dopachrome tautomerase activity by affinity chromatography, 13 µl of each eluted fraction were electrophoresed on NuPAGE® Bis-Tris 4-12% gels under reducing conditions employing NuPAGE® MES SDS running buffer (Invitrogen), blotted onto nitrocellulose and the presence of Tci-MIF-1 demonstrated as detailed above ("Detection of Tci-MIF-1 in parasite extracts").

In vitro assessment of Tci-MIF-1 cytokine activity

The influence of Tci-MIF-1 on the migration of ovine monocytes was studied with Transwell chemotaxis chambers (6.5mm diameter polycarbonate filters with 5µm pores; Costar) using a modification of methods described previously (Pastrana et al., 1998; Tan et al., 2001). Briefly, peripheral blood mononuclear cells (PBMCs) were prepared using Ficoll-Paque™ (GE Healthcare) diluted 50:50 (vol:vol) with PBS containing EDTA (500µM). The PBMC fraction was washed twice in PBS-EDTA then resuspended in medium A [RPMI 1640 medium (Gibco BRL) containing 25 mM HEPES, 2 mM L-Glutamine, 1 mM sodium pyruvate, 100U ml⁻¹ penicillin, 100µg ml⁻¹ streptomycin], then further refined by density separation using a Percoll™ (GE Healthcare) cushion (0.15M NaCl, 46% Percoll). The

monocyte fraction was washed twice in PBS-EDTA, then resuspended in medium A, counted and diluted to final concentration of 2×10^6 cells ml^{-1} . Recombinant or native purified Tci-MIF-1 preparation was diluted to a final concentration of $1 \mu\text{g protein ml}^{-1}$ with medium A, mixed with monocytes and applied to the top chamber of the Transwell apparatus. In a second set of Transwell chambers, native or recombinant Tci-MIF-1 was included in the bottom chamber. Negative controls comprised i) medium A (no MIF) and ii) heat-denatured recombinant Tci-MIF-1 (70°C for ten minutes to abolish dopachrome tautomerase activity).

Assays were prepared in quadruplicate and incubated at 37°C , 5% CO_2 for 3 h. After incubation, the media were removed and non-migrated cells on the upper surface of the filter were washed off with two changes of 37°C PBS and aspiration. The migrated cells within the filter were fixed with ice-cold methanol for ten minutes at -20°C then air dried overnight. Filters were excised from the Transwell assembly, stained using a Diff Quick kit (Dade-Behring A.G., Duedingen, Switzerland) and mounted on glass slides in Mowiol® mountant (Calbiochem). Migrated cells were counted in five random fields of view per filter. The mean numbers of migrated cells per treatment were compared using ANOVA.

In addition to the Transwell method, we also used a method based on that described by Harrington & Stastny, (1973). Briefly, one microlitre droplets of 0.8% (w/v) agarose (SeaPlaque Agarose, Cambrex BioScience) at 37°C were placed in each well of a 96-well low protein-binding multiwell plate to form hemispheres of approx. 2 mm diameter. The droplets were allowed to dry slightly before $2 \mu\text{l}$ of ovine monocyte suspension ($2 \times 10^7 \text{ ml}^{-1}$) prepared as above with or without Tci-MIF-1 were placed onto the agarose droplet before chilling the entire plate at 4°C for five minutes, allowing the cells to adhere to the droplet. The wells were then gently flooded with $250 \mu\text{l}$ medium A (as above) and incubated at 37°C , 5% CO_2 for 48h. After 24h and 48h, migration of the macrophages from the droplet was measured by capturing images using an inverted phase microscope attached to a camera. The number of macrophages in each sector was counted and expressed as a percentage of the total macrophages within the grid. A mean of eight images per treatment, at both 24 and 48h, was calculated.

