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Role of envelope *N*-linked glycosylation in Ross River virus virulence and transmission

Michelle A. Nelson,[1]† Lara J. Herrero,[2]† Jason A. L. Jeffery,[3] Marion Hoehn,[1,4] Penny A. Rudd,[2] Aroon Supramaniam,[2] Brian H. Kay,[3] Peter A. Ryan[3,5] and Suresh Mahalingam[1,2]

Correspondence
Suresh Mahalingam
s.mahalingam@griffith.edu.au

[1]Faculty of Applied Science, University of Canberra, Canberra, ACT 2601, Australia

[2]Institute for Glycomics, Griffith University, Gold Coast, QLD 4222, Australia

[3]QIMR Berghofer Medical Research Institute, Herston, Brisbane, QLD 4006, Australia

[4]Helmholtz Centre for Environmental Research, Department of Conservation Biology, Leipzig, Germany

[5]School of Biological Sciences, Monash University, Melbourne, VIC 3800, Australia

With an expanding geographical range and no specific treatments, human arthritogenic alphaviral disease poses a significant problem worldwide. Previous *in vitro* work with Ross River virus (RRV) demonstrated that alphaviral *N*-linked glycosylation contributes to type I IFN (IFN-$\alpha\beta$) induction in myeloid dendritic cells. This study further evaluated the role of alphaviral *N*-linked glycans *in vivo*, assessing the effect of glycosylation on pathogenesis in a mouse model of RRV-induced disease and on viral infection and dissemination in a common mosquito vector, *Aedes vigilax*. A viral mutant lacking the E1-141 glycosylation site was attenuated for virus-induced disease, with reduced myositis and higher levels of IFN-γ induction at peak disease contributing to improved viral clearance, suggesting that glycosylation of the E1 glycoprotein plays a major role in the pathogenesis of RRV. Interestingly, RRV lacking E2-200 glycan had significantly reduced replication in the mosquito vector *A. vigilax*, whereas loss of either of the E1 or E2-262 glycans had little effect on the competence of the mosquito vector. Overall, these results indicate that glycosylation of the E1 and E2 glycoproteins of RRV provides important determinants of viral virulence and immunopathology in the mammalian host and replication in the mosquito vector.

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INTRODUCTION

Mosquito-borne alphaviruses from the family *Togaviridae*, such as Ross River (RRV), Sindbis, Semliki Forest, chikungunya (CHIKV) and o'nyong-nyong viruses, are of significant concern worldwide, having been responsible for several major epidemics of infectious rheumatic disease in humans. Past epidemics include the 1979/80 RRV disease outbreak in the South Pacific, with more than 60 000 reported cases (Aaskov *et al.*, 1981; Rosen *et al.*, 1981; Tesh *et al.*, 1981), and the CHIKV disease outbreaks of 2005/6 in La Réunion, with over 250 000 reported cases (Renault *et al.*, 2007), 2006/7 in India, with an estimated 1.3 million reported cases (Arankalle *et al.*, 2007), and 2009 in South East Asia, with more than 100 000 people affected (Sam *et al.*, 2009; Thavara *et al.*, 2009). More recent expansion of CHIKV into the Americas has seen over 1 million cases reported so far (Weaver & Lecuit, 2015).

†These authors contributed equally to this work.

Alphaviruses are maintained in nature via transmission cycles between arthropod vectors and vertebrate hosts (Strauss & Strauss, 1994). For RRV, one of the more widely studied alphaviruses, this typically involves *Aedes vigilax*, *Culex annulirostris* and *Aedes camptorhynchus* mosquito vectors, and several Australian native marsupial hosts (Harley *et al.*, 2001; Old & Deane, 2005; Russell, 2002). Following transmission to humans, symptoms of alphaviral infection range from mild fever and malaise to more severe arthritis, arthralgia and, in some cases, encephalitis (Strauss & Strauss, 1994). RRV is endemic to Australia and the South Pacific, where it is responsible for several thousand cases of severe polyarthritis per year, with symptoms including fatigue, fever, rash, myalgia, arthralgia and arthritis (Fraser, 1986). The acute disease normally resolves within 3–6 months, with cases reported to involve chronic arthritic disease being largely associated with unrelated rheumatic conditions (Harley *et al.*, 2001; Mylonas *et al.*, 2002).

The alphaviral surface consists primarily of three envelope glycoproteins (E1, E2 and E3). Both E1 and E2 are important

for viral infection, with E1 containing a fusion domain that is thought to be involved with host cell membrane penetration (Garoff *et al.*, 1980; Rice & Strauss, 1981), and E2 containing major epitopes targeted by neutralizing antibodies and regions involved with host cell receptor recognition (Dalrymple *et al.*, 1976; Smith *et al.*, 1995; Strauss & Strauss, 1994). The smaller E3 only remains associated in some alphaviruses, such as Semliki Forest virus (Garoff *et al.*, 1974).

Alphaviral envelope glycoproteins contain several *N*-linked glycosylation sites, the specific number and location of which differ slightly between viruses. The type of attached carbohydrate can also differ, depending on the particular virus and host cell type and growth state (Hakimi & Atkinson, 1980; Hsieh *et al.*, 1983; Keegstra *et al.*, 1975; Mayne *et al.*, 1985). In vertebrate cells, high-mannose carbohydrate chains are added to all *N*-linked glycosylation sites within the lumen of the rough endoplasmic reticulum, with further modification to complex chains occurring within the Golgi apparatus, resulting in the presence of a mixture of simple and complex carbohydrates (Hsieh *et al.*, 1983). In contrast, in insect cells, the lack of processing enzymes results in all glycosylation sites containing simple chains, either high-mannose or pauci-mannose (Butters *et al.*, 1981; Hsieh & Robbins, 1984). RRV contains three glycosylation sites (residues E1-141, E2-200 and E2-262). The predominant glycan at each RRV glycosylation site has been determined, with mammalian-derived RRV found to contain a high-mannose or hybrid glycan at E2-200 and complex glycans at E1-141 and E2-262, and mosquito-derived RRV found to contain a pauci-mannose glycan at E1-141 and high-mannose at both E2-200 and E2-262 (Shabman *et al.*, 2008).

Several studies have identified a potential role for alphaviral *N*-linked glycosylation in a number of processes, including glycoprotein processing and trafficking (Leavitt *et al.*, 1977; McDowell *et al.*, 1987; Schlesinger *et al.*, 1985), replication (Knight *et al.*, 2009; Leavitt *et al.*, 1977; Shabman *et al.*, 2008), infectivity (Klimstra *et al.*, 2003), host cell interactions (Knight *et al.*, 2009) and induction of host cell antiviral responses (Davis *et al.*, 1987; Shabman *et al.*, 2007; Shabman *et al.*, 2008). The type of attached carbohydrate is also important, with differences in structure found to alter function. For example, viruses containing high-mannose *N*-linked glycans have enhanced infectivity and are poor inducers of type I IFN, whereas those containing complex *N*-linked glycans have reduced infectivity and promote high-level type I IFN induction (Klimstra *et al.*, 2003; Shabman *et al.*, 2007, 2008) In this study, we further examine the role of *N*-linked glycosylation in alphavirus replication, infection and transmission, using a panel of RRV mutant viruses, each lacking one of the three viral envelope *N*-linked glycosylation sites.

RESULTS

Mutant virus replication in mammalian and mosquito cells *in vitro*

To determine if loss of a single glycosylation site affects RRV replication in either mammalian or mosquito cells, viral replication was examined in Vero and C6/36 cells. Virus lacking the E1 glycan grew less well than WT virus in mammalian cells, reaching significantly lower titres (Fig. 1a). In contrast, no differences in viral replication were seen for viruses lacking either of the E2 glycans compared with WT virus, except at 4 h post-infection (p.i.), when E2-N262Q was found to have significantly lower titres. When grown in mosquito cells, all three mutant viruses initially grew to higher titres than WT virus; however, no significant differences in viral titre were seen from 12 h p.i. (Fig. 1b).

