Macrophage-Induced Muscle Pathology Results in Morbidity and Mortality for Ross River Virus–Infected Mice

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Ross River virus (RRV) is an Australian alphavirus that is often responsible for chronic epidemic polyarthritis and myalgia in humans. Past studies have shown severe disruption of striated muscle fibers to be prominent in RRV pathology in mice; in the present study, macrophages were directly implicated as the primary mediators of muscle damage. General immunosuppressive therapies had only minor effects on mortality and morbidity in RRV-infected mice, with no inhibition of muscle damage. Treatment of mice with macrophage-toxic agents (e.g., silica) prior to RRV infection completely abrogated disease symptoms without significantly affecting titers of virus in organs. Further studies found that clinical signs of infection and muscle damage correlated with a massive influx of macrophages into hind leg muscle, whereas no such infiltrate or damage was observed for silica-treated mice. These observations are significant for the human disease context, as monocytic cells have been detected in the synovial effusions of persons with epidemic polyarthritis.

Ross River virus (RRV) is an indigenous Australian arthropod-borne alphavirus that is responsible for outbreaks of epidemic polyarthritis in humans, not only in Australia but also in areas of the South Pacific. The predominant symptom is arthritis or arthralgia, often accompanied by rash, fever, and myalgia or lethargy [1, 2]. The incidence of RRV infection and the consequent sequelae of polyarthritis appear to be increasing. Arthritis symptoms can be severe and can persist for extended periods, even for years, resulting in significant costs to the community.

RRV antigens have been detected in synovial monocytes/macrophages soon after the onset of symptoms [2], and more sensitive molecular methods are currently being used to search for persistent virus in patients with chronic epidemic polyarthritis [3]. In a mouse model, in vitro–infected cultures of macrophages have been shown to produce a small-plaque variant that persisted for 42 days (see figure 1) [4]. On the basis of this evidence, a central role for the synovial macrophage can be proposed to account for the prolonged symptoms seen in humans through the maintenance of infectious virus.

A detailed study of the pathogenesis of RRV in mice [5, 6] found no evidence that macrophages were involved in the disease process but noted the presence of severe damage to striated muscle, ultimately leading to death in infected mice [6]. Nonetheless, there were some indications that the pathogenesis of RRV-induced disease in mice was unusual. For example, in vitro experiments that used infected cultures of mouse striated-muscle cells showed no CPE over a 30-day period [7], in contrast to the almost total destruction of striated muscle cells in vivo [5, 6]. Persistently infected muscle cell cultures produced a small-plaque variant that replaced the original parent virus by the 22nd day of culture [7], just as has been observed with infected macrophage cultures (see figure 1). Thus, RRV seems to exert very little, if any, CPE in muscle cells, and the destruction of these cells seen in vivo must be related in some way to the host response.

In this study, we reexamine the pathogenesis of RRV disease in a mouse model, with emphasis on the role of macrophage activity.

Materials and Methods

Viruses

RRV isolates used were the Nelson Bay 5092 strain and the prototype T48 strain [5]. Both viruses were used after 12 passages in suckling mouse brain and 1 passage in Vero cells.
Mouse Infection Protocols and Antiimmune Treatments

Outbred mice and CBA/H inbred mice were obtained from the animal breeding establishment in the John Curtin School of Medical Research. Litters were obtained by timed mating as required.

Mouse Infection Protocols and Antiimmune Treatments

Mice were infected with RRV via a subcutaneous inoculation. Each mouse received a dose of $10^3$ pfu of RRV (unless otherwise indicated) diluted in 100 mL of sterile PBS at 5.0 mg/mL and autoclaved (121°C) before use. Mice received 100 mL of carrageenan intraperitoneally (ip) 24 h prior to infection, control mice received 100 mL of PBS ip prior to infection.

Virus-immune sera. Hyperimmune antiviral ascitic fluids were prepared in immunized outbred mice with 180/TG ascites tumor cells [8]. Immune sera were obtained from mice at various times after injection. Blood was allowed to clot at room temperature, and serum was decanted, clarified by centrifugation, and stored at −60°C.

