Macrophage-Derived Proinflammatory Factors Contribute to the Development of Arthritis and Myositis after Infection with an Arthrogenic Alphavirus

Brett A. Lidbury,[1] Nestor E. Rulli,[1] Andreas Suhrbier,[4] Paul N. Smith,[2,3] Shaun R. McColl,[5] Anthony L. Cunningham,[6] Andrej Tarkowski,[8] Nico van Rooijen,[9] Robert J. Fraser,[1,7] and Suresh Mahalingam[1]

[1]Virus and Inflammation Research Group, Faculty of Sciences, University of Canberra, [2]Orthopedic Unit, John James Hospital, and [3]The Australian National University Medical School, Canberra, [4]Queensland Institute of Medical Research, Brisbane, [5]School of Molecular and Biomedical Science, The University of Adelaide, Adelaide, [6]Centre for Virus Research, Westmead Millennium Institute, Sydney, and [7]Department of Medicine, Royal Melbourne Hospital, University of Melbourne, Melbourne, Australia; [8]Department of Rheumatology and Inflammation Research, University of Gothenburg, Gothenburg, Sweden; [9]Department of Cell Biology and Immunology Faculty of Medicine, Vrije University, Amsterdam, The Netherlands

Alphaviruses, such as chikungunya virus and Ross River virus (RRV), are associated with outbreaks of infectious rheumatic disease in humans worldwide. Using an established mouse model of disease that mimics RRV disease in humans, we showed that macrophage-derived factors are critical in the development of striated muscle and joint tissue damage. Histologic analyses of muscle and ankle joint tissues demonstrated a substantial reduction in inflammatory infiltrates in infected mice depleted of macrophages (i.e., "macrophage-depleted mice"). Levels of the proinflammatory factors tumor necrosis factor–α, interferon–γ, and macrophage chemoattractant protein–1 were also dramatically reduced in tissue samples obtained from infected macrophage-depleted mice, compared with samples obtained from infected mice without macrophage depletion. These factors were also detected in the synovial fluid of patients with RRV-induced polyarthritis. Neutralization of these factors reduced the severity of disease in mice, whereas blocking nuclear factor κB by treatment with sulfasalazine ameliorated RRV inflammatory disease and tissue damage. To our knowledge, these findings are the first to demonstrate that macrophage-derived products play important roles in the development of arthritis and myositis triggered by alphavirus infection.

A number of viral infections can cause arthritis [1]. However, symptomatic infections with mosquito-borne alphaviruses, such as Ross River virus (RRV), chikungunya virus, and o'nyong-nyong virus, are nearly always associated with self-limiting but debilitating rheumatic disease [2, 3]. For instance, RRV causes up to 8000 cases of disease annually in Australia, where there has been a recent marked increase in the number of localities where cases have been reported [4]. During the late 1970s and early 1980s, regional island nations of the South Pacific also experienced an explosive outbreak of RRV disease (RRVD) that involved ~60,000 people [5]. In the 1960s, an epidemic of o'nyong-nyong affected 2 million people in Africa [6], and new outbreaks have been reported in recent times [7]. More recently, ~250,000 people were afflicted with chikungunya on Réunion Island in the Western Indian Ocean, and it is estimated that 1.38 million people across southern and central India also developed this disease [8–10].

RRV is a positive-sense alphavirus endemic to Australia and Papua New Guinea. Symptomatic infection results in polyarthritis or arthralgia and, in many cases, also involves myalgia, skin rash, and fatigue [5]. Symptoms of the disease are often severe at onset, but they

Received 8 May 2007; accepted 10 August 2007; electronically published 23 April 2008.

Potential conflicts of interest: none reported.

Financial support: Australian National Health and Medical Research Council (project grant 303404).

Reprints or correspondence: Dr. Suresh Mahalingam, School of Health Sciences, University of Canberra, Canberra, ACT 2601, Australia (suresh.mahalingam@canberra.edu.au).

The Journal of Infectious Diseases 2008; 197:1585–93

0022-1899/2008/19711-0015$15.00
DOI: 10.1086/587841

progressively diminish and usually resolve by 3–6 months [11, 12]. RRV RNA has been detected in synovial fluid samples obtained from patients with RRVD [13], and it is thought that viral replication within the affected joints is responsible for continued inflammation and disease [3]. Histologic studies showed that, in early human joint effusions, monocytes and macrophages are found in higher numbers than are lymphocytes, although T lymphocytes are dominant at later stages [14, 15]. RRV infection in the skeletal muscle tissue of humans has not been directly demonstrated, although 60% of patients with RRVD experience myalgia [12].

The mechanisms by which arthritogenic alphaviruses cause disease are poorly understood. Early studies in mice, although discounting the primary involvement of brain tissue damage as being responsible for disease, provided some evidence for muscle inflammation and associated damage as the likely physiological basis of RRV-induced symptoms in mice [16, 17]. We and other investigators have characterized joint and muscle pathological findings in the mouse model and have described the relevance of the model to human disease [18, 19]. We previously reported that, after infection with RRV, 17- to 21-day-old outbred mice developed severe disease characterized by inflammation of skeletal muscle tissue and associated tissue damage [18]. We demonstrated that such disease signs as hind limb dragging and muscle pathological findings were ameliorated after the mice were treated with carrageenan or silica, compounds that are toxic to macrophages. This finding suggested a critical role for these cells in mediating RRV-induced disease [18]. Given the prominent mobilization of monocytes and macrophages and the damage observed in muscle fibers, soluble mediators derived from macrophages are implicated in the pathological profile of disease.

In the present study, we used the mouse model of RRVD described elsewhere [18] to assess the role of macrophages in the pathogenesis of RRV infection. We found that mice depleted of macrophages (hereafter referred to as "macrophage-depleted mice") by use of clodronate disodium (hereafter referred to as "clondronate") do not develop inflammation of joint and skeletal muscle tissues after infection and that this observation was associated with reduced levels of proinflammatory mediators and associated transcription factors. Furthermore, we demonstrated that high levels of these proinflammatory factors are detected in the joints of humans with RRV-induced polyarthritis, suggesting that these factors may play a central role in the pathogenesis of disease. We also demonstrated that tumor necrosis factor (TNF)–α, interferon (IFN)–γ, and macrophage chemoattractant protein (MCP)–1 contribute to RRV-induced inflammatory disease. Finally, we showed that drug-based inhibition of NF-κB activity in vivo ameliorated RRV inflammatory disease and tissue damage. The present study thus implicates macrophage-derived proinflammatory mediators in RRV-induced arthritis and myositis, an observation that may also be pertinent to the immunopathological processes that lead to rheumatic disease after infection with other alphaviruses, such as chikungunya virus and o'nyong-nyong virus.