Results

Cloning and sequence analysis of *Tci-mif-1*

The cDNA representing *Tci-mif-1* (Accession number) possessed a 348 bp open reading frame (ORF) and exhibited 99.5% nucleotide identity to a previously described *T. circumcincta* EST (Accession number CB043804) across 288 of these bases. The closest homologue of Tci-MIF-1 in public databases was a MIF from the hookworm *Ancylostoma ceylanicum* (Accession number ACC54555.1) with 83% amino acid identity and 91% similarity across all 115 residues. Tci-MIF-1 possessed 41% amino acid sequence identity (62% similarity) to the sheep (*Ovis aries*) MIF (Accession number NP_001072123). When the relationships between Tci-MIF-1 and selected MIF orthologues from other nematode species were examined by neighbour joining analysis, Tci-MIF-1 clustered with MIF-1-type molecules (Vermeire et al., 2008) rather than MIF-2-type orthologues (Figure 1A). Of the “invariant” residues thought to be associated with both tautomerase activity and the MIF

substrate molecule-interaction site (reviewed in Vermeire et al., 2008), Tci-MIF-1 possessed the N-terminal catalytic proline (Pro1, Figure 1B) which is exposed following post-translational cleavage of the initiating methionine (Swope et al 1998) as well as the residues Lys32 and Ile64, but not Val106. The substitution of isoleucine for valine at position 106 is common to each of the MIF-1-type sequences shown in Figure 1B from Clade V nematodes. Clade I nematodes shown in Figure 1B have valine at this residue; however each of the Clade III nematodes has methionine at this position (Figure 1B). In addition, Prosite MIF Pattern (Accession number PS01158) [DE]-P-[CLV]-[APT]-x(3)-[LIVM]-x-S-[IS]-[GT]-x-[LIVM]-[GST] is only partly represented in Tci-MIF-1 as the motif “⁵⁴GPCGVGVLSIGGVG⁶⁸” where the residues which are not represented by the prosite pattern are underlined (Figure 1B).

Expression pattern and immunolocalisation of *Tci-mif-1*

Messenger RNA representing the Tci-MIF-1 was present in eggs, L3 and adult stages of *T. circumcincta*. The transcript was also present, but to a much lesser extent in the L4 stage (Figure 2A). Gender-specific or gender-enriched gene expression was not seen for the *Tci-mif-1* transcript (data not shown). The protein expression profile of Tci-MIF-1, determined by Western blot, demonstrated higher levels of the protein (as a proportion of total protein in the extracts) in L3 extracts than in extracts of either L4 or adults (Figure 2B). In sections of L3 worms, MIF localised to gut tissue but also, in a more diffuse manner, to material between the cuticle and gut wall (Figure 3). Sections of L4 larvae showed a similar pattern of localisation with more intense staining along the gut wall and localisation in adults was almost exclusively to gut tissues.

Dopachrome tautomerase activity of Tci-MIF-1

Aqueous extracts of *T. circumcincta* L3 contained dopachrome tautomerase activity, as measured by conversion of dopachrome to DHICA *in vitro* (Figure 4A). This activity was greatly enriched by concentration of the active enzyme during phenyl sepharose affinity chromatography (Figure 4A). The association between the presence of Tci-MIF-1 in the extracts and eluates and the dopachrome tautomerase activity was demonstrated by Western blot using serum raised in rabbits against rTci-MIF-1 (Figure 4B).

Soluble rTci-MIF-1, expressed in a bacterial expression system, displayed dopachrome tautomerase activity (Figure 5) but only if the recombinant protein was expressed in the absence of the pelB leader or any residual N-terminal amino acids remaining following removal of the pelB leader by the bacterial signal peptidase, i.e. only if the recombinant protein had a “native” N-terminal.

In vitro assessment of Tci-MIF-1 cytokine activity

Ovine monocytes, in contact with either native or recombinant Tci-MIF-1 migrated through the membrane of a Transwell chemotaxis chamber less readily than those in the control group (Figure 6A) but this effect was subtle and there were no significant differences in migration between individual treatment groups. Similarly, when the Tci-MIF-1 was in the lower chamber of the chemotaxis device (i.e. not in direct contact with the monocytes), native Tci-MIF-1 appeared to have a subtle effect on activating the movement of monocytes

but again this effect was neither dramatic nor statistically significant (Figure 6B). Using a slightly different monocyte migration assay [adapted from Harrington & Stastny, (1973)], we were unable to dissect these subtle effects any further (data not shown).