Silica. Silica was originally provided in the form of quartz dust by A. C. Allison (Clinical Research Centre, Harrow, UK) and was used as described elsewhere [9]. Saline (0.5 mL) containing 50 mg of quartz dust was given ip to treated mice 1 day before challenge with virus.

Cyclophosphamide. Cyclophosphamide (Endoxan-Asta) was obtained in powder form from Mead Johnson (Caringbah, Australia). The dry powder was dissolved in sterile distilled water immediately before use and was given to mice as a single ip injection at the rate of 0.2 mg/g of body weight.

Gamma irradiation. Radiation was provided by a cobalt source at the Commonwealth Scientific and Industrial Research Organisation laboratories (Canberra). Mice were exposed to the source at a distance of 15 cm and received 40 rad/min until a final dose of 750 rad was delivered.

Adoptive Transfer Experiments

To assess the role of immune lymphocytes in recovery from infection, 10-day-old CBA/H mice were infected with $10^3$ pfu of T48 RRV; this was followed by the transfer of immune or normal spleen cells at 8 or 28 h after infection. For some experiments, the immune lymphoid population was depleted of immunoglobulin-bearing cells and macrophages before transfer into syngeneic recipients. Transferred cells were depleted of B cells and macrophages by use of nylon wool columns [10] and of T cells by use of anti-Thy1 antisemur and complement [11]. In all cases, the recipient mice received $4 \times 10^7$ viable lymphocytes diluted in 0.2 mL of Dulbecco’s modified Eagle medium plus 10% heat-inactivated fetal calf serum by ip inoculation.

Cell Growth and Virus Assay

Vero cells (ATCC CCL-81) were used routinely for titration of virus and growing virus stocks. Cell monolayers were grown in medium 199 supplemented with 5% heat-inactivated (30 min) fetal calf serum (TRACE, Sydney) and sufficient 1.4% sodium bicarbonate solution to bring the final pH into the range of 7.2–7.4. The medium was supplemented with sodium penicillin (100 U/mL), streptomycin sulfate (100 µg/mL), and neomycin sulfate (100 µg/mL). Petri dishes (60 mm) were seeded with 1 x $10^6$ cells in 5 mL of medium and placed in a humidified atmosphere of 5% CO₂ at 37°C.

RAW 264.7 mouse macrophages (ATCC TIB-71) were cultured in Eagle’s MEM (EMEM; Life Technologies, Melbourne) supplemented with 100 mM glucose, 10 mM HEPES, 0.05%–0.1% sodium bicarbonate, 2.0 mM L-glutamine, 50 IU of penicillin/streptomycin, and 5.0% heat-inactivated fetal calf serum. For 2 weeks before infection with virus, cultures were treated with the antimycoplasma agent tylosin (Sigma) at 8.0 mg/mL. To reduce the lipopolysaccharide background in cultures, polymyxin B sulfate (Sigma) was added to EMEM at 50 µg/mL, and the macrophages were cultured in this medium for at least 1 week before infection with virus and throughout the experimental period. RAW 264.7 macrophages were grown in a humidified atmosphere of 37°C containing 5% CO₂.

Tissue samples were homogenized in 2 mL of bicarbonate-buffered gelatin saline, pH 7.2, or in Hanks’ balanced salt solution plus 1% bovine serum albumin. Serial 10-fold dilutions of homogenate (0.1 mL) were added in triplicate to monolayer cultures of Vero cells. Virus was allowed to adsorb for 1 h at 37°C in a humidified CO₂ incubator, after which an overlay containing Earle’s basic salt solution, 0.05% lactalbumin hydrolysate, 0.1% yeast extract, 1% bovine plasma albumin (fraction V), and 0.75% Bacto-
agar (Difco, Detroit) was added. Other supplements used were DEAE dextran (100 µg/mL), 0.05% sodium bicarbonate, 2% calf serum, 0.1 M Tris buffer (pH 7.6), 100 U/mL sodium penicillin, 100 µg/mL streptomycin sulfate, and 100 µg/mL neomycin sulfate. Virus plaques were made visible by staining the live cell background, during overnight incubation at 37°C, with a solution of 0.01% neutral red in water plus 0.5% agar. Results were expressed as plaque-forming units per gram of tissue.

