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MIF receptor CD74 mediates alphavirus-induced arthritis and myositis

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Abstract

Objective—Arthrogenic alphaviruses such as Ross River virus (RRV) and chikungunya virus (CHIKV) circulate worldwide. This virus class causes debilitating illnesses characterised by arthritis, arthralgia, and myalgia. We previously identified macrophage migration inhibitory factor (MIF) as a critical inflammatory factor in the pathogenesis of alphaviral disease. Here, we characterise the role of CD74, a cell surface receptor of MIF, in both RRV- and CHIKV-induced alphavirus arthritides.

Methods—Mouse models of RRV and CHIKV infection were used to investigate the immunopathogenesis of arthritic alphavirus infection. The role of CD74 was assessed using histological analysis, real time PCR, flow cytometry and plaque assay.

Results—In comparison to wild-type mice, $CD74^{-/-}$ mice developed only mild clinical features and had low levels of tissue damage. Leukocyte infiltration, characterised predominantly by inflammatory monocytes and natural killer cells, was substantially reduced in infected tissues of $CD74^{-/-}$ mice, but production of pro-inflammatory cytokines and chemokines were not decreased. CD74 deficiency was associated with increased monocyte apoptosis, but had no effect on monocyte migratory capacity. Consistent with these findings, alphaviral infection resulted in a dose-dependent up-regulation of CD74 expression in human peripheral blood mononuclear cells and serum MIF levels were significantly elevated in humans with RRV or CHIKV infections.

Conclusion—We propose that CD74 regulates immune responses to alphaviral infection through effects on cellular recruitment and survival. These findings suggest that both MIF and

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CD74 play a critical role in mediating alphaviral disease and blocking these factors with novel therapeutic agents can substantially ameliorate pathology.

Introduction

Old world alphaviruses are important causes of viral arthritis and arthralgia worldwide. Alphaviruses are members of the *Togaviridae* family and include Ross River virus (RRV), chikungunya virus (CHIKV), mayaro virus and o'nyong-nyong virus (1). These viruses circulate in both endemic and epidemic patterns and can cause widespread outbreaks of polyarthritis and arthralgia (1–3) frequently involving tens of thousands to millions of cases. With the increasing distribution of CHIKV and RRV viral vectors, these viruses pose significant global threats as emerging diseases. The most recent of these large epidemics occurred with the re-emergence of CHIKV on the island of La Réunion (4) and its subsequent spread to countries of the Indian Ocean including India and South-East Asia (5). This outbreak involved an estimated five million cases since 2006 (6–8). Currently, CHIKV continues to circulate and cause sporadic outbreaks, the most recent being the 2011/2012 outbreaks in the Republic of Congo, Brazil, Cambodia, Philippines and Papua New Guinea (9–12). A recent modelling study predicted the likelihood of outbreaks or even epidemics of CHIKV in major US cities in 2013 (13). RRV, on the other hand, circulates in Australia and the surrounding islands, with around 7000 cases reported annually (14).

The mechanisms that drive arthritis and myositis in alphavirus infections are ill-defined. Mouse models of RRV and CHIKV infection that mimic selective signs of the human disease are being used to investigate the immunopathogenesis of arthritic alphavirus infection (15–21). We previously identified a critical role for macrophage migration inhibitory factor (MIF) in the development of RRV-induced disease (21). MIF is a pleiotropic pro-inflammatory molecule with multiple roles in mediating the innate and adaptive immune responses (22), facilitating both activation and recruitment of immune cells. We showed that MIF was upregulated during acute RRV infection and that MIF-deficient mice ($MIF^{-/-}$) exhibited mild disease characterised by reductions in inflammatory infiltrates and expression of proinflammatory factors including monocyte chemotactic protein-1 (MCP-1) and tumour necrosis factor-α (TNF-α).

CD74 is a non-polymorphic type II integral membrane protein with several biological functions (23). CD74 was originally identified as a key intracellular regulator of MHC class II folding and intracellular sorting. More recently, it has been reported to have a role as *a* cell surface receptor for MIF. For example, CD74-deficiency reduces MIF-induced activation of ERK1/2 MAP kinase and NFκB (24–27) and modifies the effects of MIF on production of IL-8 and cell survival (28). MIF also directly enhances B cell survival in a CD74-dependent mechanism (24). While both MIF- and CD74-deficiency have been shown to affect macrophage chemotactic responses, CD74 is not required for leukocyte adhesion induced by combined treatment with MIF and MCP-1, suggesting that MIF and CD74 regulate cell migration in overlapping but independent fashions (29, 30). Moreover, CD74 is associated with the chemokine receptors CXCR2/4 and influences their MIF dependent activation (29, 31). Therefore, the role of MIF:CD74 interaction in the regulation of inflammatory leukocyte recruitment remains to be clarified in particular pathologic settings.

In this context, we sought to evaluate whether CD74 regulates alphaviral disease and its function in immune cell modulation during alphavirus-induced inflammation. The results indicate that CD74 is a significant contributor to disease processes initiated by alphavirus infection and suggest CD74 as a potential therapeutic target.

Methods

Virus and Cells

Stocks of the wild-type T48 strain of RRV were generated from the full-length T48 cDNA clone (kindly provided by Dr Richard Kuhn, Purdue University) (32). All titrations were performed by plaque assay on Vero cells as described previously (15).

Patient Samples

Twenty-two serum samples were obtained from patients diagnosed with acute RRV infection in Queensland and New South Wales (Australia) during the 2005–2006 period. All patients presented with arthralgia with approximately half presenting with fever. All were diagnosed as having acute RRV infection, corresponding to high levels of RRV-specific IgM antibodies and low levels of IgG antibodies.

Twenty-four patients who were admitted to the Communicable Disease Centre at Tan Tock Seng Hospital (CDC/TTSH) with acute CHIKV-infection during the outbreak from the 1st August to 23rd September 2008 were included in this study (33, 34). The clinical features and patient demographics were described previously (35). PBMC and serum were isolated from CHIKV-patients at 4 different time points: (i) acute phase (median of 4 days post illness onset), (ii) early convalescent phase (median of 10 days post illness onset), (iii) late convalescent phase (4–6 weeks post illness onset) and (iv) chronic phase (2–3 months post illness onset). PBMC samples from nine healthy volunteers were isolated and used as control.

