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Review

Mouse models of alphavirus-induced inflammatory disease

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Part of the *Togaviridae* family, alphaviruses are arthropod-borne viruses that are widely distributed throughout the globe. Alphaviruses are able to infect a variety of vertebrate hosts, but in humans, infection can result in extensive morbidity and mortality. Symptomatic infection can manifest as fever, an erythematous rash and/or significant inflammatory pathologies such as arthritis and encephalitis. Recent overwhelming outbreaks of alphaviral disease have highlighted the void in our understanding of alphavirus pathogenesis and the re-emergence of alphaviruses has given new impetus to anti-alphaviral drug design. In this review, the development of viable mouse models of Old Word and New World alphaviruses is examined. How mouse models that best replicate human disease have been used to elucidate the immunopathology of alphavirus pathogenesis and trial novel therapeutic discoveries is also discussed.

Introduction

Alphaviruses are enveloped, single-stranded, positive-sense RNA viruses of the *Togaviridae* family. As arthropod-borne viruses (arboviruses), alphaviruses are transmitted in an enzootic cycle between susceptible vertebrate hosts and arthropod vectors, primarily mosquitoes from the genera *Culex* and *Aedes*. Able to infect a large variety of vertebrates, alphaviruses are globally widespread and can cause considerable human morbidity and mortality. Clinical manifestations vary from fever and/or rashes to severe inflammatory pathologies such as debilitating arthritis and encephalomyelitis. Alphaviruses are grouped according to their geographical localization and the clinical symptoms observed during infection. Generally, Old World alphaviruses, such as Ross River virus (RRV), Chikungunya virus (CHIKV), Barmah Forest virus (BFV), O'nyong-nyong virus (ONNV), Mayaro virus (MAYV), Sindbis virus (SINV) and Semliki Forest virus (SFV), are associated with acute febrile illness, malaise, maculopapular rash and severe arthralgia (Kiwanuka *et al.*, 1999; Laine *et al.*, 2004; Muñoz & Navarro, 2012; Paquet *et al.*, 2006; Phillips *et al.*, 1990; Suhrbier & La Linn, 2004). New World alphaviruses such as Venezuelan equine encephalitis virus (VEEV), Western equine encephalitis virus (WEEV) and Eastern equine encephalitis virus (EEEV) are neurovirulent, and patients frequently experience symptoms ranging from fever, headache and myalgia to potentially fatal encephalitis (Zacks & Paessler, 2010). In addition, a number of Old World alphaviruses, including CHIKV, have been found to cause neurological disease in infected individuals (Schuffenecker *et al.*, 2006).

Alphaviruses are endemic to a number of regions, causing local and sporadic epidemics. However, recent major outbreaks of alphaviral disease have highlighted the lack of vaccines and targeted antiviral therapeutics. Currently only palliative treatment is available by administering analgesics and anti-inflammatory drugs. The re-emergence of VEEV in Venezuela and Colombia in 1995 resulted in 75 000–100 000 human cases (Weaver *et al.*, 1996). A huge outbreak of CHIKV in the islands of the Indian Ocean and India between 2005–2007 saw up to 1.4 million reported cases and recorded the first CHIKV-associated fatalities (Enserink, 2006; Mavalankar *et al.*, 2007). Viral adaptation to novel vectors has already enhanced alphaviral transmission. With climate change and an increase in urbanization, mosquito vectors have the potential to further expand their range. Additionally, as more people travel internationally an increase in the number of imported cases of alphavirus disease has been reported, particularly in Europe and the USA (Rezza *et al.*, 2007; Tsetsarkin *et al.*, 2007).

The re-emergence of alphaviruses poses a significant threat to human health worldwide. Despite this, surprisingly little is known about the pathogenesis of alphavirus infection in humans. Although clinical studies of alphavirus-infected patients offer invaluable data regarding the immunopathology of disease, human studies are often limited by factors such as sample size, ethical issues and restrictions on sample collection. Moreover, the virus strain and kinetics of infection are often unknown. Animal studies therefore serve to supplement clinical data, offering well-defined experimental parameters. As with most *in vivo* models of human disease, the animal commonly used to model alphaviral disease is the mouse. The large number of knockout or transgenic mouse strains available make it possible to dissect the immune mechanisms and viral–host interactions responsible for disease. In addition, mouse models of alphavirus infections have been used for

preclinical testing of antiviral drugs and for vaccine development studies.

Here we summarize early examples of how mice have been used in the study of alphaviral disease and discuss the development of mouse models of both Old Word and New World alphaviruses. This review will also examine how current mouse models have furthered our knowledge of alphaviral pathogenesis and have been successfully employed to trial novel therapeutic strategies against this neglected group of viruses.

Mouse models of Old World alphaviruses

Chikungunya virus

Initial *in vivo* studies of CHIKV infection and associated disease were performed by Ross in 1956. Following the large outbreak of CHIKV in Tanganyika in 1952, CHIKV was isolated and passaged in newborn albino Swiss mice (Ross, 1956). The behaviour of these mice, inoculated intracerebrally with sera from acute-phase CHIKV patients, was also monitored closely. Mice showed high mortality rates, often with an incubation period less than 48 h. This work first highlighted the difference in susceptibility to CHIKV infection between juvenile and adult mice, with 100 % mortality recorded in infected mice younger than 12 days old. Subsequent studies highlighted the neuropathogenic potential of CHIKV in infected mice. Twenty 8-day-old Swiss A_2G mice intracerebrally inoculated with CHIKV presented with disease signs of ruffled fur, lethargy, paralysis and ultimately death at 6 days post-infection (Suckling *et al.*, 1978a). Histological analysis of the brains of infected mice revealed severe necrosis and frequent lesions associated with microgliosis, leptomeningeal infiltration and perivascular infiltration.

Prior to the CHIKV outbreaks throughout 2005 and 2006, little work had been done to better understand the pathophysiology of CHIKV disease. However, the scale and severity of this series of outbreaks highlighted the lack of understanding of CHIKV pathogenesis and the need to develop suitable animal models. The symptoms most associated with CHIKV infection in humans are severe myalgia and symmetrical polyarthralgia of the distal joints. Initial studies in mice were restricted by the absence of clear disease signs in adult mouse strains infected with CHIKV by various routes of administration (Couderc *et al.*, 2008; Gardner *et al.*, 2010; Suckling *et al.*, 1978a). Consequently, numerous mouse models of acute CHIKV disease took advantage of immunologically immature neonate mice as well as adult mice with a partially or totally abrogated type-I IFN pathway (Couderc *et al.*, 2008; Schilte *et al.*, 2010; Werneke *et al.*, 2011; Ziegler *et al.*, 2008). Using CHIKV isolates from patients of the Réunion Island outbreak, Couderc *et al.* (2008) observed resistance to CHIKV infection in adult mice and lethality in neonate mice following intradermal inoculation. A robust interferon response produced in adult mice is thought to protect them from fatal CHIKV infection, as has been observed with other alphaviruses (Grieder & Vogel, 1999; Levy, 2007; Ryman *et al.*, 2000; Sourisseau *et al.*, 2007). Given the interferon sensitivity of alphaviruses, Couderc *et al.* (2008) tested the permissiveness of IFN-α/βR knockout (IFN-$\alpha/\beta R^{-/-}$) adult mice to CHIKV infection. Infected adult IFN-$\alpha/\beta R^{-/-}$ mice all developed severe disease characterized by loss of muscle tone, lethargy and death at day 3 post-infection. Interestingly, adult mice with a partially abrogated type-I IFN pathway (IFN-$\alpha/\beta R^{+/-}$) developed no disease signs upon infection with CHIKV. However, unlike WT adult mice, virus was detected in the muscles and joints of IFN-$\alpha/\beta R^{+/-}$ adult mice at day 3 post-infection. These data suggest that a dose effect of type-I IFN receptor gene alters the permissiveness of CHIKV in these animals and corroborates the importance of the innate immune system in the protection of adult mice from fatal CHIKV infection. This and similar mouse models have been used to further investigate the mechanisms of type-I IFN antiviral activity and the therapeutic value of novel treatment strategies, such as CHIKV immunoglobulins, during CHIKV infection (Couderc *et al.*, 2009; Schilte *et al.*, 2010; Werneke *et al.*, 2011).