RAW 264.7 cultures were infected with T48 at an MOI of 0.1. The virus was incubated with a 10⁻³ dilution of either normal ascitic fluid or polyclonal anti-RRV antibody for 1 h at 4°C immediately before infection. One day before infection, RAW cells were seeded into 24-well trays at a density of 3.0 × 10⁵ cells/well in 1.0 mL of medium. Virus was placed onto the confluent cell monolayer in PBS (TRACE) for 1 h at 37°C, after which the virus inoculum was removed and fresh medium was added. Supernatants for titrations of virus were collected from infected cultures at the indicated time points (figure 1) between 2 and 49 days after infection.

Histology and Immunohistochemistry

Tissue samples from control and infected mice were fixed for 48 h in 10% formalin in normal saline or in 10% paraformaldehyde and then snap-frozen for histology. Routine thin sections were stained with hematoxylin-eosin and examined microscopically.

For the identification of macrophages in situ, mice were infected at 14 days of age with T48 RRV (or sham-infected with PBS alone), and muscle (gastrocnemius; ≤1.0 cm²) was collected at the indicated day after infection. The muscle tissue was subsequently fixed in paraformaldehyde-lysine-periodate for 24 h at 4°C (paraformaldehyde, 0.25% [w/v]; lysine buffer, 0.2 M L-lysine in sodium phosphate; periodate, 0.43% [w/v]). Tissues were then transferred to a 7% sucrose (in sodium phosphate buffer) solution for 18–24 h at 4°C. Fixed samples were then placed in aluminum foil molds and covered in Tissue-Tek OCT compound (Miles, Elkhart, IN). These were snap-frozen in liquid nitrogen, and 7-µm sections were cut on a cryostat at −15°C. Sections were transferred to pregelatinized slides and stored at −20°C or −80°C before staining.

Macrophages were identified in frozen muscle sections by use of F4/80 rat monoclonal antibody [12] and peroxidase-conjugated sheep anti-rat immunoglobulin, on the basis of modification of a procedure published elsewhere [13]. To block endogenous peroxidase activity, sections were first incubated with 0.01% H₂O₂ in PBS for 10 min and then washed in PBS. Nonspecific binding of the peroxidase-conjugated second antibody was blocked by incubation of sections with goat anti-mouse IgG (1/100; code no. M30200; Caltag Laboratories, Burlingame, CA) with 0.04% sheep serum in PBS for 30 min. Sections were then immediately incubated with F4/80 culture supernatant (diluted 1/25 in PBS) or PBS (negative control) for 1 h, washed in PBS for 10 min, and treated with sheep anti-rat immunoglobulin (catalog no. NA.932; Amersham, Amersham, UK) diluted 1/25 in PBS for 1 h. After washing in PBS for 10 min, the sections were incubated in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (catalog no. D-5637; Sigma), 0.07% imidazole (pH 7.4), and 0.02% H₂O₂ in PBS for 3 min. Sections were lightly counterstained with Mayer’s hematoxylin. Frozen sections of BALB/c mouse spleen served as positive controls for localization of F4/80-positive macrophages.

Hemagglutination-Inhibiting Antibody Titration

Sera were extracted with acetone to remove nonspecific lipid hemagglutination inhibitors by the methods of Clarke and Casals [14] and Sever [15]. Hemagglutinin was prepared by standard methods, by means of sucrose acetone extraction, as described elsewhere [14].

Results

When injected subcutaneously, the T48 strain of RRV causes death in newborn mice and mice up to age 10 days. Mice infected between 10 and 21 days of age develop severe symptoms, characterized by hind leg dragging. This is due entirely to destruction of haunch and leg striated muscle, as described elsewhere [5, 6].

