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Analysis of the role of *Dirofilaria repens* macrophage migration inhibitory factors in host–parasite interactions

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Abstract

Introduction: *Dirofilaria repens* is a zoonotic parasitic filarial nematode that infects carnivores and occasionally humans. Knowledge of the host–parasite molecular interactions enabling the parasite's avoidance of the host immune response in subcutaneous dirofilariasis remains limited. Parasitic orthologues of host macrophage migration inhibitory factor (MIF) are molecules potentially involved in this process. **Material and Methods:** Complementary DNA encoding two *D. repens* MIF orthologues (r*Dre*-MIF-1 and r*Dre*-MIF-2) was cloned into a pET-28a expression vector. The recombinant proteins were produced in *Escherichia coli* and purified using affinity nickel chromatography. The reactivity of both recombinant proteins was analysed with infected dog and immunised mouse sera. **Results:** Stronger antibody production was induced by r*Dre*-MIF-1 in mice, as evidenced by significantly higher levels of anti-r*Dre*-MIF-1 total IgG, IgG2 and IgE antibodies than of anti-r*Dre*-MIF-2 immunoglobulins. Additionally, a significantly different level of antibodies specific to both proteins was noted between the sera of infected dogs and those of uninfected dogs. **Conclusion:** This study is the first attempt to characterise MIF orthologues from the filarial parasite *D. repens*, which may affect the immune response during infection.

Keywords: MIF, subcutaneous dirofilariasis, recombinant protein, humoral response, ELISA.

Introduction

Dirofilaria repens is a parasite primarily affecting carnivores, especially dogs, but displaying zoonotic potential (8). Dirofilariasis does not manifest strong, noticeable clinical symptoms, but studies suggest that it may induce a state of chronic stress in canine hosts, which may influence the outcome of the immune response (29). The most characteristic symptom is manifested by the formation of subcutaneous nodules, where the encapsulated parasite hides from the host's immune system (22). Despite the increasing threat posed by these parasites to human and veterinary health, knowledge of the molecular mechanisms of the host–parasite interaction during the course of dirofilariasis remains limited.

Parasitic nematodes modulate the host immune response to ensure their survival, and one means by which they achieve this is by secreting immunomodulatory molecules. A particularly intriguing strategy involves mimicking molecules from the host immune system. Nematodes release several orthologues of host immune components, including macrophage migration inhibitory factor (MIF) (3, 15, 17). Mammalian MIFs play a significant role in immune response regulation, serving as proinflammatory cytokines with diverse functions. One of the primary functions of MIFs is attracting cells engaged in both innate and adaptive immune responses. They can be synthesised by various cell types, including monocytes, macrophages, lymphocytes, neutrophils and endothelial and epithelial cells. Macrophage migration inhibitory factors bind to the CD74 receptor (major histocompatibility complex class II invariant chain), and form a complex with a CD44 molecule or other receptors from the CXC chemokine receptor family, leading to modulation of various intracellular signalling pathways (2, 6, 7, 24). This interference results in upregulation of Th1/Th17 type cytokine (tumour necrosis factor alpha

(TNF-α), interleukin (IL)-6, IL-1β, IL-8 and IL-12) expression as well as upregulation of expression of other proteins engaged in the immune response: Toll-like receptor 4, matrix metalloproteinases, prostaglandin E2 and cyclooxygenase 2, additionally resulting in nitric oxide release (5, 12, 16).

Two different orthologues of MIF have been identified in nematodes based on their homology to free-living *Caenorhabditis elegans* MIFs (*Ce*-MIF-1 and *Ce*-MIF-2). They both share structural similarities and catalytic properties (tautomerase and oxidoreductase activity) with mammalian MIFs (9, 27) and are expressed in various developmental stages of filarial species such as *Wuchereria bancrofti*, *Brugia malayi* and *Onchocerca volvulus* (1, 20, 23). Various data suggest their involvement in evading host immune response (4, 28, 31), but their precise molecular function remains enigmatic.

The aims of the study were production of two recombinant MIF orthologues from *D. repens*, analysis of *Dre-mif-1* and *Dre-mif*-2 mRNA expression in microfilariae and the adult stage, and evaluation of r*Dre*-MIF-1 and r*Dre*-MIF-2 immunogenicity in mice and reactivity with antibodies from infected dog sera.

Material and Methods

Expression and purification of r*Dre*-MIF-1 and r*Dre*-MIF 2. Complementary DNA encoding two proteins (GenBank accession numbers MT071087.1 and MT071088.1) was amplified using gene-specific primers containing restriction enzyme sites and cloned in the *E. coli* transformation pET28a plasmid (Novagen, San Diego, CA, USA) following *Bam*HI and *Xho*I digestion. The cloned insert was sequenced using the Sanger technique to confirm that no amino acids had changed by mutation and verify that the open reading frame was appropriate. The plasmids containing the verified insert sequences were transformed to two *E. coli* expression strains: SoluBL21 and BL21. To induce protein expression, isopropyl-1-thio-β-d-galactopyranoside (IPTG) at a final concentration of 1mM was added to the bacterial culture at the log phase of growth. After 2 h, cells were centrifuged at 5,000 × *g* for 10 min at room temperature. Bacterial pellets were either used immediately for protein purification or stored at −20°C.

The pellets were sonicated to disintegrate cell membranes and centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatants containing recombinant fusion proteins with 6 × His tags were collected and the proteins were purified using a Ni^{2+}-charged affinity chromatography column (Cytiva, Little Chalfont, UK) according to the manufacturer's protocol. The purity of the eluted proteins was assessed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Perfect Tricolor Protein Ladder molecular weight marker was used for protein sizing (EURx, Gdańsk, Poland). The two elution fractions with the highest protein concentrations were pooled, dialysed against Dulbecco's phosphate-buffered saline (Biowest, Nuaillé, France) using 2 mL Zeba Spin 7 kDa molecular weight cut-off (7K MWCO) Desalting Columns (Thermo Scientific, Waltham, MA, USA) and assessed for recombinant protein content by Western blotting using Anti-polyHistidine–Peroxidase antibody (Sigma-Aldrich, St. Louis, MO, USA). The purity and concentration of the recombinant protein solutions were determined using SDS-PAGE and a bicinchoninic acid BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA), respectively.