Using neonatal mice (strains ICR and CD-1) and subcutaneously inoculated CHIKV strain LR 2006-OPY1 (Réunion strain), Ziegler *et al.* observed arthritogenic disease manifestations in infected mice and a reduction in the high mortality rate seen in previous neonate mouse models (Ziegler *et al.*, 2008). Acute CHIKV disease signs in these mice included loss of balance, difficulty walking and dragging of the hind limbs. Histological analysis revealed dystrophic calcifications in the joint cartilage and prominent skeletal muscle necrosis in the leg. All of the mice developed viraemia that lasted 5–8 days, with CHIKV antigen staining beginning to appear focally in skeletal leg muscle on day 3 post-infection. Subsequently, staining increased in intensity and distribution, and was usually associated with muscle degeneration and necrosis. CHIKV titres were highest and persisted longest in the leg muscle, indicating a tropism of CHIKV for skeletal muscle. There was, however, little sign of collagenosis in the mouse muscle, unlike that observed in the quadriceps of acute CHIKV patients (Ozden *et al.*, 2007). To better understand the tissue tropism of replicating CHIKV, Ziegler *et al.* used *in vivo* imaging of CHIKV-luciferase in the weanling (3–4-week-old) CD-1 mouse model (Ziegler *et al.*, 2011). Virus was injected in the right rear footpad with $10^{5.5}$ $TCID_{50}$. Local replication of CHIKV-luciferase was observed as early as 12 h post-infection and up to 7 days post-infection. Viral dissemination to the hind-limb muscle began at day 3 post-infection and infectious virus remained up to day 5.

Thangamani *et al.* (2010) investigated the contrasting immune activation and cytokine expression in response to CHIKV LR 2006-OPY1 infection of 2-week-old outbred CD-1 mice by mosquito bite or intradermal needle inoculation. Looking specifically at the site of infection, they observed a skewing of host immunity towards a Th2

cytokine profile following mosquito bite compared with a significant upregulation of Th1 cytokines after needle inoculation. Downregulation of a local antiviral Th1 response following infection by mosquito would, they concluded, promote replication and dissemination of CHIKV in the host. A number of studies by Dhanwani *et al.* (2011, 2014) have used an omics approach to understand the virus-induced host response in acutely infected mice. Using 2–3-day-old mice inoculated subcutaneously with 10^6 p.f.u. Indian strain CHIKV DRDE-06, mice developed severe hind-limb disease and paralysis, which subsided by day 12 post-infection. During peak disease, tissues permissive to CHIKV replication were subject to protein profiling to identify host factors that may contribute to inflammation and myositis. Differential expression of candidate genes in CHIKV-infected tissues may reveal relevant clinical biomarkers that allow for early detection of CHIKV disease, and validation of host–CHIKV interactions could provide novel targets for therapeutic intervention.

Although these mouse models of CHIKV provided valuable information on CHIKV tissue tropism, the use of immune-deficient adult mice or immunologically immature neonate mice limits their ability to accurately mimic the immunopathogenesis of CHIKV in humans. To better replicate muscle and joint pathologies associated with acute CHIKV disease and to reduce the mortality associated with infection in newborn mice, a number of studies sought to develop juvenile mouse models (Morrison *et al.*, 2011; Rulli *et al.*, 2011). Morrison *et al.* used 14-day-old C57BL/6J mice inoculated with 100 p.f.u. CHIKV SL15649 in the left rear footpad. Mice developed swelling and oedema of the left foot, which began 2 days after inoculation and persisted for at least 1 week. At 7 days post-infection, animals developed inflammatory lesions in the joints of the foot characterized by the presence of natural killer (NK) cells, monocytes, neutrophils and lymphocytes. Infected mice displayed severe chronic active tenosynovitis and necrotizing myositis in skeletal muscle tissues, much like the debilitating rheumatic pathology that is a prominent feature of CHIKV infection in humans. Using 24-day-old C57BL/6 mice infected subcutaneously with 10^4 p.f.u. CHIKV Réunion isolate (LR2006-OPY1), Rulli *et al.* (2011) observed high levels of monocyte chemotactic protein-1 (MCP-1), TNF-α, IFN-γ in both the ankle and skeletal muscle of CHIKV-infected mice at peak disease. Increased levels of these pro-inflammatory factors were also found in the serum of acute CHIKV patients and probably have a causative role in the pathology of CHIKV disease. Treatment of infected mice with the MCP-1 synthesis inhibitor Bindarit ameliorated disease signs and reduced the influx of inflammatory cells into muscle and joint tissue. Reduced MCP-1 production contributed to decreased macrophage infiltration and therefore to the reduced tissue destruction observed in Bindarit-treated mice. Together, these data highlight the importance of the immune infiltrate, particularly inflammatory monocytes, to CHIKV pathogenesis.

Only recently has an acute mouse model of CHIKV disease been developed in adult (immunologically mature) mice. Gardner *et al.* (2010) were able to recapitulate the self-limiting arthritis, tenosynovitis and myositis seen in CHIKV-infected patients by shifting the site of infection to the ventral side of each hind foot, towards the ankle. Female C57BL/6 mice were at least 6 weeks old when inoculated subcutaneously with 10^4 $CCID_{50}$ (50 % cell culture infectious dose) Réunion Island isolate (LR2006-OPY1) or mouse-adapted Asian strain (isolated in the 1960s and closely related to the Indian isolate, IND-63-WB1, and Thai isolate, AF15561). Acute disease was characterized by development of clearly visible foot swelling that peaked 6–8 days after virus inoculation (Fig. 1). Viraemia lasted 4–5 days and viral titres in the feet peaked on day 1 post-infection. and replication-competent virus was detected in the feet until day 9. At peak disease, the feet showed immense infiltration of mononuclear cells in and around the synovial membranes and connective tissues, subcutaneous oedema and large foci of cellular infiltrates in muscle tissues. Tenosynovitis was evident with marked inflammatory cell infiltration of the connective tissues surrounding the tendons, and muscle fibres showed extensive necrosis. Disease signs were abolished in mice receiving IFN-α treatment prior to infection. However, IFN-α provided no therapeutic benefit when treatment commenced after infection. Furthermore, an inactivated CHIKV vaccine was able to protect CHIKV-challenged mice, significantly reducing viraemia and disease signs. This again illustrates the role of the type-I IFN response during the onset of infection and demonstrates the utility of the adult mouse model for testing of future vaccines and therapeutic candidates. Derivatives of this model, using alternative mouse strains and mutant or knockout mice, have largely been used to examine the innate immune response against CHIKV (Gardner *et al.*, 2012).

With no animal model currently able to reproduce the chronic rheumatic manifestations associated with CHIKV infection, a number of acute mouse models have offered clues as to why some patients experience prolonged illness. In the adult mouse footpad model, histological examination after foot swelling had subsided revealed that although tibial muscle, liver, spleen and lymph node histology had returned to normal, mononuclear cell infiltrates were

Fig. 1. Chikungunya virus-induced inflammation. Twenty-day-old C57BL/6 mice were infected subcutaneously in the footpad with 10^4 p.f.u. CHIKV or mock infected with diluent alone. Images were taken on day 3 post-infection.

observed in subcutaneous and peritendinous connective tissues from days 14 to 21 (Gardner *et al.*, 2010). Small focal infiltrates were also observed in the muscle tissue of the feet and perisynovial tissues. Similarly, signs of inflammation were observed in joint tissues of CHIKV SL15649-inoculated 14-day-old C57BL/6J mice at 21 days post-infection (Morrison *et al.*, 2011). Interestingly, CHIKV RNA was also detected in the ankles at 14 and 21 days after inoculation. Thus, although replication-competent CHIKV was cleared from peripheral tissues following acute infection, CHIKV RNA persisted in joint tissues for at least 3 weeks and was associated with ongoing inflammation within joint tissues.

The neurological complications associated with CHIKV disease, such as encephalitis and myelopathy, first reported in humans during the 2005-2006 Réunion Island outbreak, similarly drove the development of mouse models to investigate the neurovirulence potential of CHIKV *in vivo* (Chandak *et al.*, 2009; Tournebize *et al.*, 2009). Neuropathological studies began in BALB/c mice intranasally infected with the Ross strain of CHIKV (Powers & Logue, 2007). Immunohistochemical labelling of CHIKV antigen in brain sections revealed infected neurons in the anterior olfactory nucleus. Haematoxylin and eosin staining showed necrotic neurons with contracted nuclei, cytoplasmic eosinophilia and nuclear debris and by 5 days post-infection neuronal necrosis was prevalent in the pyriform cortex. Wang *et al.* developed a murine challenge model to test vaccine candidates using 5-week-old inbred C57BL/6 or outbred NIH Swiss mice infected intranasally to produce severe disease for comparative attenuation evaluations (Wang *et al.*, 2008). Both the Réunion Island and Ross CHIKV strains produced viraemia lasting 2-3 days with a peak titre of about 3 $\log_{10}$ p.f.u. ml^{-1}. However, only the high-mouse brain passage Ross strain produced clinical signs of disease after infection in C57BL/6 mice. Animals presented with ruffled fur, a hunched posture and lethargy and died by day 7 or 8 post-infection. High virus titres, over 7 $\log_{10}$ p.f.u. g^{-1}, were found in the brain on days 3 and 7 post-infection. Histopathologic evaluation of CHIKV-infected brains on day 7 showed severe multifocal inflammation and liquefactive necrosis in the cerebral cortex and hippocampus and evidence of multifocal lymphocytic leptomeningitis. The apparent enhanced neurovirulence characteristics of Réunion Island CHIKV strain in humans are still the focus of ongoing studies.

