

Critical role for macrophage migration inhibitory factor (MIF) in Ross River virus-induced arthritis and myositis

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Arthrogenic alphaviruses, such as Ross River virus (RRV), chikungunya, Sindbis, mayaro and o'nyong-nyong viruses circulate endemically worldwide, frequently causing outbreaks of polyarthritis. The exact mechanisms of how alphaviruses induce polyarthritis remain ill defined, although macrophages are known to play a key role. Macrophage migration inhibitory factor (MIF) is an important cytokine involved in rheumatoid arthritis pathogenesis. Here, we characterize the role of MIF in alphavirus-induced arthritides using a mouse model of RRV-induced arthritis, which has many characteristics of RRV disease in humans. RRV-infected WT mice developed severe disease associated with up-regulated MIF expression in serum and tissues, which corresponded to severe inflammation and tissue damage. MIF-deficient (MIF^{-/-}) mice developed mild disease accompanied by a reduction in inflammatory infiltrates and muscle destruction in the tissues, despite having viral titers similar to WT mice. In addition, reconstitution of MIF into MIF^{-/-} mice exacerbated RRV disease and treatment of mice with MIF antagonist ameliorated disease in WT mice. Collectively, these findings suggest that MIF plays a critical role in determining the clinical severity of alphavirus-induced musculoskeletal disease and may provide a target for the development of antiviral pharmaceuticals. The prospect being that early treatment with MIF-blocking pharmaceuticals may curtail the debilitating arthritis associated with alphaviral infections.

therapeutics | proinflammatory cytokine | migration inhibitory factor antagonist | immunology | viral arthritis

Mosquito-borne alphaviruses are an important cause of both encephalitic and arthritic disease in humans. Alphaviruses within the *Togaviridae* family, including Ross River virus (RRV) and chikungunya virus (CHIKV), are a major cause of debilitating arthritic disease worldwide (1). Of particular concern is the ability of this group of viruses to cause extensive epidemics of polyarthritis and arthralgia, often associated with chronic symptoms and ongoing health issues, making infection with these viruses an illness of major socioeconomic significance (1–3). Examples of such large-scale outbreaks of alphaviruses include the 1979 to 1980 outbreak of RRV in the South Pacific, which resulted in more than 60,000 reported cases (4), and the recent reemergence of CHIKV in the French island of La Réunion, which saw more than 250,000 people infected (5). From La Réunion, CHIKV rapidly spread to the Indian Ocean, India, and South East Asia, with estimates as high as 5 million reported cases since 2006 (6–8). Currently, CHIKV continues to circulate in the Asia Pacific region, causing sporadic outbreaks, the most recent being the 2009 outbreaks in South East Asia affecting more than 100,000 people (9–11); furthermore, infection with CHIKV among travelers has been reported in Europe and the United States (12).

RRV is a positive-sense RNA virus that circulates endemically in Australia and the South Pacific (4) and is maintained in transmission cycles between its mosquito vector and vertebrate

hosts (13). In Australia, roughly 5,000 cases of RRV are reported annually (4), with symptoms of arthritis, arthralgia, myalgia, fatigue, febrile illness, and rash (4, 14, 15). Acute symptoms can last for weeks to months; however, chronic arthritic disease associated with RRV infection has been reported to last up to a year or more (16). Currently, the only treatment available for patients suffering arthrogenic alphavirus infections is symptomatic care in the form of nonsteroidal anti-inflammatory drugs (4, 16).

Research into the pathogenesis of arthritis related to alphavirus infection has been aided by the development of a mouse model of RRV-induced disease (17). Aspects of the disease progression in the mouse model resemble that seen in human disease (18–20). Using this model, we have previously identified a number of host immune factors that contribute to the development of RRV-induced disease, including the critical role that macrophages play in the pathogenesis of RRV disease (19–21).

Alphaviral disease has many similarities to rheumatoid arthritis (RA), including the critical involvement of macrophages and inflammatory molecules. Macrophage migration inhibitory factor (MIF), a proinflammatory cytokine secreted by a variety of cell types, is a potent monocyte/macrophage chemoattractant and activator (22). MIF has an established role in RA (23, 24), being elevated in synovial fluid, tissues, and serum of patients with RA (25, 26). Inhibition of its function by antibody blockade, or through genetic deletion, significantly reduces the inflammation and destruction associated with mouse models of RA (27, 28). The role of MIF in viral-induced inflammatory diseases is unknown. MIF has been shown to be up-regulated in patients presenting with both Dengue virus infection (29) and West Nile virus (WNV) infection (30). Such studies show an association between MIF and viral disease, but are limited in determining its role in disease pathogenesis, leaving the involvement of MIF in viral infection largely uncharacterized.