MATERIALS AND METHODS

Virus. In the present study, the prototype T48 strain of RRV was used as described elsewhere [18].

Mice. Outbred mice were obtained from the Animal Breeding Establishment at the Australian National University (Canberra, Australia). The experiments were approved by the animal ethics committees of the Australian National University and the University of Wollongong (Wollongong, Australia).

Patient samples. Needle biopsy was performed to collect synovial fluid samples from 8 adult patients (age range, 30–45 years) residing in the Murray-Goulburn Valley (Victoria, Australia) who had acute cases of RRV-induced polyarthritis. Sample collection was conducted at the Royal Melbourne Hospital (Melbourne, Australia). Synovial fluid aspirates obtained from 5 patients with osteoarthritis were obtained from the John James Hospital (Canberra, Australia) and were used as controls. Sample collection was performed in accordance with the Australian Capital Territory Health Community Care Human Research Ethics committee, and The Royal Melbourne Hospital Human Ethics Committee. Samples were obtained at the time that knee joint arthroplasty was performed, and joints were aspirated before arthrotomy. The diagnosis given to patients was primary osteoarthritis with no evidence of an inflammatory arthropathy. Cells were separated from the joint fluid by centrifugation, and synovial fluid was stored at −80°C.

Mouse infection and clinical scales. Seventeen-day-old mice were infected with 10^3 pfu of RRV via subcutaneous inoculation in the left rear footpad. Mock-infected animals received diluent. Mice were monitored for signs of disease at 24-h intervals. Scores associated with clinical signs of disease were as follows: "0" denoted healthy mice; "1," mice with slight hind leg stiffness; "2," mice with pronounced hind leg stiffness; "3," mice with hind leg paresis; "4," mice with significant weight loss plus hind leg paresis; and "5," moribund mice. Mice with leg stiffness displayed an "uneven" gait but were still capable of locomotion. In mice with paresis, the hind legs were extended (in a flaccid state) behind the animal and did not respond to such physical stimuli as a gentle pinch by forceps. Mice that attain level-4 symptoms will often fully recover from the clinical sequelae associated with infection, including changes in the function of their hind legs. Level-5 symptoms eventually result in death; therefore, mice with symptoms scored at this level were euthanized.

In vivo macrophage depletion with Cl_2MDP (clodronate). Liposome-encapsulated clodronate and liposomes containing PBS were prepared as described elsewhere [20]. Clodronate was a gift of Roche Diagnostics GmbH (Mannheim, Germany). Each

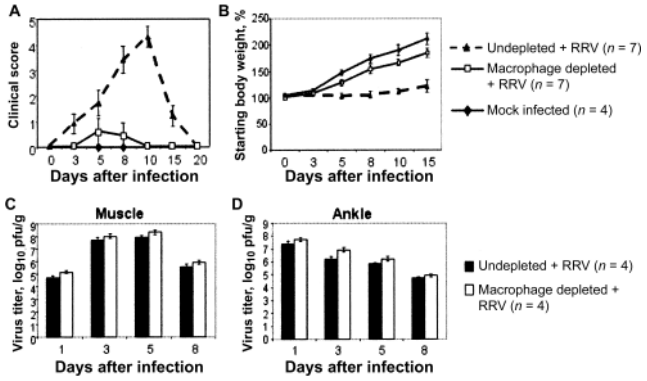

Figure 1. Ross River virus (RRV)–induced disease and viral titers in mice. Seventeen-day-old outbred mice depleted of macrophages (i.e., "macrophage-depleted mice") or mice without macrophage depletion (i.e., "undepleted mice") were infected with 10^3 pfu of RRV by injection of virus in the left rear footpad. Mock-infected mice were injected with diluent alone. *A,* Scores for the development of hind limb dysfunction and disease in mice were as follows: "0" denoted healthy mice; "1," mice with slight hind leg stiffness; "2," mice with pronounced hind leg stiffness; "3," mice with hind leg paresis; "4," mice with significant weight loss plus hind leg paresis; and "5," moribund mice. B, Monitoring of mice for weight gain or loss at 24-h intervals. *C* and *D,* Seventeen-day-old outbred macrophage-depleted mice or undepleted mice infected with 10^3 pfu of RRV by injection of virus in the left rear footpad. At various days after infection, the ankle and quadriceps muscle tissues were harvested and homogenized, and the amount of infectious virus present was quantified by plaque assay performed on Vero cells ($n = 4$).

mouse was given 200 μL of liposome Cl_2MDP intravenously in the tail vein 1 day before infection, as well as 1 day after infection. On various days after infection, mice were monitored for development of signs of disease and samples taken for virus titer determination. In a separate experiment, mice were killed on day 10 after infection, and organs were collected to determine host factors and perform histologic analyses. Control mice were given PBS liposome.

NF-κB inhibition with sulfasalazine. Mice were intraperitoneally injected with 100 mg/kg sulfasalazine (Sigma) 1 day before infection and on days 3 and 7 after infection. Control mice were given PBS. At various days after infection, mice were monitored for the development of signs of disease. On day 10 after infection, mice were killed, and muscle tissue samples were collected to determine host factors and perform histologic analyses.

Cytokine depletion with antibodies. Neutralization of TNF-α or MCP-1 was achieved by intraperitoneal injection of 200 μg of IgG-purified rabbit anti–TNF-α or rabbit anti–MCP-1. Immunoglobulin purified from the serum of a naive rabbit was used as a control antibody. IFN-γ was neutralized via intraperitoneal injection of 200 μg of monoclonal antibody to IFN-γ (clone XMG-6). Monoclonal antibody to β-galactosidase (clone GL113) was used as a control antibody. Mice were treated with antibodies 1 day before infection and on days 3 and 7 after infection.

Nitric-oxide synthase inhibition. N^{ω}-methyl-L-arginine (L-NMA; Sigma) or its inactive D-enantiomer (D-NMA) was administered (5 mg per 200 μL of PBS) via daily intraperitoneal injection.

Virus assay. Vero cells derived from African Green Monkey kidneys were used for titration of virus, as described elsewhere [18].

Histologic analysis. Quadriceps muscle and ankle joint tissue samples were collected for histologic analyses, as described elsewhere [19, 21].