O'nyong nyong virus

ONNV is antigenically related to CHIKV and produces a similar disease presentation. ONNV circulates endemically in Africa and was first isolated in the mid-1960s from both *Anopheles* mosquitoes and acute human patient sera after intracranial inoculation of suckling mice and subsequent passage (Williams *et al.*, 1965a, b). Very few studies have investigated ONNV-induced disease, and only recently have mice been used to study the pathogenesis of ONNV. Using a range of genetic knockout mice, a study was conducted to assess the immunopathogenesis of two different strains of ONNV (MP30 from the 1959 epidemic and SG650 from the 1996 epidemic) (Seymour *et al.*, 2013). This study utilized RAG1$^{-/-}$, IFNγR$^{-/-}$, STAT1$^{-/-}$ and IFNα/βR$^{-/-}$ mice and concluded that the innate immune response was critical in establishing protection against infection, whereas the adaptive immune system was not. This study further reported that IFN-γ alone is not necessary to control acute disease and the IFN-α/β response has a greater role in protection against acute disease (Seymour *et al.*, 2013). Seymour *et al.* (2013) also indicated that, as with CHIKV, to best replicate the joint swelling and myositis of human ONNV disease in mice, the site of viral inoculation is moved to the footpad.

Ross River virus

Initial studies reporting on the infection of mice with RRV were published in consecutive articles in 1973 (Mims *et al.*, 1973; Murphy *et al.*, 1973), with a subsequent publication reporting studies on the adaptation of RRV field isolates to both cell lines and laboratory mice (Seay *et al.*, 1981). Initial pathogenesis studies of RRV using the prototype T48 strain were described in two distinct parts. A focus on RRV-associated brain pathology in mice comprised the first section (Mims *et al.*, 1973), with the pathogenic effects of infection on muscle, periosteum, perichondrium and brown fat being the focus of the second report. In the first study, mice survived and recovered from the symptoms of RRV disease in spite of persistent cortical and general neurological lesions (Murphy *et al.*, 1973). By day 13 post-infection, after the animals had recovered from disease, a generalized monocyte infiltration was noted in the mouse brain suggesting that lesions were not responsible for the paralysis observed between days 7 and 10 post-infection, but that paralysis was a result of severe necrosis of the muscle. Further studies on muscle tissues showed that virus could be detected at day 3 post-infection, and by day 7 post-infection severe necrotic changes were noted, peaking at day 10 post-infection, corresponding to a peak in disease severity. Regeneration and repair of the muscle tissue was noted, returning to a normal appearance by day 34 post-infection (Murphy *et al.*, 1973). Examination of the bone perichondrium and periosteum revealed limited signs of necrosis leading the authors to conclude that there was no joint involvement in diseased mice. This observation was contradictory to the involvement of joints in infected patients suggesting little correlation between the mouse model and human disease (Murphy *et al.*, 1973). However, it was noted that monocyte involvement may vary depending on the tissue, and day 3 may have been too early to observe significant inflammatory changes in bone and joint tissues. These reports (Mims *et al.*, 1973; Murphy *et al.*, 1973) were the first to note the association of RRV-induced pathology with monocyte infiltrates, providing early evidence for the later elucidation of the central role of monocytes/macrophages in RRV disease in mice.

The early observations of RRV-induced muscle pathology associated with mononuclear infiltrates were confirmed in studies by Seay *et al.* (1981). RRV replication was detected in the serum and muscle of infected 7-day-old mice at day 1 post-infection. Virus was cleared from the serum by day 5, but was maintained in the muscle up to day 9 post-infection. Foci of muscle necrosis were observed in association with local mononuclear infiltrates at day 5 post-infection, which increased by day 7 post-infection accompanied by significant muscle necrosis. By day 11, necrosis and inflammation had almost totally replaced muscle tissue. From day 15 post-infection, inflammation had decreased such that by day 65 muscle tissue had undergone complete recovery in both structure and function (Seay *et al.*, 1981). These studies were repeated in 4-week-old mice, with no clinical signs of infection detected, confirming the age-related resistance to RRV disease that had been previously observed (Mims *et al.*, 1973; Murphy *et al.*, 1973). As humoral and cell-mediated responses to RRV were similar for the two age groups, and immunosuppressive therapies had no impact on age-related resistance to disease, Seay *et al.* (1981) concluded that RRV-induced myositis was a result of viral lysis of muscle fibres, rather than immune-mediated pathology.

The collective early RRV studies focused on very young mice with the concluding observation that depletion or neutralization of immune response did not reverse disease and hence that immunopathology was not involved in RRV disease. Studies using older outbred mice significantly bridged the gap between the mouse and human disease and resulted in an overwhelming change in opinion regarding the involvement of immunopathology in inducing RRV disease. Infected 14–17-day-old Swiss or CD-1 mice displayed signs between days 8 and 11 post-infection of ruffled fur, hunching, lethargy, hind-limb paresis and paralysis (Morrison *et al.*, 2006). Depletion of macrophages by silica or carrageenan prior to RRV inoculation was shown to ameliorate disease (Lidbury *et al.*, 2000). Further investigation of hind-limb muscle from symptomatic mice showed a strong F4/80$^+$ monocyte/macrophage phenotype in the infiltrating cell population (Lidbury *et al.*, 2000; Mims *et al.*, 1973; Murphy *et al.*, 1973; Seay *et al.*, 1981; Seay & Wolinsky, 1982, 1983) accompanied by a loss of striation and centralized nuclei. Confirming the earlier findings by Seay *et al.* (1981), mice recovered from RRV disease with muscle tissue returning to normal morphology within 30–40 days. Similar to other studies, immunosuppressive agents including cyclophosphamide and sublethal γ-irradiation resulted in the abolition of antibody production and prolonged *in vivo* virus growth, but resulted in marginal changes to morbidity and mortality in infected mice (Lidbury *et al.*, 2000). Collectively, these data firmly establish the macrophage as the cellular mediator of RRV disease in the mouse model and show clearly that while depletion of progenitor T and B cells had only very minor roles, contrary to previous opinion, the disease in the animal model can be considered to be immune mediated.

Development of the outbred mouse model to better represent the human clinical manifestations enabled further dissection of RRV pathogenesis *in vivo*. The currently utilized model of RRV disease has been developed using the outbred mouse model as a platform (Lidbury *et al.*, 2008; Morrison *et al.*, 2006, 2007). Here, 20- to 24-day-old C57BL/6 mice are subcutaneously inoculated, and virus titres peak in the first 24–48 h post-infection with viable virus detected in the serum, skeletal muscles and joints, indicative of viraemic spread. Disease onset is observed by 4–5 days post-infection with clinical disease signs that include ruffled fur and weight loss. By 7–12 days, peak disease is reached with clinical disease signs of severe hind-limb dysfunction, loss of grip strength and severe weight loss. At peak disease, viraemia is no longer detectable. The infection elicits an inflammatory response, detectable primarily within the muscle tissues, with severe myositis observed in the skeletal muscle (Fig. 2). By 15–21 days post-infection, the disease signs resolve, with mice regaining hind-limb function and beginning to gain weight. At 30 days, mice show no signs of disease.

The current RRV mouse model has provided a useful method to analyse the contribution and involvement of both soluble and cellular factors *in vivo* throughout the course of infection, and allows for a link between *in vitro* studies and the findings from RRV-infected patients. In terms of cellular factors, NK cells and CD4$^+$ and CD8$^+$ T cells were identified within the inflammatory infiltrates in addition to inflammatory macrophages. To determine the functional role of these adaptive immune cells, C57BL/6J RAG-1$^{-/-}$ mice (that lack functional T and B cells) were infected with RRV alongside WT controls, with results showing both mouse strains developed a similar disease. Muscle pathology confirmed that the adaptive immune response has a minor, if any, role in the development of RRV disease (Morrison *et al.*, 2006). Additional mouse studies that investigated macrophage products involved in RRV disease further confirmed the relevance to human disease. Macrophage-depletion studies were expanded using clodronate and demonstrated reductions in joint/muscle tissue levels of TNF-α, IFN-γ, reactive nitrogen intermediates (RNI) and MCP-1. These four pro-inflammatory proteins were elevated in synovial fluid collected from patients with active RRV polyarthritis compared with control samples from osteoarthritis patients, further demonstrating relevance of the mouse RRV disease model for human disease (Lidbury *et al.*, 2008). Further studies have shown that IFN-γ, TNF-α, IL-1β, -6 and -10, macrophage inflammatory protein-1α (MIP-1α), macrophage migration inhibitory factor (MIF), MCP-1, -2 and -3 and the enzyme arginase-1 are all upregulated in response to RRV infection, and implicate these soluble factors in the immunopathogenesis of RRV disease in the mouse model (Herrero *et al.*, 2011; Lidbury *et al.*, 2008; Rulli *et al.*, 2009; Stoermer *et al.*, 2012). The exact role that each of these factors play in the pathogenesis of RRV is still the focus of ongoing studies; however, the murine model has allowed