In the present study, we investigated the role of MIF in the pathogenesis of alphaviral arthritic disease, using the mouse model of RRV-induced disease. We found that in the absence of MIF, RRV infection resulted in reduced clinical disease severity, corresponding to a decrease in tissue inflammation and damage. These decreases were associated with reductions in cellular infiltrates, monocyte chemoattractant protein-1 (MCP-1), and IFN- γ , despite the presence of intact viral immunity. These investigations show that MIF plays a pivotal role in alphavirus-induced arthritis by regulating proinflammatory molecule expressions in the tissue

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microenvironment and not by affecting viral immunity. These investigations also highlight the therapeutic potential of MIF antagonist in the treatment of alphaviral arthropathies.

Results

MIF Is Up-Regulated Following RRV Infection. To determine if MIF was up-regulated in response to RRV infection, 20-d-old C57BL/6 WT mice were infected with 10^4 pfu RRV or mock-infected with diluent alone. At time points before disease onset [day 1 postinfection (p.i.)] and at the time of disease onset (day 5 p.i.), mice were killed, serum collected, and MIF levels determined by ELISA. At disease onset, MIF serum levels in RRV-infected mice showed a significant ($P < 0.05$) increase compared with mock-infected controls (Fig. 1).

To determine if MIF was expressed at the sites of inflammation, immunohistochemistry (IHC) was performed on quadriceps muscle and ankle joint sections of RRV-infected mice at disease onset (day 5 p.i.) and at peak disease (day 10 p.i.). Some MIF expression was present in the ankle joints of mock-infected mice (Fig. 2*B* ii and *D* ii); however, expression was markedly increased in the ankle joints of RRV-infected mice both at disease onset (Fig. 2*B* iv) and at peak disease (Fig. 2*D* iv). MIF was not detected in the quadriceps muscle of mock-infected mice (Fig. 2*A* ii and *C* ii) or during disease onset (Fig. 2*A* iv). In contrast, MIF was prominently detected in the quadriceps of RRV-infected mice at peak disease (Fig. 2*C* iv). Overall, comparison of MIF immunostaining between mock-infected and RRV-infected mice demonstrated greater staining for MIF positive cells in RRV-infected mice.

RRV-Induced Inflammatory Disease Is Less Severe in MIF^{-/-} Mice. To assess the role of MIF in RRV disease, C57BL/6 WT mice and C57BL/6 mice deficient in MIF were infected with 10^4 pfu RRV and monitored for disease signs (Fig. 3*A*). As expected, WT mice developed severe disease signs associated with severe hindlimb weakness, loss of gripping ability (Fig. 3*A*), and weight loss during stages of peak disease (Fig. 3*B*). In contrast MIF^{-/-} mice developed only mild disease signs with significantly ($P < 0.05$) lower peak disease scores (Fig. 3*A*) and no signs of weight loss (Fig. 3*B*). Although RRV-infection had a significantly diminished impact on weight gain in MIF^{-/-} mice (compared with mock-infected controls), actual weight loss was not observed at any point during the course of RRV disease (Fig. 3*B*), whereas RRV-infected WT mice lost weight at the onset of peak disease. Mock-infected mice were scored zero on the clinical scale, indicating no signs of disease for the duration of the experiment.

MIF Deficiency Does Not Compromise Antiviral Immunity. To determine if the differences in disease severity reflected a reduction in RRV replication, C57BL/6 WT and MIF^{-/-} mice were infected with 10^4 pfu RRV and killed at days 1, 3, 5, and 10 p.i. Serum, ankle joints, and quadriceps muscles were isolated and

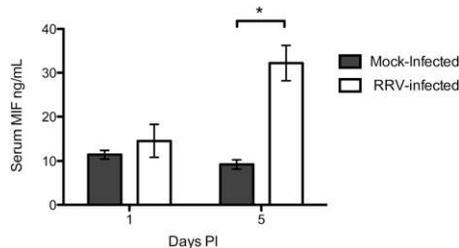


Fig. 1. MIF is up-regulated following RRV infection. MIF levels in the serum of RRV- or mock-infected C57BL/6 mice as determined by ELISA. The 20-d-old C57BL/6 WT mice were infected subcutaneously with 10^4 pfu RRV (white bars) or mock-infected (gray bars) with diluent alone. At day 1 and 5 p.i., mice were killed and serum collected. Each point is expressed as the mean \pm the SEM for three to five mice and is representative of two independent experiments. * $P < 0.05$ compared with mock-infected using a Student *t* test.

assayed for viral titers by plaque assay. Serum titers were comparable at all days tested, indicative of equivalent systemic replication. Serum titers fell below the limit of detection by day 5 (Fig. S1*A*). At day 1 p.i., RRV titers recovered from quadriceps muscles and ankle joints of WT and MIF^{-/-} mice were comparable (Fig. S1*B*), as were the titers at days 3, 5, and 10 p.i. from quadriceps muscles. In contrast, despite the initial infection reaching the same titers in ankle tissues, viral titers at later stages of infection were slightly lower in MIF^{-/-} mice compared with WT mice (Fig. S1*C*), suggesting that MIF deficiency does not adversely affect the host response to viral infection.