Bioinformatic analysis. Alignment of human MIF (hMIF), dog MIF (dMIF), *Dre*-MIF-1 and *Dre*-MIF-2 protein sequences was performed using the Multalin tool (10). The GenBank accession numbers of the analysed sequences were CAG30406.1 (hMIF), XP_038293371.1 (dMIF), MT071087.1 (*Dre*-MIF-1) and MT071088.1 (*Dre*-MIF-2). The protein identity (%) between them was calculated using ClustalW (25). The tertiary structures of *Dre*-MIF-1 and *Dre*-MIF-2 were predicted using Phyre2 (18). The structures were visualised and superimposed using Protein Imager (26).

***Dre-mif-1* and *Dre-mif-2* mRNA expression in microfilariae and adult *D. repens* stages**. Total RNA was isolated from microfilariae and adult worms using a Total RNA purification kit (A&A Biotechnology, Gdańsk, Poland) according to the manufacturer's instructions. Genomic DNA contamination was removed using DNAseI (Thermo Scientific) and the efficiency of DNA cleavage was assessed using PCR. When free of DNA contamination, the RNA solution was used for cDNA synthesis using a RevertAid RT Reverse Transcription Kit (Thermo Scientific).

Table 1. Primers used in the reverse-transcriptase quantitative PCR to amplify *Dirofilaria repens* macrophage migration inhibitory factor (*Dre-mif*)*-1* and *-2*

Primer	Sequence
*Dre-mif-1*_F	5′ GGCTGATGAACT CAAAAT CCC 3′
*Dre-mif-1*_R	5′ ACCCATTGCCGAAGCACTAATA 3′
*Dre-mif-2*_F	5′ GATTGGATCATTTTCGGCTGATA 3′
*Dre-mif-2*_R	5′ CGTACCATTGCATCCCACATTT 3′

F – forward; R – reverse

The qPCR was performed in a 12 µL volume in a 96-well PCR plate. The reaction components were as follows: 2 µL of cDNA template, 1 × Maxima SYBR Green qPCR Master Mix (Thermo Scientific), ROX passive reference dye (10 nM) and a mixture of the primers *Dre-mif-1*_F and *Dre-mif-1*_R or *Dre-mif-2*_F and *Dre-mif-2*_R at 0.3 µM (Table 1). Due to the lack of data regarding a suitable reference gene for quantitative real-time PCR analyses in *D. repens*, the direct copy number was estimated for reaction evaluation. To achieve standard curves for both genes, pET28a/*Dre-mif-1* or pET28a/*Dre-mif-2* recombinant plasmid was 10-fold

serially diluted to contain from 10^8 to 10^2 copies per reaction and used as a matrix for qPCR. The PCR was performed as follows: 50°C for 2 min, 95°C for 10 min, 45 cycles of 95°C for 15 s and 60°C for 1 min and a disassociation curve stage. The reaction was performed in triplicate.

Generation of anti-r*Dre*-MIF-1 and anti-r*Dre*-MIF-2 polyclonal mouse sera and analysis of antibody cross-reactivity. The antibodies were generated by subcutaneous injection into the neck tissue of two 10-week-old male BALB/c mice of 100 µg of either r*Dre*-MIF-1 or r*Dre*-MIF-2 precipitated with Imject Alum (Thermo Scientific) in a final volume ratio of 1:2, followed by two boosts at 14-day intervals. The experiments were conducted following the guidelines and regulations of the 2nd Local Ethics Committee for Animal Experimentation in Warsaw (Permit No. WAW2/142/2021). Polyclonal sera were collected from mice 14 days after the last immunisation.

Serum reactivity against the recombinant proteins was assessed using ELISA and Western blot. Ninety-six-well plates (Wuxi NEST Biotechnology, Wuxi, China) were incubated with r*Dre*-MIF-1 or r*Dre*-MIF-2 (2.5 µg/mL) diluted in bicarbonate buffer (100 µL/well of 0.015 M Na_2CO_3 and 0.035 M $NaHCO_3$ at pH 9.5) overnight at 4°C. The plates were rinsed three times with 250 µL of phosphate-buffered saline (PBS) supplemented with 0.05% Tween-20 and then blocked with 200 µL of 5% filtered skimmed milk in PBS buffer for 90 min at 20°C. The plates were rinsed as described above, and subsequently 100 µL of anti-r*Dre*-MIF-1, anti-r*Dre*-MIF-2 or negative control mouse serum (n = 2) appropriately diluted in PBS (1:51,200 for total IgG, IgG1, IgG2 and IgM and 1:5,120 for IgE) was added to the wells. Dilutions were established based on preliminary data obtained using a serum dilution series. The plates were incubated at room temperature for 1.5 h and washed three times. The subsequent step was a 1-h incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG solution (1:30,000), goat anti-mouse IgG1 (1:4,000), goat anti-mouse IgG2 (1:4,000), goat anti-mouse IgM (1:4,000) or goat anti-mouse IgE (1:4,000) (all from AbD Serotec, now Bio-Rad, Kidlington, UK). The reaction was developed with the TMB Substrate Kit (Thermo Scientific) and stopped after 30 min using 2 M H_2SO_4. Absorbance readings were recorded at 450 nm using a Synergy H1 microplate reader (BioTek, Winooski, VT, USA).

