



Interferon-alpha/beta deficiency greatly exacerbates arthritogenic disease in mice infected with wild-type chikungunya virus but not with the cell culture-adapted live-attenuated 181/25 vaccine candidate

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ABSTRACT

In humans, chikungunya virus (CHIKV) infection causes fever, rash, and acute and persisting polyarthralgia/arthritis associated with joint swelling. We report a new CHIKV disease model in adult mice that distinguishes the wild-type CHIKV-LR strain from the live-attenuated vaccine strain (CHIKV-181/25). Although eight-week old normal mice inoculated in the hind footpad developed no hind limb swelling with either virus, CHIKV-LR replicated in musculoskeletal tissues and caused detectable inflammation. In mice deficient in STAT1-dependent interferon (IFN) responses, CHIKV-LR caused significant swelling of the inoculated and contralateral limbs and dramatic inflammatory lesions, while CHIKV-181/25 vaccine and another arthritogenic alphavirus, Sindbis, failed to induce swelling. IFN responses suppressed CHIKV-LR and CHIKV-181/25 replication equally in dendritic cells in vitro whereas macrophages were refractory to infection independently of STAT1-mediated IFN responses. Glycosaminoglycan (GAG) binding may be a CHIKV vaccine attenuation mechanism as CHIKV-LR infectivity was not dependent upon GAG, while CHIKV-181/25 was highly dependent.

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Introduction

First recognized in 2005, a pandemic of mosquito-borne chikungunya (CHIK) fever of unprecedented magnitude has been afflicting inhabitants of the Indian Ocean territories. An estimated six million people have been infected in a large geographic area encompassing the East-African equatorial coasts, the Indian Ocean islands of La Réunion, Madagascar, Mauritius and the Seychelles, most of the Indian peninsula, Singapore, and Thailand (Powers, 2010; Staples et al., 2009). Many CHIK fever cases have been diagnosed in travelers returning home to Europe and the Americas from known outbreak areas (Tilston et al., 2009). In Southern Italy and France, local disease transmission has resulted, controlled by intensive mosquito abatement (Gould et al., 2010; Nicoletti et al., 2008; ProMED-mail, 2011). Since 2006, 106 laboratory-confirmed CHIK cases have occurred in the United States and public health officials have been warned to be vigilant for evidence of local transmission by indigenous mosquitoes; however to-date all cases appear to be travel-associated (Gibney et al.,

2011). Disturbingly, chikungunya virus (CHIKV), the etiologic agent of CHIK fever, has adapted to allow transmission by a more promiscuously biting mosquito vector, *Aedes albopictus* (Reiter et al., 2006), by the mutation of a single amino acid in the E1 envelope protein (Tsetsarkin et al., 2007, 2009). This has facilitated human-mosquito-human spread without the necessity for non-human primate (NHP) reservoir hosts and lifted a major restriction on endemic establishment.

CHIKV, an enveloped positive-sense RNA virus in the genus *Alpha-virus*, family *Togaviridae*, was first isolated during an explosive epidemic in Tanzania in 1952. Infection is characterized by the abrupt onset of fever, headache and skin rash, almost invariably accompanied by excruciating myalgia and polyarthralgia with swollen ankles, knees and wrists (Powers and Logue, 2007). Higher viremic titers in the acute phase have been associated with lymphopenia, monocytopenia, neutrophilia, CD8⁺ T cell activation and higher level induction of inflammatory cytokines including interferon-alpha (IFN-α) and IFN-γ (Ng et al., 2009; Wauquier et al., 2011). Although infection is usually self-limiting and patients recover within 2 weeks, painful polyarthralgia can persist long after other symptoms disappear, shifting from joint to joint with possible post-illness destructive arthropathy (Brighton and Simson, 1984). Chronic polyarthralgia is associated with elevated serum IL-6 and GM-CSF (Chow et al., 2011). CHIK fever is rarely fatal in the absence of underlying complications, but the

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severity of infection is strongly age-dependent with febrile convulsions observed in infected children, associated with neurologic sequelae and occasional mild to severe hemorrhagic manifestations (Brighton and Simson, 1984; Halstead et al., 1969a, b; Nimmannitya et al., 1969).

Although no licensed vaccine is available, a live-attenuated CHIKV vaccine candidate was produced two decades ago at USAMRIID by performing 18 serial passages of wild-type CHIKV-15561 strain on human MRC-5 fibroblasts (Levitt et al., 1986). CHIKV-181/25 (TSI-GSD-218) was selected as the vaccine seed based upon homogeneous small-plaque phenotype, avirulence in suckling mice, reduced viremia in NHPs, and genetic stability. Furthermore, a pilot lot of the vaccine elicited neutralizing antibody, and protected mice and NHPs against challenge (Levitt et al., 1986). Except for transient arthralgia in approximately 8% of human vaccinees, this live-attenuated vaccine was safe, produced well-tolerated side-effects, and was highly immunogenic in Phase II clinical trial (Edelman et al., 2000). However, the molecular mechanism(s) underlying attenuation have not been defined.