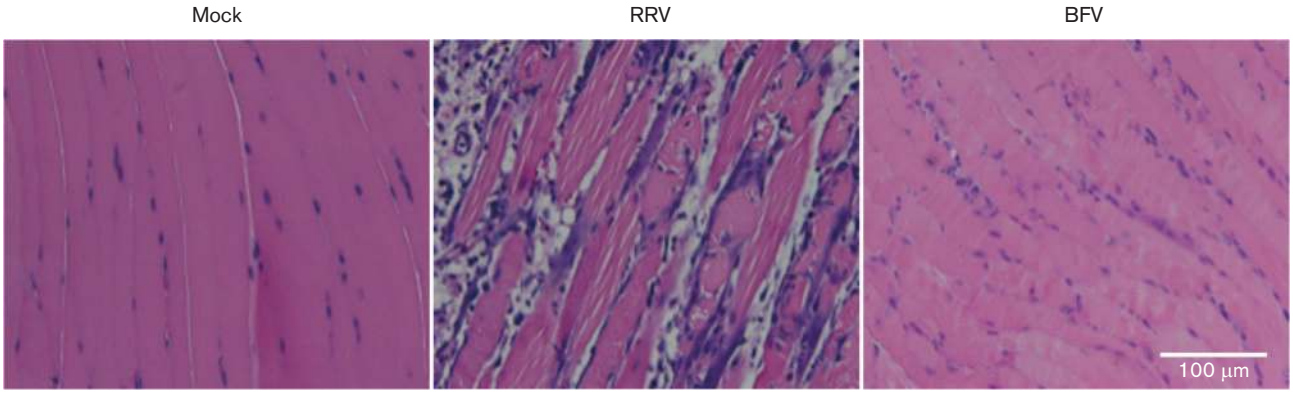

Fig. 2. Muscle pathology of RRV- and BFV-infected mice. Twenty-day-old C57BL/6 mice were infected subcutaneously with 10^4 p.f.u. RRV, BFV or mock infected with diluent alone. Representative images show the pathology of skeletal muscle tissues at peak disease. Quadriceps were removed on day 10 post-infection for processing and stained with haematoxylin and eosin. Magnification, ×200 .

for detailed analysis of several of these pathways. The soluble factors MIF, TNF-α, MCP-1 and IL-10 have been extensively studied demonstrating critical and unique functions for each. MCP-1 has been shown to be involved in macrophage recruitment during RRV-induced inflammation, whereas MIF is critical in the upregulation of MCP-1, which in turn is instrumental in disease severity (Herrero *et al.*, 2011; Rulli *et al.*, 2009). Neutralization of TNF causes a decrease in antiviral immunity by downregulating IFN, thereby resulting in increased RRV titres and virus-induced mortality in the mouse (Zaid *et al.*, 2013). RRV infection of murine bone cells was confirmed using a reporter RRV able to express enhanced green fluorescent protein (EGFP) during replication. Infection results in bone loss by disrupting the receptor activator of nuclear factor *κβ* ligand (RANKL) and osteoprotegerin (OPG) ratio in an IL-6-dependent manner such that neutralization of IL-6 protects from RRV-induced bone loss (Chen *et al.*, 2014). Arginase-1 was found to be a key factor in RRV-induced myeloid cell activation inducing prolong ation of viral infection and musculoskeletal disease (Stoermer *et al.*, 2012). These studies are being furthered to determine the potential of these soluble factors to act as novel therapeutic targets in treating alphaviral disease.

Using the RRV mouse model, studies have also demonstrated that complement activation contributes to RRV inflammatory disease and assists in the production of severe inflammatory arthritis and myositis (Gunn *et al.*, 2012; Morrison *et al.*, 2007, 2008). C3 activation products, such as the complement receptor 3 (CR3) ligand iC3b, were detected at the sites of RRV-induced inflammation, and C3 was found to be critical for the tissue destruction phase of RRV-induced inflammatory disease (Morrison *et al.*, 2007). Complement activation can occur via several different pathways, and studies have further determined that the lectin pathway and mannose-binding lectin are instrumental in RRV-induced complement activation and RRV disease (Gunn *et al.*, 2012).

Since 2000, dramatic progress has been made to establish a mouse model that, as described above, has consistently shown relevance to human disease associated with RRV infection and provided a platform to develop and test novel therapeutics. To date there have been a number of studies on a range of potential new treatments including the indazole derivative, Bindarit, the antimetabolite methotrexate and the rheumatoid arthritis drug etanercept (Rulli *et al.*, 2009; Taylor *et al.*, 2013; Zaid *et al.*, 2013). Bindarit-treated mice had less severe RRV disease, reduced tissue damage and lower intensity of inflammatory cell recruitment through downregulation of MCP-1, -2 and -3. Alternatively, both methotrexate and etanercept increased RRV-induced disease severity by affecting antiviral immunity.

Barmah Forest virus

BFV shares an analogous distribution to RRV and displays similar disease signs. Due to these similarities it is believed that BFV is largely underdiagnosed (Jacups *et al.*, 2008; McGill, 1995). To date, most studies on BFV infection have been sero-epidemiological. A recently published paper analysed the susceptibility of both newborn Swiss mice and adolescent C57BL/6 mice, showing distinctions from the widely published RRV models (Herrero *et al.*, 2014). Similar to RRV, Swiss outbred mice were found to be susceptible to BFV. The BFV prototype strain, BFV2193, was identified as the most virulent strain in Swiss mice inducing disease characterized by hind-leg paresis and leg extension with high mortality (Herrero *et al.*, 2014). Tissue tropism demonstrated efficient viral replication resulting in high viraemia and high titres in the muscle and brain (Herrero *et al.*, 2014). Subcutaneous infection of C57BL/6 mice with the prototype BFV2193 was found to induce

moderate arthritic disease, in contrast to the severe myositis and arthritis caused by RRV in these mice. Observed disease signs included lethargy, ruffled fur and a hunched posture but did not include hind-limb dysfunction, which is classically observed in RRV-infected C57BL/6 mice (Herrero *et al.*, 2014). Correlating with disease severity, histological studies demonstrated that, in contrast to RRV, BFV replicated poorly in muscle and did not cause severe myositis (Fig. 2). Analysis of inflammatory mediators further supported the mild disease signs, as BFV did not induce high levels of TNF-α and IL-6 in the muscle tissue as seen with RRV (Herrero *et al.*, 2014). The study concluded that although BFV is highly pathogenic and displays tropism for many tissues, it is distinct from RRV causing only moderate disease in mice with limited muscle tropism (Herrero *et al.*, 2014).

Mayaro virus

MAYV was first isolated in 1954 and is endemic to South America where it causes sporadic cases and smaller outbreaks of disease that manifest with fever, headaches, arthralgia and myalgia (Anderson *et al.*, 1957; Halsey *et al.*, 2013). To date, there have been very few studies on mouse infections with MAYV. A study in 2009 described infection of newborn mice with acute-phase serum samples collected from febrile patients to isolate MAYV, but no clinical signs were described in these mice (Azevedo *et al.*, 2009). However, unpublished data described by Ziegler in a 2008 report suggest that subcutaneously infected newborn and 14-day-old ICR mice present skeletal muscle and connective tissue changes similar to those described for CHIKV and RRV (Ziegler *et al.*, 2008). A recent study used A129 mice, which lack functional type-I IFN receptors, to assess the level of attenuation of MAYV vaccines (Weise *et al.*, 2014). Five 8-week-old mice were inoculated intradermally in the left footpad with 10^4 p.f.u. of MAYV or vaccine and monitored for survival, changes to body weight and viraemia. Although vaccines were well tolerated, all MAYV-infected mice succumbed between 4 and 5 days post-inoculation.

Semliki Forest virus

Although most human SFV infections are asymptomatic, SFV has been found to be responsible for febrile outbreaks associated with severe persistent headache, myalgia and arthralgia (Mathiot *et al.*, 1990). Following the initial isolation of SFV from mosquitoes in Uganda, Smithburn & Haddow (1944) first analysed the pathogenic properties of SFV in mice. Lethal encephalitis was observed in adult mice intracerebrally inoculated with SFV. Using the same virus isolate (L10), Seamer *et al.* (1967) described the progression of a 'highly fatal disease' in all mice infected either intracerebrally or peripherally. Paralysis was observed in a number of mice prior to death, and survival was prolonged in older mice. In 25-day-old mice inoculated with $10^{1.6}$ p.f.u. in the footpad, productive viral replication was found in the spleen and central nervous system (CNS). Furthermore, histological analysis of the CNS revealed signs of encephalitis and myelitis, demonstrating the neurovirulence of the L10 strain in mice.