To confirm the cross-reactivity between the sera, Western blot analyses were performed. A 5-µg mass of either r*Dre*-MIF-1 or r*Dre*-MIF-2 was resolved in polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS (w/v) overnight with continuous shaking at 4°C. The membranes were probed with anti-r*Dre*-MIF-1 or anti-r*Dre*-MIF-2 sera (diluted 1:5,000), rinsed with PBS/0.05% Tween 20 buffer and incubated for 60 min with HRP-conjugated anti-mouse IgG solution (1:5,000, Sigma-Aldrich). The immunoreactive bands were developed using West Pico Chemiluminescent substrate (Thermo Scientific) and visualised on radiography films.

Immune recognition of r*Dre*-MIF-1 and r*Dre*-MIF-2 by sera from dogs naturally infected with *D. repens*. Samples from naturally infected and uninfected dogs were collected during the study by Wysmołek *et al.* (29). Infected dogs were classified for the study based on a positive Knott's test result. The reactivity of r*Dre*-MIF-1 and r*Dre*-MIF-2 with sera from infected (n = 17) and uninfected (n = 6) dogs with total IgG, IgG1, IgG2, IgE and IgM antibodies was measured using an indirect ELISA.

The secondary antibodies for this ELISA were HRP-conjugated rabbit anti-dog IgG (total) (Jackson ImmunoResearch, Cambridge, UK), goat anti-dog IgG1, goat anti-dog IgG2, goat anti-dog IgM and goat anti-dog IgE (all from AbD Serotec). The ELISA procedure was the same as for the mouse sera except that the dog sera were diluted 1:400 for total IgG, IgG1 and IgG2; 1:20 for IgE; and 1:3,200 for IgM, and the secondary antibodies were diluted 1:30,000 for rabbit anti-dog IgG; 1:10,000 for goat anti-dog IgG1 and IgG2; and 1:1,000 for goat anti-dog IgM and IgE.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 9 software (GraphPad, Boston, MA, USA).

Results

Expression and purification of r*Dre*-MIF-1 and r*Dre*-MIF-2. Expression studies were conducted in two distinct *E. coli* strains: BL21 and SoluBL21. In both cases, cells produced soluble recombinant proteins with an approximate size of 15 kDa–17 kDa. However, there were differences between the purification efficiency from one strain and the efficiency from the other. Purification of r*Dre*-MIF-1 was more efficient from the SoluBL21 strain, while better results for r*Dre*-MIF-2 were achieved using the BL21 strain. Analyses by SDS-PAGE indicated that the purified r*Dre*-MIF-1 and r*Dre*-MIF-2 were electrophoretically homogeneous and without impurities (Fig. 1).

***Dre-mif-1* and *Dre-mif-2* mRNA expression in microfilariae and adult *D. repens* stages**. A significantly higher expression of both genes was observed in the adult stage. Interestingly, the expression level of *Dre-mif-1* was double that of *Dre-mif-2* in the adult stage (Fig. 2).

Bioinformatic analysis. The sequence comparison analysis (Fig. 3) revealed that *Dre*-MIF-1 had higher identity (40 %) to host MIFs (human and dog) than *Dre*-MIF-2 (27%). Despite *Dre*-MIF-1 and *Dre*-MIF-2 amino acid sequences having shown low identity of 27.8 % (Table 2), their potential tertiary structures showed a high level of similarity (Fig. 4).

Generation of anti-r*Dre*-MIF-1 and anti-r*Dre*-MIF-2 polyclonal sera and analysis of antibody cross-reactivity. The results suggest that r*Dre*-MIF-1 was

more immunogenic, as the levels of anti-r*Dre*-MIF-1 total IgG, IgG2 and IgE antibodies were much higher than those of anti-r*Dre*-MIF-2 immunoglobulins (Fig. 5). At the same time, antibodies raised against r*Dre*-MIF-1 were less specific than these produced after r*Dre*-MIF-2 immunisation. Western blot analysis revealed that antibodies specific to r*Dre*-MIF-1 recognised both molecules with similar affinity, whereas anti-r*Dre*-MIF-2 IgG antibodies showed only weak reactions with r*Dre*-MIF-1 (Fig. 6). However, as shown in Fig. 5, only IgG1 antibodies were responsible for this cross-reactivity. Our results show that immunisation with MIFs favours the production of IgG1 subclass antibodies. In turn, IgG2 antibodies are produced only after r*Dre*-MIF-1 immunisation and do not cross-react with r*Dre*-MIF-2 molecules. A similar observation was made for the IgE class. Antibodies of the IgM class were found to be the least specific and the most cross-reactive of all the analysed classes.

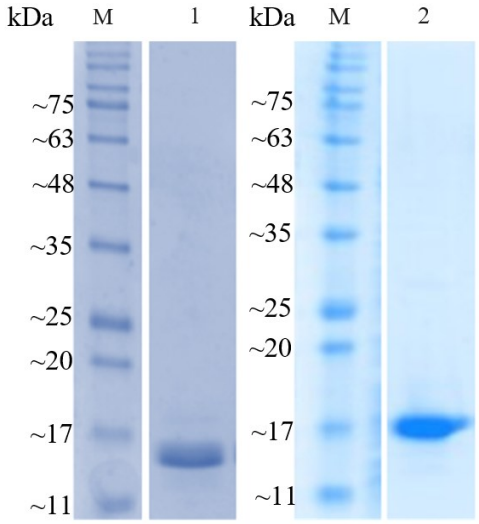

Fig. 1. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis analysis of purified recombinant *Dirofilaria repens* (r*Dre*)-macrophage migration inhibitory factor (MIF)-1 (left) and r*Dre*-MIF-2 (right). Lane M – molecular weight marker; Lane 1 – r*Dre*-MIF-1 (10 µL); Lane 2 – r*Dre*-MIF-2 (10 µL)