Mortality rates in mice infected with the T48 strain of RRV: In table 1, we present survival times and antibody titers following infection with RRV in mice between 1 and 21 days of age. Production of antibody did not necessarily provide protection from virus-associated morbidity or mortality. For example, 8-day-old mice infected with the T48 strain of RRV produced hemagglutination-inhibiting antibody by day 5 after infection, 3 days before symptoms developed and 8 days before death. Severe symptoms that developed 8 days after infection, followed by recovery, were routinely observed in 17- to 21-day-old mice infected with the T48 strain of RRV. Mice of this age were, therefore, chosen for further study. Groups of mice were immunosuppressed with cyclophosphamide, gamma irradiation, and either powdered silica or carrageenan. Data in table 2 show that average survival time of 17-day-old mice was reduced by 1.5 days after cyclophosphamide treatment. Mortality rates were increased from 75% in untreated to 100% in treated mice. Quite unexpectedly, mice treated with powdered silica showed no virus-specific morbidity or mortality, an effect that

| Table 1. Hemagglutination-inhibiting antibody titers in blood of mice infected with Ross River virus (T48). |

<table>
<thead>
<tr>
<th>Age at infection (days)</th>
<th>Day (after infection) blood sample was obtained</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
</tr>
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<td>0</td>
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<td>18</td>
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</tr>
<tr>
<td>21</td>
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NOTE. Data are reciprocal of dilution giving 50% inhibition in assay. *100% mortality.
was reproduced in 4 subsequent experiments that used a total of 48 mice. The macrophage-cytotoxic drug carrageenan was used to reproduce the effect of silica. Mice treated with this drug prior to infection with virus showed no obvious morbidity, and no deaths were recorded. Control mice that did not receive carrageenan before infection showed 100% morbidity, with 90% of mice finally dying from RRV-induced symptoms.

Various organs previously shown to support growth of RRV (brain, muscle, blood, brown fat) were assayed for the presence of virus. Treatment of mice with general immunosuppressive agents such as cyclophosphamide or gamma irradiation (figure 2), which primarily affect progenitor cells of the myeloid and immune systems, abolished antibody production and significantly prolonged virus growth. Persistence of virus was associated with only very small changes in morbidity and mortality, and virus titers were clearly falling, even in treated mice, by the sixth day of infection.

In a second series of experiments that used mice treated with silica (figure 3), no difference in virus titers could be detected in spleen or brown fat over a 10-day period. In silica-treated mice, virus was cleared from muscle between 5 and 6 days after infection, coincident with the appearance of antibody. Control mice that did not receive carrageenan before infection showed 100% morbidity, with 90% of mice finally dying from RRV-induced symptoms.

**Virus clearance in mice infected with the T48 strain of RRV**

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**RRV persistently infects RAW 264.7 macrophages in vitro.**

Figure 1 describes the in vitro growth of RRV in macrophages over a period of several weeks. The initial growth of virus (days 1–2 after infection) was similar for both the control infection (RRV plus normal ascitic fluid) and RRV infection in the presence of subneutralizing antiviral antibody. The RRV infection in the presence of antibody was cleared to below detectable levels by day 21 after infection, whereas the control RRV infection persisted at a concentration detectable by plaque assay until day 42 after infection. Also of significance was the observation that by day 15 after infection, the large-plaque parent virus had been replaced by a small-plaque variant. Only the small-plaque virus was detected for the remainder of the culture period. Other studies [7] have also demonstrated the production of a persistent small-plaque variant in primary cultures of striated muscle cells.

**Histology of muscle from RRV-infected mice.**

Histologic examination was done on striated muscle from the hind legs of symptomatic mice infected at 17 days of age with the T48 strain of RRV and on control tissue from normal mice, as well as on tissue from nonsymptomatic silica-treated mice. Muscle fiber destruction was seen in muscle tissue from control RRV-infected mice, as observed elsewhere [6]. Muscle fiber damage was absent in silica-treated and control mice.