Immunohistochemical analysis. For the identification of macrophages in situ, muscle tissue samples were collected and subsequently were fixed in paraformaldehyde-lysine-periodate for 24 h at 4°C. Tissues were then transferred to a 7% sucrose solution for 18–24 h at 4°C. Fixed samples were then placed in aluminum foil and covered in Tissue-Tek OCT compound (Miles). These samples were then snap-frozen in liquid nitrogen, and 7-μm sections were cut on a cryostat at −15°C. Sections were transferred to slides and stored at −20°C before staining.

Because decalcification interfered with our ability to detect antigens within the joint tissues, we stained cells prepared from total joint digests. Ankle joint tissue samples obtained from 5 infected mice were pooled, mechanically disrupted, and subjected to sequential treatment with collagenase type IV (0.5 mg/mL; Sigma). The cells were adhered to glass slides by means of cytocentrifugation and were stored at −20°C before staining. Macrophages were identified in sections by use of F4/80 rat monoclonal antibodies [22] and peroxidase-conjugated sheep anti–rat Ig, according to a procedure published elsewhere [23].

Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. RT-PCR was performed as described elsewhere, to determine relative quantities of mRNA for various cytokines and chemokines [24, 25].

ELISAs performed for the detection of mouse and human cytokines. Concentrations of IFN-γ, TNF-α, and MCP-1 in mouse tissue samples or human synovial fluid samples were determined by ELISA (R&D Systems) performed as specified by the manufacturer.

Measurement of reactive nitrogen intermediates (RNIs). The concentrations of nitrite and nitrate, the nitric-oxide breakdown products in the supernatants, were determined using the Griess reaction, as described elsewhere [26].

Nuclear extraction. Mice were inoculated as described above, killed at 10 days after infection, and perfused for 10 min with PBS. Muscle and joint tissues were collected, snap-frozen in liquid nitrogen, and pulverized. The mouse paws were cut just above and below the ankle, and the skin was removed. Nuclear extracts were prepared according to the method described by Gerlag et al. [27]. The protein concentration was determined using the Bradford method [28].

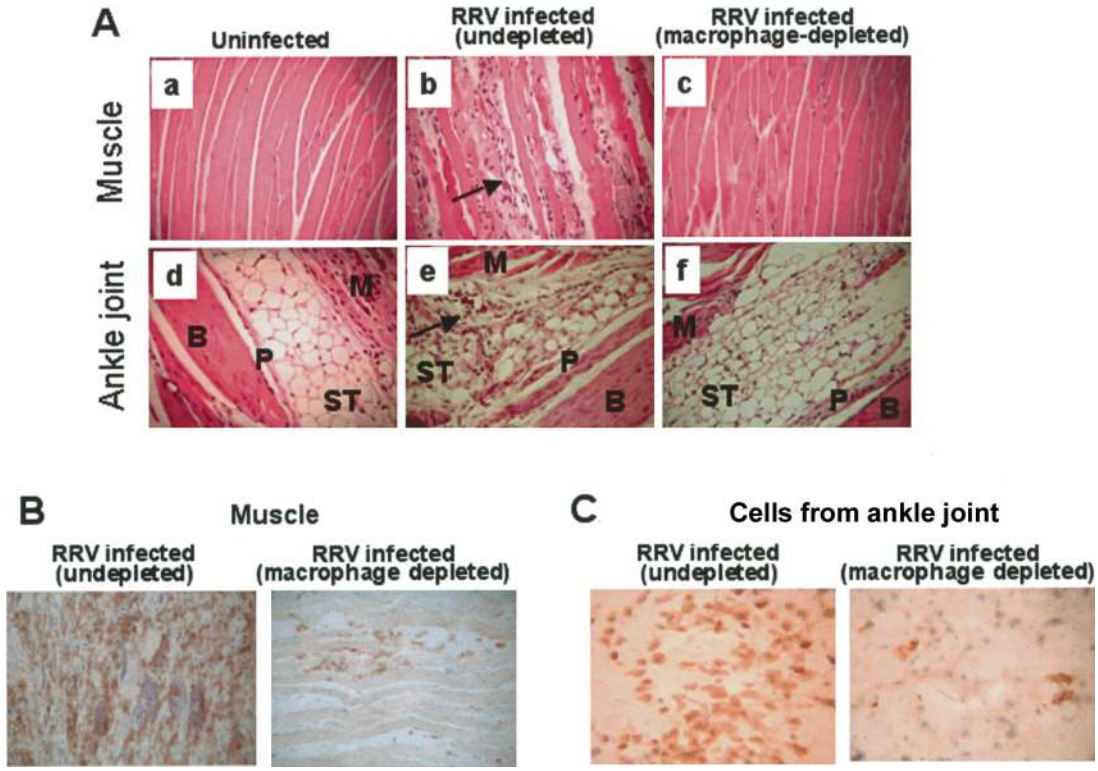

Figure 2. Histologic analyses of hind limb joint tissue and skeletal muscle samples obtained from Ross River virus (RRV)–infected mice. Seventeen-day-old outbred mice depleted of macrophages (i.e., "macrophage-depleted mice") or mice without macrophage depletion (i.e., "undepleted mice") were infected with 10^3 pfu of RRV by injection of virus in the left rear footpad. Mock-infected mice were injected with diluent alone. *A,* At 10 days after infection, mice were perfused with 4% paraformaldehyde, and 5-μmol/L-thick paraffin-embedded sections generated from quadriceps muscle were stained with hematoxylin-eosin (mock-infected mice *[a];* RRV-infected, undepleted mice *[b];* and RRV-infected, macrophage-depleted mice *[c]).* After decalcification, 5-μmol/L-thick, paraffin-embedded sections generated from ankle joints were stained with hematoxylin-eosin (mock-infected mice *[d];* RRV-infected, undepleted mice *[e];* and RRV-infected, macrophage-depleted mice *[f]).* M, muscle; B, bone; P, periosteum; ST, synovial tissue. *B* and *C,* Immunostaining for F4/80-positive cells in muscle tissue section *(B)* and ankle joint tissue (cytospin) *(C)* obtained from RRV-infected mice at day 10 after infection. Images are representative of data for at least 3 mice per group.

Electrophoretic mobility shift assay (EMSA). The oligonucleotide sequences and the EMSA protocol that were used have been described elsewhere [29]. In brief, double-stranded oligonucleotides were labeled with ^{32}P-deoxynucleotide (Amersham) by use of Klenow polymerase (Roche). The reaction mixture contained nuclear extract (10 μg), 5 μg of poly(dI-dC), 1 mmol/L dithiotreitol, and 1 μL of ^{32}P-labeled oligonucleotides (0.1 μg/μL) dissolved in binding buffer (20 mmol/L Tris-HCl, 30 mmol/L NaCl, 5 μmol/L EGTA, and 50% glycerol) supplemented with 0.2 μg/mL bovine serum albumin. After incubation, electrophoresis was performed in Tris-borate EDTA buffer at 175 V. The gels were dried and were analyzed by autoradiography.