Mice

C57BL/6 WT mice were obtained from the Animal Resources Centre (Perth, Australia), CD74-deficient ($CD74^{-/-}$) and MIF-deficient ($MIF^{-/-}$) mice on the C57BL/6 background (36) were obtained from Monash University (Melbourne, Australia) and bred in-house. All animal experiments were performed in accordance with the guidelines set out by the Griffith University Animal Ethics Committee. Twenty-day-old C57BL/6 male and female mice, of equal distribution, were inoculated subcutaneously (s.c.) in the thorax below the right forelimb with 10^4 pfu RRV diluted in PBS to a volume of 50 μL as described previously (21), or in the ventral side of the footpad with 10^4 pfu CHIKV diluted in PBS to a volume of 20 μL. Mock-infected mice were inoculated with PBS alone. Mice were weighted and scored for disease signs every 24h. RRV disease scores were assessed based on animal strength and hind-leg paralysis as outlined previously (18). CHIKV-induced footpad swelling was assessed by measuring the height and width of the perimetatarsal area of the hind foot using Kincrome digital vernier calipers.

Histology

Mice were sacrificed and perfused with 4% paraformaldehyde (PFA). Tissues were collected and fixed in 4% PFA, followed by paraffin-embedding. Ankles were decalcified prior to embedding. Five-micron sections were prepared and stained with haematoxylin and eosin (H & E) (Histology facility, ANU, Canberra, Australia).

ELISA

The level of MIF in RRV human serum samples was measured by ELISA (R&D systems) according to the manufacturer's instructions. Plasma MIF levels from CHIKV-infected patients were measured using the multiplex microbead immunoassay (Human Sepsis Panel 1, Milipore) according to the manufacturer's instructions. Data was acquired using a

Luminex 200™ (Biorad) and analysed using the Bio-plex Manager™ 6.1 software (Biorad).

Real-Time PCR

Transcriptional analysis of cytokines was performed as described previously using commercially available QuantiTec primers for MCP-1, TNFα, IFNγ, IL-1β, IL-4, IL-10 and IL-6 (Qiagen) with FastStart SYBR-Green Master (Roche) (21, 34). Results are expressed as fold change relative to mock-infected controls, calculated with the ΔΔCt method. Briefly, ΔΔCt = ΔCt(RRV-infected) − ΔCt(mock-infected) with ΔCt = Ct(gene-of-interest) −Ct(housekeeping gene-HPRT1).

Transcriptional analysis of CD74 expression in human PBMC was performed by extracting total RNA using RNeasy® Mini Kit (QIAGEN) according to the manufacturer's instructions. For human PBMCs; extracted total RNA (10 ng/μL) was subjected to reverse transcription PCR using QuantiFast™ SYBR® Green RT-PCR Kit (QIAGEN) according to manufacturer's recommendations in 12.5 μL reaction volume. Real-time PCR (qRT-PCR) was performed in Applied Biosystems (ABI) 7900HT Fast real-time PCR System in 384-well plates, with the following thermal cycling conditions: (i) Reverse transcription step: 50°C for 10 min, 1 cycle; (ii) PCR initial activation step: 95°C for 5 min, 1 cycle; (iii) 2-step cycling: 95°C for 10 sec, follow by 60°C for 30 sec, 40 cycles. The forward and reverse primers used are as follows: GAPDH_forward (5′→3′): CCA CAT CGC TCA GAC ACC AT, GAPDH_reverse (5′→3′): GGC AAC AAT ATC CAC TTT ACC AGA GT, CD74_forward (5′→3′): AGC ATC ACT CCC CAA GGA AGA, CD74_reverse (5′→3′): TGT GAA CCA TGG CCC TGA AA. The fold is normalised to the housekeeping gene GAPDH calculated as ΔCt = Ct(gene-of-interest) −Ct(housekeeping gene-GAPDH).

Detection of leukocyte infiltrates in quadriceps

Quadriceps were removed following PBS perfusion. Quadriceps were weighed and minced, followed by enzyme digestion. Briefly, tissues were incubated with 3 mg/mL collagenase IV (Worthington, Lakewood, NJ) and 1 mg/mL DNase I (Sigma Aldrich) in 100 μL RPMI 1640 at 37°C for 1.5 h. Tissue mass was mixed with 5 mL RPMI, vigorously pipetted for 10 sec, and passed through a 40 μm cell strainer. Cells were washed, pelleted and treated with 1× RBC lysis buffer for 5 min, and counted. To determine percentages and numbers of specific leukocyte populations, cells were treated with Fc Block (2.4G2; BD) for 5 min at 4°C and labelled with fluorochrome-conjugated anti-mouse antibodies, including anti-CD3-FITC (145-2C11, BD), anti-CD19-APC (MB19-1, eBioscience), anti-CD11b-PE (M1/70, BD), anti-Gr1-APC (RB6-8C5, eBioscience) and anti-pan-NK/NKT antigen-PE (U5A2-13, BD) in various combinations in the presence of biotinylated anti-CD45 (30-F11, eBioscience), followed by treatment with streptavidin PE-Cy7 at 4°C for 30 min. To determine inflammatory monocytes, we found that CD11b was a more distinguishable marker than F4/80 in quadriceps. Cells were resuspended in 500 μL PBS containing 2% FCS and 1 μg/mL propidium iodine (PI), and analysed by the CyAn ADP flow cytometer (Beckman Coutler) with Kaluza software.

Peritoneal cell recruitment and apoptosis evaluation

Mice were infected intraperitoneally with 10^5 pfu RRV. Mice were sacrificed and peritoneal lavages collected with 5 mL PBS. To define inflammatory leukocyte populations, cells were treated with Fc Block at 4°C for 5 min and stained with anti-F4/80-PE (BM8, eBioscience) and anti-Gr1-APC at 4°C for 30 min. In contrast to quadriceps, peritoneal cell populations were highly identifiable by F4/80 labelling. In some experiments, cells were further treated with 5 μL of Annexin V FITC in 100 μL 1× binding buffer (BD Biosciences) at RT for 15 min to evaluate apoptosis. Cells were washed and analysed by flow cytometry as described.