To assess if the origin of the virus could have any effect on the rate of viral replication, WT and mutant viruses were propagated using mosquito cell culture (C6/36 cells), and replication of the resultant mosquito-derived viruses was examined in Vero and C6/36 cells. In both cell lines, results were similar to that seen for mammalian cell-derived virus, with virus lacking the E1 glycan growing to significantly lower titres in Vero cells after 12 h p.i. than WT virus (Fig. 1c), and all mutant viruses showing significantly higher initial titres in C6/36 cells than WT virus up to 6 h p.i., after which no differences were seen (Fig. 1d). Early replication in Vero cells, however, deviated from what was seen for the mammalian cell-derived virus, with all mutant viruses showing significantly higher titres up to 6 h p.i. than WT virus, differences immediately following the attachment phase being the most significant. In addition, viruses lacking either of the E2 glycans were also seen to have significantly lower titres at 48 h p.i. than WT virus (Fig. 1c).

RRV-induced disease severity *in vivo* is reduced in the absence of the E1 glycan

To assess the role of envelope glycans in RRV disease, C57BL/6 mice were infected with 10^4 p.f.u. WT or mutant RRV and monitored for disease signs. Mice infected with WT RRV developed severe disease signs, with a significant reduction in weight gain compared with mock-infected mice (Fig. 2). Similar, although slightly less severe, disease progression was seen for mice infected with RRV lacking either of the two E2 glycans. No significant differences in either clinical disease signs or weight gain were seen as compared with WT RRV-infected mice, except for mice infected with E2-N262Q, where the decrease in severity of disease signs was significant at days 6 and 7 p.i. (Fig. 2). In contrast, mice infected with RRV lacking the E1 glycan developed significantly less severe disease signs than those infected with WT RRV and gained weight at a similar rate to the mock-infected controls, with significantly higher weight gain than T48-infected mice by 8 days p.i. In addition to an overall decrease in disease severity, disease recovery occurred earlier in E1-N141Q-infected mice, with mice moving into the recovery phase by 10 days p.i. (Fig. 2a).

Viral clearance *in vivo* is increased in the absence of the E1 glycan

To determine if the reduction in disease severity seen in the absence of the E1 glycan corresponded with a reduction in

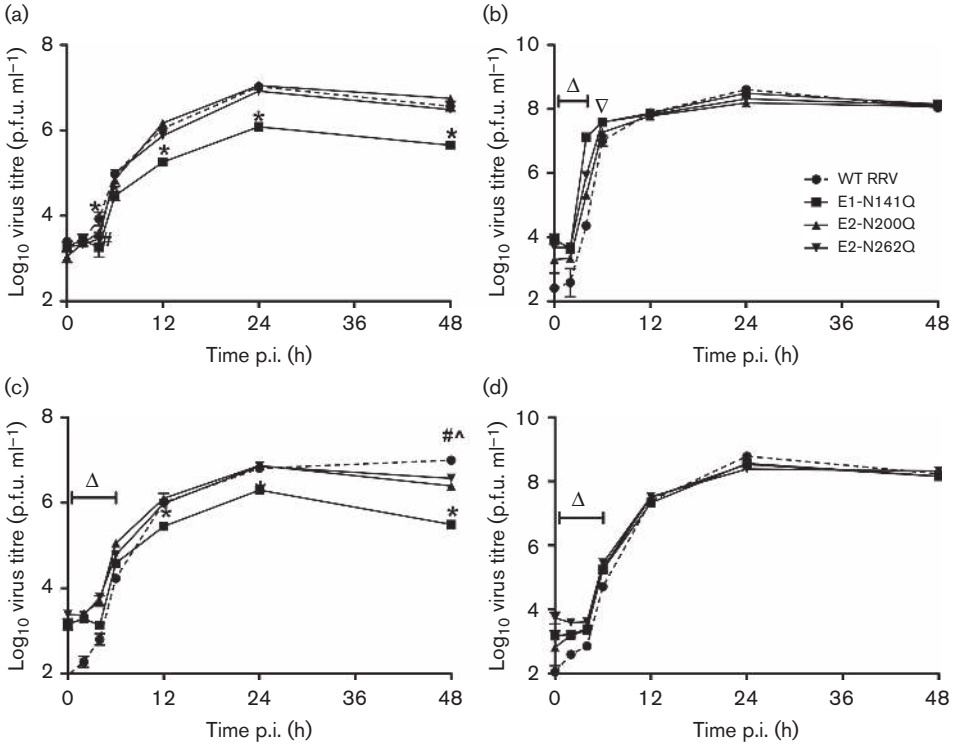


Fig. 1. Effect of viral envelope glycosylation loss on virus replication in mammalian and mosquito cells. Both Vero-derived (a, b) and C6/36-derived (c, d) WT and mutant viruses were used to infect Vero (a, c) and C6/36 (b, d) cells at an m.o.i. of 0.1; replication was measured by plaque assay. Replication curves are shown, with data points representing mean ± SE for three independent samples. Compared with WT RRV: *E1-N141Q, ^E2-N200Q or #E2-N262Q titres were significantly lower, while ΔE1-N141Q, E2-N200Q and E2-N262Q titres were all significantly higher, or ∇E1-N141Q and E2-N262Q titres were significantly higher ($P<0.05$, using a two-way ANOVA followed by a Bonferroni post-test).

viral load, C57BL/6 mice were infected with 10^4 p.f.u. WT or E1-N141Q RRV and sacrificed at 1, 3, 5 and 10 days p.i. Serum, quadriceps muscle and ankle joint were isolated and assayed for viral titre. The loss of the E1 glycan significantly increased the rate of viral clearance in the serum (Fig. 3a). Viral replication peaked at 1 day p.i. in mice infected with either WT RRV or RRV lacking the E1 glycan, with no significant differences seen between titres. At 3 days p.i., viral titres were significantly lower in mice infected with RRV lacking the E1 glycan than in those infected with WT virus; however, both forms of RRV were completely cleared by 5 days p.i. Viral replication and clearance showed similar trends in both the quadriceps muscle and ankle joint, with an increased rate of viral clearance in mice infected with RRV lacking the E1 glycan. In the quadriceps muscle, an increased rate of viral clearance was seen in E1-N141Q-infected mice from 3 days p.i.; however, this was only significant at 5 days p.i. (Fig. 3b). In contrast, no differences in viral titre were seen in the ankle joint until 10 days p.i., at which point viral titres were significantly lower in mice infected with RRV lacking the E1 glycan (Fig. 3c).

RRV-induced inflammation and tissue damage is reduced in the absence of the E1 glycan

Severe inflammation of the quadriceps muscle is associated with RRV disease in C57BL/6 mice (Morrison *et al.*, 2006). To determine if loss of viral envelope glycans alters the level of inflammation and tissue damage caused by RRV, C57BL/6 mice were infected with 10^4 p.f.u. WT or mutant RRV, and quadriceps muscle tissue was collected at 10 days p.i., sectioned and stained with haematoxylin and eosin (H&E). Mice infected with WT RRV and RRV lacking either of the E2 glycans all showed a similar level of inflammation, with widespread muscle tissue damage and extensive infiltration of inflammatory cells (Fig. 4b, d–f). In contrast, inflammation was much less severe in mice infected with RRV lacking the E1 glycan, with less damage and fewer inflammatory infiltrates in the quadriceps muscle tissue (Fig. 4c, f). No inflammation was seen for mock-infected mice (Fig. 4a).