Statistical analysis. For assessment of virus titers and mouse cytokines, data were analyzed using Student's unpaired *t* test. For assessment of clinical scores and human cytokines, data were analyzed using the Mann-Whitney *U* test. Differences in mean values were considered to be statistically significant if $P < .05$.

RESULTS

Development of mild disease after RRV infection in macrophage-depleted mice. Seventeen-day-old outbred mice were depleted of macrophages by use of liposomes containing clodronate [20], and mice were subsequently infected with RRV by means of subcutaneous injection of virus into the footpads. Liposomes containing clodronate provide higher efficacy for depletion through enhanced specificity and killing, mediated by the natural phagocytosis of liposomes by macrophages and the subsequent intracellular degradation of liposome membranes to release the clodronate toxin [20]. Control mice without macrophage depletion (hereafter referred to as "undepleted mice"; the mice were treated with PBS liposomes) were also infected with RRV. Mock-infected mice received injections of diluent. Both groups were monitored for the development of signs of disease.

In undepleted mice, RRV infection resulted in severe disease, with peak clinical scores observed at day 10 after infection (figure *1A*). In contrast, the signs of disease were mild in RRV-infected

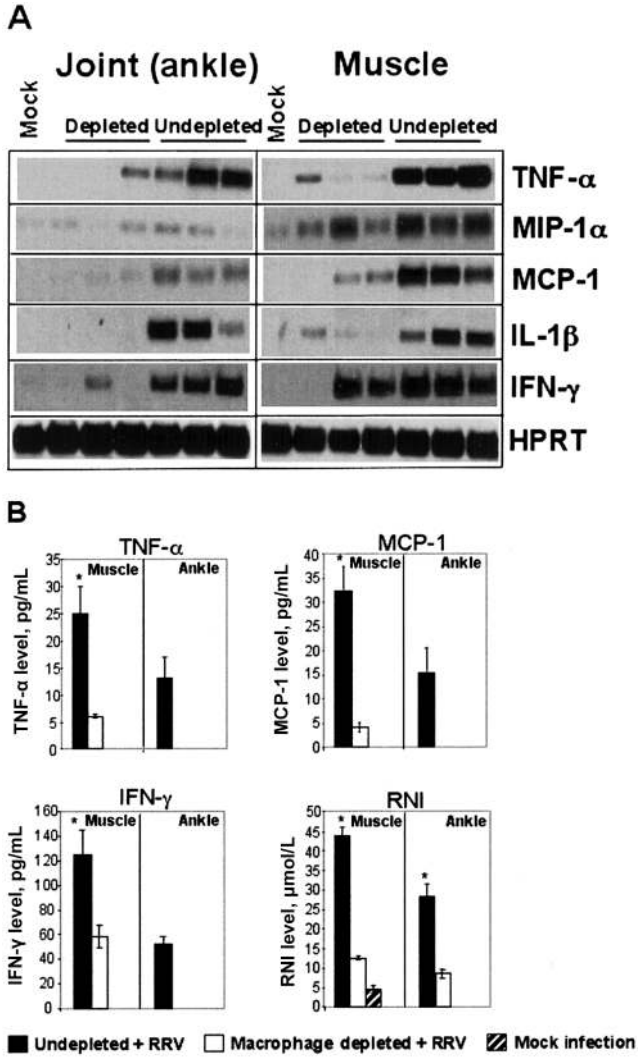

Figure 3. Analysis of proinflammatory cytokines and chemokines in Ross River virus (RRV)–infected mice. Seventeen-day-old outbred mice depleted of macrophages (i.e., "macrophage-depleted mice") or mice without macrophage depletion (i.e., "undepleted mice") were infected with 10^3 pfu of RRV by injection of virus in the left rear footpad. *A,* At 10 days after infection, 3 mice were euthanized and perfused with PBS, and ankle and quadriceps muscle tissue samples were homogenized in RNAwiz solution. Total RNA was also prepared from tissues of mock-infected mice. Specific mRNA was amplified by reverse-transcriptase polymerase chain reaction (PCR). The amplification products were blotted onto nylon membranes and hybridized to fluorescein-labeled oligonucleotide probes specific for the PCR product. *B,* At 10 days after infection, 3 mice were euthanized and perfused with PBS, and ankle and quadriceps muscle tissue samples were homogenized in PBS. Cell debris was pelleted by centrifugation, and the supernatant was stored at −80°C. Protein levels for each cytokine were determined by ELISA performed on supernatants from these tissues. Mock-infected mice were used as controls. *$P < .05$ (for macrophage-undepleted vs. macrophage-depleted mice). IL, interleukin; IFN, interferon; HPRT, hypoxanthine guanine phosphoribosyl transferase; MCP, macrophage chemoattractant protein; MIP, macrophage inflammatory protein; RNI, reactive nitrogen intermediate; TNF, tumor necrosis factor.

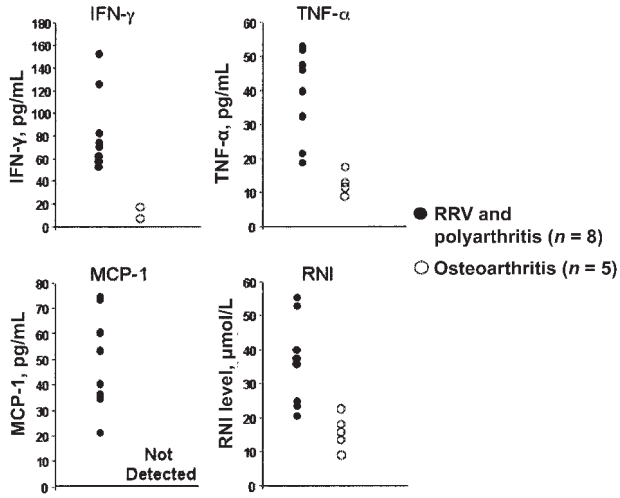

Figure 4. ELISA analysis of proinflammatory factors in the synovial fluid of Ross River virus (RRV)–infected patients who had polyarthritis. Synovial fluid samples obtained from 8 individuals with diagnosed acute RRV-induced polyarthritis and from 5 individuals with osteoarthritis assessed for interferon (IFN)-γ, tumor necrosis factor (TNF)–α, macrophage chemoattractant protein (MCP)–1, and reactive nitrogen intermediate (RNI) levels. The differences in levels of proinflammatory factor in samples from patients with RRV vs. those in samples from patients with osteoarthritis were statistically significant, as determined by the Mann-Whitney *U* test ($P < .05$). ND, MCP-1 was not detected in patients with osteoarthritis.