Many years after CHIKV pathogenesis was first studied in rodents (Weiss et al., 1965), investigators have revisited murine models of CHIKV infection and disease in response to the ongoing pandemic to understand the pathophysiology of CHIKV infection and reveal determinants of disease severity and attenuation. The susceptibility of immunocompetent mice to wild-type CHIKV is strongly age-dependent, reminiscent of studies with wild-type strains of related arthritogenic Old World alphaviruses, Sindbis (SINV; (Klimstra et al., 1999; Ryman et al., 2007a)), Semliki Forest (SFV; (Fleming, 1977)), and Ross River (RRV; (Seay et al., 1981)). Neonatal mice are highly susceptible to CHIKV-induced fatality (Couderc et al., 2008; Weiss et al., 1965; Ziegler et al., 2008), but as mice mature average survival times (ASTs) and survival rates quickly rise (Couderc et al., 2008; Ziegler et al., 2008). Similar to SINV (Ryman et al., 2000, 2007b) and SFV (Fragkoudis et al., 2007), STAT1-dependent type I interferon (IFN- α/β) responses protect adult mice from fatal CHIKV infection initiated by peripheral inoculation (Couderc et al., 2008; Schilte et al., 2010). However, these severe, fatal infections likely have little relevance to the majority of CHIK fever and polyarthritis/polyarthralgia cases in humans.

It has recently been shown that adult (six-week-old) C57BL/6 mice develop mild musculoskeletal inflammatory disease following wild-type CHIKV infection with some striking similarities to human infection (Gardner et al., 2010). Moreover, younger C57BL/6 animals (two-week-old) develop more severe inflammatory disease, with evidence of arthritis, tenosynovitis and myositis, and viral persistence (Morrison et al., 2011). Here, we demonstrate for the first time that clinical signs of CHIKV-induced musculoskeletal disease are tremendously exacerbated in the absence of STAT1-dependent and type I IFN receptor signaling-dependent responses, leading to polyarthritis/myositis, with edema in the hind limbs, correspondingly increased CHIKV replication in joint-associated tissues, tissue pathology and inflammatory cell infiltration. Interestingly, severe inflammatory disease manifestations are specific to wild-type CHIKV infection and not observed in animals infected with SINV or with the live-attenuated CHIKV-181/25 vaccine strain, suggesting a pathology specific to wild-type CHIKV infection. Our findings support the contention that relative IFN- α/β response deficiency may underlie severity of human articular disease, whether the result of host genetics or the virus' ability to evade/antagonize IFN- α/β responses (Fros et al., 2010).

Although clinical signs of CHIKV-181/25 infection become more severe in the absence of STAT1-dependent IFN responses, increased sensitivity to IFN-mediated antiviral responses does not appear to be the primary mechanism of severe inflammatory disease amelioration as proposed by Partidos et al. (2011). Instead, we propose that the acquisition of positive-charge mutation(s) in the E2 glycoprotein leading to glycosaminoglycan (GAG)-mediated cell-surface attachment may, at least in part, underlie amelioration of musculoskeletal disease, inflammatory pathologies, and virus replication.

Results

STAT1-dependent IFN signaling is critical for protection of mice against wild-type CHIKV infection, but not for attenuating the CHIKV-181/25 vaccine strain

Innate immune responses, particularly those mediated by type I IFN, are known to be pivotal for the protection of mice against fatality following peripheral inoculation of wild-type Old World alphaviruses (Ryman and Klimstra, 2008), including SINV (Ryman et al., 2000, 2002, 2007b), SFV (Fragkoudis et al., 2007) and CHIKV (Couderc et al., 2008; Schilte et al., 2010). To further investigate this phenomenon and extend the observations to type II IFN responses, we compared the independent and combined ability of IFN- α/β and IFN- γ receptor-mediated signaling to protect mice from morbidity and mortality caused by wild-type CHIKV. Two cDNA clone-derived, minimally cell culture-passaged, CHIKV strains were used: CHIKV-LR (from a traveler returning from La Réunion, 2006; Tsetsarkin et al., 2006) and CHIKV-37997 (from a Senegalese mosquito, 1983; Vanlandingham et al., 2005), to ensure the phenotype observed was not virus strain-dependent. Groups of eight-week-old control mice (WT129), mice lacking receptors for IFN- α/β (A129), IFN- γ (G129) or both IFNs (AG129), or mice deficient in the STAT1 signal transduction factor involved in signaling of both type I and type II IFN responses (STAT129) were inoculated subcutaneously in one hind-leg footpad with 10^3 plaque-forming units (pfu) of either CHIKV-LR or CHIKV-37997 virus (Fig. 1). Inbred, immunocompetent WT129 mice exhibited no mortality (Figs. 1A–B) or clinical signs of infection (Figs. 1D–E) following inoculation with either virus. Furthermore, although Gardner et al. (2010) showed that IFN- γ is rapidly induced in CHIKV-infected mice, in the absence of IFN- γ signaling, G129 mice remained resistant to CHIKV-induced morbidity/mortality and were indistinguishable from WT129 controls (Figs. 1A–B; D–E).

In striking contrast, A129 mice lacking IFN- α/β receptor-mediated signaling were highly susceptible to infection with either CHIKV-LR (Fig. 1A) or CHIKV-37997 (Fig. 1B), consistent with the results obtained by Couderc et al. for the biological CHIKV-LR isolate (Couderc et al., 2008). These animals displayed clinical signs of illness within 2 days post-infection (d p.i.) including lethargy, rapid weight loss (Figs. 1D–E), hunched posture, ruffling of the fur, and generalized cachexia. CHIKV-infected A129 mice deteriorated quickly, typically reaching euthanasia criteria within 4 d p.i. We further showed that the combined lack of type I and II IFN receptor-mediated signaling in AG129 mice significantly reduced the AST ($p < 0.05$ versus A129; Figs. 1A–B), suggesting a compensatory role for IFN- γ in the absence of IFN- α/β , similar to our prior observations for SINV infection (Ryman et al., 2007b). Interestingly, mice deficient in STAT1-dependent IFN responses had a significantly extended AST ($p < 0.005$ versus A129 or AG129), surviving several days beyond CHIKV-infected A129 or AG129 animals, but eventually reached euthanasia criteria around 7 d p.i. (Figs. 1A–B; D–E). We infer that substantial IFN-mediated anti-CHIKV activity remains in the STAT129 animals, although the STAT1-dependent IFN- α/β responses are critical for protection. No clinical signs of neurologic disease were apparent even in moribund animals.