In order to further characterize the role of viral *N*-linked glycans on both lymphoid and myeloid infiltrating cells, we analysed the cell populations in the quadriceps muscles

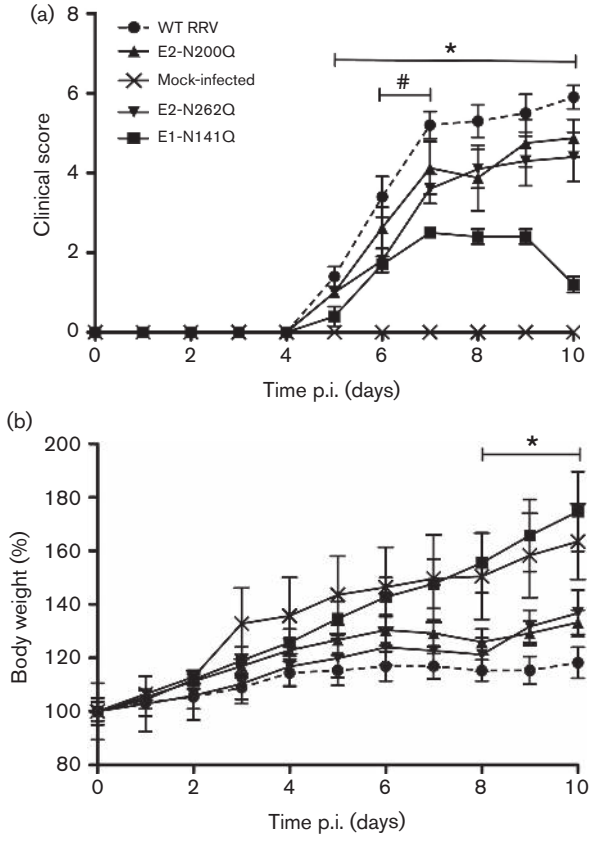


Fig. 2. Effect of viral envelope glycosylation loss on disease progression *in vivo*. C57BL/6 mice, 21 days old, were infected subcutaneously with 10^4 p.f.u. WT or mutant RRV, or mock-infected with PBS, and disease progression was monitored daily. (a) Clinical disease signs were scored according to the following scale: 0, no disease signs; 1, ruffled fur; 2, very mild hind-limb weakness; 3, mild hind-limb weakness; 4, moderate hind-limb weakness and dragging of hind-limbs; 5, severe hind-limb weakness/dragging; 6, complete loss of hind-limb function; 7, moribund; and 8, death. Data points represent mean ± SE for four to six mice. Compared with WT-infected mice, $P<0.05$ for *E1-N141Q or #E2-N262Q-infected mice, using a Mann–Whitney test. (b) Weight gain shown as a percentage of starting body weight. Data points represent mean ± SE for four to six mice and are representative of three independent experiments. *$P<0.05$ for mice infected with E1-N141Q when compared with WT-infected mice using a two-way ANOVA followed by a Bonferroni post-test.

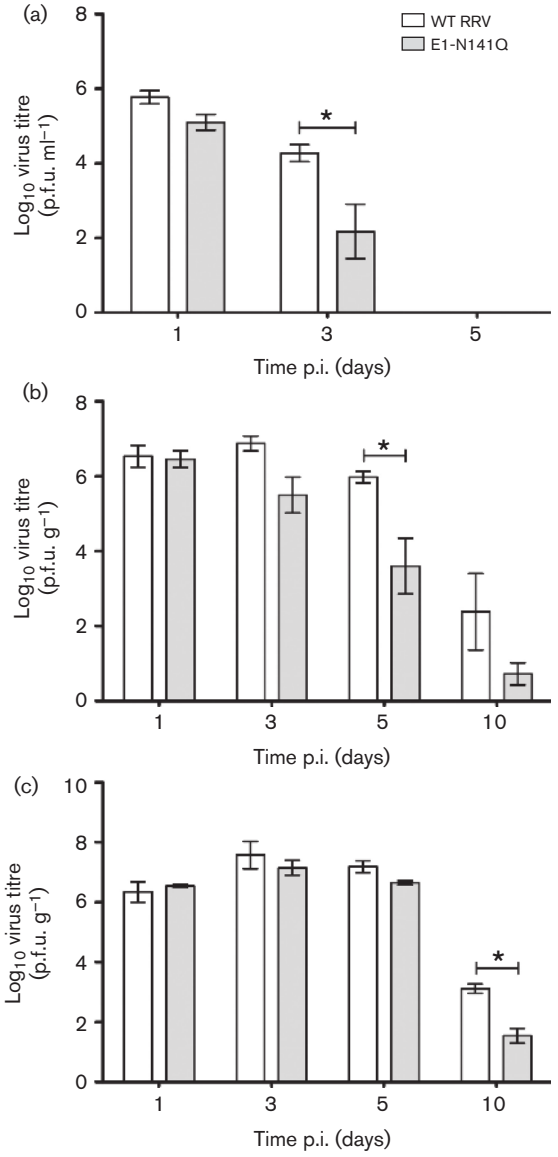


Fig. 3. RRV is cleared more rapidly *in vivo* in the absence of the E1 glycan. C57BL/6 mice, 21 days old, were infected subcutaneously with 10^4 p.f.u. WT or E1-N141Q RRV. At days 1, 3, 5 and 10 p.i., the serum (a), quadriceps muscle (b) and ankle joint (c) were harvested, and the amount of virus present was determined by plaque assay. Data points represent mean ± SE of at least four mice. *$P<0.05$ using a two-way ANOVA followed by a Bonferroni post-test.

at day 10 p.i. by flow cytometry. The absence of the E1 glycan uniformly reduced the numbers of all $CD45^+$ infiltrating leukocytes, including reductions ($P<0.05$) in the inflammatory monocyte, neutrophil and NK cell populations (Fig. 5) corresponding to an overall reduction in percentage population compared with total leukocytes (data not shown). While absence of the E1 glycan also reduced T-cell numbers, this reduction was not statistically significant. Interestingly, whereas the E1-N141Q mutant showed a decrease in the total NK cell populations ($P<0.05$), the corresponding decrease in $CD45^+$ cells resulted in an alteration of the NK cell to total cell ratio, resulting in an increase in the overall percentage of NK cells in the quadriceps muscle (data not shown). The absence of either E2 glycan had no significant effect on the numbers of infiltrating immune cells, with the exception of the E2-N200Q mutant, which showed a significant increase in overall T-cell numbers. Despite this, there was