macrophage-depleted mice at various days after infection (figure 1*A*). Furthermore, RRV-infected undepleted mice did not gain weight between 5 and 10 days after infection (figure 1*B*), which was the period that correlated with peak clinical scores, whereas RRV-infected, macrophage-depleted mice and mock-infected mice consistently gained body weight during the 20-day experimental period (figure 1*B*). Macrophage removal achieved using liposomes containing clodronate is significantly more effective and is associated with fewer nonspecific effects than is macrophage removal achieved using silica [20]. These results confirm the observations previously made in association with the use of carrageenan or silica [18], and they illustrate the importance of macrophages in the immunopathological profile of RRV-induced disease. Interestingly, viral titers were not significantly different between the 2 groups at all time points after infection (figure 1*C* and 1*D*). Thus, although macrophages can be readily infected in vitro [30–32], they do not appear to be a major source of virus in vivo.

Inflammation and pathological findings not triggered by RRV infection in the muscle and joint tissues of macrophage-depleted mice. To determine the effects of macrophage depletion on tissue inflammation and pathological findings, histologic analyses of the hind limb joint and skeletal muscle tissues were performed on RRV-infected mice in the absence or presence of macrophages. Inflammation was completely abolished in the synovial tissue and skeletal muscle tissue of RRV-infected

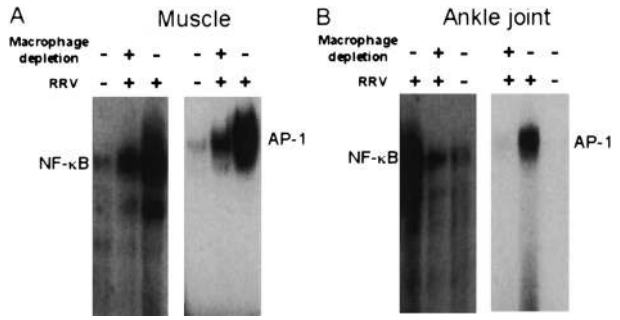

Figure 5. Activation of nuclear factor (NF)–κB and AP-1 in Ross River virus (RRV)–infected mice. Seventeen-day-old outbred mice depleted of macrophages (i.e., "macrophage-depleted mice") or mice without macrophage depletion (i.e., "undepleted mice") were infected with 10^3 pfu of RRV by injection of virus in the left rear footpad. At 10 days after infection, mice were killed and perfused for 10 min with PBS. Quadriceps muscle and ankle joint tissue samples were collected and processed for electrophoretic mobility shift assay, as described in Materials and Methods. NF-κB and AP-1 activity in the muscle *(A)* and ankle joints *(B)* of mice.

macrophage-depleted mice (figure 2*A [c, f]*). In contrast, severe inflammation was observed in the synovial tissue and skeletal muscle of the RRV-infected control group at 10 days after infection (figure 2*A [b, e]*). Extensive disruption of striated muscle fibers was associated with the monocyte/macrophage infiltrate. Muscle tissue collected from infected macrophage-depleted mice exhibited no detectable damage. The majority (90%) of inflammatory infiltrate in the muscle and synovial tissues stained positive for the macrophage marker F4/80 (figure 2*B* and 2*C*). These results are consistent with the mild disease signs observed in RRV-infected, macrophage-depleted mice, and they further establish an important role for macrophages in mediating RRV-induced inflammatory disease in the muscle and joint.

Reduction of proinflammatory factors in RRV-infected, macrophage-depleted mice. Given the crucial roles that cytokines and chemokines play in the inflammatory process, it was important to establish the expression and production of these soluble mediators in infected mice in the presence and absence of macrophages after infection. Expression of the proinflammatory mediators IFN-γ, TNF-α, interleukin (IL)–1β, MCP-1, macrophage inflammatory protein (MIP)–1α, and RNIs was analyzed, because these factors have been shown to be produced at high levels and play important roles in rheumatoid arthritis (RA) [33]. RT-PCR analyses revealed that the levels of expression of TNF-α, MIP-1α, MCP-1, IL-1β, and IFN-γ mRNA at day 10 after infection were clearly higher in the muscle and joint tissues of RRV-infected and undepleted mice than in those of macrophage-depleted mice (figure 3*A*). We also detected elevated levels of these factors in $CD11b^+$ cells (macrophages) isolated from the inflamed skeletal muscle of infected mice. Together, these results clearly demonstrate that, among the groups of mice evaluated, macrophages from infected mice express higher levels of several proinflammatory cytokine genes associated with immunopathological inflammatory responses.

ELISAs were also performed on supernatants of homogenized skeletal muscle and joint tissues. Supernatants were analyzed for TNF-α, MCP-1, and IFN-γ. The amount of these mediators produced by RRV-infected, macrophage-depleted mice was significantly lower than that produced by infected, undepleted mice (figure 3*B*) ($P < .05$, by Student's *t* test). Furthermore, RNI levels were significantly lower in RRV-infected, macrophage-depleted mice than in RRV-infected, nondepleted mice (figure 3*B*).

Detection of proinflammatory cytokines in the synovial joints of RRV-infected humans. The data presented to date suggest that the macrophage-derived soluble mediators may contribute to the severity of RRV-induced disease in mice; however, whether these factors contribute to RRV-induced disease in humans is not known. The levels of proinflammatory cytokines in synovial fluid samples obtained from RRV-infected patients with RRV-induced polyarthritis were assessed and compared with those in synovial fluid samples obtained from humans with noninflammatory osteoarthritis. All RRV-infected patients were RRV IgM^+/RRV IgG^+ and exhibited severe joint swelling, rash, fever, and myalgia (consistent with known symptoms of RRVD). Synovial fluid samples showed high numbers of monocytes and macrophages, low numbers of lymphocytes, and detectable RRV antigen in macrophages (data not shown) [15, 34]. Synovial fluid samples obtained from persons with acute RRV-induced polyarthritis showed significantly higher levels of TNF-α, IFN-γ,

Table 1. Clinical scores for Ross River virus (RRV)–infected mice with depletion of the host factor.