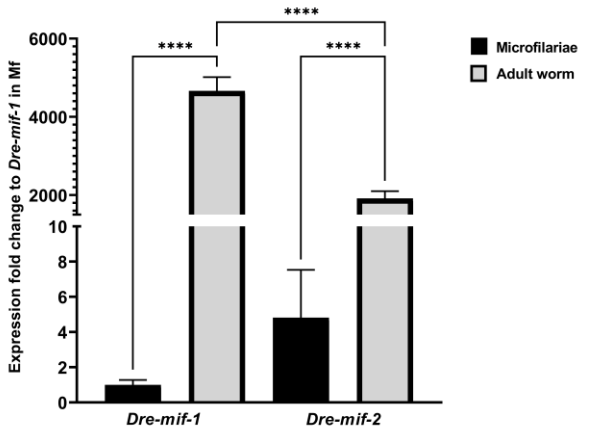

Fig. 2. *Dirofilaria repens* macrophage migration inhibitory factor (*Dre-mif*)-*1* and *Dre-mif*-2 gene expression in microfilariae and the adult stage of *D. repens*. The fold increase in expression is shown over the level of expression of *Dre-mif*-1 in microfilariae. Statistical analysis was performed using one-way analysis of variance; **** – P-value <0.001

Table 2. The protein identity between human macrophage migration macrophage migration inhibitory factor (hMIF), dog MIF (dMIF), *Dirofilaria repens* (*Dre*)-MIF-1 and *Dre*-MIF-2

Protein	hMIF	dMIF	*Dre*-MIF-1	*Dre*-MIF-2
hMIF	-	93.9%	41.7%	27.8%
dMIF	93.9%	-	40.0%	26.9%
Dre-MIF-1	41.7%	40.0%	-	27.8%
Dre-MIF-2	27.8%	26.9%	27.8%	-

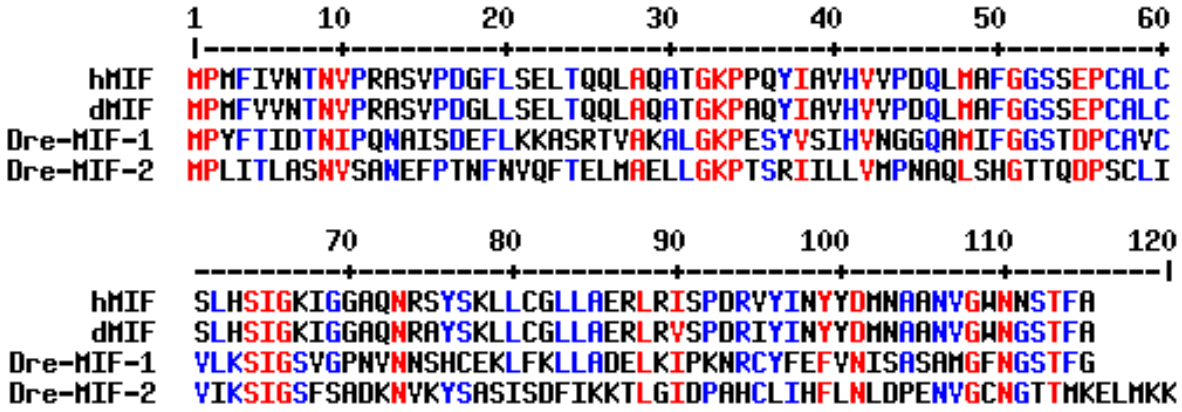

Fig. 3. Alignment of human macrophage migration inhibitory factor (hMIF), dog MIF (dMIF), *Dirofilaria repens* (*Dre*)-MIF-1 and *Dre*-MIF-2 amino acid sequences

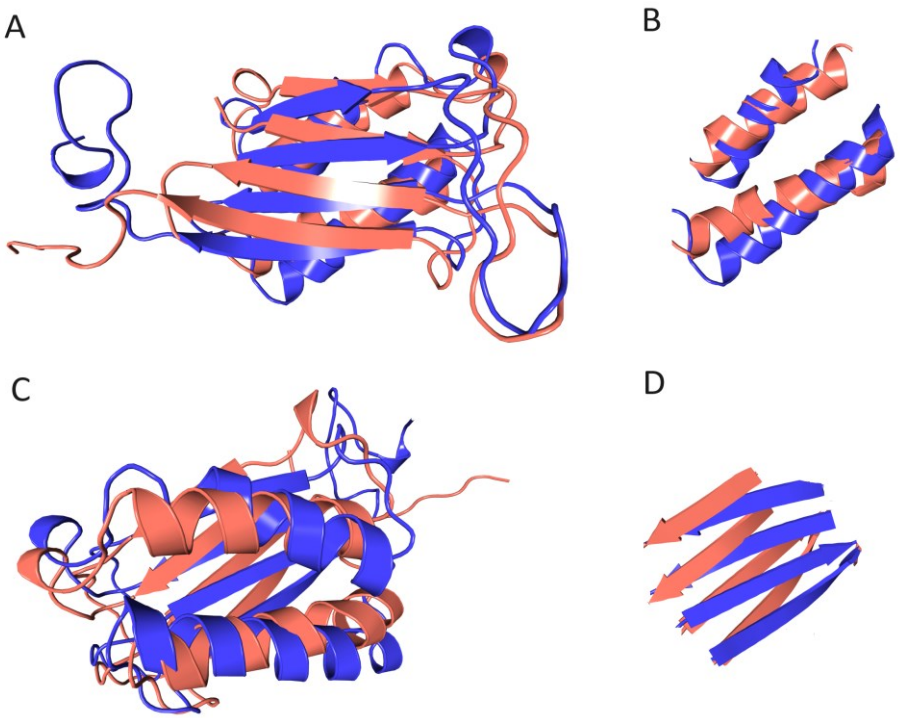

Fig. 4. The visualisation of superimposed potential structures of *Dirofilaria repens* (*Dre*)-macrophage migration inhibitory factor (MIF)-1 (red) and *Dre*-MIF-2 (blue). The complete structures are shown on fragments A) and C), whereas matching helices and strands are respectively shown on fragments B) and D)