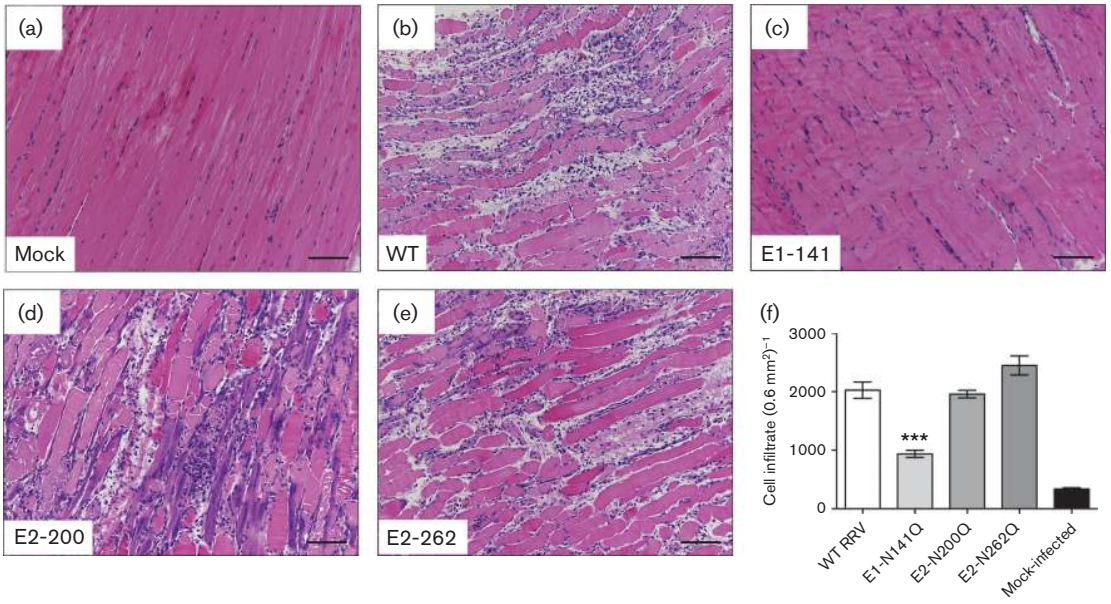


Fig. 4. Inflammation and tissue damage following RRV infection *in vivo* is less severe in the absence of the E1 glycan. C57BL/6 mice, 21 days old, were mock infected with PBS (a), or infected subcutaneously with 10^4 p.f.u. WT RRV (b), E1-N141Q (c), E2-N200Q (d) or E2-N262Q (e). At 10 days p.i., mice were sacrificed and perfused with 4 % paraformaldehyde, and quadriceps muscle was removed. Tissue was paraffin-embedded and 5 μm sections were stained with H&E. Images are representative of at least four mice per group (bars, 100 μm). Quantification of immune infiltrates was performed using ImageJ (f) and analysed using a one-way ANOVA followed by Dunnett's post-test comparing with WT RRV; ***$P<0.001$.

no corresponding increase in the ratio of T-cells to total leukocytes, with the percentage of T-cells being equivalent to that observed in WT RRV (data not shown).

Expression of RRV-induced soluble factors is altered in the absence of the E1 glycan

Several proinflammatory cytokines and soluble factors are known to contribute to RRV disease pathogenesis. To determine the effect of virus envelope glycans on the expression of proinflammatory factors, C57BL/6 mice were infected with 10^4 p.f.u. WT or mutant RRV and gene expression was measured in the quadriceps muscle at 10 days p.i.

The expression of monocyte chemotactic protein 1 (MCP-1), TNF-α, IFN-γ, IL-6, IL-1β and arginase-1 was significantly increased following infection with WT RRV compared with mock-infected mice. Infection with RRV lacking the E1 glycan resulted in a significant reduction in MCP-1 and arginase-1 expression and a significant increase in the expression of IFN-γ compared with that seen for WT RRV (Fig. 6a, c, h). In contrast, no significant differences were seen in the expression of any of the soluble factors assessed in mice infected with RRV lacking either of the two E2 glycans when compared with WT RRV infection (Fig. 6).

RRV-induced interferon expression *in vivo* is affected by the expression of *N*-linked glycans

Previous studies have shown murine dendritic cells to be robust producers of type I IFN in response to RRV infection, and this is affected by viral glycan expression. In order to further assess the role of *N*-linked glycans in early IFN production *in vivo*, quadriceps muscles and ankle joints were collected from RRV WT- and mutant-infected mice at 40 h p.i. and analysed for IFN gene expression. Whereas loss of either of the E2 glycans had little effect on the induction of type I or type II IFN, loss of the E1 glycan resulted in a decrease in type I IFN gene expression and no significant effect on the expression of type II IFN (Fig. 7).

RRV infectivity in the mosquito is impaired in the absence of the E2-200 glycan

To determine the importance of viral envelope glycans in the mosquito vector, the rates of infection, viral dissemination and ability of virus to be transmitted were examined following oral exposure of *A. vigilax* to blood containing 1.5×10^5 p.f.u. ml^{-1} WT or mutant RRV. At 4 days p.i., mosquitoes were snap-frozen, and legs and bodies were processed separately. For those mosquitoes containing viral RNA in the legs, salivary glands were first dissected from the body before each was processed individually.

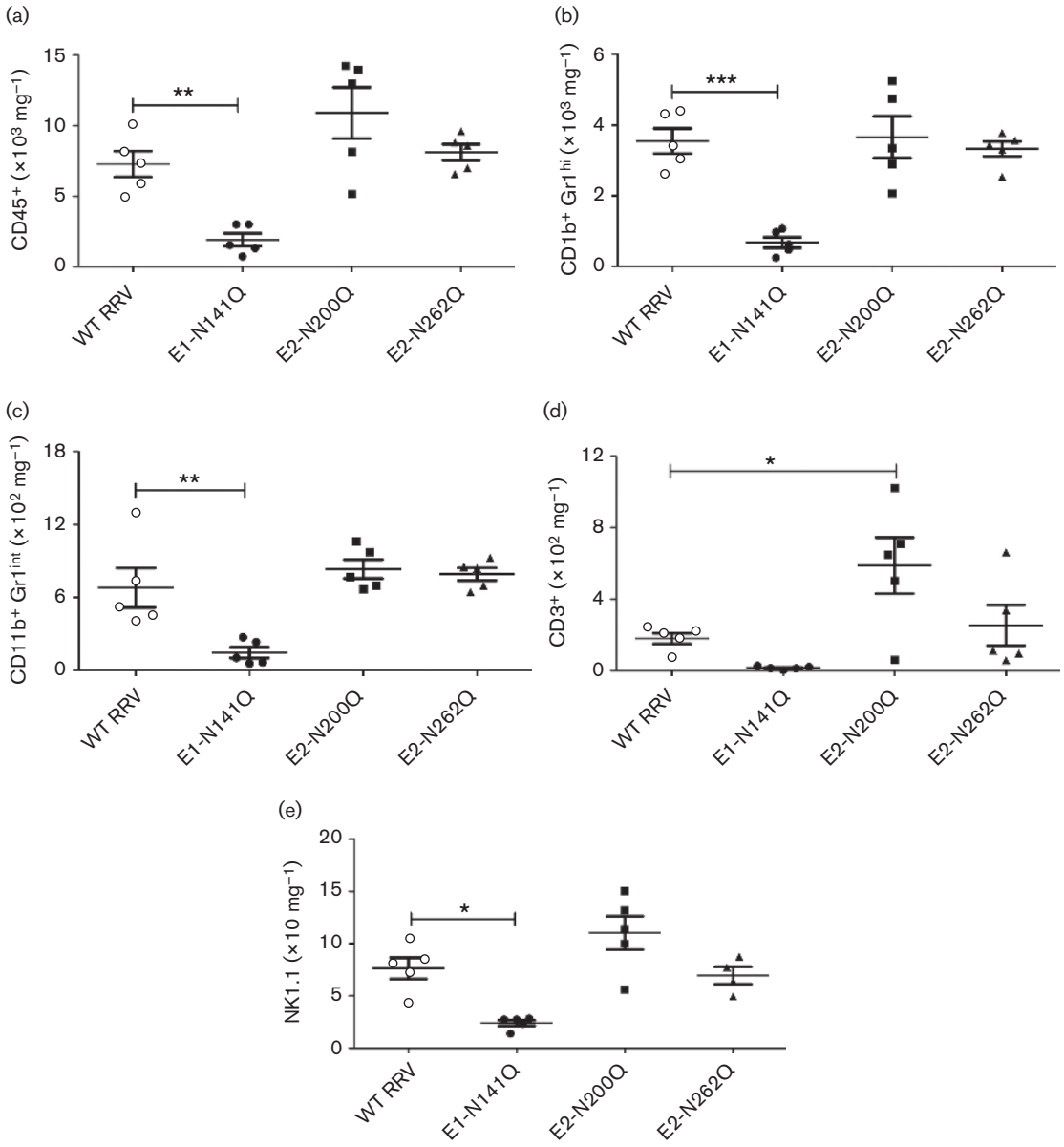


Fig. 5. Absence of the RRV E1 glycan reduces total number of infiltrating leukocytes at peak disease *in vivo*. Quadriceps muscles were removed from RRV WT- and mutant-infected mice at days 7 and 10 p.i. Cells were isolated, counted and stained for CD45, Gr1, CD11b, pan-NK/NKT, CD3 and CD19 expression. Total leukocyte ($CD45^{hi}$) (a), inflammatory monocyte ($Gr1^{hi}CD1b^{hi}$) (b), neutrophil ($Gr1^{int}CD11b^{hi}$) (c), and T-cell ($CD3^{hi}$) (d) and NK cell ($NK1.1^{hi}$) (e) populations were determined among total live (propidium iodide-negative) infiltrated cells using various gating strategies. Compared with WT-infected mice, $*P<0.05$, $**P<0.01$, $***P<0.001$ using a one-way ANOVA followed by Dunnett's post-test.