Statistical analysis

Weights and plaque assay were analysed using two-way ANOVA with Bonferroni post-test or a one-way ANOVA with a Dunnetts post-test. Real-time PCR and flow cytometry data were analysed using unpaired Students *t*-test or a one-way ANOVA with a Dunnetts post-test. All data was tested for normality using the D'Agostino–Pearson normality test prior to analysis with these parametric tests. Clinical scores and ELISA were analysed using the non-parametric Mann-Whitney test. Statistics were performed with GraphPad Prism 5.02.

Results

CD74 regulates the severity of RRV-induced disease

To assess the role of CD74 in alphaviral disease, C57BL/6 WT mice and C57BL/6 mice deficient in CD74 ($CD74^{-/-}$) were infected with 10^4 pfu RRV and monitored for disease signs. RRV-infected WT mice developed severe manifestations of disease with severe hind limb weakness, loss of gripping ability (Figure 1A) and weight loss during stages of peak disease (Figure 1B). In contrast, $CD74^{-/-}$ mice developed only mild disease, with significantly ($p< 0.05$) lower peak disease scores (Figure 1A) and no weight loss (Figure 1B). Mock-infected mice scored zero on the clinical scale, indicating no signs of disease, for the duration of the experiment.

To determine if the reduced clinical disease severity observed in $CD74^{-/-}$ mice was accompanied by a reduction in inflammation and tissue damage, tissues from RRV-infected mice were collected at the peak of disease (day 10 p.i.) for histological analysis. No inflammation was observed in the quadriceps muscle (Figure 1C i, ii) or ankle joint (Figure 1D i, ii) of mock-infected WT or $CD74^{-/-}$ mice. RRV-infected WT mice showed extensive inflammation and tissue damage in quadriceps muscle (Figure 1C iii) and around the ankle joint (Figure 1D iii). In contrast, RRV-infected $CD74^{-/-}$ mice showed reduced inflammation in the quadriceps muscle (Figure 1C iv) and ankle joint (Figure 1D iv), with a markedly reduced number of infiltrating cells compared to WT mice.

Reduced RRV disease in CD74-deficient mice is not due to decreased viral burden

To determine if the reduction in disease severity in CD74-deficient mice was due to an effect on virus replication, C57BL/6 WT and $CD74^{-/-}$ mice were infected with 10^4 pfu RRV and sacrificed at days 1, 3, 5 and 10 p.i. Serum, ankle joints and quadriceps muscles were assayed for viral titers. Viral titers in the serum of WT and $CD74^{-/-}$ mice were comparable at all days tested, indicative of equivalent systemic replication (Figure 2A). Peak RRV titers (day 1 p.i.) recovered from quadriceps muscles of WT and $CD74^{-/-}$ mice were comparable (Figure 2B). In contrast, peak RRV titers were significantly higher in the ankle tissues of $CD74^{-/-}$ mice compared to WT mice (Figure 2C). At later stages of infection, RRV titres were slightly elevated in the quadriceps and ankles of $CD74^{-/-}$ mice compared to WT mice (Figure 2B, 2C) suggesting that CD74 may have an effect on viral clearance within these tissues. Nonetheless, these results indicate that the reduced disease in $CD74^{-/-}$ mice was not due to decreased viral load in key target tissues.

CD74 deficiency does not affect the expression of pro-inflammatory cytokines and chemokines

A range of pro-inflammatory factors have been shown to mediate or contribute to alphaviral disease (16). Our group and others have demonstrated that MCP-1, TNF-α and IFN-γ play major roles in the development of RRV-induced arthritis. To elucidate whether the reduced disease in $CD74^{-/-}$ mice was due to decreased production of pro-inflammatory cytokines and chemokines, we examined the expression of IL-1p, IL-6, IL-4, IL-10 TNF-α, IFN-γ and MCP-1. At the peak of disease (day 10 p.i.), quadriceps muscle and ankle joint tissues were

isolated and selected mRNA transcripts were measured by real-time PCR. The data obtained were normalised against the housekeeping gene HPRT1 and expressed relative to tissues from mock-infected mice. Expression of the pro-inflammatory mediators in RRV-infected mice was increased compared to mock-infected controls (Figure 2D). Interestingly, only IL-10 was reduced in the absence of CD74. IFN-γ in the joint and TNF-α in both quadriceps and joint tissues were elevated, whereas IL-6, IL-1β, IL-4 and MCP-1 were unaffected by CD74 deficiency. These results suggest that the reduced disease severity in CD74-deficient mice was not due to impaired production of pro-inflammatory cytokines and chemokines.

RRV-infected CD74$^{-/-}$ mice exhibit a significant reduction of leukocyte infiltration in quadriceps

Although virus titres and cytokine levels were comparable or even higher in CD74$^{-/-}$ mice, these mice showed significantly reduced RRV disease and pathology. We next determined whether there was a difference in leukocyte accumulation in the infected tissues of WT and CD74$^{-/-}$ mice. As shown in Figure 3A, compared to WT mice, there was a substantial reduction in CD45^{+} (pan-leukocyte) cell accumulation in quadriceps muscles of RRV-infected CD74$^{-/-}$ mice. In line with previous studies (17, 18), three major leukocyte subsets, including inflammatory monocytes (Gr1^{hi}CD11b^{hi}), NK/NKT cells (panNK^{hi}) and T cells (CD3^{+}) were identified in the tissues of infected mice. Each of these populations was significantly reduced in the tissues of infected CD74$^{-/-}$ mice (Figure 3A–B). In addition, CD74$^{-/-}$ mouse quadriceps demonstrated much less immune accumulation, evidenced by lower percentages (Figure 3C) and total numbers (Figure 3D) of CD45^{+} cells, inflammatory monocytes, NK/NKT and T cells.