In 1961, an avirulent strain of SFV, A7, was isolated from mosquitoes in Mozambique (McIntosh *et al.*, 1961). The early stages of infection with A7 and the more virulent L10 strain were indistinguishable in weanling mice (Bradish *et al.*, 1971; Pusztai *et al.*, 1971). However, there were differences in the replication kinetics of virulent and avirulent strains of SFV in the brain tissues of mice, with virulence linked to the ability of strains to replicate faster and to a higher titre in neuronal cells (Gates *et al.*, 1985). This leads to rapid and extensive damage of neurons in the CNS of mice infected with virulent SFV (Balluz *et al.*, 1993). Infection of mice over 17 days old with the A7 strain proved to be largely non-lethal. Animals did, however, develop neurological disease manifestations over the course of infection, including virus-induced demyelination in the CNS. Murine models and *in vivo* studies of SFV disease have therefore sought to understand the immunopathology and virus–host interactions responsible for viral encephalitis.

Early identification of demyelination as a feature of SFV infection in mice was investigated in greater detail by a number of subsequent studies (Chew-Lim, 1975; Mackenzie *et al.*, 1978; Zlotnik *et al.*, 1972). Using avirulent strains, Chew-Lim *et al.* (1977) traced the course of SFV infection in the mouse brain. Mice infected at 3–4 weeks of age were used to evaluate changes in the immunopathology of CNS inflammation during infection. Histological analysis revealed lesions, including perivascular cuffing composed largely of lymphocytes, in the medullary substance of the cerebellum. Acute encephalitis peaked at 18 days post-inoculation and gradually resolved by day 42. Similar pathological studies revealed that in addition to cellular degeneration and infiltration, focal spongiform lesions irregularly distributed throughout the CNS characterized infection with avirulent SFV. These lesions were associated with peripheral astrocytic hypertrophy and intense activity of glycoside hydrolases (Mackenzie *et al.*, 1978).

Suckling *et al.* (1978b) undertook a comprehensive examination of the inflammatory pathology associated with SFV infection of a number of mouse strains (random outbred Swiss/A_2G mice, outbred BSVS, inbred BALB/c, CBA and SJL/J mice), with particular emphasis on the demyelination observed in the cerebellar white matter. Mice 4–7 weeks of age were inoculated intraperitoneally with 10^4 LD_{50} suckling mouse intracerebral avirulent SFV. While seldom producing clinical signs of disease, several mouse strains displayed mild encephalitis and focal microcystic spongiform lesions were evident, as was a loss of myelin within the white matter of the CNS. SJL/J mice appeared to be much less affected.

Historically, the exact cause of SFV-induced demyelination has been a topic of debate, often with conflicting data. Virus-induced myelin loss was generally attributed to one

of two distinct pathways: the cytotoxic effect of viral replication on the host cell or immunopathological myelin loss, during which the immune response to infection induces demyelination. A number of studies used immune-compromised mice to assess the contribution of the immune system to demyelination and SFV disease. In irradiated mice, Chew-Lim *et al.* (1977) observed an increase in SFV titre and extensive demyelination. Similarly, an increased number of demyelinative plaques were found in the cerebellum of infected nude (T-cell-deficient) mice compared with immune-competent controls (Chew-Lim, 1979). Hence, it was suggested that demyelination during SFV infection was due to direct viral cytotoxicity of the CNS rather than an immune-mediated mechanism. However, concurrent studies and a large body of subsequent data highlighted the important role of the immune system in mediating acute demyelination during SFV infection. Several studies noted the absence or decreased incidence of demyelination in SFV-infected nude mice (Fazakerley *et al.*, 1983; Gates *et al.*, 1984; Jagelman *et al.*, 1978). Mackenzie *et al.* (1978) also noted that histopathological changes to the brain of infected mice occurred subsequent to the appearance of infiltrating cells at day 5 post-infection. Further studies have also demonstrated the importance of CD8+ T cells to SFV infection in the development of demyelination (Smith *et al.*, 2000; Subak-Sharpe *et al.*, 1993). Although required for viral clearance, the role of antibodies during demyelination is less clear. Anti-myelin antibodies may contribute to myelin loss, but studies indicate that antibodies are not required for the formation of demyelinating lesions during SFV infection (Fragkoudis *et al.*, 2008). It is now widely accepted that the immune system plays a pivotal role in SFV-induced demyelination; however, the exact viral/host interactions and immune mechanisms required to stimulate demyelination remain unclear.

Subsequently, mouse models of SFV encephalomyelitis have been used to test the application of novel therapeutic strategies to treat alphaviral infections of the CNS. Coppenhaver *et al.* (1995) infected weanling outbred ICR mice intracranially with SFV, causing lethal encephalitis. Measuring survival, viraemia and brain viral titres as outcomes of disease, Coppenhaver *et al.* (1995) showed a combination therapy of anti-SFV hyperimmune serum and interferon inducing poly I:CLC, effectively protected mice from alphaviral infection of the CNS. Treated animals had a 50 % survival rate and reduced viraemia and brain viral load over the course of infection. Treatment was also effective when administered after viral dissemination to the target organs. The model of SFV encephalomyelitis established by Fazakerley *et al.* (1993) has the advantage over a number of other models of encephalitis that SFV is efficiently neuro-invasive. Indeed, the susceptibility of mouse neuronal cells to SFV infection is thought to reflect, in part, the maturation state of neurons (Fazakerley *et al.*, 1993). Following intraperitoneal inoculation of SFV A7(74) strain, the virus is highly neuro-invasive, crossing the blood–brain barrier to initiate perivascular foci of infection in neurons and oligodendrocytes (Fazakerley, 2004). This method of extraneural inoculation produces lesions of inflammatory demyelination and allows the pathology of the CNS during infection to be examined without direct intracerebral inoculation and consequent disturbance of the blood–brain barrier.

The SFV mouse model established by Mokhtarian *et al.* (1994) uses a scale of disease score to measure the severity of paralysis in infected mice (Mokhtarian & Swoveland, 1987). Developed over a number of years, the current model uses female C57BL/6 mice 5–6 weeks of age at the time of inoculation. Mice are infected intraperitoneally with 10^4 p.f.u. A7(74) SFV strain and scored daily for the development of disease signs on a scale of 0–6, where 0 represents no disease signs and 6 represents the most severe form of disease, manifesting as hind-limb paralysis or quadriplegia. Peak disease is seen at day 7 post-infection, coinciding with high viral titres in the brain. Mice develop a transient encephalomyelitis, with demyelination in the cerebellum and white matter vacuolation, from which they recover (Mokhtarian & Swoveland, 1987; Mokhtarian *et al.*, 1994; Smith-Norowitz *et al.*, 2000). More recent studies using this model have used knockout mouse strains to further dissect the immune-pathogenesis of demyelinating lesions (Safavi *et al.*, 2011).

The development of a number of mouse models of SFV disease has provided insight into the pathogenesis of infections with encephalitic viruses and the mechanisms that drive associated neuropathy. The neuronal sequelae in SFV-infected mice have been likened to the demyelination observed in patients with multiple sclerosis. Demyelination within SFV-infected mice extends to the optic nerve (Fleming *et al.*, 1982), an observation that has been successfully modelled by infecting Swiss A_2G mice at 4–6 weeks of age and performing electrophysiological recordings. The visual latencies of both single-cell and field potential responses of infected mice were found to be longer than those of the controls (Tremain & Ikeda, 1983). Using the same flash stimuli, visual evoked potentials were recorded from patients with demyelinating disease. Patients with multiple sclerosis showed a clear increase in peak latency of the visual evoked potential and a significantly reduced flicker fusion frequency, as observed in SFV-infected mice (Tremain & Ikeda, 1983). As a result, it has been proposed that mouse models of SFV disease may also be used to model other demyelinating diseases such as multiple sclerosis. Furthermore, increased understanding of SFV disease pathogenesis may advance the understanding of other viral encephalitides.

Sindbis virus

SINV was first isolated from mosquitoes in Egypt in 1952. SINV causes outbreaks of arthritis in humans, but in mice SINV serves as a model for the study of alphaviral neurological pathogenesis. Early studies showed infection

of suckling mice resulted in fatal encephalitis. Initial studies therefore investigated the neuro-invasive nature of SINV (Johnson, 1965). Inoculated virus was found to replicate in striated, smooth and cardiac muscle and in the vascular endothelium. High plasma viraemia also developed rapidly. SINV disseminated rapidly within cerebral parenchyma infecting both neural and glial cells and spread within the CNS. As in other mouse models of alphavirus disease, resistance to SINV infection is age dependent (Reinarz *et al.*, 1971). Furthermore, Thach *et al.* (2000) demonstrated that certain strains of mice have genetic determinants of resistance to fatal encephalitis caused by SINV infection. Using neuro-adapted Sindbis virus (NSV) delivered intranasally, Thach *et al.* (2000) saw fatal encephalitis in all infected C57BL/6 mice up to 10 weeks of age. However, by 4–5 weeks of age, BALB/cBy mice no longer developed fatal disease. C57BL/6 mice also developed more severe disease than BALB/cBy mice, and the severity of neurological disease signs was age dependent. Both mouse strains developed kyphoscoliosis, hindlimb paralysis, and muscular atrophy. C57BL/6 mice lost up to 30 % of their body mass and died 7–9 days after infection, yet at 5 weeks of age only ruffled fur and mild paralysis was observed in infected BALB/cBy mice. Interestingly, although remaining susceptible to NSV infection, C57BL/6 mice survived longer as the age at infection was increased.