Four mosquitoes had very low levels of RRV RNA in the legs but no detectable RRV RNA in either the body or salivary gland. Levels were confirmed by retesting and, owing to the questionable nature of these results, these four mosquitoes were excluded from the analysis. Similar results have been noted elsewhere, although the reason for such observations remains unknown (Richards *et al.*, 2012).

Of the mosquitoes fed on blood containing WT RRV, 100 % were infected, with viral RNA present in their bodies (Fig. 8d). This was not significantly different from the body infection rates in mosquitoes fed on blood containing RRV that lacked the E1 (95.0 %) or the E2-262 (94.1 %) glycans; however, the body infection rate was significantly reduced in mosquitoes fed the E2-N200Q glycan (75.0 %). Disseminated infection rates were not significantly different in mosquitoes fed RRV that lacked any of the glycans (65.0–95.0 %) compared with mosquitoes fed WT RRV (84.2 %). Similarly, the salivary gland infection rates were not significantly

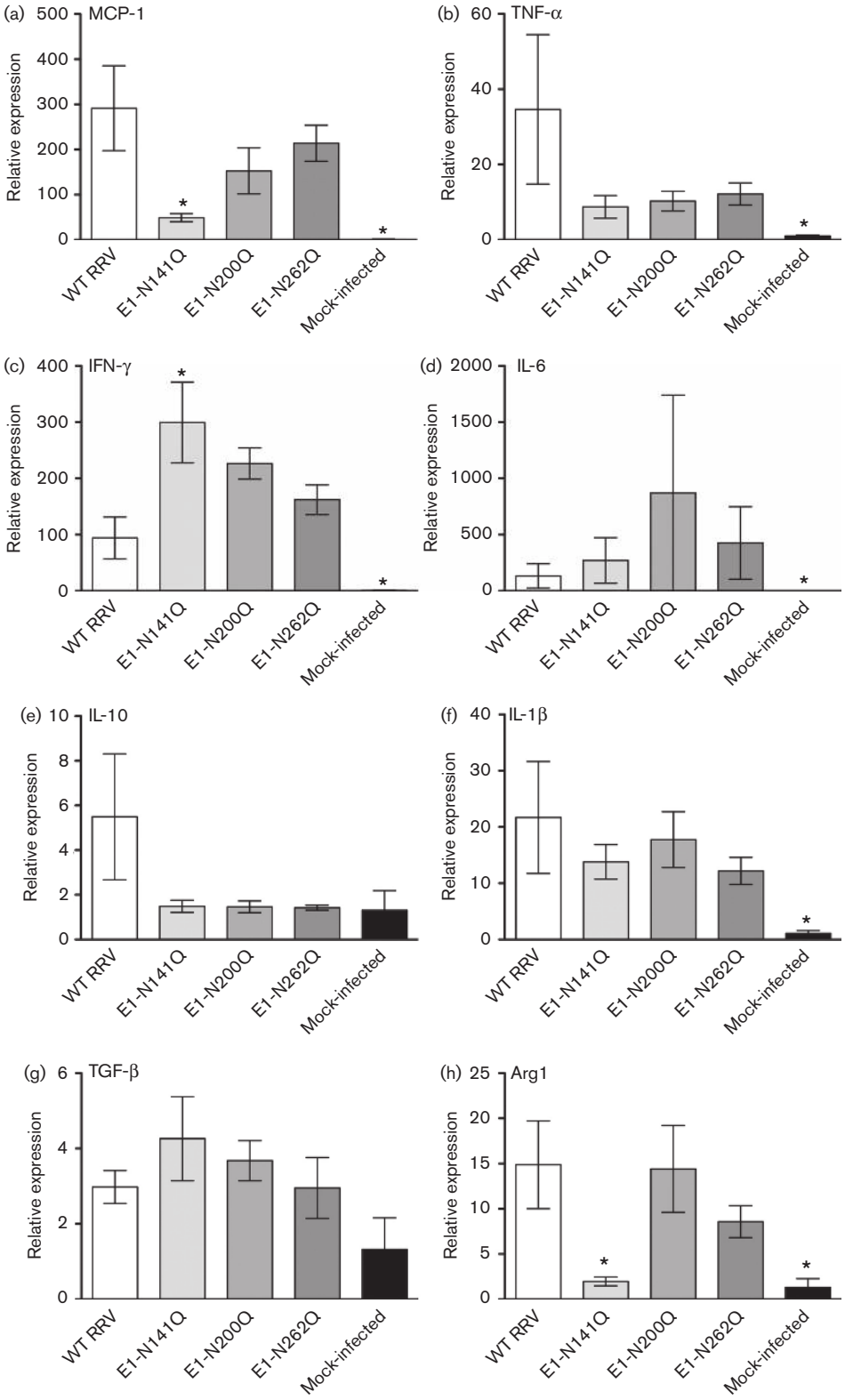


Fig. 6. RRV-induced cytokine expression is altered in the absence of the E1 glycan. C57BL/6 mice, 21 days old, were infected subcutaneously with 10^4 p.f.u. WT or mutant RRV, or mock-infected with PBS. At 10 days p.i., mice were sacrificed and total RNA was isolated from quadriceps muscle. MCP-1 (a), TNF-α (b), IFN-γ (c), IL-6 (d), IL-10 (e), IL-1β (f), TGF-β (g) and arginase 1 (h) mRNA expression was analysed by quantitative real-time PCR (qRT-PCR), with data normalized to the housekeeping gene HPRT1 and shown as relative expression. Data points represent mean$\pm$SE of four to ten mice per group. Compared with WT-infected mice, *$P<0.05$, using a one-way ANOVA followed by Dunnett's post-test.