CD74-deficient inflammatory monocytes migrate normally but exhibit increased apoptotic potential in response to RRV infection

We sought to understand why RRV-infected CD74$^{-/-}$ mice showed reduced immune accumulation. CD74-deficient monocytes have been shown to exhibit reduced migration responses to chemokines *in vivo* and *in vitro* (30). CD74 regulates cellular migration and apoptosis through its interaction or association with MIF (27, 30), and MIF was previously shown to enhance RRV disease (21). Moreover, inflammatory monocytes/macrophages are critical contributors to RRV disease (16). We therefore investigated RRV-induced cellular migration using a peritoneal exudate model in WT and CD74$^{-/-}$ mice, and used MIF$^{-/-}$ mice for comparison. Following the intraperitoneal RRV challenge, the migratory pattern of F4/80^{+}Gr1^{hi} inflammatory monocytes and F4/80^{hi}Gr1$^{lo\text{-}int}$ macrophages over time was determined with 22 hours p.i. selected for further analysis (data not shown). Cellular recruitment in mice was compared showing no significant difference in migration between cells from WT and CD74$^{-/-}$ mice (Figure 4A). This was also confirmed in vitro using bone marrow derived macrophages cultured in transwell plates and stimulated with RRV (data not shown). In contrast, MIF$^{-/-}$ mice exhibited significantly reduced F4/80^{+}Gr1^{hi} monocyte migration in response to RRV (Figure 4A). Because CD74$^{-/-}$ monocytes did not show a migratory defect, we investigated whether alterations of survival of these cells could contribute to the reduced numbers of leukocyte infiltrates in the tissues during RRV infection. As shown in Figure 4B, we observed significantly higher Annexin V staining in cells from CD74$^{-/-}$ mice in comparison to WT mice.

CD74 mediates the severity of CHIKV-induced inflammation

To extend the analysis of the role of CD74 in arthrogenic alphavirus infection, we next examined the mouse model of CHIKV (37). Similar to RRV infection, CHIKV-infected CD74-deficient mice demonstrated little swelling in either the footpad or the ankle joint in the course of infection, while infected WT mice showed significant swelling peaking at day 3 p.i (Figure 5A and 5B). Similarly, CHIKV-infected WT mice showed extensive cellular

infiltration, tissue damage and enlarged cavities (associated with plasma leakage) in both tissues (Figure 5C ii and 5D ii). In contrast, CHIKV-infected $CD74^{-/-}$ ankle and footpads exhibited minimal inflammation and tissue damage at day 3 (Figure 5C iv and 5D iv). No inflammation was observed in the ankle joint or the footpad tissues of mock-infected WT or $CD74^{-/-}$ mice (Figure 5C (i and iii) and 5D (i and iii).

MIF and CD74 are upregulated in patients with alphaviral polyarthritis

In order to determine if CD74 expression is upregulated in the monocytes/macrophages of mice with alphavirus infection, RRV-infected mouse splenocytes were harvested at day 6 and 10 p.i. and stained with anti-CD74, anti-CD11b and anti-Ly6C. CD74 expression was found to be upregulated correlating with disease severity reaching highest levels of expression at peak disease (Figure 6A).

In humans, infection with RRV or CHIKV causes a febrile illness associated with joint inflammation and infiltration of synovial macrophages (38). We measured the levels of MIF in serum samples from RRV- and CHIKV-infected individuals (Figure 6B and 6C). MIF was significantly elevated in the serum samples from alphavirus-infected patients compared to healthy donors. Finally, to determine if alphavirus infection resulted in an upregulation of CD74 in peripheral blood mononuclear cells (PBMC), CD74 mRNA expression in CHIKV-infected patients was determined (Figure 6D). Alphavirus infection resulted in an upregulation of CD74 correlating with the stage of disease with the highest levels of CD74 expression being detected during acute and early convalescent stages of infection.

Discussion

The mechanisms by which alphaviruses trigger arthritis and myositis are not well understood. Alphaviral disease has many similarities to rheumatoid arthritis (RA), including the key involvement of both macrophages and MIF (21). CD74 is a key MIF receptor, acting to recruit CD44 and initiate MAPK activation and prolong cell survival (24, 27). Furthermore, CD74 forms a receptor complex with CXCR2 and CXCR4 that can contribute to leukocyte recruitment in a MIF-dependent manner (29, 31). It has been implicated in the pathogenesis of a number of diseases, predominantly autoimmune diseases and cancer (23). However, CD74 has been little studied in the context of infectious disease. We now propose that MIF-dependent CD74 signalling regulates the alphavirus-induced inflammatory responses whereby CD74 deficiency results in reduced tissue inflammation and disease severity.

The innate immune response is critical in the pathogenesis of alphaviral disease, mediating cell recruitment, viral clearance and inflammation (16, 17, 19). In particular, monocytes and macrophages are the major cellular contributors to disease progression and severity (16), with adaptive T and B cell immunity playing no role in the development of inflammation (18). Hence, as the invariant chain of CD74 regulates MHC-class II transport and its deficiency leads to impaired thymic selection of CD4 T cells and a reduced CD4 T cell number (39), such a defect in T cell phenotype was expected to have little effect on alphavirus disease manifestations in CD74 deficient mice. However, while CD74 deficiency resulted in the relief of clinical manifestations and reduced immunopathology, virus titers were not reduced in $CD74^{-/-}$ mice and were increased in the ankle and quadriceps, suggesting a role for CD74 in viral clearance. It is possible that the MHC function of the invariant chain, the intracellular form of CD74 and its regulatory function for T/B cell responses were required for optimal elimination of virus. In this regard, virus titres were significantly higher in ankle and quadriceps of CD4 and CD8 deficient mice compared to WT mice (data not shown), indicating that T cells may be important for clearance of RRV

infection and that the T cell defect in CD74$^{-/-}$ mice could have contributed to the higher virus loads in some tissues.