To determine the contribution of STAT1-dependent IFN signaling to the attenuation of the CHIKV-181/25 vaccine strain, eight-week-old WT129, A129, and STAT129 mice were infected with 10^3 pfu of CHIKV-181/25 subcutaneously in the hind footpad. No mortality (Fig. 1C), weight loss (Fig. 1F), or ruffling of the fur were observed in CHIKV-181/25-infected WT129 or A129 mice. Interestingly, STAT129 and AG129 mice were more susceptible to CHIKV-181/25 infection than the A129 mice with substantial weight loss in all infected mice (Fig. 1F) and 50% or 100% mortality rates, respectively (Fig. 1C). The AST of CHIKV-181/25-infected STAT129 and AG129 mice that succumbed to infection was significantly extended compared to mice infected with wild-type CHIKV ($p < 0.005$), corresponding to a 1–2 day

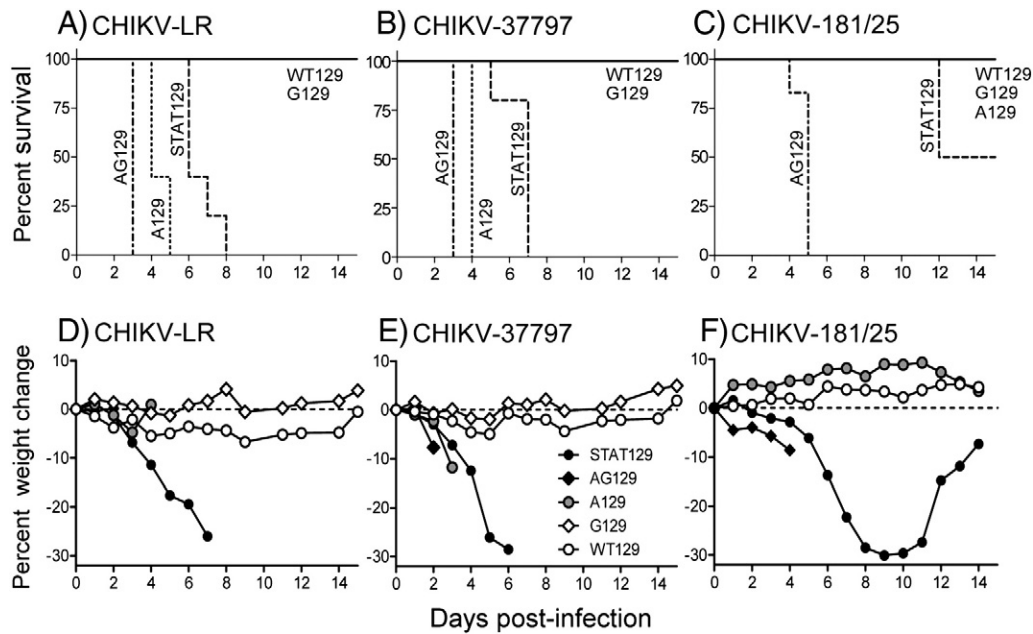


Fig. 1. Morbidity and mortality following subcutaneous infection of IFN-deficient mice with wild-type or live-attenuated CHIKV strains. WT129 (solid line/open circle); G129 (solid line/open diamond); A129 (dotted line/gray circle); AG129 (dot-dash line/closed diamond); STAT129 (dashed line/closed circle) mice were inoculated subcutaneously in the hind foot-pad with 10^3 pfu of CHIKV-LR (A and D), CHIKV-37997 (B and E) or CHIKV-181/25 (C and F). Percent survival was calculated (A–C) and daily weight change was plotted (D–F) as an indicator of morbidity (percent weight change compared to day 0). $n = 4$ or 5 mice per treatment group and data are representative of two to three independent experiments.

delay in weight-loss kinetics. We infer that the lethality of the CHIKV-181/25 vaccine strain is attenuated primarily by mechanism(s) independent of IFN- α/β sensitivity; however, the severity of this virus infection is increased in the absence of STAT1-dependent IFN pathways in our studies and even further in the combined absence of IFN- α/β and IFN- γ receptor-mediated signaling in keeping with the observations of Partidos et al. (2011), implicating IFN- γ in protection from lethality primarily via STAT1-dependent pathways in the absence of IFN- α/β activity.