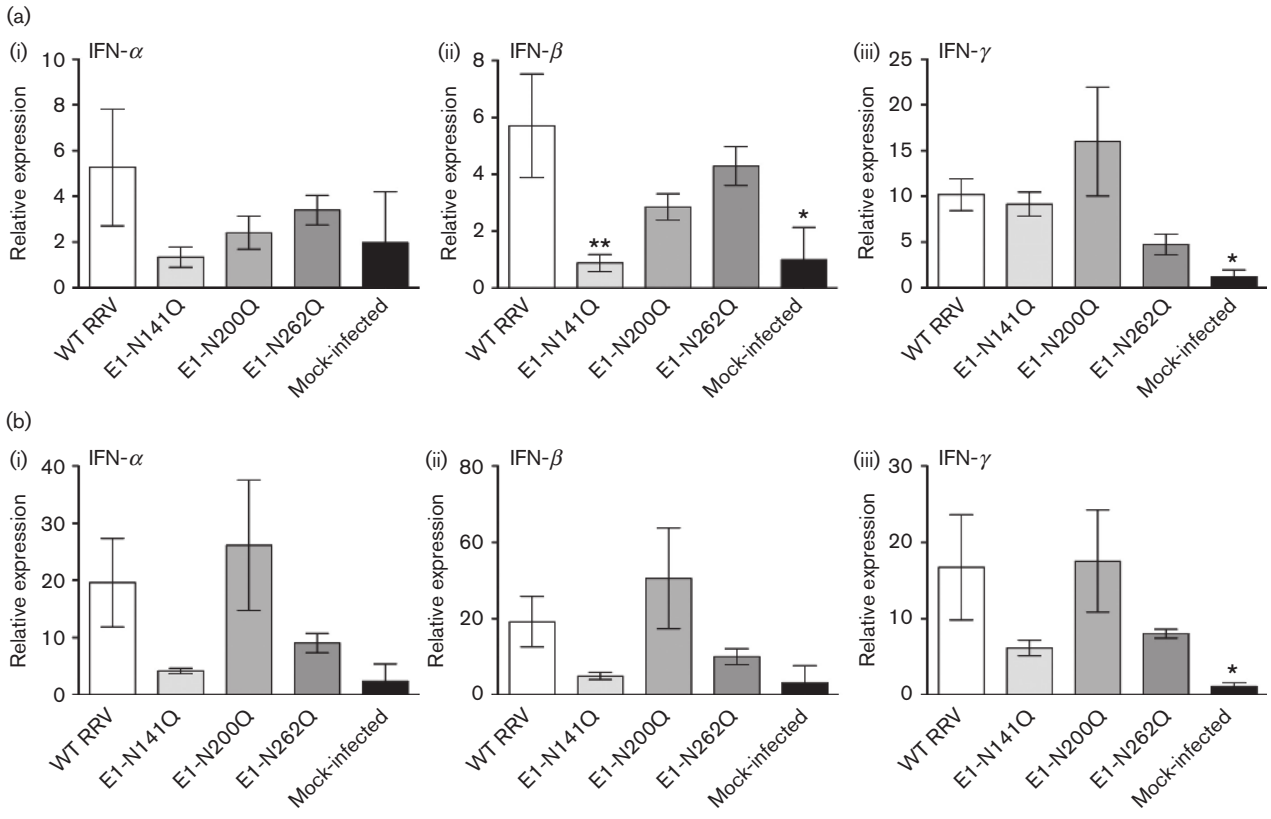


Fig. 7. RRV-induced IFN expression *in vivo* is affected by the expression of *N*-linked glycans. C57BL/6 mice, 21 days old, were infected subcutaneously with 10^4 p.f.u. WT or mutant RRV, or mock-infected with PBS. At 40 h p.i., mice were sacrificed and total RNA was isolated from quadriceps muscles (a) and ankle joints (b). IFN-α (i), IFN-β (ii) and IFN-γ (iii) mRNA expression was analysed by qRT-PCR, with data normalized to the housekeeping gene HPRT1 and shown as relative expression. Data points represent mean $\pm$ SE of four to ten mice per group. Compared with WT-infected mice, $^*P<0.05$, $^{**}P<0.01$ using a one-way ANOVA followed by Dunnett's post-test.

different in mosquitoes fed RRV that lacked any of the glycans (45.0–90.0 %) compared with mosquitoes fed WT RRV (63.2 %). No infection of bodies or legs was seen in mosquitoes fed on sheep blood alone.

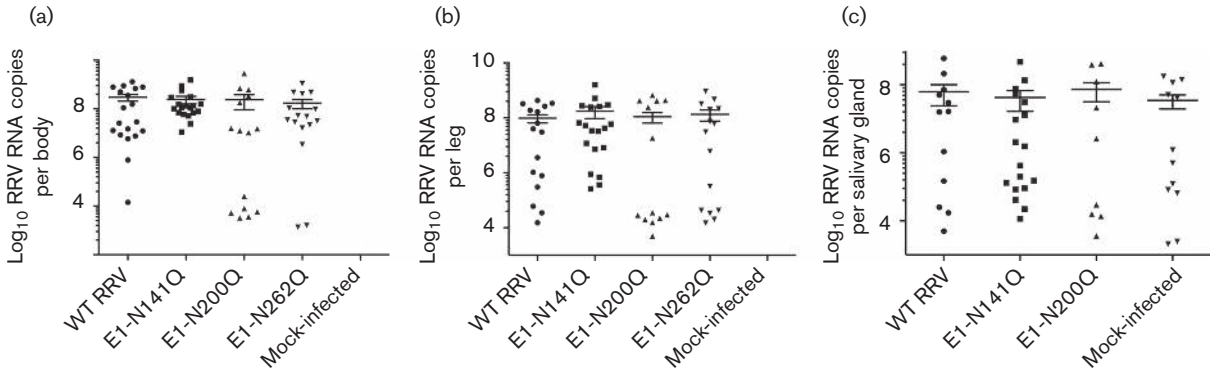
The total number of viral RNA copies present in the body, leg and salivary gland samples varied substantially within each group. As a result, no significant differences were seen in the total number of viral RNA copies within mosquito bodies, legs or salivary glands when mosquitoes fed on blood containing WT RRV were compared with those fed on blood containing any of the mutant viruses (Fig. 8).

DISCUSSION

Alphavirus envelope protein *N*-linked glycosylation plays a potential role in a number of processes, including viral replication and infectivity, and is an important determinant of both tissue tropism and host antiviral responses (Rogers & Heise, 2009). In this study, we used three single-site asparagine to glutamine glycosylation mutants to determine the role of each glycan in RRV replication *in vitro*, pathogenesis *in vivo* and replication, dissemination and transmission in the mosquito vector.

As the type of glycans expressed on the E1 and E2 glycoproteins are species-specific, we used mutants generated in both mammalian cell and mosquito cell culture to thoroughly investigate the potential of envelope glycosylation to affect viral replication *in vitro*. We found that, despite binding at similar levels to WT, the loss of *N*-linked glycans at the E1-141 site resulted in a reduction in virus output in mammalian cells only, regardless of whether the source of the virus was initially of mammalian or mosquito origin. The E1 protein directs the membrane fusion process, while E2 is postulated to function as a cell receptor-binding domain for several alphaviruses (Strauss & Strauss, 1994), which may help to explain the distinction in replication between the E1-141 mutant and the E2 mutants.

A study by Shabman *et al.* (2008) found that mosquito-derived RRV lacking E1 glycosylation displayed a similar growth defect in mammalian cells, further supporting the



(d)

	% Infected (number infected/number engorged)		
Virus	**Body**	**Legs**	**Salivary glands**
WT	100.0 (19/19)	84.2 (16/19)	63.2 (12/19)
E1-N141Q	95.0 (19/20)	95.0 (19/20)	30.0 (18/20)
E1-N200Q	75.0* (15/20)	65.0 (13/20)	45.0 (9/20)
E1-N262Q	94.1 (16/17)	88.2 (15/17)	70.6 (12/17)
Negative control	0.0* (0/16)	0.0* (0/16)	ND

Fig. 8. Viral RNA present in *A. vigilax* mosquitoes following ingestion with RRV. Mosquitoes were orally infected with 10^5 p.f.u. ml^{-1} WT or mutant RRV diluted in sheep blood or mock-infected with sheep blood alone. After 4 days, mosquitoes were harvested and viral RNA was extracted from the bodies and legs; or bodies, legs and separated salivary glands for those with disseminated infections, as defined by the presence of viral RNA in the legs. RNA was reverse transcribed and cDNA was quantified using qRT-PCR. Data are expressed as the total number of RRV RNA copies per body (a), leg (b) or salivary gland (c) or as the number of virus-positive bodies or legs compared with the total number engorged, to give the rate of infection or dissemination, respectively (d). The potential for transmission was determined by the salivary gland infection rate, expressed as the number of virus-positive salivary glands compared with the total number engorged. No significant differences were seen between sample groups using a Kruskal–Wallis test followed by Dunn's multiple comparison post-test; however, all groups had significantly higher RRV RNA than mock-infected controls for body and leg samples (a, b, c). *$P<0.05$, using Fisher's exact test (d). ND, Not done.

requirement for the glycan at E1-141 for optimal virion production. Whether the E1 glycan is important for the fusion event required to release the virus capsid and genome from the entry lysosome or for the fusion event during virus particle release is yet to be determined.