MIF Deficiency Reduces RRV-Induced Inflammation and Tissue Destruction. We have previously shown that peak disease in RRV-infected WT C57BL/6 mice corresponds to severe inflammation of the quadriceps muscle and ankle joints (20). To determine if the reduction in disease severity in MIF^{-/-} mice corresponded to a reduction in inflammation, quadriceps muscles and ankle joint tissues of WT and MIF^{-/-} RRV-infected mice were collected at peak disease (day 10 p.i.) for histological analysis. No inflammation was observed in the quadriceps muscle (Fig. 4*A* i and ii) and ankle joint (Fig. 4*B* i and ii) tissues of the mock-infected WT or MIF^{-/-} mice. RRV-infected WT mice showed extensive inflammation in both quadriceps muscle (Fig. 4*A* iii) and ankle joint (Fig. 4*B* iii), with large numbers of inflammatory infiltrates and extensive disruption of striated muscle fibers. In contrast, RRV-infected MIF^{-/-} mice showed reduced inflammation in quadriceps muscle (Fig. 4*A* iv) and ankle joint (Fig. 4*B* iv), with fewer infiltrating cells compared with tissues from WT mice and no apparent disruption of striated muscle fibers.

To further investigate whether RRV infection of MIF^{-/-} mice show reduced tissue destruction compared with WT mice, damage to quadriceps muscle was assessed by myofiber permeability to Evan's blue dye (EBD). At day 10 p.i., corresponding to peak inflammatory disease, RRV-infected WT and MIF^{-/-} mice were injected intraperitoneally with 1% EBD before being killed. Quadriceps muscles were collected, snap-frozen in OCT compound, cryosectioned, and EBD⁺ cells identified by fluorescence microscopy. Mock-infected WT and MIF^{-/-} mice showed only background staining, with no EBD⁺ cells evident (Fig. S2*A* and *B*). The quadriceps muscle of RRV-infected WT mice displayed abundant EBD⁺ cells (Fig. S2*C*), indicative of extensive muscle tissue damage, whereas MIF^{-/-} mice showed substantially less EBD⁺ cells (Fig. S2*D*). Taken together, these results demonstrate a role for MIF in the development of inflammation and tissue damage that contribute to the pathogenesis of RRV-induced disease.

MIF Modulates Proinflammatory Cytokines in RRV-Induced Disease. It is well established that cytokines play crucial roles in inflammatory disease processes. Given that RRV-infected MIF^{-/-} mice develop mild disease compared with infected WT mice, it was important to determine the expression and production of proinflammatory factors in the absence and presence of MIF. At peak disease (day 10 p.i.), quadriceps muscle and ankle joint tissues were isolated and cytokine levels measured by real-time PCR. MCP-1 and IFN- γ mRNA expression were significantly lower in both quadriceps muscles and ankle joints of MIF^{-/-} mice compared with WT mice (Fig. S3*A*). However, TNF- α was not affected by MIF deficiency, with no significant difference in expression between MIF^{-/-} and WT mice in both tissues (Fig. S3*A*).

To assess whether the mRNA expression changes are observed at the protein level, quadriceps muscle and ankle joint tissues were isolated from infected WT and MIF^{-/-} mice at peak disease (day 10 p.i.) and tissues homogenized for cytokine measurement by ELISA. The effects of MIF deficiency on cytokine production largely complimented mRNA-expression analysis, with MIF^{-/-} mice exhibiting significantly lower MCP-1 and IFN- γ protein levels in ankle joint tissue, significantly lower MCP-1 protein levels in quadriceps muscle, and no significant difference in tissue TNF- α protein levels (Fig. S3*B*). We also assessed the affect of MIF deficiency on tissue MCP-1, IFN- γ , and TNF- α protein levels