Disruption of STAT1-dependent type I IFN-mediated responses exacerbates joint-associated swelling following wild-type CHIKV-LR infection

It has been previously reported that six-week-old C57BL/6 mice develop a mild swelling of the ipsilateral (inoculated) hind footpad following subcutaneous inoculation with wild-type CHIKV (Gardner et al., 2010), with increased severity in younger animals (Morrison et al., 2011). However, no swelling of the contralateral hind footpad was detectable in either study. Perhaps not unexpectedly, we detected no swelling in the ipsilateral or contralateral hind footpads of eight-week-old WT129 or G129 mice following subcutaneous inoculation with CHIKV-LR (Fig. 2B). However, in infected A129 mice swelling commenced within 24 h p.i. in the inoculated footpad, progressing to involve the entire hind limb (Fig. 2B) including the toe, ankle, and knee joints. Moreover, contralateral footpads in CHIKV-LR-infected A129 animals were significantly swollen by 48 h p.i. compared with the mock-inoculated footpads of uninfected controls (Fig. 2C). CHIKV-LR-infected STAT129 mice developed swelling of the ipsilateral (Figs. 2A and B) and contralateral (Fig. 2C) hind limbs with similar kinetics to the A129 mice, persisting until these animals reached euthanasia criteria. In both CHIKV-LR-infected A129 and STAT129 mice, the swelling appeared to cause considerable discomfort, evidenced by altered gait and reduced tendency to grip with the hind feet, suggestive of a polyarthralgia. CHIKV-37997 infections were indistinguishable from CHIKV-LR (data not shown). To our knowledge, this is the first description of disseminated CHIKV-induced joint inflammation

and signs of polyarthralgia in mice, progressing beyond the inoculated limb and more accurately reproducing human symptoms.

In terms of morbidity and mortality, the virulence phenotype of another arthritogenic Old World alphavirus, SINV, in IFN-deficient mice (Ryman et al., 2000, 2005), is very similar to our findings for CHIKV. To determine whether or not fatal SINV infection of A129 or STAT129 mice was associated with arthralgia, we inoculated these mice with 10^3 pfu of wild-type SINV strain TR339 subcutaneously in the hind footpad. TR339 infection caused no detectable hind footpad swelling in the infected mice, although mice rapidly lost weight and infection was fatal within 3–4 d p.i. (data not shown). Thus, joint-associated swelling is not a ubiquitous trait of severe Old World alphavirus infection in the IFN-deficient models, but is specific to CHIKV infection, whereas lethality is common to different virus infections (Couderc et al., 2008; Frangkoudis et al., 2007; Ryman et al., 2000, 2005) and likely disconnected from severity of arthropathy.

To determine whether the attenuated phenotype of the CHIKV-181/25 vaccine strain in IFN-deficient animals extends to amelioration of clinical signs of joint inflammation and musculoskeletal disease in mice, the hind footpads of CHIKV-181/25-infected WT129, A129 and STAT129 mice were monitored. As observed in WT129 mice infected with wild-type CHIKV, no clinical signs of arthralgia were detectable in CHIKV-181/25-infected WT129 mice (Fig. 2D). In contrast to wild-type infection, no evidence of hind footpad swelling was measurable in CHIKV-181/25 infection of A129 or STAT129 mice and only mild swelling was observed in CHIKV-181/25-infected AG129 animals, including those STAT129 and AG129 animals for which the infection was fatal (Fig. 2D). From these data we infer that selection of the CHIKV-181/25 vaccine strain by MRC-5 fibroblast passage has dramatically attenuated the tendency to cause swelling of the limbs, and that altered sensitivity to IFN responses is not responsible for this phenotype. Furthermore, we found that three weeks after “immunization” with CHIKV-181/25, A129 and surviving STAT129 mice were completely protected not only against generalized morbidity and death following subcutaneous challenge with 10^3 pfu CHIKV-LR in the hind footpad, but also against the development of disseminated joint swelling.

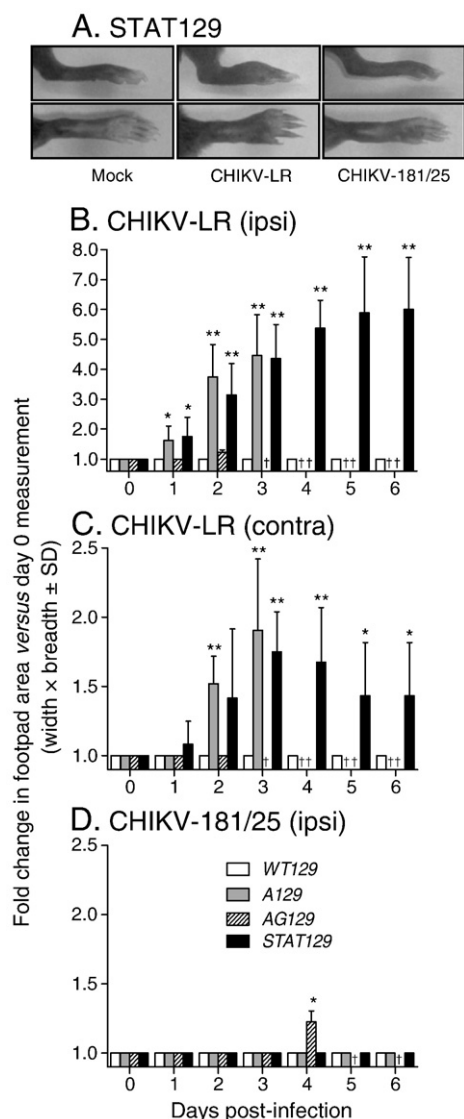


Fig. 2. Hind limb swelling following subcutaneous infection of IFN-deficient mice with wild-type or live-attenuated CHIKV strains. WT129 (white bar); A129 (gray bar); AG129 (hatch bar); and STAT129 (black bar) mice were inoculated subcutaneously in one hind foot-pad (ipsilateral) with 10^3 pfu of CHIKV-LR (A, B and C) or CHIKV-181/25 (A and D). Ipsilateral footpads of mock- and virus-infected STAT129 mice were photographed (A). Swelling in ipsilateral (B) and contralateral (C) footpads of CHIKV-LR-infected mice and ipsilateral footpads of CHIKV-181/25-infected mice (D) was measured with calipers (width $\times$ height = area) and calculated as fold-change from preinfection area $\pm$ standard deviation (SD). Cross symbol indicates that animals had died prior to this time-point. * $p < 0.05$; ** $p < 0.01$ compared to mock-inoculated, uninfected control. $n = 4$ or 5 mice per treatment group and data are representative of two to three independent experiments.