Discussion

Here, we have shown the presence of an enzymically active MIF-like molecule, Tci-MIF-1, in the somatic tissues of the ovine abomasal nematode parasite *T. circumcincta*. The expression levels of both the gene encoding Tci-MIF-1 and the protein itself were highest in the pre-parasitic, infective larval stage (L3), though both the transcript and its protein product were present in all stages analysed. The possible functional significance of a host cytokine mimic in a free-living stage is perhaps difficult to reconcile, though evidence from other species of nematode gives indications of potential multiple functions of their MIF orthologues: In *B. malayi*, *Bm-mif-1* is also expressed in all developmental stages, with transcript levels in microfilariae and adults being approximately double those in L3 and L4 stages (Pastrana et al., 1998). Bm-MIF-1 protein was also present in both somatic extracts and ES material from each of the stages investigated and immunolocalisation showed the presence of the protein in the uterine lining, hypodermis and the surface of muscle bundles (Pastrana et al., 1998). In *C. elegans*, MIF homologues are expressed in muscle bundles, hypodermis, reproductive tissues and intestine (data available from WormBase WS201 www.wormbase.org). The presence and association of mammalian MIF with a number of different cell and tissue types (e.g. T cells, macrophages, pituitary gland corticotrophic cells, eye lens cells, keratinocytes etc.) suggests that it also carries out multiple roles (Pastrana et al., 1998). *C. elegans* is a free-living nematode with no requirement to influence a host immune system, yet this species possesses four MIF-encoding genes (Marson et al., 2001). At least two plant-parasitic nematodes (*Meloidogyne* and *Heterodera* spp.) also possess MIF homologues. While gene silencing by RNAi has not demonstrated phenotypes for three of the four homologues, silencing of *Ce-mif-2* by RNAi suggests a key role for this gene in embryogenesis (Lehner et al., 2006), consistent with the observations of MIF's importance in rapidly differentiating cells and embryos (Marson et al., 2001). Interestingly, the transcription of both *Ce-mif-2* and *Ce-mif-3* was increased by more than 100 fold in dauer larvae compared to the expression levels in L2, suggesting an additional role in cellular maintenance during developmental arrest (Marson et al., 2001). Some studies have suggested that parasitic nematode infective stage larvae represent a “dauer-like” stage of the lifecycle (e.g. Brand et al., 2005) and this may partly explain the higher levels of transcript and protein in L3 *T. circumcincta* compared with L4 and adult, though more recent studies suggest significant divergent evolution between the processes involved in dauer formation and those linked to infective larval development in parasitic nematodes (Elling et al., 2007).

Although these studies suggest roles for MIF in nematode physiology and a potential for the molecule to act as a cytokine within the nematode itself, it is also thought that parasitic helminths have adapted these ancestral genes to mimic host cytokines and modulate the host immune system (Maizels and Yazdanbakhsh, 2003). The presence of Tci-MIF-1 in the gut of the parasitic stages of *T. circumcincta* suggested that the molecule may be excreted and involved in influencing monocyte taxis and activation. If this were the case the interaction may be, at least in part, a contributing factor to the eosinophilia associated with *T.*

circumcincta infection and the resulting relationship between an increased host eosinophilia and larger worm length (Henderson and Stear, 2006). In the current study we present some evidence for Tci-MIF-1 possibly acting as a host cytokine mimic, but the effect was subtle.

In addition to inhibiting the random migration of monocytes and macrophages, mammalian MIF is a functional tautomerase (EC 5.3.2.3) interconverting the enol and keto forms of phenylpyruvate and (*p*-hydroxyphenyl)pyruvate and converting dopachrome to 5,6-dihydroxyindole-2-carboxylic acid [DHICA (Rosengren et al, 1996)]. We have demonstrated functional tautomerase activity for Tci-MIF-1 in both the native enzyme preparations and a recombinant version of the protein. The possible links between MIF's ability to act as a tautomerase and its role as an inhibitor of monocyte chemotaxis and random migration have been investigated using murine MIF mutants with an altered N-terminal proline. These mutated MIFs were found to have minimal or no enzymatic activity but did not differ from wild-type MIF in monocyte chemotaxis and random migration assays (Hermanowski-Vosatka et al., 1999). In contrast, mutation of Pro1 to Gly had profound negative effects on both dopachrome tautomerase activity and cytokine activity of MIFs from *Brugia* (Zang et al., 2002).