Treatment	Clinical score,[a] mean ± SD
Anti–IFN-γ	1.8±0.6[b]
Control IgG	4.2±0.4
Anti–MCP-1	2.0±0.6[b]
Anti–TNF-α	1.7±0.5[b]
Control IgG	3.9±0.6
L-NMA	4.4±0.5
D-NMA	4.3±0.4

NOTE. Scores rating the development of hind limb dysfunction and disease in mice were as follows: "0" denoted healthy mice; "1," mice with slight hind leg stiffness; "2," mice with pronounced hind leg stiffness; "3," mice with hind leg paresis; "4," mice with significant weight loss plus hind leg paresis; and "5," moribund mice. D-NMA, inactive D-enantiomer; IFN, interferon; L-NMA, N^{ω}-methyl-L-arginine; MCP, macrophage chemoattractant protein; TNF, tumor necrosis factor.

[a] At day 10 after infection.

[b] The difference between mice with host factor depletion and mice without host factor depletion was statistically significant ($P < .05$, by Mann-Whitney *U* test) ($n = 12$).

MCP-1, and RNIs than did samples obtained from patients with osteoarthritis (figure 4) ($P < .05$, by Mann-Whitney U test).

Poor induction of transcription factors associated with proinflammatory factors in infected macrophage-depleted mice. To determine the role of transcription factors in regulating macrophage activity in RRV-triggered inflammation, the role of NF-κB and activator protein–1 (AP-1) was investigated. These transcription factors are critical in the regulation of proinflammatory cytokine genes at the transcriptional level and are also known to play an important role in RA [35, 36]. The influence of RRV infection on the activation of transcription factors in the muscle and ankle joints was assessed by EMSA. Clearly, NF-κB and AP-1 levels were significantly higher in the muscle and ankle joints of infected, undepleted control mice than in the tissues of infected, macrophage-depleted mice (figure 5).

Reduced severity of RRVD in mice in the absence of TNF-α, IFN-γ, or MCP-1. Because high levels of proinflammatory factors were detected after RRV infection, we next investigated their role in disease. To establish the functional significance of IFN-γ, TNF-α, or MCP-1 in RRV-induced disease, mice were treated with neutralizing antibodies to specific cytokines, and the severity of disease was determined. In addition, the functional role of nitric oxide in RRVD was determined by treating mice with NOS-2 inhibitor (L-NMA) or D-NMA. We demonstrated that depletion of IFN-γ, TNF-α, or MCP-1—but not NO—partially reduced the development of severe RRVD in mice (table 1).

Inhibition of RRV inflammatory disease in mice by blocking NF-κB activity. NF-κB activation and subsequent cytokine production are known to play a role in RA, as well as in animal models of inflammatory arthritis [37]. In figure 5, we show that, in this model, NF-κB activity is prominent after infection and corresponds with the production of proinflammatory factors. To determine the role of NF-κB in RRV-induced inflammatory disease, we inhibited the activity of this transcription factor by use of sulfasalazine, a drug that is known to block nuclear translocation of NF-κB via inhibition of IκBα degradation [36]. Sulfasalazine is commonly used for the treatment of inflammatory diseases, such as RA and Crohn disease [36]. RRV infection of sulfasalazine-treated mice resulted in a disease course that was less severe than that noted in untreated mice (figure 6*A*). Like macrophage-depleted mice, sulfasalazine-treated mice exhibited less severe tissue destruction and lower levels of inflammatory cell recruitment than did untreated mice

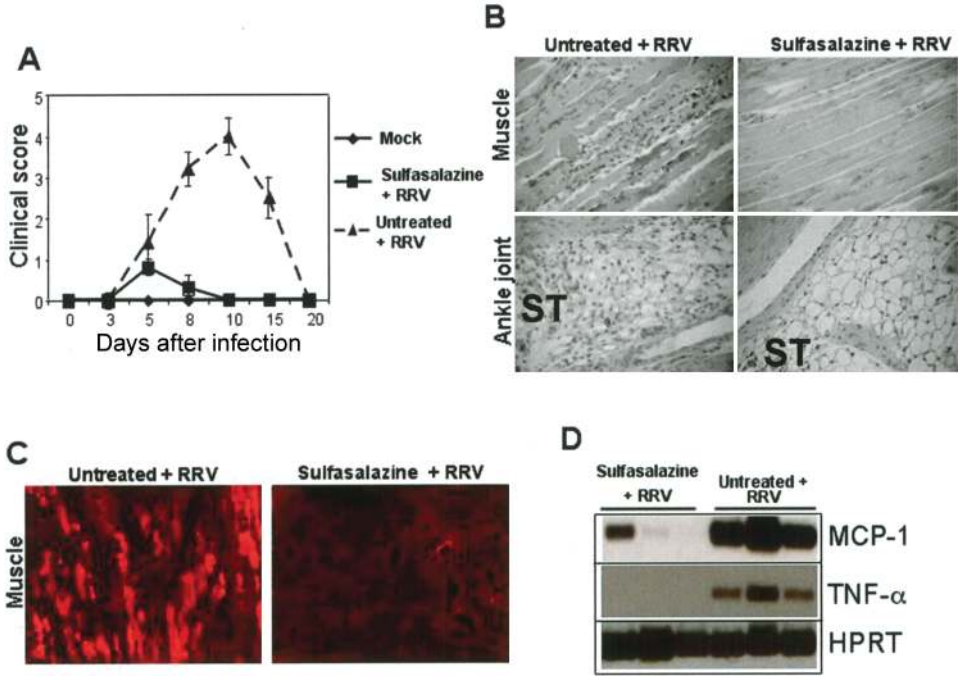

Figure 6. Blocking nuclear factor (NF)–κB activity ameliorates Ross River virus (RRV) disease (RRVD) in mice. Seventeen-day-old, outbred, sulfasalazine-treated or -untreated mice were infected with 10^3 pfu of RRV by injection of virus in the left rear footpad. *A,* Scoring of development of hind limb dysfunction and disease in 5 mice, according to the scale described in the figure 1 legend. *B,* At 10 days after infection, mice were perfused with 4% paraformaldehyde, and 5-μm-thick paraffin-embedded sections generated from quadriceps muscle and ankle joint tissues were stained with hematoxylin-eosin. ST, synovial tissue. *C,* Mice injected intraperitoneally with 1% Evans blue dye (EBD) and, 6 h later, perfused with 4% paraformaldehyde [21]. Uptake of EBD was visualized by fluorescent microscopy (magnification, ×100). EBD is taken up by cells with disrupted sarcolemmal membranes. *D,* Reverse-transcriptase polymerase chain reaction analysis of tumor necrosis factor (TNF)–α and macrophage chemoattractant protein (MCP)–1 in the muscle of sulfasalazine-treated or -untreated mice at day 10 after RRV infection ($n = 3$). HPRT, hypoxanthine guanine phosphoribosyl transferase.