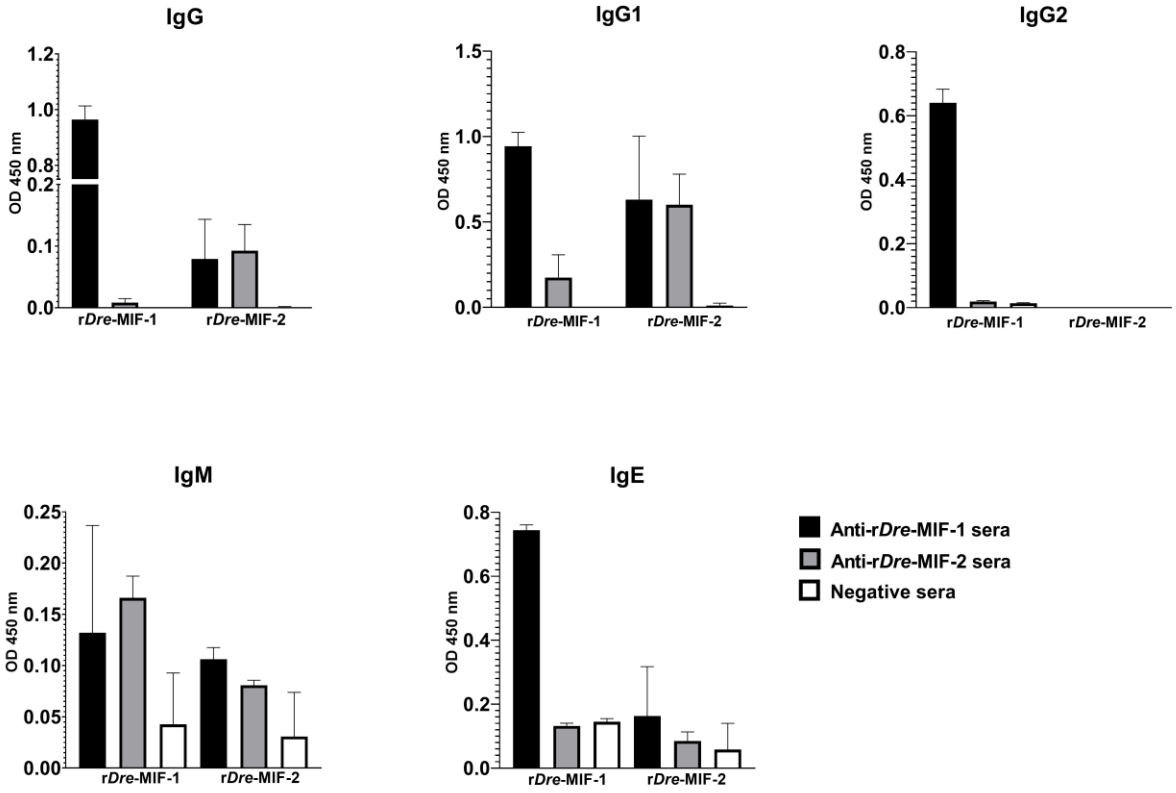

Fig. 5. Reactivity of mouse anti-recombinant *Dirofilaria repens* (r*Dre*)-macrophage migration inhibitory factor (MIF)-1 and anti-r*Dre*-MIF-2 sera with r*Dre*-MIF-1 and r*Dre*-MIF-2. Serum dilutions for detection: immunoglobulin (Ig)G, IgG1, IgG2 and IgM – 1:51,200; IgE – 1:5,120. OD – optical density

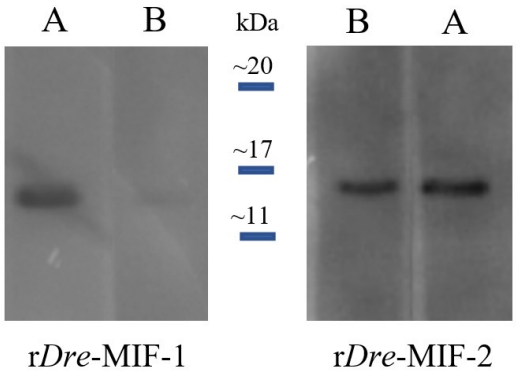

Fig. 6. Western blot cross-reactivity analysis of mouse anti-recombinant *Dirofilaria repens* (r*Dre*)-macrophage migration inhibitory factor (MIF)-1 (A) and anti-r*Dre*-MIF-2 (B) antibodies with r*Dre*-MIF-1 and r*Dre*-MIF-2