It is worthwhile to note that the level of CD74 expression on different leukocyte populations may differ. In the context of this study, it is known that macrophages and monocytes are instrumental in determining alphaviral disease severity (16). Therefore, the level of expression of CD74 on monocytes and macrophages was the major focus with the results showing increased CD74 in inflammatory monocytes corresponding to disease severity (Figure 6A).

Despite the significant reduction of immune infiltration in susceptible tissues of CD74-deficient mice, the equal or higher viral load in these mice actively stimulated equal or higher production of the majority of inflammatory cytokines and chemokines. CD74 deficiency has been previously reported to result in an increase in IFN-γ production, potentially due to preferential skewing towards a Th1 response (40, 41). However, we cannot rule out a negative feedback loop established by the MIF-CD74 axis particularly as it has been shown to regulate TNF receptor expression (42). Disruption of this loop might result in dysregulated production of various inflammatory mediators, including TNF-α. Pro-inflammatory mediators other than those measured in this study may be important in determining the protection of CD74$^{-/-}$ mice from alphaviral arthritis. In particular, other mediators such as complement and mannose binding lectin are known to contribute to the pathogenesis of alphavirus arthritis and therefore will be further investigated in CD74$^{-/-}$ mice (43, 44).

An alternate explanation for the effects of CD74 deficiency on alphavirus arthritis is through effects on leukocyte recruitment. We recently showed that macrophage recruitment responses to MCP-1 were impaired in CD74$^{-/-}$ mice (30) and the current results also suggest impairment of monocyte recruitment in CD74$^{-/-}$ mice. We therefore investigated potential mechanisms of leukocyte recruitment to explain the observed reduction in immune infiltrates in the tissues of infected CD74-deficient mice. Infection with RRV induced a series of changes in the cellular populations in the peritoneal cavity. The early but transient appearance of Gr1^{hi}F4/80^{-} neutrophils suggests that these cells are unlikely to play a role in controlling RRV infection or in long-term RRV-induced inflammation. The recruitment and persistence of Gr1^{hi}F4/80^{+} monocytes in the peritoneum appears to correspond to the Gr1^{hi}CD11b^{hi} population induced by virus infection in quadriceps (17, 45), while a Gr1^{int}F4/80^{hi} macrophage population also emerged. Although it remains to be clarified whether Gr1^{int}F4/80^{hi} macrophages are involved in control of RRV infection, they did not correspond to any of the cell populations in RRV-infected quadriceps. Unexpectedly, CD74$^{-/-}$ mice did not exhibit reductions in recruitment of Gr1^{hi}F4/80^{+} inflammatory monocytes from blood in response to alphavirus infection of the peritoneum. In contrast, MIF deficiency, which has been shown to result in impaired monocyte adhesion to the arterial wall (29), did result in impaired monocyte recruitment in response to alphavirus infection. In contrast to the lack of effect on migration, cells from CD74-deficient mice exhibited accelerated apoptosis among inflammatory monocytes relative to wild-type monocytes, showing apoptosis within as little as 30 hours post infection. This finding phenocopies MIF deficiency (46) whereby MIF may act as an antagonist to p53-mediated apoptosis. Furthermore the role of CD74 in cell survival has been demonstrated both *in vitro* in primary macrophages and *in vivo* in CD74-deficient mice (27). Interestingly, it has also been shown that the role of CD74 in cell survival is at least partially dependent on signalling through CD44 (27), which may explain why mice deficient in both MIF and CD74 show greater apoptotic potential than mice deficient in solely MIF or CD74 (data not shown). Overall, an increase in leukocyte apoptosis may explain the reduced numbers of leukocytes and reduced pathology in alphavirus-infected CD74$^{-/-}$ mice.

The factors affecting leukocyte migration are complex and we cannot conclusively rule out the impact that CD74-deficiency has in disrupting the CD74-CXCR2/CXCR4 receptor complex, which has been shown to induce leukocyte recruitment in a MIF dependent manner (29, 31). Irrespective, the observation of reduced leukocyte survival has critical significance as an overall reduction of functional monocytic cells has been shown to reduce alphavirus-induced inflammation (16). Overall, this is the first study illustrating an impact of CD74 on apoptosis in an infectious disease model.

Here we demonstrate an increase in both serum MIF levels and an upregulation of CD74 expression in PBMCs during CHIKV infection. These findings, together with our observations from the mouse models, strongly suggest important roles for MIF and CD74 in mediating alphaviral disease in humans. Further studies are required to decipher how signalling mechanisms of the MIF-CD74 axis control cellular survival and death in viral infection.

To date, non-steroidal anti-inflammatory drugs are the primary therapeutic means to alleviate the symptoms of alphaviral disease. These drugs can be associated with undesired side effects and may compromise immunity in treated patients (47, 48). Cell-surface CD74 may be a tractable specific target for the application of anti-CD74 (milatuzamab) to limit inflammatory responses to viral infection. Targeting CD74 at the cell surface would avoid any potential side effects associated with the function of CD74 as invariant chain to regulate MHC-class II complex formation. Nonetheless, the effect of CD74 deficiency on viral clearance requires greater understanding before clinical trials can be contemplated.