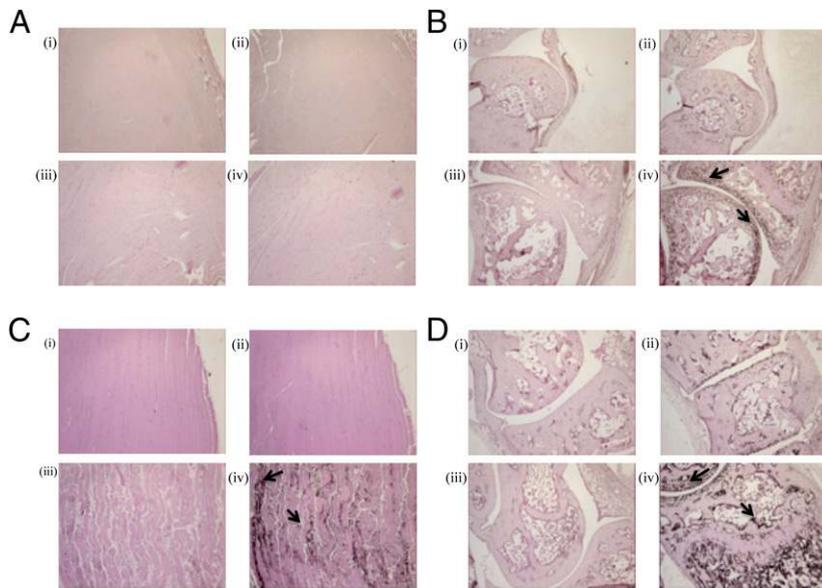


Fig. 2. MIF is detected in the tissues of RRV-infected mice. MIF expression in RRV- or mock-infected C57BL/6 WT mice. At 5 and 10 d p.i., ankle and quadriceps tissues were fixed in 4% PFA, paraffin-embedded, and 5- μ m sections obtained. IHC was performed with either primary goat anti-human MIF mAb or isotype control IgG1. Arrows point to areas of MIF staining. (A) Quadriceps muscle at day 5 p.i., (B) ankle tissues at day 5 p.i., (C) quadriceps muscle at day 10 p.i., (D) ankle tissues at 10 p.i. Panels: (i) mock-infected IgG control stained, (ii) mock-infected MIF stained, (iii) RRV-infected IgG control stained, (iv) RRV-infected MIF stained. (Magnification, 100 \times .)

during the early stages of RRV infection (days 1, 3, and 5 p.i.) (Fig. S4). MCP-1 protein levels were consistently and significantly lower in both quadriceps muscle and ankle joints of MIF^{-/-} mice compared with WT mice, but no significant differences in the levels of TNF- α were seen. IFN- γ protein levels were significantly lower at day 3 p.i. in MIF^{-/-} mice compared with WT mice but levels were not significantly different at day 5 p.i. Interestingly, IFN- γ protein levels were increased in MIF^{-/-} mice at day 1.

Reintroduction of Recombinant MIF into Mice Exacerbates Disease.

To further confirm the role of MIF in the development of severe RRV-induced inflammatory disease, we assessed the effects of reintroducing recombinant murine MIF (rMIF) into RRV-infected MIF^{-/-} mice on disease signs and changes in body weight. RRV-infected MIF^{-/-} mice were either administered with 50 ng rMIF or given vehicle control. Uninfected MIF^{-/-} mice, both untreated and treated with rMIF, were included as additional controls. As expected RRV-infected MIF^{-/-} mice without MIF treatment developed mild disease; however, RRV-infected MIF^{-/-} mice treated with rMIF developed severe disease signs, with mice exhibiting moderate to severe hindlimb weakness and dragging (Fig. 5A). In addition, RRV-infected MIF^{-/-} mice treated with rMIF showed significantly less weight gain (Fig. S5A).

Experiments were repeated in RRV-infected WT mice to determine whether the administration of rMIF can increase disease severity in WT mice. Consistent with earlier observations, WT mice infected with RRV developed severe disease. Strikingly, RRV-infected WT mice treated with rMIF developed more pronounced disease with mice showing complete loss of hindlimb function (Fig. 5B). Interestingly, the addition of rMIF in WT mice appeared to prolong disease signs, with mice showing severe disease signs past day 18, the point at which the disease had resolved in mice that were RRV-infected alone. Treatment of mice with rMIF also affected body weight, with weights decreasing at day 7 and remaining low for the duration of the experiment. (Fig. S5B).

Treatment with ISO-1 MIF Antagonist Ameliorates RRV Arthritic Disease.

To determine the potential of MIF to act as a pharmaceutical target to treat alphaviral disease, WT C57BL/6 mice were infected with RRV as previously shown. At the onset of clinical signs, mice were treated with 50 μ L of either the MIF ISO-1 antagonist (equivalent to 50 μ g) or diluent via the intraperitoneal route, with treatments continuing every 24 h up to day 14 p.i. Treated mice developed only mild disease compared with the mock-treated control mice, with a significant decrease in

arthritic disease from days 11 to 15 p.i. (Fig. 6). MIF antagonist-treated mice failed to develop the hindlimb dysfunction seen in the mock-treated mice and MIF antagonist treatment resulted in less disease induced weight reduction (Fig. S6).