As Tci-MIF-1 may have only subtle effects as a host cytokine mimic its primary role may lie elsewhere in nematode physiology, acting as a dopachrome tautomerase. Dopachrome, a derivative of tyrosine, plays a major role in the production of melanin in both mammals and invertebrates and melanotic encapsulation is a key process in innate immunity to invading pathogens in a number of invertebrates. Melanin biosynthesis as a defence response in insects is initiated, via the prophenoloxidase cascade, by the hydroxylation of tyrosine to DOPA and subsequent oxidization to dopaquinone and dopachrome. Each of these three reactions is catalysed by the enzyme phenoloxidase and further re-arrangement of dopachrome to the melanin precursor 5,6-dihydroxyindole is performed by dopachrome conversion enzyme [DCE (Han et al., 2002; Yuang et al., 2005)]. In mammals, which lack the prophenoloxidase cascade reaction as part of their innate immune response, the conversion of dopachrome during melanogenesis is by dopachrome tautomerase, which converts dopachrome to DHICA.

In *C. elegans*, a soil-dwelling nematode, frequent exposure to pathogenic organisms would be expected and the immune system of *C. elegans* has been studied widely and used as a model for innate immunity in other organisms (Schulenburg et al., 2004). Although nematode coelomocytes possess the ability to phagocytose bacteria (reviewed in Tahseen, 2009) it is not known whether the phagocytosed pathogens are then melanised, as is the case in arthropods. *C. elegans*, at least, lacks the enzymes necessary for a prophenoloxidase cascade (Schulenburg et al., 2008) and, if nematodes do use melanisation as part of their immune repertoire, it is probable that the conversion of tyrosine to melanin is dependent on tyrosinases (Cerenius and Söerhäll 2004) and dopachrome tautomerase in a similar manner to that seen in vertebrates rather than the phenoloxidase/DCE system seen in arthropods.

An alternative role for melanisation may be protection from ultraviolet light (UV) exposure, a subject which has received recent attention because of the potential impact of climate change on the epidemiology of parasitic nematodes (van Dijk et al., 2009). Increased

exposure to UV under both laboratory and field conditions resulted in substantial increases in mortality of *T. circumcincta* larvae (van Dijk et al., 2009). This observation may, in part, explain why both transcript and protein levels of Tci-MIF-1 are substantially higher in the infective L3 stage compared to those stages which exist within the host (Figure 2).

In conclusion, Tci-MIF-1 acts as a dopachrome tautomerase and is enriched in the free-living, infective stage of *T. circumcincta*. Although it is present in the parasitic stages of the nematode, we were not able to unequivocally demonstrate a functional role for Tci-MIF-1 as a cytokine mimic acting on host monocytes, in an *in vitro* system.

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Figure 1.

Relationship between *Tci-MIF-1* and other selected nematode MIF orthologues.

Panel A: The neighbour joining tree was bootstrapped 1000 times using Clustal X (Jeanmougin et al. 1998) and the resulting tree viewed with TreeView (Page, 1996). Each node is annotated with a figure indicating the degree of bootstrap support for each branch. Abbreviations and accession numbers are as follows: *Trichuris*: [*Trichuris trichiura* (CAB46355)]; *Trichinella*: [*Trichinella spiralis* (CAB46354)]; *Ascaris*: [*Ascaris suum* (BAD24819)]; *Brugia1*: [*Brugia malayi* (AAB60943)]; *Brugia2*: [*B. malayi* (AAF91074)];

Ostertagia: [*Ostertagia ostertagi* EST (BQ457911)]; *Haemonchus*1: [*Haemonchus contortus* EST (CB012470)]; *Haemonchus*2: [*H. contortus* EST (CB015598)]; *Onchocerca*1 [*Onchocerca volvulus* (AAK66563)]; *Onchocerca*2 [*O. volvulus* (AAK66564)]; *Ancylostoma* [*Ancylostoma caninum* EST (AW626839)]; *CeMIF-1* [*Caenorhabditis elegans* (NP_499536)]; *CeMIF-2* [*C. elegans* (NP_506003)].

* Designated as described in Vermeire et al. (2008).