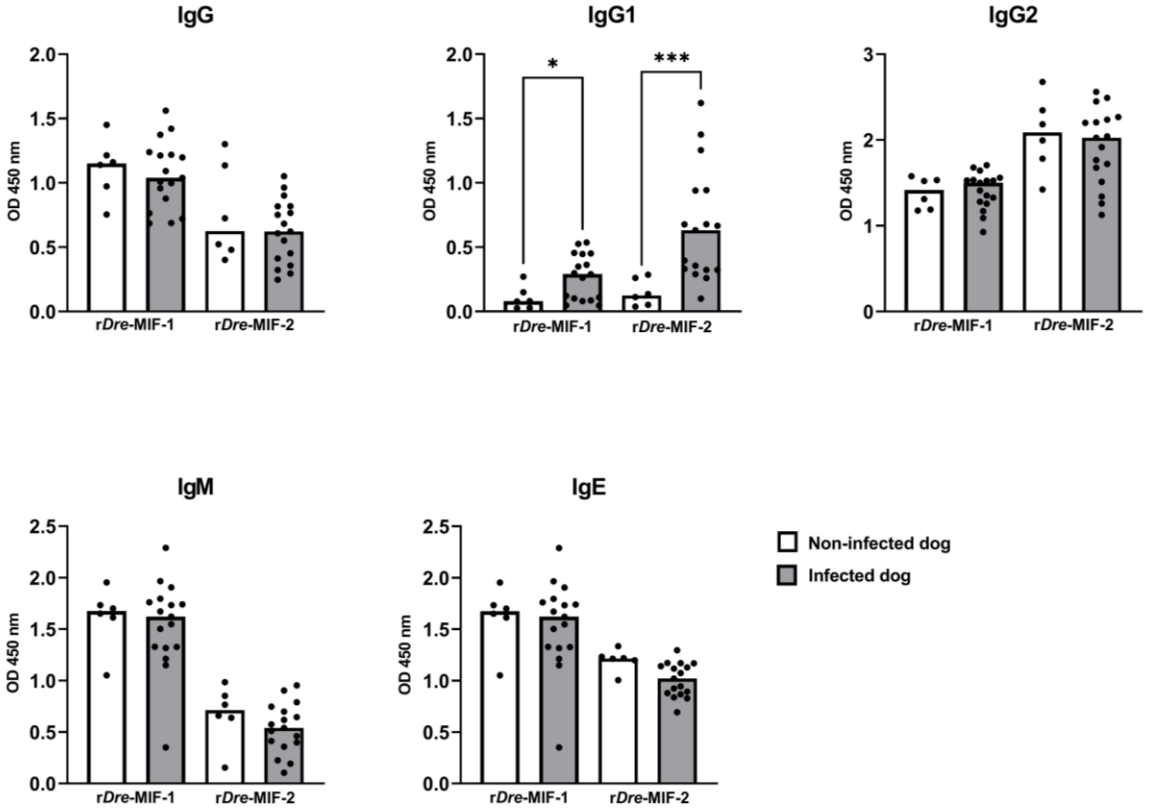

Fig. 7. Reactivity of different serum antibody classes in infected and non-infected dogs with recombinant *Dirofilaria repens* (r*Dre*)-macrophage migration inhibitory factor (MIF)-1 and r*Dre*-MIF-2. Serum dilutions for detection: immunoglobulin (Ig) G, IgG1, and IgG2 – 1:400; IgM – 1:3,200; IgE – 1:20. Statistical analysis was performed using the Mann–Whitney test; * – P-value <0.05; *** – P-value <0.001

Immune recognition of r*Dre*-MIF-1 and r*Dre*-MIF-2 by sera from dogs naturally infected with *D. repens*. The reactivity of r*Dre*-MIF-1 and r*Dre*-MIF-2 was tested with sera of naturally infected and uninfected dogs. An elevated level of IgG1-subclass antibodies recognising both r*Dre*-MIF-1 and r*Dre*-MIF-2 was noted in infected dogs' sera (Fig. 7). No significant differences were observed in the levels of total IgG, IgG2, IgE or IgM antibodies between infected dogs' and uninfected dogs' sera.

Discussion

Subcutaneous dirofilariasis is a relatively new problem in human and veterinary medicine. Since the diagnosis of this infection is currently imperfect, the zoonosis is spreading uncontrollably throughout the world (8). The molecular interactions between *D. repens* and the host immune system remain unknown.

Nematodes of various species secrete MIF homologues to influence and modulate the immune response of their hosts. Novel technologies allowing for rapid identification of MIF genes and their corresponding cDNA, coupled with the production of recombinant proteins, have facilitated comprehension of homologue expression patterns and functions in nematode parasitical infection. In the present study, two MIF paralogues from the parasitic nematode *D. repens* were described for the first time. As other nematode MIF orthologues also do, *Dre*-MIF-1 showed a higher range of amino acid similarity to mammalian host MIFs than *Dre*-MIF-2 (27). The amino

acid sequence similarity between *Dre*-MIF-1 and human or canine MIF is approximately 40%, while *Dre*-MIF-2 exhibits a similarity of about 27%, suggesting their unalike abilities to stimulate the host's immune system. Studies show that MIF-2 homologues from *B. malayi* and *O. volvulus* should rather be considered as D-dopachrome tautomerase homologues, which would explain the lower degree of similarity to their mammalian counterparts (19).

In our study, we observed highly upregulated expression of *Dre-mif-1* and *Dre-mif-2* in the adult worm compared to their expression in microfilariae. The results are in line with those of similar experiments of Pastrana *et al.* (20), who confirmed MIF expression in all *B. malayi* developmental stages, and of research by Ajonina-Ekoti *et al.* (1), who proved MIF expression in the adult stage of the parasitic filarial nematode *O. volvulus* using immunolocalisation. However, in *B. malayi* the expression in the adult stage was only slightly increased from that in microfilariae. The reason for this observation is not explained, but high MIF expression in various adult filarial nematodes suggests that these proteins may play a significant role in the course of filarial infection when adult nematodes reside in the host's subcutaneous tissue.

In the present study, we observed that mice immunised with r*Dre*-MIF-2 generated a more specific response, while mice immunised with r*Dre*-MIF-1 produced serum with cross-reactivity with r*Dre*-MIF-2, which may be explained by similar predicted tertiary structures of the proteins. Such cross-reactions were not noted in the case of *O. volvulus* or *C. elegans*; however, similarly to our results, *Ov*-MIF-1 was more immunogenic in rats than *Ov*-MIF-2 for Ajonina-Ekoti *et al.* (1). Another group of researchers also excluded cross-reactivity between antibodies specific to recombinant *Strongyloides ratti* MIF and human MIF (31).