Discussion

The impact that alphaviral disease has on quality of life, combined with the fact that alphaviruses circulate worldwide—frequently causing large epidemics—makes this group of viruses a global concern and highlights the importance of research into

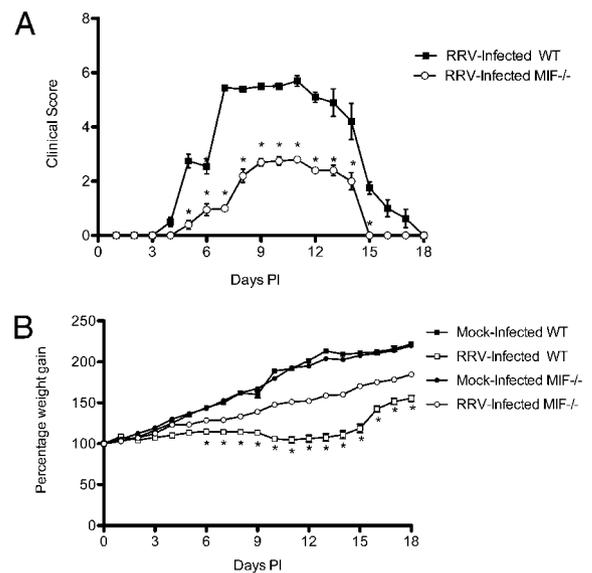


Fig. 3. RRV-induced disease is less severe in MIF^{-/-} mice. The 20-d-old C57BL/6 WT or MIF^{-/-} mice were infected subcutaneously with 10⁴ pfu RRV or mock-infected with diluent alone. (A) Mice were scored for the development of hindlimb dysfunction. (B) Mouse weight was monitored at 24-h intervals. Mock-infected mice were scored zero for the duration of the experiment. Each datapoint represents the mean \pm SEM of 5 to 10 mice and is representative of four independent experiments. * P < 0.05 using a Mann-Whitney test (A). * P < 0.05 compared with RRV-infected MIF^{-/-} using two-way ANOVA with Bonferroni posttest and RRV infection significantly reduced weight gain compared with mock-infected controls, with P < 0.05 for all time points after day 5.

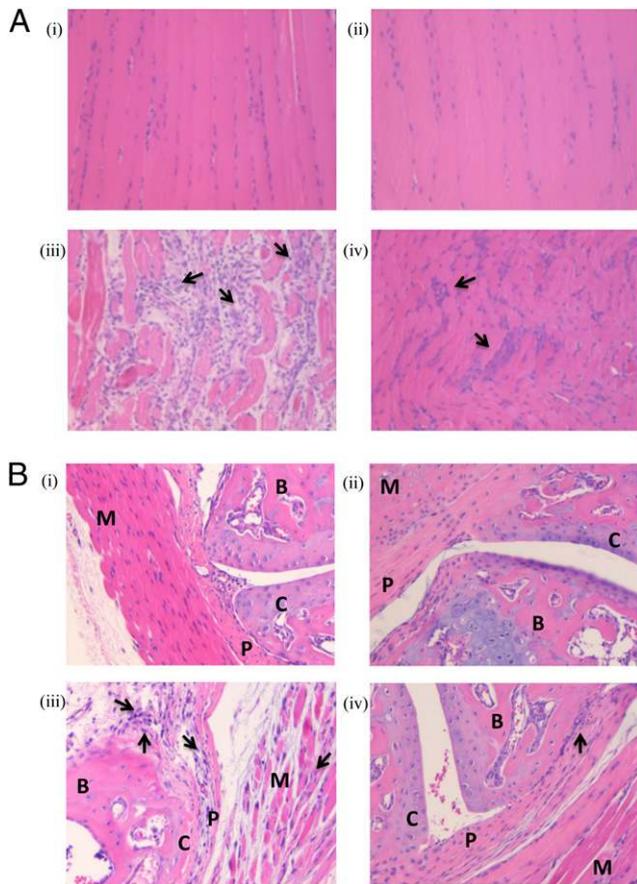


Fig. 4. RRV-induced inflammation is less severe in $MIF^{-/-}$ mice. The 20-d-old C57BL/6 WT or $MIF^{-/-}$ mice were infected subcutaneously in the right thorax with 10^4 pfu RRV or mock-infected with diluent alone. At 10 d p.i., mice were killed, perfused with 4% PFA, (A) quadriceps and (B) ankle tissues removed, paraffin-embedded, and 5- μ m sections generated. Sections were stained with H&E. Panels: (i) mock-infected WT, (ii) mock-infected $MIF^{-/-}$, (iii) RRV-infected WT, (iv) RRV-infected $MIF^{-/-}$. B, bone; C, cartilage; M, muscle; P, periosteum. Images are representative of at least five mice per group (magnification 100 \times) and arrows indicate abundance of inflammatory infiltrates.

potential anti-alphaviral therapies. Ongoing research into the pathogenesis of alphaviral disease has revealed numerous similarities between alphaviral-induced arthritic disease and RA including an important role of macrophages and the contribution of proinflammatory cytokines and chemokines (16–18, 21).