(figure 6*B* and 6*C*). Furthermore, sulfasalazine-treated mice had markedly reduced TNF-α and MCP-1 mRNA levels, compared with untreated mice (figure 6*D*).

DISCUSSION

The mechanisms by which alphaviruses cause musculoskeletal and rheumatic disease are poorly understood, and understanding the processes by which these arthrogenic viruses cause disease is a prerequisite to the quest for better treatments [3]. In the present study, we showed that macrophages are a major cause of muscle and joint immunopathological findings after RRV infection, with clinical symptoms correlating with pronounced F4/80-positive infiltrates into hind-limb muscle and synovial tissue at 10 days after infection. The macrophages themselves are not a major source of virus replication, but they appear to be responsible for the production of a series of proinflammatory cytokines and chemokines and NO. However, virus was mainly concentrated in muscle- and joint-associated cells, such as muscle fibers, synovial tissue, tendons, and ligaments [19]. We also demonstrated that proinflammatory factors are present in synovial effusions from RRV-infected patients exhibiting polyarthritis, suggesting that the mouse model is representative of disease in humans. Induction of many proinflammatory mediators involves activation of NF-κB and AP-1, and these transcription factors are strongly activated in RRV-infected mice, albeit significantly less so in macrophage-depleted and RRV-infected animals. Furthermore, the use of sulfasalazine to inhibit NF-κB activity significantly ameliorated RRVD in mice. Taken together, these data suggest that RRVD is an immunopathological finding arising from excessive macrophage activation rather than a pathological finding induced by replicating virus.

Macrophages and their proinflammatory products have been implicated in the pathogenesis of a number of inflammatory diseases, such as RA [38]. It is probable that the induction of MCP-1 (figures 3 and 4), IL-8 [39], and IFN-α/β [31, 40] by RRV infection contributes to the recruitment and activation of macrophages. Furthermore, RRV antigen has been detected in synovial macrophages [15, 34], suggesting that RRV infection of macrophages may trigger the production of some of these soluble factors. T cell–derived IFN-γ (figures 3 and 4) [40, 41] may further activate the macrophages to secrete TNF-α, IL-1β, and RNIs. Of interest is the observation that depletion of IFN-γ, TNF-α, and MCP-1—but not NO—partially reduced the severity of RRVD in mice, suggesting that the first 3 factors contribute to RRVD in humans (table 1).

Activation of NF-κB and AP-1 has been implicated in the development of many inflammatory diseases, such as colitis, glomerulonephritis, and asthma, and these elements are known to function in concert to enhance the production of proinflammatory cytokines [36]. The role of NF-κB activation in inflammation has also been demonstrated in rats with streptococcal cell wall–induced arthritis and in mice with collagen-induced arthritis (CIA) [35, 42]. In RA, NF-κB is overexpressed in the inflamed synovium and may contribute to the recruitment of inflammatory cells and the production of proinflammatory mediators [35]. The identification of NF-κB as a key player in the pathogenesis of many inflammatory diseases has prompted studies investigating NF-κB–targeted therapeutics. For instance, the incidence and severity of CIA were significantly reduced in transgenic mice expressing a constitutive inhibitor of NF-κB (IκBα) [43]. In addition, blocking NF-κB by use of NF-κB decoy oligonucleotides inhibited the development of streptococcal cell wall–induced arthritis and CIA in rats [42, 44]. Our findings show that RRV activates NF-κB and AP-1 in the muscle and ankle joints and that the NF-κB inhibitor sulfasalazine ameliorates disease, suggesting that NF-κB activation is central to the disease process. The concentration used in the present study has also been shown to ameliorate disease in other models of inflammatory disease [45, 46]. Thus, our findings highlight the potential for NF-κB inhibitors in the treatment of alphaviral arthritides.

References

1. Franssila R, Hedman K. Infection and musculoskeletal conditions: viral causes of arthritis. Best Pract Res Clin Rheumatol **2006**; 20:1139–57.
2. Rulli NE, Suhrbier A, Hueston L, et al. Ross River virus: molecular and cellular aspects of disease pathogenesis. Pharmacol Ther **2005**; 107:329–42.
3. Suhrbier A, La Linn M. Clinical and pathologic aspects of arthritis due to Ross River virus and other alphaviruses. Curr Opin Rheumatol **2004**; 16: 374–9.
4. Tupanceska D, Zaid A, Rulli N, et al. Ross River virus: an arthritogenic alphavirus of significant importance in the Asian Pacific. In: Lal SK, ed. Emerging viral diseases of Southeast Asia. Vol 4. Switzerland: Karger, **2007**:94–111.
5. Harley D, Sleigh A, Ritchie A. Ross River virus transmission, infection, and disease: a cross-disciplinary review. Clin Microbiol Rev **2001**; 14: 909–32.
6. Williams MC, Woodall JP, Corbet PS, Gillett JD. O'nyong-nyong fever: an epidemic virus disease in East Africa. 8. Virus isolations from *Anopheles* mosquitoes. Trans R Soc Trop Med Hyg **1965**; 59:300–6.
7. Posey DL, O'Rourke T, Roehrig JT, Lanciotti RS, Weinberg M, Maloney S. O'nyong-nyong fever in West Africa. Am J Trop Med Hyg **2005**; 73: 32.
8. Josseran L, Paquet C, Zehgnoun A, et al. Chikungunya disease outbreak, Reunion Island. Emerg Infect Dis **2006**; 12:1994–5.
9. Yergolkar PN, Tandale BV, Arankalle VA, et al. Chikungunya outbreaks caused by African genotype, India. Emerg Infect Dis **2006**; 12:1580–3.
10. Kalantri SP, Joshi R, Riley LW. Chikungunya epidemic: an Indian perspective. Natl Med J India **2006**; 19:315–22.
11. Mylonas AD, Brown AM, Carthew TL, et al. Natural history of Ross River virus-induced epidemic polyarthritis. Med J Aust **2002**; 177:356–60.
12. Harley D, Bossingham D, Purdie DM, Pandeya N, Sleigh AC. Ross River virus disease in tropical Queensland: evolution of rheumatic manifestations in an inception cohort followed for six months. Med J Aust **2002**; 177:352–5.
13. Soden M, Vasudevan H, Roberts B, et al. Detection of viral ribonucleic acid and histologic analysis of inflamed synovium in Ross River virus infection. Arthritis Rheum **2000**; 43:365–9.
14. Hazelton RA, Hughes C, Aaskov JG. The inflammatory response in the synovium of a patient with Ross River arbovirus infection. Aust N Z J Med **1985**; 15:336–9.