Using our well-established mouse model we have shown for the first time, we believe, that RRV envelope glycans are important in disease pathogenesis *in vivo*. To date, studies have demonstrated a role for RRV *N*-linked glycans *in vitro*, showing induction of high levels of type I IFN by RRV in myeloid dendritic cells is dependent on envelope protein complex *N*-linked glycans (Shabman *et al.*, 2008). In our study we showed that despite a reduction in replication *in vitro*, RRV lacking the complex E1-141 glycan replicated to similar peak titres in mice, with a subsequent increase in viral clearance corresponding to an increase in IFN-γ. The E1 complex glycan was required for the development of severe musculoskeletal disease, and its ablation resulted in a decrease in myositis in parallel with a decrease in arginase-1 and MCP-1. We have previously shown both arginase-1 and MCP-1 to be elevated in RRV infection. Arginase-1 has been shown to be associated with alternatively activated macrophages and tissue repair, and is elevated in RRV-infected WT mice at peak disease in a complement C3-dependent manner (Morrison *et al.*, 2008). MCP-1 is required for the development of severe alphavirus-induced musculoskeletal disease, including myositis, osteoclastogenesis and bone loss (Chen *et al.*, 2015; Rulli *et al.*, 2009). We hypothesize that the E1 complex glycan contributes to viral activation of the mammalian innate immune response, resulting in upregulation of MCP-1 and a subsequent increase in chemotaxis of inflammatory cells to sites of viral infection, as well as assisting in fibrosis and tissue repair by alternatively activated macrophages. In addition, our results confirm previous *in vitro* work demonstrating a role for alphaviral *N*-linked glycans in the production of IFN; however, our *in vivo* results suggest that E1 glycans contribute to IFN

induction more than either of the single E2 glycans. We also show that the complex sugar on the E1 glycoprotein of RRV may act to suppress IFN-γ induction as RRV E1-N141Q induced higher levels of IFN-γ *in vivo* compared with WT RRV at peak disease.

In a previous study, we showed that activation of the complement system by the mannose-binding lectin (MBL) pathway is required for RRV disease development (Gunn *et al.*, 2012). MBL deposition was increased on the surface of RRV-infected cells, and in a subsequent study we found that RRV can bind directly to MBL, most likely through a glycan/lectin (Foo *et al.*, 2015). Therefore, we hypothesize that, in this study, *N*-linked glycans contributed to disease severity by acting as ligands for MBL binding, activating complement and contributing to the activation and/or recruitment of inflammatory macrophages. However, further studies are needed to support this hypothesis and determine the exact role of RRV envelope glycans in the activation of the MBL pathway.

Arbovirus transmission from vector to vertebrate host is a crucial process that determines whether the virus will successfully establish an infection. For a virus to be transmitted, it must first infect the mosquito and multiply within the midgut (mesenteron), before escaping to disseminate throughout the rest of the body. Once outside the midgut, the virus must infect and replicate within the salivary glands, releasing virus into the saliva, and thereby enabling transmission to a host during feeding (Hardy *et al.*, 1983). For successful transmission, the virus must overcome several barriers, including the mesenteronal infection, mesenteronal escape, salivary gland infection and salivary gland escape barriers (Hardy *et al.*, 1983). Using *A. vigilax*, one of the major vectors responsible for the transmission of RRV, we showed that a loss of the E2 *N*-linked glycan at the E2-200 position reduces the ability of mosquitoes to become infected with RRV. However, once infected, the loss of the E2-200 glycan may not significantly affect the mosquito's potential to transmit RRV, as seen by the similar rate of salivary gland infection.

In the past decade there have been a number of studies focused on the role of envelope proteins in arbovirus replication in the mosquito vector and virus transmission to the mammalian host, although the majority of these have focused on mutations that are unlikely to affect glycan expression (Ashbrook *et al.*, 2014; de Lamballerie *et al.*, 2008; Jupille *et al.*, 2013; Ng & Hapuarachchi, 2010; Silva *et al.*, 2014; Tsetsarkin & Weaver, 2011). A number of studies, however, have revealed a role for virus glycosylation in both mammalian virulence and transmission via the mosquito vector. Studies on West Nile virus have shown envelope-glycosylated strains have increased neuroinvasiveness (Beasley *et al.*, 2005; Shirato *et al.*, 2004) and that removal of glycosylation results in a significantly attenuated virus that may serve as a vaccine candidate (Whiteman *et al.*, 2010). Similar to our findings, removal of viral envelope protein glycans decreased the vector competence of West Nile virus in *Culex tarsalis* and *Culex pipiens* mosquitoes (Moudy *et al.*, 2009; Van Slyke *et al.*, 2013). A recent structural study by Crispin *et al.* (2014) found that the alphavirus E1 glycan is heavily affected by the host species whereas the glycan processing of E2 is more restricted, displaying heterogeneous glycosylation. Further studies are required in order to determine if the differences observed in this study can be partially explained by the difference in glycan processing of the E1 versus E2 glycoproteins.

In summary, RRV *N*-linked glycans are critical determinants of mammalian virulence *in vivo* and viral replication in the mosquito vector. Loss of the E1 glycan (whether the pauci-mannose or the complex oligosaccharide) resulted in reduced production of infectious virions *in vitro* and a reduction in disease severity *in vivo*. This reduction suggests a critical role for the E1 glycan in virion production and in inducing RRV-associated musculoskeletal disease, which in part appears to be due to its role in stimulating the production of MCP-1 and suppressing the production of IFN-γ. Loss of the E2 glycans had little effect on virus replication and virulence in the mammalian host; however, a loss of glycosylation at E2-200 resulted in a significant reduction in the infectivity of *A. vigilax*, thereby potentially affecting its efficiency as a vector for RRV transmission. Despite this reduction, no differences were observed in the rate of salivary gland infection, and therefore additional studies are required to fully assess the role of the E2-200 glycan in RRV transmission by the *A. vigilax* vector. Overall, the results of this study demonstrate that viral glycans play a critical role in the pathology of RRV disease and in transmission by the mosquito vector, suggesting viral glycans as a potential target for the development of therapeutic intervention.

METHODS

Cells and viruses. Vero cells (ATCC CCL-81) were grown at 37 °C with a 5 % CO_2 atmosphere in Opti-MEM I Reduced-Serum Medium supplemented with 3 % FCS. C6/36 *Aedes albopictus* mosquito cells (ATCC CRL-1660) were grown at 29 °C with a 5 % CO_2 atmosphere in RPMI 1640 supplemented with 10 % FCS.

WT (T48 strain) RRV was generated from the full-length T48 cDNA clone, kindly provided by Dr Richard Kuhn (Kuhn *et al.*, 1991). The full-length cDNA clone was also used to generate recombinant strains of RRV, each containing a single mutation at one of the three viral envelope *N*-linked glycosylation sites. Asparagine (N) residues at either E1-141, E2-200 or E2-262 were converted to glutamine (Q) residues using site-directed mutagenesis, with mutations confirmed by sequence analysis.

WT and mutant cDNA clones were linearized by *Sac*I digestion, and full-length RNA transcripts were generated by *in vitro* transcription using SP6 RNA polymerase (Ambion). All transcripts were transfected into Vero cells by electroporation, and supernatants were collected after 24 h. Supernatants were either stored at −80 °C or concentrated through a 20 % (w/v) sucrose cushion at 75 000 ***g***, 4 °C for 5 h and resuspended in PBS before storage. Viral titres were determined by standard plaque assay on Vero cells, in which 10-fold serial dilutions of supernatants were added to Vero cell monolayers and incubated for

1 h at 37 °C, 5 % CO_2. Virus dilutions were then removed and cells were overlaid with growth medium containing 1 % agarose. After 48 h incubation at 37 °C, 5 % CO_2, semi-solid medium was removed and cells were visualized with 0.1 % crystal violet in 20 % ethanol. Titres were expressed as p.f.u. ml^{-1}. To confirm that each of the introduced mutations corresponded with a loss of glycosylation site, concentrated virions were analysed using SDS-PAGE, with all showing an E1 or E2 mobility shift indicative of the loss of an *N*-linked glycan (data not shown).