Joint-associated pathology is exacerbated in wild-type CHIKV infection and/or STAT1-dependent IFN response deficient mice

In hind limb sections from the infected limbs of eight-week-old WT129 mice at 5 d p.i., CHIKV-LR-infected and CHIKV-181/25 vaccine-infected animals showed little evidence of edema or other gross inflammatory changes in comparison with mock-infected animals (Figs. 3A–C). This was consistent with the lack of detectable swelling after footpad measurement (Fig. 2B). However, with CHIKV-LR-infected WT129 animals, occasional polymorphonuclear and mononuclear infiltrates were observed in the hypodermis and associated with skeletal muscle and tendons (Fig. 3C). After infection of STAT129 mice, the vaccine strain caused slight thickening of the dermis with mild edema (Fig. 3D versus E), minor protein (likely

fibrin) deposition limited to the hypodermis (Fig. 3H) and marked infiltration of polymorphonuclear and mononuclear cells into the dermis, hypodermis, peri-muscle and peri-tendon areas (Figs. 3E and H). Notably, CHIKV-LR-infected STAT129 animals exhibited edema and striking, disseminated protein deposition in the dermis, hypodermis, and underlying tissues associated with inflammatory cell infiltration (Figs. 3F and I). Erythrocytes were often observed in the protein depositions indicative of local hemorrhage. Interestingly, the inflammatory cell infiltration in CHIKV-181/25-infected STAT129 mice appeared to be similar (Fig. 3E versus F) or perhaps greater (Fig. 3H versus I) than that observed with CHIKV-LR, yet swelling was undetectable by morphometric analysis (Fig. 2D) and edema/protein deposition was very mild. Finally, unlike similar studies using younger C57BL/6 mice sampled at 7 d p.i. (Gardner et al., 2010; Morrison et al., 2011), infiltration of mononuclear cells into associated skeletal muscle was not readily observable with any treatment, suggesting that maximal edema occurs in STAT129 animals in the absence of or prior to frank myositis. These analyses indicate that the CHIKV-181/25 strain elicits a robust inflammatory infiltrate into the infected limb; however, this is not associated with detectable swelling. In contrast, swelling due to CHIKV-LR is associated with inflammatory cell infiltration, dramatic protein deposition, and edema.

STAT1-dependent type I IFN responses control the replication and dissemination of wild-type CHIKV in mice

Since type I, and to a lesser extent type II, IFNs control replication and dissemination of Old World alphaviruses (Couderc et al., 2008; Frangkoudis et al., 2007; Ryman et al., 2000, 2002, 2005; Schilte et al., 2010), we reasoned that the increased susceptibility of IFN-deficient A129 and STAT129 mice to wild-type CHIKV infection would correspond to more rapidly disseminated, higher titer virus, and potentially to altered virus tropism compared with infection of less susceptible WT129 mice. WT129, A129, and STAT129 mice were inoculated subcutaneously with 10^3 pfu CHIKV-LR in each hind foot-pad. Infectious viral titers were measured by plaque assay of serum and PBS-perfused tissues at various times p.i. to establish the kinetics and extent of replication and dissemination (Fig. 4). In subclinical infection of WT129 mice, early CHIKV-LR replication in the popliteal lymph node, draining the inoculation site (DLN), was followed by a low-level serum viremia and disseminated replication in peripheral, and subsequently CNS, tissues. Virus replicated most efficiently in muscle (Fig. 4E), but WT129 mice were able to control and clear the infection from all tissues within 120 h p.i.

In the absence of IFN- α/β receptor-mediated antiviral responses, CHIKV replication and dissemination were poorly controlled, with significantly higher levels of replication in the DLN by 48 h p.i. (Fig. 4A), correspondingly higher serum viremia (Fig. 4B), and rapid seeding of tissues in the lymphoid compartment, including spleen and liver (Figs. 4C and D, respectively) and musculoskeletal compartment including gastrocnemius muscle, bone epiphyses and aspirate marrow (Figs. 4E, F and G, respectively), demonstrating tropism similar to human disease (Ozden et al., 2007). Peak replication in musculoskeletal tissues of the hind limb occurred 48 h p.i., coincident with the onset of severe hind limb swelling. However, although virus levels in these tissues began to decline after 48 h, swelling worsened. Titration of spinal cords and whole brain homogenates revealed exponential virus replication in the absence of IFN- α/β receptor- or STAT1-dependent signaling without evidence of control/clearance (Figs. 4H and I, respectively). Virus replication may be predominately restricted to the meninges and/or endothelial cells, sparing the brain parenchyma, as demonstrated by Couderc et al. (2008). It seems likely that systemic replication and/or CNS infection are responsible for mortality, disconnected from arthralgic manifestations.