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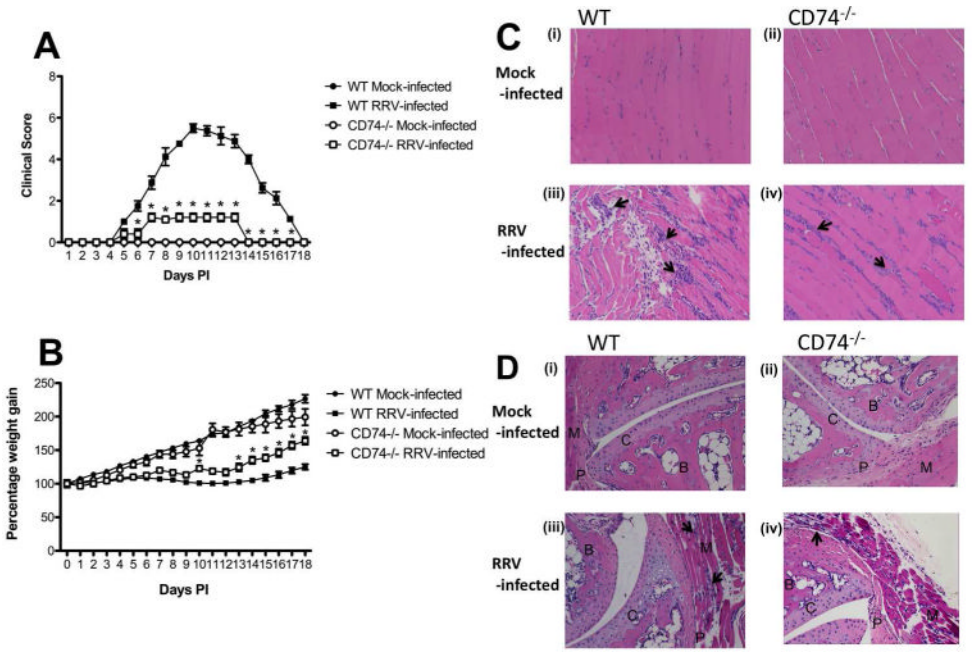

Figure 1. RRV-induced disease is less severe in CD74$^{-/-}$ mice

20-day-old C57BL/6 WT or CD74$^{-/-}$mice were infected s.c. with 10^4 pfu RRV or mock-infected with diluent alone. (A) Mice were scored for the development of hind-limb dysfunction. (B) Mouse weight was monitored at 24-hour intervals. Mock-infected mice were scored zero for the duration of the experiment. Each data point represents the mean +/− SEM of 5 to 10 mice and is representative of 4 independent experiments. *$p < 0.05$ using a Mann-Whitney test (A). *$p < 0.05$ compared to RRV-infected CD74$^{-/-}$using two-way ANOVA with Bonferroni post test and RRV infection significantly reduced weight gain compared to mock-infected controls, with $p < 0.05$ for all time points after day 3 (WT) or day 7 (CD74$^{-/-}$) (B). For histological analysis, mice were sacrificed at 10 days p.i., perfused with 4% PFA, (C) quadriceps and (D) ankle tissues removed, paraffin-embedded and 5 μm sections generated. Sections were stained with H&E. Panels (i) mock-infected WT, (ii) mock-infected CD74$^{-/-}$, (iii) RRV-infected WT, (iv) RRV-infected CD74$^{-/-}$. Annotations; (B) bone, (C) cartilage, (P) periostem, (M) muscle. Images are representative of at least 5 mice per group (magnification 100X) and arrows indicate abundance of inflammatory infiltrates.

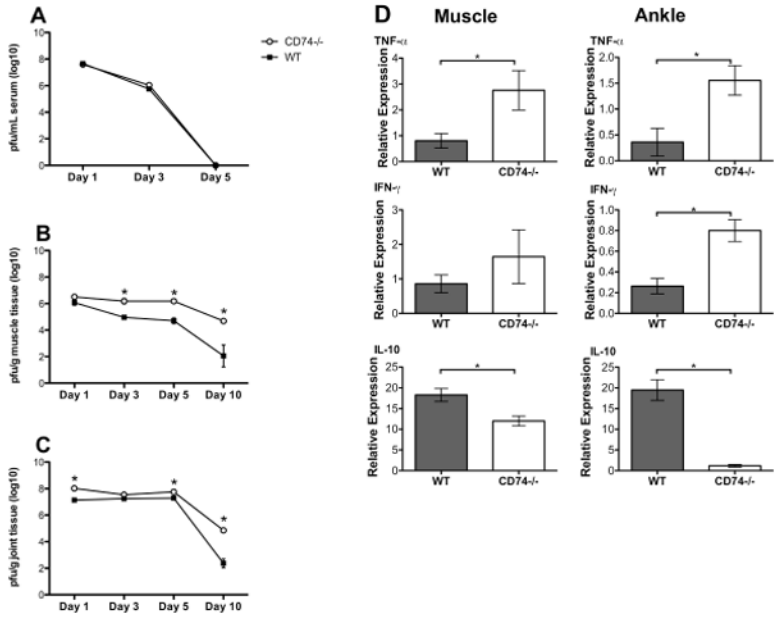

Figure 2. CD74 deficiency does not reduce systemic RRV viraemia or pro-inflammatory cytokine expression

20-day-old C57BL/6 WT or CD74$^{-/-}$mice were infected s.c. with 10^4 pfu RRV. At days 1, 3, 5 and 10 p.i. the (A) serum, (B) quadriceps and (C) ankle tissues were harvested, homogenised and the amount of infectious virus determined by plaque assay on Vero cells. Each data point represents the mean +/− standard error of 5 to 6 mice. *p < 0.05 using two-way ANOVA with Bonferroni post test. Similarly, At day 10 p.i. total RNA from (C) quadriceps muscle and (D) ankle joint tissues was isolated and analysed for mRNA expression by qRT-PCR. Data was normalized to the housekeeping gene HPRT1 and expressed as relative expression *$p < 0.05$ Students t test. Each bar represents the mean +/− the SEM for 5–6 mice per group.