Muscle tissue was stained for the presence of F4/80-positive cells (figure 4). More than 90% of the infiltrating cells in muscle tissue collected from mice displaying clinical signs (e.g., ruffled fur, cachexia, hind leg stiffness) of RRV disease at day 8 after infection were F4/80-positive, suggesting a functional association of cells of the monocyte/macrophage lineage with RRV pathology. The extent of inflammatory infiltrate was supported by cell counts (per ×400 field) that showed that by day 8 after infection, RRV-infected mice had dramatically increased numbers of F4/80-positive cells (185) compared with those in infected mice at day 4 after infection (3), sham-treated controls (1), and silica-treated animals on day 8 after infection (0). Extensive disruption of striated muscle fibers was associated with the monocyte/macrophage infiltrate. Muscle tissue collected on day 4 and day 8 after infection from sham-infected control mice showed only occasional F4/80-positive cells and no damage to muscle fibers. Muscle tissue collected from RRV-infected mice at day 4 after infection exhibited no detectable damage and a presence of F4/80 cells equivalent to that found for the sham controls; RRV-infected mice at day 4 also displayed no clinical symptoms. In silica-treated mice, the mononuclear infiltrate was completely abolished, and damage to muscle fibers was undetectable.

In RRV-infected mice treated with cyclophosphamide or gamma irradiation, inflammation was slightly reduced, but muscle fiber damage remained extensive (data not shown).

**Protection by adoptive transfer of immune cells.**

Symptoms were abolished by transfer of immune cells 8 h after infection of CBA/H mice with RRV, and this correlated with failure of virus to grow in target tissues. When immune spleen cells were transferred 28 h after infection, virus was already present at high titers in target tissue, but rapid clearance of virus was achieved. Both T cells and B cells contributed to this effect. Transfer of normal cells had, if anything, an enhancing effect on virus growth, but the removal of adherent cells from the transferred immune population increased the protective effect of the cells (data not shown).

These results indicate that if timed correctly, there was a role for the immune response in clearance of RRV infection, and

<table>
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<th>Age (days), treatment</th>
<th>Survival (days)</th>
<th>Mortality (%)</th>
<th>Morbidity (%)</th>
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</tr>
<tr>
<td>Cyclophosphamide</td>
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<tr>
<td>Irradiation</td>
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**NOTE.** For irradiation, 750 rad of gamma irradiation was delivered by a 60Co source.

* Proportion of nonspecific deaths among silica-treated Ross River virus-infected mice. No morbidity was observed due to silica treatment alone.
Figure 2. Growth of T48 Ross River virus in tissues of 17-day-old outbred mice after treatment with cyclophosphamide (■) or radiation (▲). Error bars are not included, although each point represents mean value from groups of 5 mice. No significant difference in virus titer was seen between irradiated and cyclophosphamide-treated mice (□; P > .05). On and after day 4 after infection, both treatments caused increased concentrations of virus to be detected in all tissues, compared with untreated controls (●; P < .01). Hemagglutination-inhibiting antibody was found in all control mouse sera on day 4 after infection, coinciding with elimination of viremia.

hence protection from disease, overcoming the effect of macrophages in exacerbating RRV-induced pathology.

Discussion

The data presented here clearly implicate the macrophage as a key mediator of pathology in RRV-induced disease of mice. Symptoms and death in RRV-infected mice were abolished by the use of two macrophage-specific toxins but not by general immune-suppressive therapy. The model system that we described can readily be adapted for more detailed studies on the role of the macrophage in inducing damage to striated muscle fibers both in vivo and in vitro.

The host response to RRV in mice has been shown to involve, to varying degrees, both adaptive and nonadaptive immune activities. If timed correctly, transfer of either anti-RRV antibody or immune spleen cells provided protection from symptoms and/or reduced virus titers. The transfer of immune cells or antibody had to be very early after infection to result in protection. Although the immune response can certainly clear virus, it does not appear to be responsible for resistance to the development of RRV-induced pathology.

When RRV infection of mice was assessed as a possible model for human disease, the role of macrophages in the development of RRV pathology captured particular interest. Depletion of peritoneal macrophages by use of either silica or carrageenan treatment eliminated RRV-induced morbidity and mortality in outbred mice. Our results indicate that this abrogation of disease was not due to a silica-mediated decrease in virus titers (see figure 3) but was related to the blocking of monocyte/macrophage infiltration into infected muscle tissue. Treatments aimed at eliminating the immune response (gamma irradiation...
and cyclophosphamide) did not significantly reduce muscle damage in RRV-infected mice, nor were there any significant effects on peak virus titers in target organs. Traditionally, silica has been used to deplete macrophages from mice before virus infection, resulting in the enhancement of virus-induced morbidity and mortality [9]. Although it is possible that silica treatment induces a variety of antiviral responses in macrophages, there was no effect on virus titers in target organs of RRV-infected, silica-treated mice compared with titers in the organs of controls.