Climate change and increased migration with pet dogs have led to dirofilariasis being more frequently described in Central and Eastern European countries: Germany, Poland, Slovakia and Ukraine. According to the latest data, cases of dirofilariasis have been reported in Northern Europe and Baltic countries, particularly in Lithuania, Latvia, and Finland (8, 13, 14). Moreover, the number of infections in humans is increasing because of the increased prevalence among dogs. This urges the characterisation of molecular interaction mechanisms between the worm and its natural host, the dog, which is a reservoir of the disease for humans. The knowledge will result in development of novel diagnostics, prophylaxis and treatment procedures (21). In our previous study, we confirmed that microfilaraemic infections were associated with higher levels of IgG1 antibodies specific to *D. repens* somatic antigens than of IgG2 immunoglobulins, whereas in occult infections IgG2 predominated over IgG1 (30). Here, we evaluated the presence of IgG, IgG1, IgG2, IgM and IgE antibodies specific to *D. repens* MIF molecules in naturally infected dog sera. A significant difference between infected and uninfected dogs was found only in the case of IgG1 antibodies specific to both proteins. This corresponds to the findings reported by other authors who observed that in the sera of dogs naturally infected with *D. immitis*, although both parasite-specific IgG1 and IgG2 antibodies were present, IgG1 appeared to be the predominant type (11). Our data show that different IgG subclasses show different affinity for and specificity to the analysed antigens. This should be therefore taken into consideration in the development of diagnostic tests, which are usually based on total IgG detection.

Scientific research is still underway to create a serological test that would be particularly useful in the prepatent period of invasion. Novel approaches have been reported, such as the use of a phage display library to search for diagnostic peptides (21). In our study we used recombinant *D. repens* MIF molecules, but these were found to be non-specific and cannot be considered as potential diagnostic antigens.

Conclusion

The present study describes the first attempt at the characterisation of MIF homologues from *D. repens* as a significant filarial parasite of dogs. The proteins share low similarity at the amino acid level, but appear to have very similar tertiary structures. The noted cross-reactivity rather excludes their use in diagnostic tests, but the observation of the different serological responses they raise in the host provides new insights into naturally occurring events during dirofilariasis.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: The experiment was approved by the 2nd Local Ethical Committee for Animal Experiments in Warsaw (approval no. WAW2/142/2021). All procedures were carried out according to the Polish Act of 15 January 2015 on the protection of animals used for scientific or educational purposes (Official Journal of Laws 2015, item 266 based on Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

References

1. Ajonina-Ekoti I., Kurosinski M.A., Younis A.E., Ndjonka D., Tanyi M.K., Achukwi M., Eisenbarth A., Ajonina C., Lüersen K., Breloer M., Brattig N.W., Liebau E.: Comparative analysis of macrophage migration inhibitory factors (MIFs) from the parasitic nematode *Onchocerca volvulus* and the free-living nematode *Caenorhabditis elegans*. Parasitol Res 2013, 112, 3335–3346, doi: 10.1007/s00436-013-3513-1.