MIF has long been recognized as an important proinflammatory cytokine in RA. Given the similarities between alphaviral-induced arthritis and RA, we hypothesized a similarly critical role for MIF in RRV-induced arthritic disease. In this study we have used the mouse model of RRV disease to show that MIF plays a pivotal role in the development of inflammatory arthritis in alphaviral-induced disease. Mice deficient in MIF, either through gene knockout or treatment with a MIF antagonist, were significantly protected from the severe arthritic disease observed in RRV-infected WT mice, corresponding to a significant reduction in inflammation in the joint and the muscle tissues of RRV-infected mice. MIF deficiency reduced MCP-1 mRNA and protein expression following RRV infection, and decreased macrophage infiltration into muscle and joint tissues, resulting in reduced tissue damage and significant amelioration of disease signs. Furthermore, the reintroduction of MIF into mice with rMIF reestablished the susceptibility of these mice to severe RRV-induced arthritic disease. The amelioration of signs in this model provides compelling evidence that MIF is a viable target for therapy, and that MIF antagonists, such as the MIF ISO-1

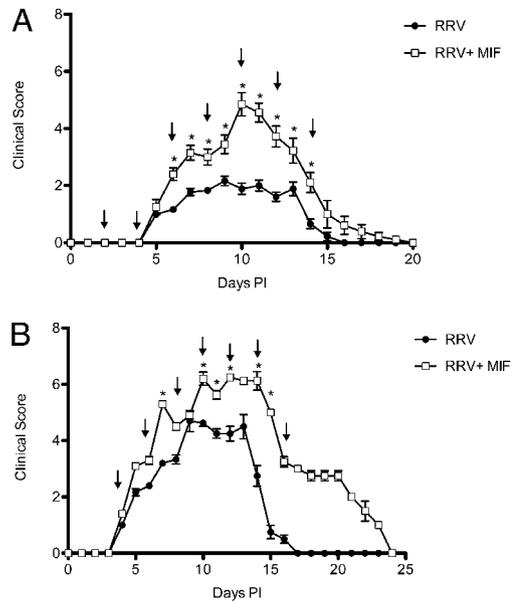


Fig. 5. Reconstitution of rMIF into WT and $MIF^{-/-}$ mice exacerbates RRV disease. The 20-d-old (A) $MIF^{-/-}$ or (B) C57BL/6 WT mice were infected with 10^4 pfu RRV alone, 50 ng recombinant mouse MIF alone, or 10^4 pfu of RRV plus 50 ng MIF. All injections were by the subcutaneous route into the right thorax. Mice were re-injected with 50 ng MIF every 48 h, as indicated by arrows. Mice were scored for the development of hindlimb dysfunction. Group infected with MIF alone showed no disease signs for the duration of the experiment. Each datapoint represents the mean \pm SEM of up to five mice and is representative of two independent experiments. * $P < 0.05$ using a Mann-Whitney test; note, statistical differences were unable to be determined after 15 d p.i. of 10^4 pfu RRV because of the loss of too many mice, reducing the RRV+MIF-infected mice sample size to two.

antagonist, may find application in the treatment of alphaviral arthritides in humans.

MIF is a cytokine with critical functions in both the innate and adaptive immune systems (23, 24, 31). Of particular interest is the role MIF plays in the innate immune system, where it has been identified as a key regulator (32). The innate immune system has been shown to play an important role in the pathogenesis of RA (33), with the role of MIF attributed to several key functions, including as a proinflammatory cytokine, as an inducer of metalloproteinases and as an activator and recruiter of immune cells including macrophages (34, 35).

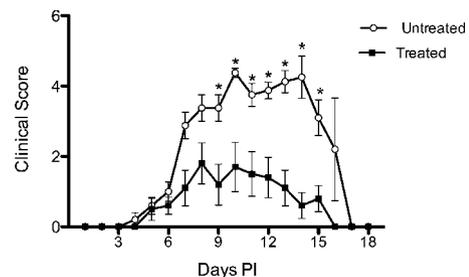


Fig. 6. Treatment with ISO-1 MIF antagonist ameliorates RRV-arthritic disease. The 20-d-old C57BL/6 WT mice were infected subcutaneously with 10^4 pfu RRV or mock-infected with diluent alone. At the onset of disease and at 24-h intervals thereafter, mice were treated with 50 μ L of either the MIF ISO-1 antagonist (1 mg/mL) or ISO-1 diluent intraperitoneally. Mice were weighed and scored for disease symptoms every 24 h, as outlined above. Each datapoint represents the mean \pm SEM of up to five mice and is representative of at least two independent experiments. * $P < 0.05$ using a Mann-Whitney test.