Recent work has shown that the mouse-virulent T48 RRV strain is capable of replication in macrophages in vitro, and, furthermore, T48 could persist in these cells for protracted periods. This work also demonstrated that infection of macrophages was enhanced by subneutralizing concentrations of antiviral antibody, as well as showing that infection of macrophages was possible only in the presence of anti-viral antibody [4] (figure 1). In vitro evidence demonstrates that RRV can persist in both mature muscle cells and macrophages and that antibody, when present at low levels, enhances monocyte/macrophage infection. This may, in part, explain why infant mice make antibody before symptoms develop, yet go on to die at a later time after infection. In mice, pathology could be increased by the presence of enhancing antibodies that facilitate the virus’s entry into cells and also by anti-viral antibodies that allow macrophages to recognize antibody-coated infected cells through Fc receptor binding.

Macrophages have been shown to be a factor in the pathogenesis of arthritis for animal models [16], and the monokine tumor necrosis factor has been shown to be a primary disease mediator in rheumatoid arthritis of humans [17] and in mouse models of this disease [18]. Tumor necrosis factor was not de-
Macrophages Mediate Muscle Pathology of RRV

Figure 4. Immunostaining for F4/80-positive cells in muscle sections from Ross River virus (RRV)-infected mice collected at either day 4 (A) or day 8 (B) after infection and from sham-infected control mice collected at day 4 (C) or day 8 (D) after treatment. Muscle sections from mice treated with silica before RRV infection were also examined by F4/80 immunostaining at day 8 after infection (E). All sections were photographed at ×400 magnification by use of Fuji ASA 100 color film and an Olympus microscope.

ected in RRV-infected macrophage cultures [4], indicating an alternative mechanism for symptoms of epidemic polyarthritis caused by RRV, although there have not been any in situ cytokine studies on macrophages from RRV-infected mice. A caprine lentivirus has been shown [19] to be associated with arthritis in its host species, and this was found to be mediated by dysfunctional macrophage-cytokine activity. This study found that transcription of tumor necrosis factor in infected cells was depressed, giving further support to the notion that virus-induced arthritis involves a more subtle mechanism. T cell cytokine activity has been shown to vary according to whether the biologic basis of arthritis was reactive (i.e., me-
diated by an infectious agent) or autoimmune [20]. Although both conditions were associated with interleukin-10 and interferon-γ transcription, a greater proportion of reactive arthritis samples were found to have detectable interleukin-4 transcription, indicating that differences in T cell help were a factor. Our future RRV studies will consider the in situ role of T cell cytokines in modulating macrophage-induced pathology after infection.

In conclusion, we have shown that depletion of macrophages prior to RRV infection abolishes RRV-induced symptoms and death in mice. This evidence suggests a primary role for macrophages in RRV pathology, a view further supported by the presence of large numbers of F4/80-positive cells in muscle sections from symptomatic, RRV-infected mice. More general forms of immunosuppression (gamma irradiation and cyclophosphamide) did not have the same dramatic effect on the clinical outcome of this infection, most likely because these treatments exert their efficacy by preventing progenitor cell proliferation rather than depleting mature cells of the monocyte/macrophage lineage. Infection of muscle in vitro has previously been shown not to result in CPE [7], but the results described in this study showed extensive muscle damage in vivo 8 days after RRV infection. This was found to be associated with a massive influx of F4/80-positive cells, whereas silica-treated mice displayed neither muscle damage nor enhanced macrophage infiltration. Studies have shown that RRV can persist in both mouse muscle cells and macrophages, suggesting that in the in vivo context, there may be the capacity for the formation of a chronic infection cycle because of the presence of persistent virus and the migration of naive macrophages into the site, which could further support RRV infection and produce additional muscle damage. Future studies will focus on the in situ profile of cytokines and cytokine-producing cells in muscle sections from RRV-infected mice, as well as consider subtle roles for T cells in modulating the pathogenic activities of macrophages after infection.

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References