Panel B: Amino acid alignment of *Tci-MIF-1* and other MIF-1-type orthologues. Annotation is as described for Panel A. “Invariant” residues of the MIF active site are solid boxed.

Prosite MIF Pattern (Accession number PS01158) [DE]-P-[CLV]-[APT]-x(3)-[LIVM]-x-S-[IS]-[GT]-x-[LIVM]-[GST] is shown as a dotted box.

*Amino acid residue numbering relative to Pro1 of *Tci-MIF-1*, which is exposed after post-translational cleavage of the initiating methionine.

Figure 2.

Expression pattern of *Tci-mif-1*

Panel A: Approximately 5 ng ss-cDNA derived from each stage were used as template in semi-quantitative PCR reactions using primers specific for *Tci-mif-1*. Equal loading and integrity of each of the ss-cDNA preparations was verified by PCR using primers designed to amplify a 320 bp fragment of the *T. circumcincta* β -tubulin gene (Accession number Z69258; Elard et al., 1996). Negative controls were performed where ss-cDNA was omitted from the reactions. The analysis was performed in duplicate with identical results.

Panel B: *T. circumcincta* homogenates from L3, L4 and adults (12 μ g total protein each) and pure rTci-MIF-1 were electrophoresed and transferred to a nitrocellulose membrane, incubated with serum raised in rabbits against rTci-MIF-1 followed by goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate. Peroxidase activity was revealed using 3,3'-Diaminobenzidine (DAB) as substrate.

Figure 3.

Immunolocalisation of Tci-MIF-1

Sections of *T. circumcincta* [L3,L4 (day 8 p.i.) and adult (day 28 p.i.)] were incubated with pre-immunisation serum (panel A) or serum raised in rabbits against rTci-MIF-1 (panel B). Goat anti-rabbit IgG-HRP conjugate followed by DAB substrate (Sigma) were used to detect binding.

Figure 4.

Dopachrome tautomerase activity and purification from *T. circumcincta* L3 extracts

Panel A: Tci-MIF-1 was extracted (“L3 Ext”) and enriched from L3 extracts by phenyl-agarose chromatography. The tautomerisation of dopachrome to 5,6-dihydroxyindole-2 carboxylic acid (DHICA) was measured using L-dopachrome methyl ester as a substrate. Recombinant MIFs of *B. malayi* [“Bm-MIF-1” (enzymically active) and “Bm-MIF-1G” (inactive) (Zang et al., 2002)] were used as positive and negative controls. All assays were performed in triplicate and repeated three times. Spontaneous decay of L-dopachrome methyl ester as a result of decarboxylation of the substrate was measured by omitting enzyme, L3 extract, or its chromatographic fractions, from the assay (“blanks”). *Enzyme activity expressed as mOD (A₄₇₄) min⁻¹. Error bars represent SEM.

Panel B: The association between the presence of Tci-MIF-1 in the extracts and eluates and the dopachrome tautomerase activity, demonstrated by Western blot using serum raised in rabbits against rTci-MIF-1.

Figure 5.

Enzymically active rTci-MIF-1

Recombinant *Tci*-MIF-1 was generated in a bacterial expression system (BL21-CodonPlus®) using the expression vector pET-22b(+), purified by nickel affinity chromatography and dopachrome tautomerase activity determined. Recombinant MIFs of *B. malayi* [*Bm*-MIF-1 (enzymically active) and *Bm*-MIF-1G (inactive) (Zang et al., 2002)] were used as positive and negative controls. All assays were performed in triplicate. Data shown are means of triplicate measurements in a single repetition of the experiment $\pm$ SEM. Enzyme activity is expressed as $\text{mOD } (A_{474}) \text{ min}^{-1} \mu\text{g protein}^{-1}$.

Figure 6.

In vitro assessment of Tci-MIF-1 cytokine activity

Panel A: Sheep monocytes were incubated with Tci-MIF-1 ($1\mu\text{g protein ml}^{-1}$) in the upper chamber of a Transwell chemotaxis chamber for 3h prior to counting migration into the dividing filter. * Values represent mean ($n=4$) $\pm$ SEM, values annotated with the same letter are not significantly different ($P<0.05$).

Panel B: As for panel A but with MIF in lower chamber of Transwell chamber