Following intranasal infection, NSV is observed in olfactory neurons and infection is thought to proceed on a neuron-to-neuron basis via axonal transport. Veckenstedt *et al.* (1985) infected 4–6-week-old ABD2F1 mice intranasally with 10 LD_{50} neuro-adapted SINV strain AR86. With this model, they observed acute encephalomyelitis and lesions in the CNS following histological analysis. Furthermore disease signs such as ruffled fur, arched back and paralysis of one or more limbs was evident prior to death at approximately day 4 post-infection. With lethality as a measure of disease outcome, this model was used to test the effectiveness of novel therapeutic strategies, such as the anti-Parkinson drug Norakin, in the treatment of SINV encephalomyelitis (Veckenstedt *et al.*, 1985).

Cook & Griffin (2003) used *in vivo* imaging to identify viral and host determinants of SINV virulence in mice. Chimeric viruses containing gene segments derived from NSV and AR339 SINV strains were generated to determine the viral strain- specific contribution to virulence. The recombinant viruses were engineered to express the reporter gene luciferase under the control of a second subgenomic promoter. Infecting 4-week-old albino C57BL/6-Tyr^{c-2J} mice and BALB/cAnNCrlBR mice, Cook & Griffin (2003) also examined the location and extent of virus replication in susceptible and resistant mice. The luciferase *in vivo* imaging system was able to differentiate between susceptible and resistant mouse strains and could also be used to phenotype different strains of SINV. Furthermore, imaging suggested that SINV entry into the nervous system could occur by retrograde axonal transport either from neurons innervating the initial site of replication or from the olfactory epithelium after viraemic spread (Cook & Griffin, 2003). Not only does this model have the benefit of being able to monitor viral infection over time in a single animal, but it also allows SINV disease progression to be directly linked to virus replication.

Mouse models of New World alphaviruses

Eastern equine encephalitis virus

EEEV was first described in 1933 in infected horses in Delaware, Maryland and Virginia (Giltner & Shahan, 1933). In humans, a disease similar to that seen in equines develops with headache, fever, seizures and coma (Deresiewicz *et al.*, 1997). EEEV is the most pathogenic encephalitic alphavirus, with human mortality rates of 30–70 %. Some of the earliest mouse studies of EEEV infection aimed to better understand a variety of disease characteristics such as general immune responses, host factors determining resistance/susceptibility, adequate routes of immunization, the development of novel diagnostic tests for encephalitides and basic histopathology of infected mice (Casals & Palacios, 1941; King, 1940a, b; Morgan, 1941; Olitsky & Cox, 1936; Olitsky & Harford, 1938). These early reports did not focus on the mouse model per se but rather utilized a lethal model to test various hypotheses regarding protection after immunization. Most strains of EEEV kill 70–100 % of mice infected regardless of the route of inoculation. This, together with the high lethality rate in many mouse strains, can account for the wide variety of murine models of EEEV.

By extensively studying the histopathology of EEEV in mice, early work by King showed that mice display signs of neuro-inflammation and neurodegeneration upon infection. Inoculating infant mice either intraperitoneally or subcutaneously in the periphery, King observed inflammatory brain pathology as early as 36 h post-infection. Inflammation spread quickly over time and infant mice inoculated intracranially showed particularly accelerated disease. King also noted the reduced sensitivity to infection of juvenile and adult mice, with inflammation being less pronounced in these animals and disease incubation longer following peripheral infection. Vacuolation and neurodegeneration were also key disease features in infected animals (King, 1940b). Using a highly mouse-adapted strain of EEEV and Rockefeller albino mice (of Swiss origin), Morgan (1941) examined the influence of age on the susceptibility to EEEV infection. Animals that were 14 days, 22 days, 1 month or 6 months of age were inoculated either intraperitoneally or intracranially. The average incubation period until death and the number of animals surviving after intraperitoneal injection increased with age. Results from this study also suggested that susceptibility to EEEV following intraperitoneal injection correlates with levels of viraemia.

Murphy & Whitfield (1970) inoculated 1-day-old Swiss ICR mice with EEEV and studied the CNS using light

and electron microscopy. Mice were inoculated either intraperitoneally or intracranially with 1000 LD_{50} of EEEV and encephalitis was seen as early as 48 h post-infection; animals were moribund by 60 h. Initial observations included intercellular and perivascular oedema. Virus was observed within the lumina of vessels of the basement membranes beneath the endothelium. Neurons underwent cytoplasmic swelling, retraction and vacuolation as early as 24 h post-infection, ultimately leading to significant neuronal loss. Glial cells were infected, with signs of cytoplasmic chromatolysis. Infant NIH Swiss mice usually succumb to intracranial infection with WT EEEV between days 3–4 post-infection, making it a highly stringent model for neurovirulence. Seven 8-week-old mice infected intranasally or intraperitoneally with a North American strain of EEEV FL93-939 went on to develop clinical signs of encephalitis by days 3 or 4 post-infection and to die by days 4 or 5. High viral titres were found in the brains of these adult mice and also in the serum, heart, liver, spleen and kidneys. Interestingly, the authors reported that adult mice were refractory to the South American strain BeAr436087 (Wang *et al.*, 2007). This is in contrast to studies reporting that 5–7-week-old NIH Swiss mice were susceptible to both North and South American strains given subcutaneously. In this study, all mice became viraemic and mortality rates reached 70–90 %. It is possible that by using different South American EEEV strains to BeAr436087, the observed difference in pathogenicity may be strain specific (Aguilar *et al.*, 2005).

To examine the early events in the pathogenesis of EEEV, 5-week-old C57BL/6 mice were inoculated in the footpad with 10^5 p.f.u. EEEV North American strain FL91-4679 (Vogel *et al.*, 2005). Early after infection mice were lethargic and ruffled and by day 4 post-infection, they were hunched and exhibited tremors and were subsequently euthanized. Viraemia peaked at 12 h post-infection and was still measurable when experimental end points were reached. Importantly, EEEV replication was evident in fibroblasts, macrophages and dendritic cells, and through haematogenous spread reached peripheral tissues. Virus was also detected in bones of the nasal turbinates, hard palate and skull. Osteoblasts present in the femur and tibia displayed widespread necrosis and cell loss. Not unlike infection with VEEV, subsequently these mice also showed multifocal necrosis and loss of odontoblasts, ameloblasts and tooth pulp. After 24 h, virus-positive neurons were detected in the CNS. Once in the CNS, rapid and random replication of EEEV was seen, supporting the notion of haematogenous neuro-invasion. Infected areas included, but were not limited to, the cerebellum, lateral olfactory tract and the cerebral and secondary motor cortices. By day 4 post-infection, widespread necrosis of neurons was detected and infiltration of inflammatory cells was limited. Glial cells were also infected and virus could be found throughout most regions of the brain.

Current studies regularly use CD-1 Swiss mice in their investigations of EEEV. Gardner *et al.* (2008) used CD-1 mice to investigate the difference in disease aetiology and tissue tropism after peripheral injection of both VEEV and EEEV. Animals infected with 10^3 p.f.u. VEEV strain ZPC738 displayed signs of disease much quicker than those infected with the same amount of EEEV strain FL93-393. VEEV mice developed ruffled fur, ataxia and weight loss within the first 72 h and showed signs of CNS disease by days 4–6 post-infection, which progressed to paresis, paralysis and death. EEEV-infected animals appeared normal until day 4, developing signs of illness including seizures only shortly before death. VEEV was seen to induce a strong lymphotropism due to its ability to infect both macrophages and dendritic cells. In contrast, EEEV has a propensity to infect fibroblasts and osteoblasts, which are of mesenchymal lineage. Importantly, being unable to infect lymphoid tissues allows EEEV to avoid induction of a strong IFN response. Gardner *et al.* (2011) later used the CD-1 Swiss mouse model to demonstrate the importance of heparan sulfate as an attachment receptor for EEEV E2 protein. Interestingly, binding was shown to contribute to certain pathological aspects of EEEV disease including seizures, neurovirulence and limited prodromal cytokine induction. Together, these findings are particularly important to the development of new antivirals or vaccines against EEEV.

Studies of EEEV vaccine candidates have used various mouse strains as challenge models. Most recently, a novel pan-alphavirus vaccine comprising cationic lipid nucleic acid complexes and the ectodomain of WEEV E1 protein was shown to provide 100 % protection in CD-1 mice challenged with WEEV and to significantly increase their survival following challenge with EEEV, irrespective of the route of infection (Phillips *et al.*, 2014).