Unless otherwise stated, experiments were carried out using virus obtained from mammalian cell culture (Vero); however, some were carried out using virus obtained from mosquito cell culture. For these viral stocks, mammalian cell-derived virus was passaged through C6/36 cells using an m.o.i. of 0.1. After 24 h, virus was harvested, concentrated, titrated and stored as described for mammalian cell-derived virus.

Viral replication *in vitro*. For *in vitro* replication studies, Vero and C6/36 cells were infected at an m.o.i. of 0.1. Virus was suspended in serum-free growth medium and added to cells for 1 h at either 37 °C (Vero) or 29 °C (C6/36). Following the attachment phase, virus was removed, cells were washed three times with PBS, and 1 ml fresh, serum-containing growth medium was added. At 0, 2, 4, 6, 12, 24 and 48 h p.i., supernatant was collected and cell monolayers were washed three times with a final volume of 450 µl PBS. Supernatant and washes were pooled, and centrifuged to remove any remaining cells, and viral titres were determined by plaque assay on Vero cells.

Viral infection of mice. C57BL/6 mice were obtained from the Animal Resource Centre (Perth, Australia) and bred in-house. All animal experiments were performed according to methods and conditions approved by the Animal Ethics Committee of Griffith University. Twenty-day-old mice with equal sex distribution were inoculated subcutaneously in the thorax below the right forelimb with 10^4 p.f.u. WT or mutant virus diluted in PBS to a final volume of 50 µl. Mock-infected mice were inoculated with 50 µl PBS as a negative control. Mice were weighed and examined daily for signs of disease, scoring disease symptoms based on animal strength and hind-leg paralysis, as described previously (Morrison *et al.*, 2006), according to the following scale: 0, no disease signs; 1, ruffled fur; 2, very mild hind-limb weakness; 3, mild hind-limb weakness; 4, moderate hind-limb weakness and dragging of hind-limbs; 5, severe hind-limb weakness/dragging; 6, complete loss of hind-limb function; 7, moribund; and 8, death.

Infections with RRV mutants that differed significantly from that seen following WT RRV infection were further investigated to assess viral clearance. At 1, 3 and 5 days p.i., serum, quadriceps muscle and ankle joint were harvested and weighed, the tissues were homogenized in 1 ml PBS, and the amount of infectious virus was determined by plaque assay on Vero cells [expressed as p.f.u. $(g\ tissue)^{-1}$].

Histology. To assess inflammation, mice were sacrificed at 10 days p.i. and perfused with either 4 % paraformaldehyde (PFA) or PBS. For histology, quadriceps muscles were collected and fixed in 4 % PFA before paraffin-embedding. Five-micrometre sections of tissues were prepared and stained with H&E (QIMR Histochemistry Laboratory).

Detection of leukocyte infiltrates in quadriceps. Quadriceps muscles were removed and processed as described previously (Herrero *et al.*, 2013). In brief, muscles were harvested, digested with buffer containing 2 mg collagenase IV (Worthington)ml^{-1}, 0.1 mg DNase I (Sigma) ml^{-1} and RMPI containing 10 % FCS, then filtered with 70 µm (Corning Falcon) cell strainers and counted with trypan blue to determine viable population. Non-specific binding to Fc receptors was blocked by incubating samples with anti-mouse FcγRII/III (clone 2.4G2; BD Pharmingen) and cells were then stained with a panel of fluorochrome-conjugated anti-mouse antibodies inclusive of CD45-PE-Cy5 (clone 30-F11; eBioscience), NK1.1-PE-Cy7 (clone PK136; eBioscience), $CD3_\varepsilon$-FITC (clone 145-2C11; BD Pharmingen), CD11b-FITC (clone M1/70; BD Pharmingen) and Ly-6C-APC (clone HK1.4; eBioscience) before being read on a CyAn ADP flow cytometer (Beckman Coulter). All subsequent post-analyses were performed using the Kaluza program.

Real-time PCR. For cytokine and viral RNA analysis, total RNA was isolated from quadriceps muscle or ankle joints using TriReagent (Ambion) according to the manufacturer's instructions. RNA was reverse transcribed using random primers (Promega) and cDNA was generated with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Sigma). Quantitative real-time PCR (CFX96 Touch; Bio-Rad) was performed on 50 ng cDNA using commercially available QuantiTec primers for MCP-1, TNF-α, IFN-γ, IL-6, arginase 1, IL-10, IL-1β, IFN-α and IFN-β (Qiagen) or primers purchased from Sigma-Aldrich with the sequences TGF-β F: 5′-CAACGCCATCTATGAGAAAACC-3′, and TGF-β R: 5′-AAGCCCTGTATTCCGTCTCC-3′ with FastStart SYBR Green Master Mix (Roche).

Viral infection in mosquitoes. Three-day-old *A. vigilax* mosquitoes were orally infected with WT or mutant virus diluted in sheep blood to give a final concentration of 1.6×10^5 p.f.u. ml^{-1}. Mosquitoes fed on sheep blood containing no virus were used as a negative control. Mosquitoes were allowed to feed through a porcine intestinal membrane for 1.5 h, and fully engorged females were held under insectary conditions (27 °C, 70 % relative humidity, photoperiod of 12 h L : 12 h D with 1 h crepuscular periods, fed on a 15 % sucrose solution) for 4 days. The legs and bodies of each mosquito were then snap-frozen separately in 140 µl grinding medium (RPMI with L-glutamime, GlutaMAX, 5 % FBS, 2 % penicillin/streptomycin, 0. 1 % Fungizone) and stored at −80 °C until processing.

To detect virus, samples were thawed on ice, and homogenized using a motorized micropestle, and viral RNA was extracted using a QIAamp Viral RNA Mini kit (Qiagen) with a DNase set (Qiagen) to remove mosquito genomic DNA, according to the manufacturer's protocol. RNA was reverse transcribed using random primers (Promega) and cDNA was generated with M-MLV Reverse Transcriptase (Sigma). Viral cDNA was quantified by real-time PCR (Bio-Rad) with a primer set targeted against the Nsp4 gene region (F, 5′-ACCCGACAGTGGCTAGTTAC-3′; R, 5′-CGGTTGGTGGTAAGCATGAT-3′), using FastStart SYBR Green Master Mix (Roche). Quantification was achieved by comparing against a standard curve generated from the T48 infectious clone, with a limit of detection of 64 copies per qPCR run, equivalent to a total of 1400 copies of viral RNA in the original mosquito sample.

Mosquito legs were initially analysed, with those containing viral RNA indicating a disseminated infection. Intact bodies from non-disseminated infections were analysed for the presence of viral RNA in order to confirm initial infection. For samples with disseminated infections, salivary glands were dissected from bodies and processed separately from the body remnants for the presence of viral RNA, with the presence of viral RNA in the salivary gland indicating the ability of the virus to be transmitted. The mosquito body infection rate was determined as the number of virus-positive bodies compared with the total number engorged; the rate of dissemination was determined as the number of virus-positive legs compared with the total number engorged; and the rate of salivary gland infection was determined as the number of mosquitoes with virus-positive salivary glands compared with the total number engorged.

Statistical analysis. Viral replication *in vitro*, mouse weight gain and *in vivo* viral clearance were analysed using a two-way ANOVA followed by a Bonferroni post-test, and cytokine gene expression was

analysed using a one-way ANOVA followed by Dunnet's post-test. Clinical score data were analysed using a Mann–Whitney test. In mosquitoes, the body infection, dissemination and salivary gland infection rates were analysed using Fisher's exact test, and viral RNA quantity was analysed using a Kruskal–Wallis test followed by a Dunn multiple comparison post-test. All statistics were carried out using GraphPad Prism 5.02. A P value of ≤ 0.05 was considered to be statistically significant.

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