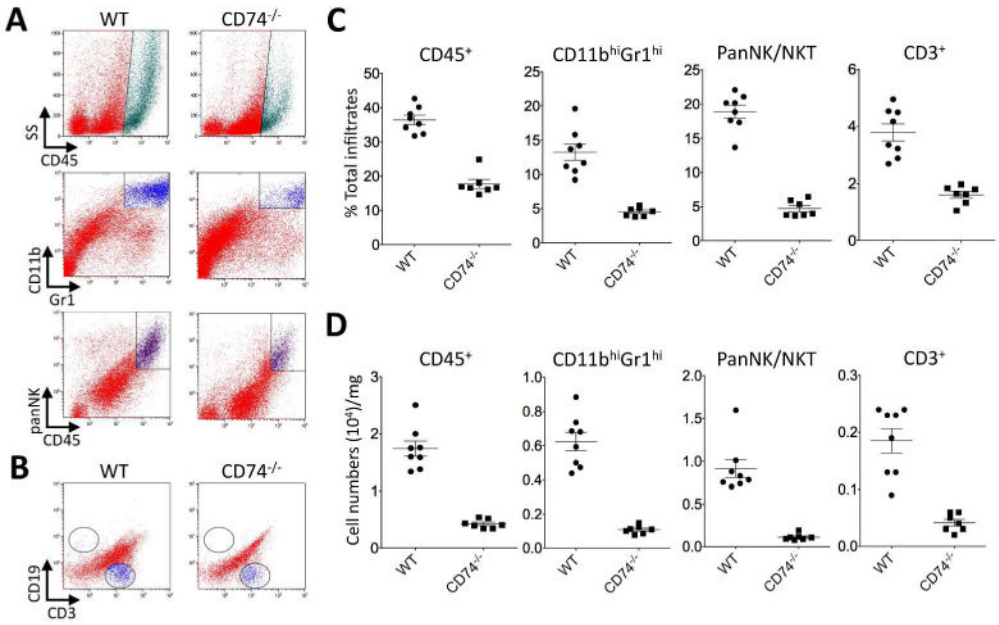

Figure 3. Immune infiltration is largely reduced in quadriceps of infected CD74$^{-/-}$ mice

Quadriceps muscles were removed from infected WT (n = 8) and CD74$^{-/-}$ (n = 7) mice at day 10 p.i.. Following tissue digestion, cells were isolated, counted and stained for CD45, Gr1, CD11b, pan NK/NKT, CD3 and CD19 expression. (A) Total leukocyte (CD45^{hi}), inflammatory monocyte (Gr1^{hi}CD11b^{hi}), NK/NKT (CD45^{hi}pan NK^{hi}) populations of infected WT and CD74$^{-/-}$ mice were determined among total live (PI-negative) infiltrated cells using various gating strategies. Representative plots of infected WT and CD74$^{-/-}$ mice are shown. (B) T and B cells were also determined based on the live and CD45^{hi} gate. (C, D) The percentages and cell numbers of leukocyte populations in total cell infiltrates from each individual mouse were compared in scatter plots (means ± SEM), of which all demonstrated statistical significance between WT and CD74$^{-/-}$ ($p<0.005$), using the Student *t* test. Data are representative of two separate experiments. SS; side scatter.

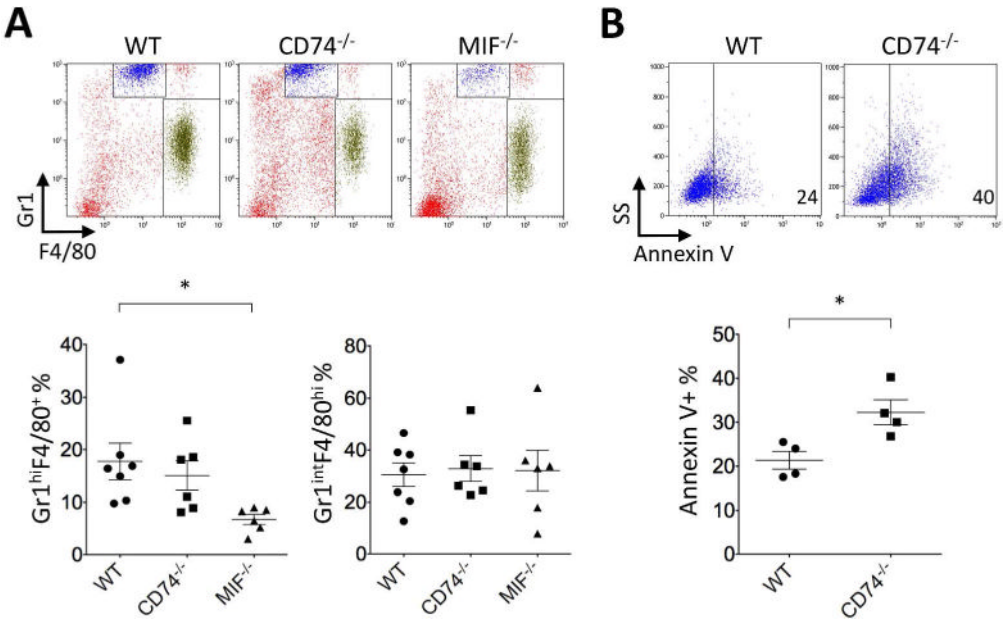

Figure 4. CD74$^{-/-}$ mice demonstrate an enhanced apoptosis potential, but not defective migration, of inflammatory monocytes, in response to the RRV challenge

(A) WT (n = 7), CD74$^{-/-}$ (n = 6), MIF$^{-/-}$ (n = 6) and CD74/MIF$^{-/-}$ (n = 6) mice were injected with 10^5 pfu RRV i.p.. At 22 h, peritoneal cells were collected and stained with anti-Gr1-APC and anti-F4/80-PE. The percentages of live Gr1^{hi}F4/80^{+} and Gr1^{int}F4/80^{hi} cells of WT, CD74$^{-/-}$ and MIF$^{-/-}$ mice were determined. A representative dot plot of each strain is shown (upper panel). Cell percentages are collectively compared in scatter plots. Statistical significance was determined by one-way ANOVA. (B) Similarly, WT (n = 4) and CD74$^{-/-}$ (n = 4) mice were injected with RRV i.p., and, at 30 h, peritoneal cells were collected and labelled with anti-Gr1-APC and anti-F4/80-PE, followed by Annexin V FITC stain. Cells were analyzed by flow cytometry. Live Gr1^{hi}F4/80^{+} cells were gated to determine Annexin V binding, based on non-Annexin V-treated cells. Statistical significance was determined by the Student t test. Data are representative of at least two separate experiments. SS; side scatter.