Western equine encephalitis virus

In humans, WEEV typically causes mild disease in adults such as fever, headache and malaise. Children are more inclined to develop severe neurological disease following infection, such as encephalomyelitis and coma. Human fatality rates from WEEV are approximately 3–7 % (Reeves *et al.*, 1958). Initial investigations into WEEV disease in mice used virus recovered from the brain of a child who died of encephalitis following the 1938 Californian epidemic (Howitt, 1938). The majority of inoculated young Swiss mice died between 3 and 5 days post-infection and those that survived longer displayed signs of CNS disease, including tremors and hyperexcitability. Infant and weanling Swiss Rockefeller mice were also used to investigate age-related pathological changes *in vivo* during infection (Aguilar, 1970). Infected infant mice succumbed to subcutaneous inoculation 48 h post-infection. No significant changes were seen in the brain except for focal softening and discoloration. Interestingly, severe pathological changes and cellular infiltration in muscle, cartilage and bone marrow were found in infected infant mice. In contrast, weanling mice developed meningo-encephalitis 10 days post-infection and displayed pathological changes

in organs such as the heart, liver and brain. Changes in skeletal muscle were only seen at the site of inoculation. Another group using adult NIH Swiss mice infected by subcutaneous inoculation examined WEEV tissue tropism, viraemia and mortality (Monath *et al.*, 1978). High viral titres were observed in cardiac muscle, and mortality rates reached 40 % with all animals developing illness (ruffled fur, hunched posture, fever). Mice that did not recover died between 7 and 14 days post-infection.

Virulence markers have been investigated using both suckling and adult outbred Swiss albino mice (Bianchi *et al.*, 1993). Highly virulent strains of WEEV like McMillan (McM), Cba 87 and Cba CIV 180 were found to be neuro-invasive and neurovirulent when inoculated intraperitoneally in adult mice. A number of low-virulence strains such as HJV and Y62-33 had intermediate phenotypes causing neurovirulence but were not neuroinvasive. The remaining low-virulence strains studied were neither neuro-virulent nor neuro-invasive in the same adult model. Furthermore, the low-virulence strain AG80-646 failed to replicate in the brain after intracranial injection. Results generally correlated with the known ability of these WEEV strains to cause disease in humans and horses, affirming the validity of the mouse model and its importance to future studies. Similar studies of WEEV virulence have been carried out in adult CD-1 mice and an adult BALB/c model with comparable findings (Logue *et al.*, 2009; Nagata *et al.*, 2006). Mice were challenged with 10^3 p.f.u. of either high-virulence (McM) or low-virulence (IMP) WEEV (Logue *et al.*, 2009). When infected subcutaneously all mice developed viraemia regardless of the viral strain used, unlike the clinical outcome. The route of inoculation affected organ viral titres and survival of the mice. Animals infected subcutaneously, intracranially and via aerosol with McM did not survive, while intravenous infections had a 10 % survival rate. With IMP, all animals survived unless inoculated by aerosol challenge, where 10 % succumbed to infection. Furthermore, histopathology differed greatly between mice infected with McM or IMP. McM-infected mice had signs of necrotic neurons, gliosis and oedema throughout the forebrain. Infiltrating lymphocytes were observed in the meninges and neuropathology worsened with time. In contrast, ~66 % of IMP-infected mice showed no abnormal brain pathology on days 7 and 13 post-infection. Animals that did show neurovirulence had similar pathological changes to those seen in McM-infected mice, albeit less severe. Taken together, these findings suggest that strain-specific determinants alter the neurotropism of WEEV and its capacity to cause disease. Currently an area of intense research, it is suggested that genetic determinants and their capacity to activate the innate immune response may play a role in the strain-specific differences of WEEV neuro-invasion and neurovirulence. Further understanding of WEEV strain-specific pathogenicity will probably identify novel targets for therapeutic intervention and aid vaccine development.

A more recent study of WEEV neurotropism used *in vivo* and *ex vivo* bioluminescent imaging to characterize neuro-invasion in 4–5-week-old CD-1 mice (Phillips *et al.*, 2013). This technique used a recombinant McM strain expressing firefly luciferase (LUC). Animals were infected intranasally with 10^4 p.f.u. recombinant WEEV. All mice showed signs of lethargy and/or depression and motor deficits. As observed by Logue *et al.* (2009), all animals succumbed to McM.LUC between days 3 and 4 post-infection. Despite a similar disease outcome, McM.LUC did replicate to lower titres compared with the parental virus and induced lower levels of MCP-1 and IP-10 at early time points. The olfactory pathway appeared to be the main route of entry to the CNS although, as with VEEV, the trigeminal nerve was infected. This platform for examining WEEV infection *in vivo* is useful not only for studying viral pathogenesis but also for testing prophylactics or antivirals aimed at reducing viral replication and/or dissemination. As proof of principle, 4–6-week-old mice were vaccinated with cationic liposome–nucleic acid complexes of WEEV E1 ectodomain using a prime–boost regimen and then challenged with 10^4 p.f.u. McM.LUC. No luciferase activity was detected in vaccinated mice and all were protected from disease.

In the 1970s, 3-week-old CD-1 mice were used to test WEEV vaccine candidates (Robinson *et al.*, 1972). In weanling mice, intracranial challenge was found to be 100 % lethal. Animals were vaccinated intraperitoneally and challenged intracranially with 100–1000 LD_{50} of a virulent WEEV (B-11). With vaccine candidates found to have the same efficiency in mice as in guinea pigs (the gold standard at that time), the mouse model would provide a quicker, cheaper and more statistically relevant assay. More recently, much effort has been made in developing WEEV therapeutics or vaccine candidates. The susceptibility of multiple mouse strains to WEEV and the responsiveness of mice to various routes of inoculation has given researchers great flexibility when choosing how to model WEEV disease in mice. The relative ease of replicating aspects of human WEEV disease in mice has allowed testing of a variety of vaccine candidates, including adenovirus vectors, DNA-based vaccines and live chimeric recombinant and subunit-based vaccines produced in *E. coli* (Atasheva *et al.*, 2009; Barabé *et al.*, 2007; Das *et al.*, 2004, 2007; Gauci *et al.*, 2010; Wu *et al.*, 2007). In particular, 5–8-week-old C57BL/6 mice have been used extensively to test the efficacy of WEEV antivirals and vaccines. Both the pyrazinecarboxamide derivative, T-705 (Favipiravir) and Antimycin A derivatives have shown therapeutic potential in these animals, reducing the mortality rate and ameliorating disease signs associated with WEEV infection (Julander *et al.*, 2009; Raveh *et al.*, 2013).

Venezuelan equine encephalitis virus

Since the first isolation of VEEV in 1938, mice have been used to gain insights into its pathogenesis, neurovirulence, neuro-invasion and vaccine development (Beck & Wyckoff, 1938). From these early studies VEEV was quickly

identified as a new virus, being immunologically different from the then known Eastern and Western viruses. Initial *in vivo* studies saw mice develop paralysis and succumb to infection between 3 and 5 days post-infection following intracerebral inoculation (Beck & Wyckoff, 1938). Subsequent mouse models of VEEV disease have been found to develop a very similar course of disease following infection. High mortality is a common feature of infection in most mouse strains.

Early studies sought to examine the viraemic spread and tissue tropism of VEEV. Using Swiss Webster (CFW) suckling mice, Kundin *et al.* (1996) showed that all mice infected subcutaneously or intracranially succumbed to VEEV after extensive CNS infection. VEEV was able to replicate actively in the brain and, throughout the course of infection, viral antigen was readily detected in nerve cells, spinal cord, ganglia and tooth buds (Kundin *et al.*, 1966). Similarly, later studies used adult C57BL/6 mice to further characterize CNS involvement in VEEV pathogenesis. Here, virus reached the CNS by day 3 post-intraperitoneal injection. Animals were hunched by days 3–4 post-infection and developed bilateral hind-limb paralysis by day 6, progressing to quadriplegia and death. VEEV antigen was first detected in scattered neurons of the cerebral cortex. As the disease progressed, infection spread in neurons and glial cells to the hippocampus, thalamus, brainstem and grey matter of the spinal cord. Infiltration of inflammatory cells was also reported in the parenchyma and leptomeninges (Fig. 3). Perivascular cuffing and neuronal degeneration and loss were also observed during infection (Jackson *et al.*, 1991). Five-week-old CD-1 mice were also used to model how VEEV gains access to the CNS. Charles *et al.* (1995) showed that VEEV can disseminate to the brain via two distinct routes: the olfactory system and trigeminal nerves. These routes are important portals of entry for many other neurotropic viruses. Following subcutaneous inoculations, virus was detected in the olfactory epithelium. VEEV disseminates along the olfactory nerves to the olfactory bulb where it can spread via the glomeruli to adjacent structures such as the lateral olfactory tracts, pyriform cortex, hypothalamus and hippocampus. VEEV was shown to spread to the periodontal membranes followed by its presence in certain branches of the trigeminal nerves. Furthermore, ablation of the olfactory bulbs did not hinder neuro-invasion, demonstrating the independence of the two routes of infection.