Given the role of the innate immune system in viral infections, the central role of macrophages in the pathogenesis of alphaviral infection and the similarities between RA and alphaviral-induced arthritis, it is reasonable to speculate a role for MIF in the modulation of alphaviral disease. MIF's involvement in RA was first identified using a murine collagen-induced arthritis model in 1997 (36) and then in a more severe rat adjuvant-induced arthritis model in 1999 (37). In the latter study, MIF expression was increased in the synovial tissues and serum of rats with arthritis. Similarly, in the murine RRV disease model, serum MIF concentration was increased in WT mice following RRV infection. The increase in serum MIF coincided with the onset of clinically evident disease, suggesting that the actions of MIF exacerbate the disease but do not contribute to the establishment of infection. MIF concentration was also found to be up-regulated in the tissues of the ankle joint at day 5 and 10 p.i. and the quadriceps muscles at day 10 p.i. As both these tissues are primary sites of RRV infection, the results suggest that there may be an association between the presence of RRV and the expression of MIF.

The impact of MIF on alphaviral disease was assessed using RRV infection of MIF^{-/-} mice, in which arthritic features were found to be almost entirely abrogated compared with WT mice. Histological analysis revealed mild inflammation and corresponding mild muscle-tissue damage in the RRV-infected MIF^{-/-} mice, in contrast to the more severe inflammation and damage seen in RRV-infected WT mice. Despite a reduction in disease severity, peak viral titers were unaffected, suggesting that any immunocompromise associated with MIF deficiency did not result in impaired viral clearance, an important issue in the consideration of anti-MIF therapy in viral disease. MIF^{-/-} mice have been used in similar studies of RA, showing a reduction of arthritis in MIF-deficient mice, which was found to correspond to reduced histological arthritis and cartilage damage (27). Given that viral replication and load in the RRV-infected MIF^{-/-} mice was comparable to WT mice, we interpret the reduction in disease severity in MIF^{-/-} mice as an immunopathological effect of the MIF deficiency.

MIF is known to act as a proinflammatory cytokine, stimulating the expression of TNF, IL-6, IL-1, and IL-8 (24). In this study, IFN- γ and MCP-1 were up-regulated in the ankle joint and quadriceps muscle of RRV-infected WT mice, compared with mock-infected mice, as has been previously reported (18, 21). The down-regulation of MCP-1 expression in the absence of MIF is of particular interest. MCP-1 is a potent monocyte chemoattractant (38) and has been previously shown to play an important role in RRV disease, with the inhibition of MCPs by the indazole derivative drug Bindarit, resulting in a significant decrease in the severity of RRV-induced disease (21). MIF has been shown to regulate MCP-1 expression in its promotion of macrophage recruitment in vivo (22) and leukocyte endothelial interactions in vitro (39). In the present study, the increase in MCP-1 expression and concentration seen at peak RRV-disease in WT mice was significantly diminished in MIF^{-/-} mice, a result that was also seen during the earlier stages of infection. These results suggest that an important aspect of the MIF-induced response to RRV infection is the induction of MCP-1 expression, thereby contributing to the marked macrophage infiltration observed in RRV-infected mice.

In addition, at peak disease, MIF^{-/-} mice showed a significant decrease in the expression of IFN- γ in the ankle joint, with a corresponding decrease in the quadriceps muscle. In support of previous studies, which have shown that the functions of MIF and IFN- γ are closely linked (32, 40, 41), the present data suggest a role for MIF in the regulation of IFN- γ within joints during RRV infection. However, the absence of MIF did not cause a uniform reduction of cytokine expression. There were no significant differences seen in the expression of TNF- α between MIF^{-/-} and WT mice following RRV-infection. Overall, the results suggest that the reduction of disease severity observed in MIF-deficient mice may be explained by reductions in MCP-1 and IFN- γ expression in these mice, with MCP-1 being particularly relevant because of its role in monocyte/macrophage recruitment, a key process known to be involved in RRV pathogenesis.

Several studies have investigated the role of MIF in viral infection; however, this remains an area of limited knowledge. A number of in vitro studies on the role of MIF in viral infections, including influenza, human cytomegalovirus, and Sindbis virus (42–44), have shown an increase in the level of MIF following infection. These findings, however, are limited because of the absence of corresponding in vivo studies. On the other hand, WNV and Japanese encephalitis virus, both arboviruses from the *Flaviviridae* family, have been shown to cause a significant increase in MIF mRNA in mouse brains (30, 45), and in the case of the WNV study, in vivo antagonism of MIF by genetic deletion, antibody, and antagonist treatment all resulted in decreased viral neuroinvasion. Moreover, Dengue virus, another flavivirus, was shown to up-regulate MIF in response to viral infection, with MIF-deficient mice showing increased survival compared with WT mice (46). Although these studies show a role for MIF in response to flavivirus infection and outcome, they are limited in their ability to demonstrate a role for MIF in the development of clinical signs and disease. Taken together, these studies suggest a previously unappreciated role for MIF in viral infection.