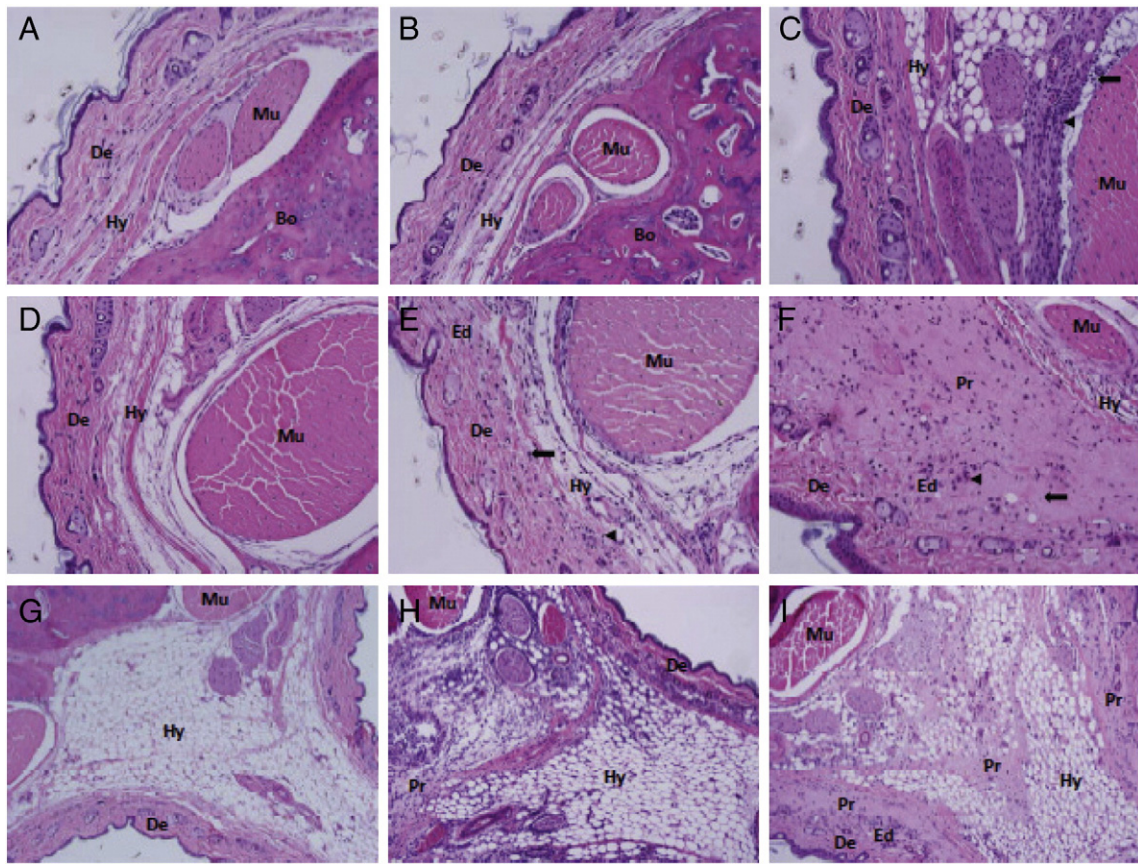


Fig. 3. Histopathology in CHIKV-infected hind limb tissues. Hematoxylin and eosin stained sections from the footpads of mock-infected (A,D,G), CHIKV-181/25-infected (B, E, H) and CHIKV-LR-infected (C,F,I) WT129 (A–C) and STAT129 (D–I) mice. Within panels, bone (Bo), dermis (De), hypodermis (Hy), muscle/tendon (Mu), edema (Ed), polymorphonuclear cells (arrowheads), and mononuclear cells (arrows) are indicated. Original magnification was 200 $\times$ (A–F) or 100 $\times$ (G–I). $n = 3$ mice per treatment group.

Replication and dissemination of the CHIKV-181/25 vaccine virus is compromised even in IFN-deficient animals

Reasoning that reduced ability of the CHIKV-181/25 vaccine to replicate and disseminate in mice may contribute to the amelioration of hind limb swelling, WT129, A129, and STAT129 mice were inoculated subcutaneously with 10^3 pfu of either CHIKV-LR or CHIKV-181/25 in each hind footpad and infectious viral titers were determined in serum and PBS-perfused tissues 48 h p.i. This time-point was selected as peak titers were measured in CHIKV-LR-infected mice above. In WT129 infected mice, CHIKV-181/25 replication was greatly reduced compared to CHIKV-LR, with virus titers below the limit of detection for serum and all tissues except DLN and muscle (Fig. 5A). Interestingly, CHIKV-LR and CHIKV-181/25 titers in the DLNs of A129 and STAT129 mice (Figs. 5B and C, respectively), were within 10-fold, suggesting that these viruses spread from the site of inoculation and seed the DLN similarly, but differ in their ability to fuel a serum viremia and disseminate systemically. CHIKV-181/25 replication in the tissues of A129 and STAT129 mice was similar at 48 h p.i. ($p > 0.05$), although mortality differed tremendously, suggesting that CNS replication at later times may be significantly different. Overall, it is evident that IFN- α/β receptor-mediated antiviral responses dramatically retard the replication and dissemination of both the wild-type and vaccine viruses, primarily via STAT1-dependent pathways. However, STAT1-dependent IFN response deficiency only restores CHIKV-181/25 vaccine replication to levels equivalent to CHIKV-LR infection of WT129 mice. Thus, the reduced replication potential of CHIKV-181/25 in peripheral tissues may be responsible, either directly or indirectly, for the lack of hind limb swelling and inflammation,

and is largely independent of IFN sensitivity. However, STAT1-dependent host responses are important for protection from lethality.