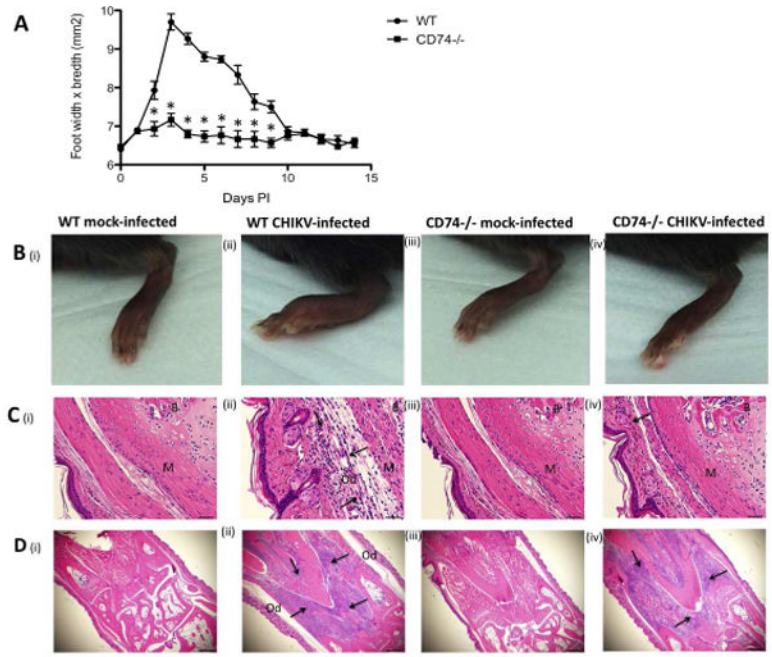

Figure 5. CHIK-induced inflammation is reduced in CD74$^{-/-}$mice

20-day-old C57BL/6 WT or CD74$^{-/-}$mice were infected s.c. in the footpad with 10^4 pfu CHIKV or mock-infected with diluent alone. (A) Mice were monitored daily for joint inflammation with foot width and breadth measured over time. Each data point represents the mean +/− SEM of 5 to 10 mice and is representative of 2 independent experiments. (A) *$p < 0.05$ using a Mann-Whitney test. (B) peak swelling at day 3 shown in (i and ii) WT and (iii and iv) CD74$^{-/-}$ mice. For histological analysis, mice were sacrificed at 3 days p.i., perfused with 4% PFA, (C) ankle tissues and (D) footpads removed, paraffin-embedded and 5 μm sections generated. Sections were stained with H&E. Panels (i) mock-infected WT, (ii) RRV-infected WT, (iii) mock-infected CD74$^{-/-}$, (iv) RRV-infected CD74$^{-/-}$. Annotations; (B) bone, (M) muscle. (Od) subcutaneous oedema. Images are representative of at least 5 mice per group (magnification (C) 200X (D) 40X) and arrows indicate abundance of inflammatory infiltrates.

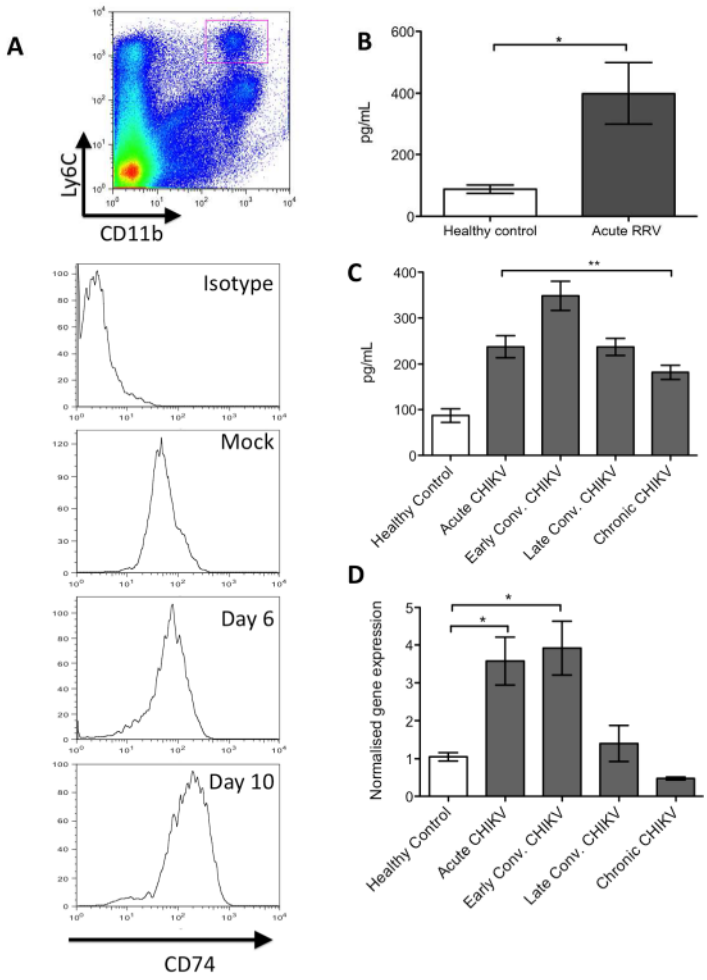

Figure 6. MIF/CD74 is up-regulated in alphaviral-induced disease

(A) CD74 expression on inflammatory monocytes isolated from RRV-infected mouse splenocytes harvested at day 6 and 10 p.i. stained with anti-CD74-FITC, anti-CD11b-PE-Cy7 and anti-Ly6C-APC. Acute serum samples from (B) RRV-acute positive patients (n = 10) and (C) CHIKV-positive patients (n = 24) were measured by MIF cytokine ELISA and compared to healthy controls (n = 9). Each point represents a single patient sample with the mean +/− SEM. The difference in MIF serum levels were statistically significant *$p < 0.05$ as determined by Mann-Whitney test. (D) qRT-PCR analysis of CD74 gene expression on CHIKF patients' PBMCs (n = 24) and normal healthy controls (n = 9). Data are presented as mean relative CD74 gene expression fold change against mean healthy controls ± SEM. *, $p < 0.05$, **, $p < 0.01$, ***, $p<0.001$, Mann-Whitney test, two tail. CHIKV samples come from a case-control longitudinal study performed on 24 laboratory confirmed adult CHIKV-infection patients across four collection time points see methods for details.