Using BALB/c and C3H/HeN mice strains Steele *et al.* (1998) compared neurovirulence and tissue tropism between WT and attenuated strains of VEEV. Studies showed that C3H/HeN mice are more susceptible than BALB/c mice to VEEV infection regardless of whether the animals receive a WT strain (TrD), a vaccine strain (TC-83) or an attenuated infectious clone (V3526). The authors also determined that VEEV tropism is strain specific and that neuro-invasion does not always correlate with neurovirulence. Viral antigen was detected in the brains of all mice, but BALB/c mice survived infection with TC-83 and

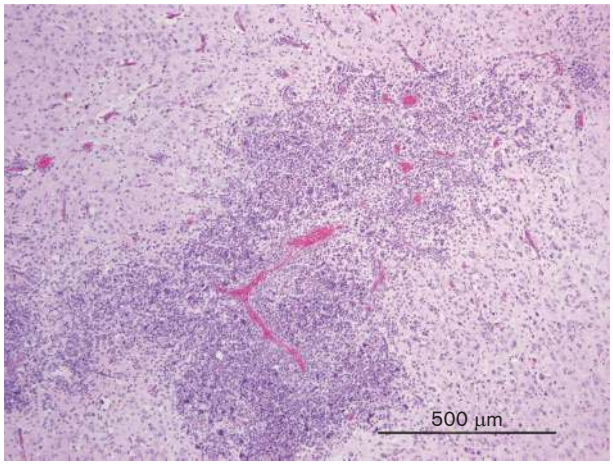

Fig. 3. Histopathological analysis of the brain tissue of VEEV-infected mice. Juvenile CD1 mice were infected intracranially with 10^7 p.f.u. VEEV. Representative image showing the inflammatory cell infiltrate of the brain tissue. Brains were removed on day 7 post-infection for processing and stained with haematoxylin and eosin. Magnification ×100.

both BALB/c and C3H/HeN mice survived infection with V3526 (Steele *et al.*, 1998). To examine the immunopathology associated with infection by avirulent and virulent strains of VEEV, LeBlanc *et al.* (1978) used congenitally athymic (nude) mice along with BALB/c mice. Twenty-eight- to 35-day-old mice were inoculated subcutaneously with avirulent TC-83 or virulent 69Z1 or 68U201 strains. In the absence of T cells, nude mice generally succumb to virulent strains faster than immunocompetent controls. T cells restricted viral replication in the brain. Nude mice demonstrated higher viral loads in the CNS compared with BALB/c mice when infected with avirulent strains and had lower and transient antibody responses without succumbing to the infection. Therefore, factors other than T cells contribute to VEEV virulence. Similarly, Dal Canto & Rabinowitz (1981) infected BALB/c mice, nude mice (homozygotes) and heterozygous nude mice subcutaneously and examined pathological changes in the brain. The BALB/c controls and heterozygous nude mice developed lethargy and hind-limb paralysis by days 6–7 post-infection and succumbed to infection by day 12 post-infection. Nude mice showed a 2-day delay in the development of clinical signs and succumbed between days 12 and 15 post-infection. Demyelinating lesions in the spinal cords of the immunocompetent mice were reported at day 9 post-infection, and became extensive over time. This was the first report of an arbovirus inducing prominent demyelination at late times post-infection. Interestingly, nude mice still developed lesions in the grey matter, albeit to a lesser degree, but did not show any signs of infiltrating cells or demyelination in the white matter, suggesting an important role for host immune factors in VEEV pathogenesis (Dal Canto & Rabinowitz, 1981).

Four- to 12-week-old BALB/c mice are commonly used in VEEV vaccine studies. A diverse range of vaccination strategies has been investigated along with a variety of vaccine candidates including replicons and adenovirus- and DNA-based vaccines (Schäfer *et al.*, 2009; Tretyakova *et al.*, 2013; Williams *et al.*, 2009). Studies have examined the immunological profile of BALB/c mice following vaccination with live attenuated VEEV strain (TC-83) given at 10^5 p.f.u. via subcutaneous injection. BALB/c mice developed a robust immune response, with IgM titres reaching maximal values on days 7–9 post-vaccination. The IgG response was Th1 biased, with a predominant production of IgG2a antibodies. Activated monocytes/macrophages and NK cells were prominent and IFN-γ and TNF mRNA were upregulated. These findings were replicated in 5–6-week-old C57BL/6 and μMT mice (Elvin *et al.*, 2002). B cells were also strongly activated along with early activation of CD4+ and CD8+ T cells in draining lymph nodes. CD-1 mice have also been used to analyse immune responses to vaccine candidates. Charles *et al.* (1997) showed that upon subcutaneous administration of a live attenuated recombinant vaccine candidate V3014, spread occurs to lymphoid tissues without causing viraemia and induces a VEEV-specific IgA response sufficient for protection against aerosol challenge with the virulent V3000 infectious clone. Rossi *et al.* (2013) also used these mice as a model to test second-generation IRES-based vaccine candidates. Here, mice infected with virulent 68U201 strain by subcutaneous inoculation developed paralysis and extreme lethargy by day 2 post-infection and did not survive. Overt signs of disease included hunched posture, hind-limb paralysis and over 20 % loss in initial body weight. Most vaccinated animals developed neutralizing antibodies within 3 weeks and maintained a high level of antibody titres for up to a year post-vaccination, sufficient to protect them from lethal challenge. Rossi *et al.* (2013) also developed a highly sensitive *in vivo* model to test the attenuation of vaccine candidates, in which 6-day-old CD-1 mice received vaccines intracranially. Although vaccines were well tolerated in adult mice, all pups died after inoculation. However, the mean day of death varied depending on the degree of attenuation of the vaccine candidate.

The outbred Porton TO mouse strain is occasionally used in VEEV studies as the genetic diversity of outbred strains better reflects the diversity of the human population. When receiving 10^4 LD_{50} via aerosols these mice develop ruffled fur, hunched posture and lethargy. Disease caused by epizootic TrD or other strains from serogroup 1 A/B is more acute and severe than that from enzootic strains. All animals succumbed between 100 and 150 h post-infection (Wright & Phillpotts, 1998). Six 8-week-old Porton TO mice were used to assess protection after vaccination with live attenuated vaccine TC-83. Mice were vaccinated subcutaneously with 10^4 p.f.u. TC-83. By day 4 post-vaccination, mice had obtained maximum immunity to both subcutaneous and aerosol challenge by TrD. However, some animals did not survive aerosol challenge. Breakthrough infections were more frequent with the highly virulent serogroup 1A/B viruses following aerosol challenge (Phillpotts & Wright, 1999).

C3H/HeN mice are particularly sensitive to VEEV TC-83 infection when receiving the virus intranasally, intracranially or by aerosol exposure (Hart *et al.*, 1997; Julander *et al.*, 2008; Ludwig *et al.*, 2001). Like most strains, the clinical features of disease in these mice include lethargy, hunched posture and weight loss. This commonly used model also exhibits a similar CNS disease to that seen in other animal models, such as the development of meningo-encephalitis, gliosis and multifocal neurophil vacuolation (Julander *et al.*, 2008). Differences do exist, however. TC-83 replicates less efficiently in lymphatic tissues, and viraemia is generally lower than in other models. Despite these limitations, this model has been used since the 1970s to test the efficacy of new therapeutics (Kuehne *et al.*, 1977), and was chosen recently as a model to examine novel anti-VEEV compounds that specifically target the nsP2 protein (Chung *et al.*, 2014).

Summary

Alphaviruses are capable of causing sudden and significant human morbidity and mortality. Only in recent years has considerable progress been made in understanding the molecular, cellular and viral–host interactions responsible for the pathogenesis of both Old and New World alphaviruses *in vivo*. Advances in our understanding of the role of innate immune mechanisms as mediators of alphavirus-induced disease have already aided therapeutic design and can be expected to continue to identify potential drug targets. These advances owe much to studies performed in animal models, with mouse models being the most prominent system used in alphavirus research. Although variability in disease pathogenesis still exists between models, numerous mouse models of alphaviral disease are now able to successfully replicate the immunopathology observed in infected patients and reliably mimic disease signs associated with symptomatic infection in humans.

In addition to resurgence in the development of viable mouse models, recent alphavirus outbreaks have also highlighted the lack of targeted therapeutics and the need for cost-effective vaccines. Mouse models that parallel human disease have thus provided a platform to allow preclinical testing of novel therapeutics and vaccines against alphaviruses. Although able to successfully replicate acute disease, no mouse model is currently able to simulate the chronic manifestations associated with alphaviral disease. As such there is little knowledge of alphavirus-induced pathogenesis during the chronic phase of disease. The challenge remains therefore to replicate in mice the chronic arthralgia and long-term neurological sequelae often observed in patients infected with Old World and New World alphaviruses, respectively.

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