STAT1-dependent responses determine the permissivity of dendritic cells, but not macrophages, to CHIKV-LR and CHIKV-181/25 infection

To determine whether or not wild-type CHIKV-LR and CHIKV-181/25 vaccine strains differ in their ability to infect myeloid dendritic cells (mDCs) and macrophages, to assess the relative contribution of the STAT1-dependent IFN response to permissiveness of these cells to the wild-type versus vaccine virus, primary bone marrow-derived mDCs and macrophages from WT129 and STAT129 mice were infected at low multiplicity (0.1 pfu/cell). As anticipated, neither virus replicated efficiently within WT129-derived mDCs, but STAT1-deficient mDCs were highly permissive to both (Fig. 6A), suggesting that mDCs are protected from CHIKV by inherent and/or induced STAT1-dependent IFN responses and that attenuation of the CHIKV-181/25 vaccine does not correlate with increased sensitivity to or induction of IFN responses *in vitro*. Interestingly, neither virus replicated above input levels in immature (3 d-cultured; Fig. 6B) or mature (7 d-cultured; Fig. 6C) macrophages, even in the absence of STAT1, or in the RAW264.7 monocyte/macrophage cell line (Fig. 6D). These data further support our contention that amelioration of arthralgic disease in CHIKV-181/25-infected STAT129 mice is not due to increased IFN sensitivity/induction, but also suggest that increased permissivity of STAT1-deficient DCs to CHIKV replication *in vitro* is not necessarily correlated with the development of inflammatory disease.

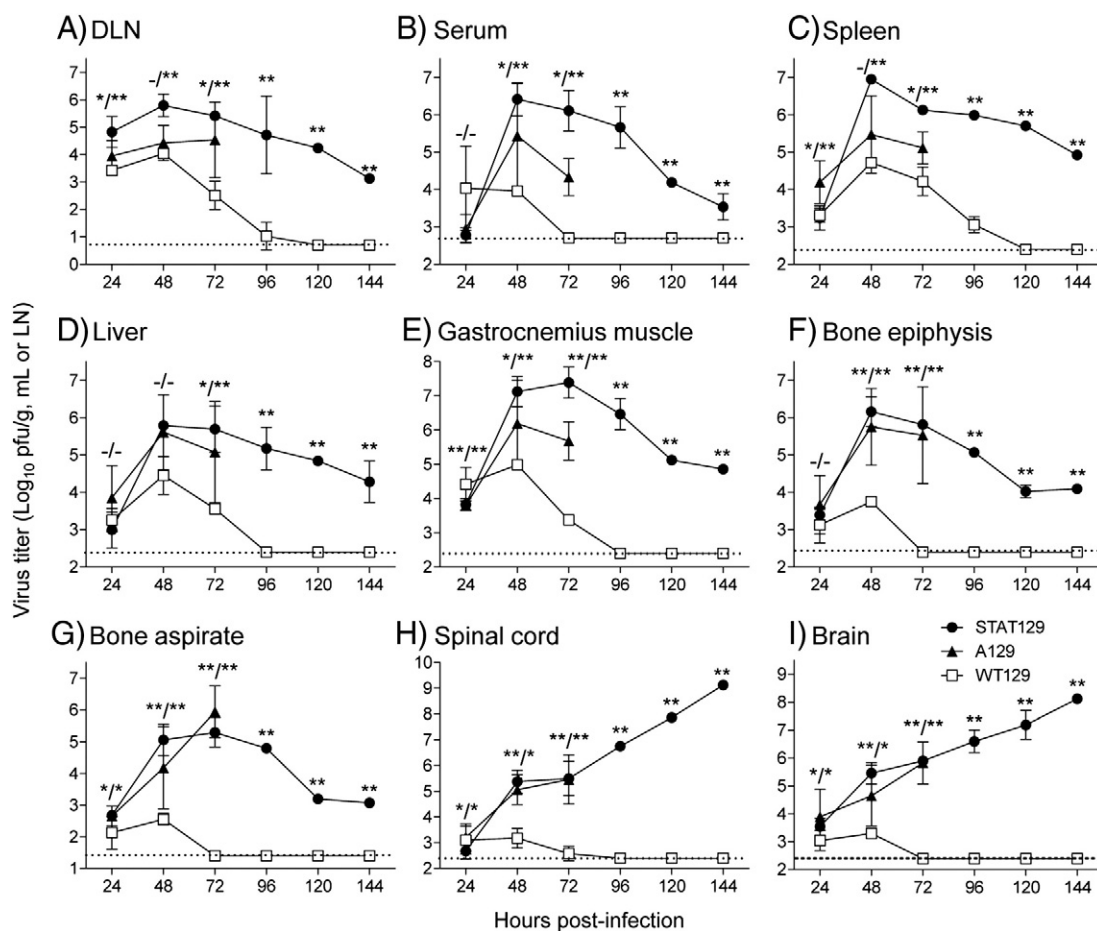


Fig. 4. Replication in IFN-deficient mice infected with CHIKV-LR. WT129 (open square), A129 (closed triangle), and STAT129 (closed circle) mice were inoculated subcutaneously in both foot-pads with 10^3 pfu of CHIKV-LR. Infectious virus titers in sera and PBS-perfused tissue homogenates were determined by standard plaque assay on BHK-21 cells and presented as pfu/DLN, mL (sera and bone aspirate) or g (all other tissues) $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; - $p > 0.05$ for WT129 versus A129/WT129 versus STAT129. $n = 3$ mice per treatment group and time-point.