2. Bacher M., Metz C.N., Calandra T., Mayer K., Chesney J., Lohoff M., Gemsa D., Donnelly T., Bucala R.: An essential regulatory role for macrophage migration inhibitory factor in T-cell activation. Proc Natl Acad Sci U S A 1996, 93, 7849–7854, doi: 10.1073/pnas.93.15.7849.
3. Behnke J., Barnard C., Wakelin D.: Understanding chronic nematode infections: evolutionary considerations, current hypotheses and the way forward. Int J Parasitol 1992, 22, 861–907, doi: 10.1016/0020-7519(92)90046-n.
4. Cai J., Huang L., Tang H., Xu H., Wang L., Zheng M., Yu H., Liu H.: Macrophage migration inhibitory factor of *Thelazia callipaeda* induces M2-like macrophage polarization through TLR4-mediated activation of the PI3K-Akt pathway. FASEB J 2021, 35, e21866, doi: 10.1096/fj.202100676R.
5. Calandra T.: Macrophage migration inhibitory factor and host innate immune responses to microbes. Scand J Infect Dis 2003, 35, 573–576, doi: 10.1080/00365540310016277.
6. Calandra T., Bernhagen J., Mitchell R.A., Bucala R.: The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. J Exp Med 1994, 179, 1895–1902, doi: 10.1084/jem.179.6.1895.
7. Calandra T., Roger T.: Macrophage migration inhibitory factor: a regulator of innate immunity. Nat Rev Immunol 2003, 3, 791–800, doi: 10.1038/nri1200.
8. Capelli G., Genchi C., Baneth G., Bourdeau P., Brianti E., Cardoso L., Danesi P., Fuehrer H.-P., Giannelli A., Ionică A.M., Maia C., Modrý D., Montarsi F., Krücken J., Papadopoulos E., Petrić D., Pfeffer M., Savić S., Otranto D., Poppert S., Silaghi C.: Recent advances on *Dirofilaria repens* in dogs and humans in Europe. Parasit Vectors 2018, 11, 663, doi: 10.1186/s13071-018-3205-x.
9. Chauhan N., Hoti S.L.: Role of cysteine-58 and cysteine-95 residues in the thiol di-sulfide oxidoreductase activity of Macrophage Migration Inhibitory Factor-2 of *Wuchereria bancrofti*. Acta Trop 2016, 153, 14–20, doi: 10.1016/j.actatropica.2015.09.017.
10. Corpet F.: Multiple sequence alignment with hierarchical clustering. Nucl Acid Res 1988, 16, 10881–10890, doi: 10.1093/nar/16.22.10881.
11. Deplazes P., Smith N.C., Lutz H., Eckert J.: Specific IgG1 and IgG2 antibody responses of dogs to *Leishmania infantum* and other parasites. Parasite Immunol 1995, 17, 451–458, doi: 10.1111/j.1365-3024.1995.tb00914.x.
12. Flaster H., Bernhagen J., Calandra T., Bucala R.: The Macrophage Migration Inhibitory Factor-Glucocorticoid Dyad: Regulation of Inflammation and Immunity. Mol Endocrinol 2007, 21, 1267–1280, doi: 10.1210/me.2007-0065.
13. Fuehrer H.-P., Morelli S., Unterköfler M.S., Bajer A., Bakran-Lebl K., Dwużnik-Szarek D., Farkas R., Grandi G., Heddergott M., Jokelainen P., Knific T., Leschnik M., Miterpáková M., Modrý D., Huus Petersen H., Skírnisson K., Vergles Rataj A., Schnyder M., Strube C.: *Dirofilaria* spp. and *Angiostrongylus vasorum*: Current Risk of Spreading in Central and Northern Europe. Pathogens 2021, 10, 1268, doi: 10.3390/pathogens10101268.
14. Genchi C., Kramer L.H.: The prevalence of *Dirofilaria immitis* and *D. repens* in the Old World. Vet Parasitol 2020, 280, 108995, doi: 10.1016/j.vetpar.2019.108995.
15. Ghosh S., Jiang N., Farr L., Ngobeni R., Moonah S.: Parasite-Produced MIF Cytokine: Role in Immune Evasion, Invasion, and Pathogenesis. Front Immunol 2019, 10, 1995, doi: 10.3389/fimmu.2019.01995.
16. Harris J., VanPatten S., Deen N.S., Al-Abed Y., Morand E.F.: Rediscovering MIF: New Tricks for an Old Cytokine. Trends Immunol 2019, 40, 447–462, doi: 10.1016/j.it.2019.03.002.
17. Hewitson J.P., Grainger J.R., Maizels R.M.: Helminth immunoregulation: The role of parasite secreted proteins in modulating host immunity. Mol Biochem Parasitol 2009, 167, 1–11, doi: 10.1016/j.molbiopara.2009.04.008.
18. Kelley L.A., Mezulis S., Yates C.M., Wass M.N., Sternberg M.J.E.: The Phyre2 Web Portal for Protein Modeling, Prediction and Analysis. Nat Protoc 2015, 10, 845–858, doi: 10.1038/nprot.2015.053.
19. Miska K.B., Fetterer R.H., Lillehoj H.S., Jenkins M.C., Allen P.C., Harper S.B.: Characterisation of macrophage migration inhibitory factor from *Eimeria* species infectious to chickens. Mol Biochem Parasitol 2007, 151, 173–183, doi: 10.1016/j.molbiopara.2006.10.020.
20. Pastrana D.V., Raghavan N., FitzGerald P., Eisinger S.W., Metz C., Bucala R., Schleimer R.P., Bickel C., Scott A.L.: Filarial Nematode Parasites Secrete a Homologue of the Human Cytokine Macrophage Migration Inhibitory Factor. Infect Immun 1998, 66, 5955–5963, doi: 10.1128/IAI.66.12.5955-5963.1998.
21. Pękacz M., Basałaj K., Kalinowska A., Klockiewicz M., Stopka D., Bąska P., Długosz E., Karabowicz J., Młocicki D., Wiśniewski M., Zawistowska-Deniziak A.: Selection of new diagnostic markers for *Dirofilaria repens* infections with the use of phage display technology. Sci Rep 2022, 12, 2288, doi: 10.1038/s41598-022-06116-8.
22. Petrocheilou V., Theodorakis M., Williams J., Prifti H., Georgilis K., Apostolopoulou I., Mavrikakis M.: Microfilaremia from a *Dirofilaria*-like parasite in Greece. APMIS 1998, 106, 315–318, doi: 10.1111/j.1699-0463.1998.tb01352.x.
23. Sharma R., Hoti S.L., Meena R.L., Vasuki V., Sankari T., Kaliraj P.: Molecular and functional characterization of macrophage migration inhibitory factor (MIF) homolog of human from lymphatic filarial parasite *Wuchereria bancrofti*. Parasitol Res 2012, 111, 2035–2047, doi: 10.1007/s00436-012-3051-2.
24. Shimizu T.: Role of macrophage migration inhibitory factor (MIF) in the skin. J Dermatol Sci 2005, 37, 65–73, doi: 10.1016/j.jdermsci.2004.08.007.
25. Thompson J.D., Higgins D.G., Gibson T.J.: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl Acid Res 1994, 22, 4673–4680, doi: 10.1093/nar/22.22.4673.
26. Tomasello G., Armenia I., Molla G.: The Protein Imager: A full-featured online molecular viewer interface with server-side HQ-rendering capabilities. Bioinformatics 2020, 36, 2909–2911, doi: 10.1093/bioinformatics/btaa009.
27. Vermeire J.J., Cho Y., Lolis E., Bucala R., Cappello M.: Orthologs of macrophage migration inhibitory factor from parasitic nematodes. Trends Parasitol 2008, 24, 355–363, doi: 10.1016/j.pt.2008.04.007.
28. Wang Y., Lu M.-M., Wang S., Ehsan M., Yan R.-F., Song X.-K., Xu L.-X., Li X.-R.: Characterization of a secreted macrophage migration inhibitory factor homologue of the parasitic nematode *Haemonchus Contortus* acting at the parasite-host cell interface. Oncotarget 2017, 8, 40052–40064, doi: 10.18632/oncotarget.16675.
29. Wysmołek M.E., Dobrzyński A., Długosz E., Czopowicz M., Wiśniewski M., Jurka P., Klockiewicz M.: Hematological and Biochemical Changes in Dogs Naturally Infected With *Dirofilaria repens*. Front Vet Sci 2020, 7, 590, doi: 10.3389/fvets.2020.00590.
30. Wysmołek M.E., Klockiewicz M., Długosz E., Wiśniewski M.: Canine antibody response against *Dirofilaria repens* in natural occult and microfilaremic infections. Comp Immunol Microbiol Infect Dis 2022, 86, 101818, doi: 10.1016/j.cimid.2022.101818.
31. Younis A.E., Soblik H., Ajonina-Ekoti I., Erttmann K.D., Luersen K., Liebau E., Brattig N.W.: Characterization of a secreted macrophage migration inhibitory factor homologue of the parasitic nematode *Strongyloides* acting at the parasite–host cell interface. Microbes Infect 2012, 14, 279–289, doi: 10.1016/j.micinf.2011.09.006.