Currently, the only treatment available for alphaviral-induced disease is the use of broad range anti-inflammatory drugs, with no specific prophylactics available. Similarly, anti-inflammatory drugs, such as glucocorticoids (GC), are currently the main treatment used for other inflammatory diseases, including RA. Interestingly, although GCs act to reduce inflammation, studies have found that expression of MIF can actually be induced by GCs, suggesting that MIF can act as a physiological antagonist to the anti-inflammatory effects of GCs (28, 47, 48). There have been a number of studies focused on the potential of MIF antagonists in the treatment of inflammatory diseases (reviewed in ref. 47), with the particular aim of developing MIF-antagonists as a form of “steroid-sparing” treatment. Whereas the focus remains on immunoinflammatory diseases, such as RA, systemic lupus erythematosus, and atherosclerosis, this study illustrates that an application of anti-MIF pharmaceuticals to inflammatory infectious diseases, such as alphaviral arthritis, remains promising.

Materials and Methods

Virus and Cells. Stocks of the WT T48 strain of RRV were generated from the full-length T48 cDNA clone (kindly provided by Richard Kuhn, Purdue University, West Lafayette, IN) (49). Transcripts were electroporated into Vero cells (ATCC; CCL-81) and supernatants collected 24 h p.i. All titrations were performed by plaque assay on Vero cells as described previously (17).

Mice. C57BL/6 WT mice were obtained from the Animal Resource Centre (Perth, Australia) and MIF-deficient mice (MIF^{-/-}) (on the C57BL/6 background) (50) were obtained from Monash Medical Centre (Melbourne, Australia) and bred in-house. All animal experiments were performed in accordance with the guidelines set out by the University of Canberra Committee for Ethics in Animal Experimentation. The 20-d-old C57BL/6 male and female mice, of equal distribution, were inoculated subcutaneously in the thorax below the right forelimb with 10⁴ pfu virus, diluted in PBS to a final volume of 50 μ L. Mock-infected mice were inoculated with PBS diluent alone. Mice were weighted and scored for disease signs every 24 h. Disease scores were assessed based on animal strength and hind-leg paralysis, as outlined previously (20), using the following scale: 0, no disease signs; 1, ruffled fur; 2, very mild hindlimb weakness; 3, mild hindlimb weakness; 4, moderate hindlimb weakness and dragging of hindlimbs; 5, severe hindlimb weakness/dragging; 6, complete loss of hindlimb function; 7, moribund; and 8, death.

For treatment with rMIF (R & D Systems; reconstituted with 0.2% BSA in PBS), 20-d-old MIF^{-/-} or C57BL/6 WT mice were either infected with 10⁴ pfu RRV alone, diluent alone, treated with 50 ng rMIF alone, or infected with 10⁴ pfu RRV and treated with 50 ng rMIF. Infected and uninfected mice treated with rMIF were reinjected with 50 ng of rMIF every 48 h up to day 14 p.i. All injections were subcutaneous in the thorax in a 50- μ L volume, using 0.2% BSA in PBS as the diluents. For treatment with MIF ISO-1 antagonist (Merck; reconstituted in 10% DMSO in PBS to a final concentration of 1 mg/mL), 20-d-old C57BL/6 WT mice were either infected with 10⁴ pfu RRV or mock-infected with PBS. At the first signs of disease (ruffled fur and lethargy), mice were treated with 50 μ L of either the MIF ISO-1 antagonist or the 10% DMSO in PBS diluent via the intraperitoneal route, every 24 h up to day

14 p.i. In all experiments, mice were weighed and scored for disease signs every 24 h, as outlined above.

For histology, mice were killed and perfused with 4% paraformaldehyde (PFA). Tissues were collected, fixed in 4% PFA, ankles decalcified, followed by paraffin-embedding. Five-micrometer sections were prepared and stained with H&E (Histology facility, Australian National University, Canberra, Australia).

For EBD methods, see *SI Materials and Methods*.

For IHC, 5- μ m paraffin-embedded sections were dewaxed, rehydrated, then incubated with rabbit serum for 20 min. Sections were incubated with primary goat anti-human MIF mAb (R & D Systems) or isotype control IgG1 overnight at 4 °C. Endogenous peroxidase was blocked with 0.75% hydrogen peroxide in methanol and sections then stained with rabbit anti-goat IgG horseradish peroxidase (R & D Systems) for 30 min. Color was developed with 3, 3'-diaminobenzidine black (DAB; Vector Laboratories) enzyme substrate according to the manufacturer's instructions and counter-stained with fast red.

Real-Time PCR. For real-time PCR, see *SI Materials and Methods*.

ELISAs. For ELISA, see *SI Materials and Methods*.

Statistical Analysis. Viral titers and ELISA analysis were statistically evaluated by unpaired *t* tests. Weights, plaque assay, and real-time PCR data were analyzed using one-way ANOVA with Tukey's posttest or two-way ANOVA with Bonferroni posttest. All data were tested for normality using the D'Agostino–Pearson normality test before analysis with these parametric tests. Clinical scores were analyzed using the nonparametric Mann-Whitney test. Statistics were performed with GraphPad Prism 5.02.

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