15. Fraser JR, Cunningham AL, Clarris BJ, Aaskov JG, Leach R. Cytology of synovial effusions in epidemic polyarthritis. Aust N Z J Med **1981**; 11: 168–73.
16. Mims CA, Murphy FA, Taylor WP, Marshall ID. Pathogenesis of Ross River virus infection in mice. I. Ependymal infection, cortical thinning, and hydrocephalus. J Infect Dis **1973**; 127:121–8.
17. Murphy FA, Taylor WP, Mims CA, Marshall ID. Pathogenesis of Ross River virus infection in mice. II. Muscle, heart, and brown fat lesions. J Infect Dis **1973**; 127:129–38.
18. Lidbury BA, Simeonovic C, Maxwell GE, Marshall ID, Hapel AJ. Macrophage-induced muscle pathology results in morbidity and mortality for Ross River virus-infected mice. J Infect Dis **2000**; 181:27–34.
19. Morrison TE, Whitmore AC, Shabman RS, Lidbury BA, Mahalingam S, Heise MT. Characterization of Ross River virus tropism and virus-induced inflammation in a mouse model of viral arthritis and myositis. J Virol **2006**; 80:737–49.
20. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applicabilities. J Immunol Methods **1994**; 174:83–93.
21. Morrison TE, Fraser RJ, Smith PN, Mahalingam S, Heise MT. Complement contributes to inflammatory tissue destruction in a mouse model of Ross River virus-induced disease. J Virol **2007**; 81:5132–43.
22. Austyn JM, Gordon S. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Eur J Immunol **1981**; 11:805–15.
23. Simeonovic CJ, Ceredig R, Wilson JD. Effect of GK1.5 monoclonal antibody dosage on survival of pig proislet xenografts in $CD4^+$ T cell-depleted mice. Transplantation **1990**; 49:849–56.
24. Mahalingam S, Chaudhri G, Tan CL, John A, Foster PS, Karupiah G. Transcription of the interferon γ (IFN-γ)-inducible chemokine Mig in IFN-γ-deficient mice. J Biol Chem **2001**; 276:7568–74.
25. Herd KA, Mahalingam S, Mackay IM, Nissen M, Sloots TP, Tindle RW. Cytotoxic T-lymphocyte epitope vaccination protects against human metapneumovirus infection and disease in mice. J Virol **2006**; 80:2034–44.
26. Rolph MS, Mahalingam S, Cowden WB. Nonspecific antiviral immunity by formalin-fixed *Coxiella burnetii* is enhanced in the absence of nitric oxide. Virology **2004**; 326:1–5.
27. Gerlag DM, Ransone L, Tak PP. The effect of a T cell-specific NF-κB inhibitor on in vitro cytokine production and collagen-induced arthritis. J Immunol **2000**; 165:1652–8.
28. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **1976**; 72:248–54.
29. Zare F, Bokarewa M, Nenonen N, et al. Arthritogenic properties of double-stranded (viral) RNA. J Immunol **2004**; 172:5656–63.
30. Linn ML, Aaskov JG, Suhrbier A. Antibody-dependent enhancement and persistence in macrophages of an arbovirus associated with arthritis. J Gen Virol **1996**; 77:407–11.
31. Mahalingam S, Lidbury BA. Suppression of lipopolysaccharide-induced antiviral transcription factor (STAT-1 and NF-κB) complexes by antibody-dependent enhancement of macrophage infection by Ross River virus. Proc Natl Acad Sci USA **2002**; 99:13819–24.
32. Way SJ, Lidbury BA, Banyer JL. Persistent Ross River virus infection of murine macrophages: an in vitro model for the study of viral relapse and immune modulation during long-term infection. Virology **2002**; 301: 281–92.
33. Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. Annu Rev Immunol **1996**; 14:397–440.
34. Cunningham AL, Fraser JR. Ross River virus infection of human synovial cells in vitro. Aust J Exp Biol Med Sci **1985**; 63:199–204.
35. Han Z, Boyle DL, Manning AM, Firestein GS. AP-1 and NF-κB regulation in rheumatoid arthritis and murine collagen-induced arthritis. Autoimmunity **1998**; 28:197–208.
36. Tak PP, Firestein GS. NF-κB: a key role in inflammatory diseases. J Clin Invest **2001**; 107:7–11.
37. Firestein GS. NF-κB: Holy Grail for rheumatoid arthritis? Arthritis Rheum **2004**; 50:2381–6.
38. Szekanecz Z, Koch AE. Macrophages and their products in rheumatoid arthritis. Curr Opin Rheumatol **2007**; 19:289–95.
39. Mateo L, La Linn M, McColl SR, Cross S, Gardner J, Suhrbier A. An arthrogenic alphavirus induces monocyte chemoattractant protein-1 and interleukin-8. Intervirology **2000**; 43:55–60.
40. Antalis TM, La Linn M, Donnan K, et al. The serine proteinase inhibitor (serpin) plasminogen activation inhibitor type 2 protects against viral cytopathic effects by constitutive interferon α/β priming. J Exp Med **1998**; 187:1799–811.
41. Linn ML, Mateo L, Gardner J, Suhrbier A. Alphavirus-specific cytotoxic T lymphocytes recognize a cross-reactive epitope from the capsid protein and can eliminate virus from persistently infected macrophages. J Virol **1998**; 72:5146–53.
42. Miagkov AV, Kovalenko DV, Brown CE, et al. NF-κB activation provides the potential link between inflammation and hyperplasia in the arthritic joint. Proc Natl Acad Sci USA **1998**; 95:13859–64.
43. Seetharaman R, Mora AL, Nabozny G, Boothby M, Chen J. Essential role of T cell NF-κB activation in collagen-induced arthritis. J Immunol **1999**; 163:1577–83.
44. Tomita T, Takeuchi E, Tomita N, et al. Suppressed severity of collagen-induced arthritis by in vivo transfection of nuclear factor κB decoy oligodeoxynucleotides as a gene therapy. Arthritis Rheum **1999**; 42:2532–42.
45. Axelsson LG, Landström E, Bylund-Fellenius AC. Experimental colitis induced by destran sulphate sodium in mice: beneficial effects of sulfasalazine and olsalazine. Aliment Pharmacol Ther **1998**; 12:925–34.
46. Hunneyball IM, Crossley MJ, Spowage M. Pharmacological studies of antigen-induced arthritis in BALB/c mice. II. The effects of second-line antirheumatic drugs and cytotoxic agents on the histopathological changes. Agents Actions **1986**; 18:394–400.