CHIKV-181/25 vaccine, but not wild-type CHIKV-LR, strain exhibits glycosaminoglycan-dependence for infectivity

Recently, we have demonstrated that wild-type strains of encephalitic New World alphaviruses exhibit varying degrees of dependency upon cell-surface glycosaminoglycan (GAG), heparan sulfate (HS), for infectivity. In contrast, the infectivity of wild-type Old World

alphaviruses, including CHIKV-LR, is almost entirely HS-independent (Gardner et al., 2011). Since HS binding can be conferred upon Old World alphaviruses by adaptation to cell culture and the accumulation of positively charged amino acid substitutions in the E2 envelope glycoprotein (Heil et al., 2001; Klimstra et al., 1998; Smit et al., 2002; Zhang et al., 2005), resulting in attenuation in vivo (Byrnes and Griffin, 1998, 2000; Klimstra et al., 1999; Ryman et al., 2000), we

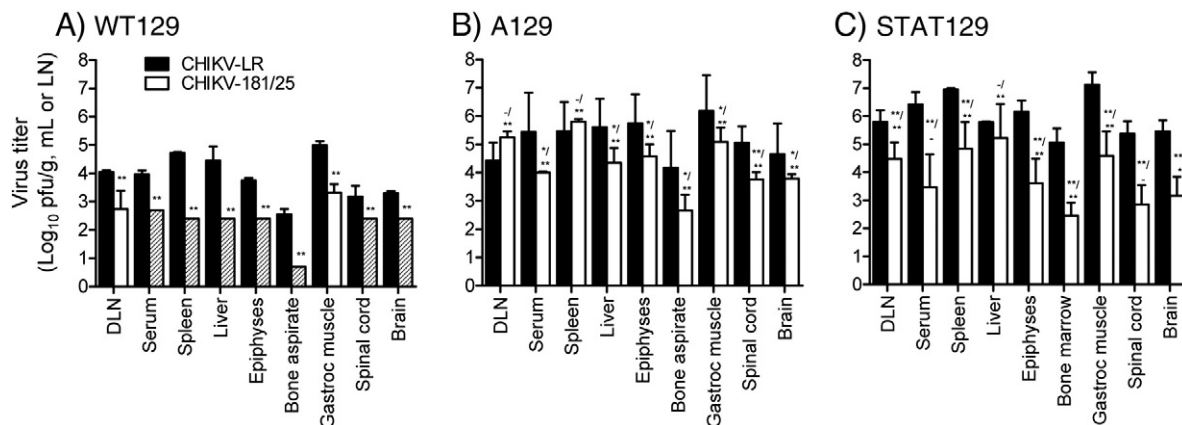


Fig. 5. Comparative replication in IFN-deficient mice infected with CHIKV-LR or CHIKV-181/25. WT129 (A), A129 (B), and STAT129 (C) mice were inoculated subcutaneously in both foot-pads with 10^3 pfu of CHIKV-LR (black bar) or CHIKV-181/25 (white bar; hatching indicates titer was below limit of detection). Infectious virus titers in sera and PBS-perfused tissue homogenates harvested 48 h p.i. were determined by standard plaque assay on BHK-21 cells and presented as pfu/DLN, mL (sera and bone aspirate) or g (all other tissues) $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; - $p > 0.05$ for CHIKV-LR versus CHIKV-181/25/ versus WT129. $n = 3$ mice per treatment group and time-point.

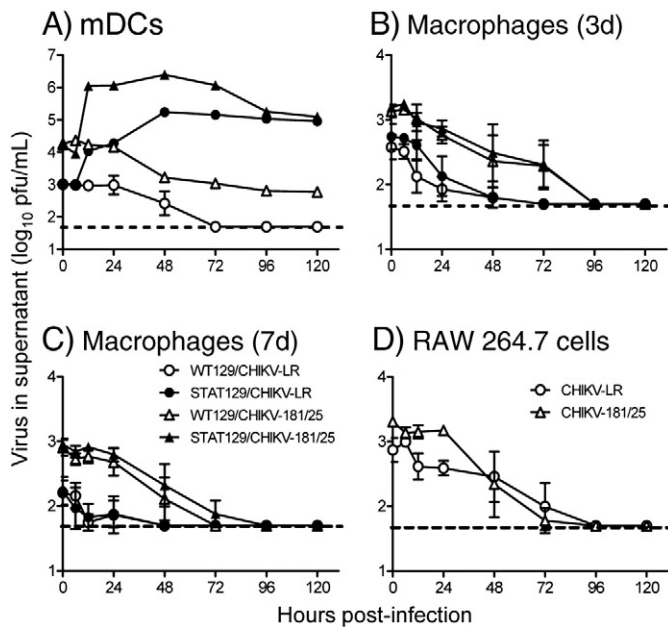


Fig. 6. CHIKV replication in myeloid cells. Bone marrow was harvested from WT129 (open symbols) and STAT129 (closed symbols) mice and differentiated into mDCs (A), 3 d immature macrophages (B), or 7 d mature macrophages (C), and compared to mouse RAW 264.7 monocyte/macrophage cells (D). Cells were infected at a multiplicity of infection (MOI) of 0.1 pfu/cell with CHIKV-LR (circles) or CHIKV-181/25 (triangles). Supernatant was collected at various times p.i. titrated by standard plaque assay. Datum points are $n = 3 \pm \text{SD}$ pfu/mL. Dotted line indicates limit of detection.

compared the relative infectivity of CHIKV-LR and CHIKV-181/25 for wild-type CHO-K1 versus GAG-deficient pgsA745 cells. Whereas the infectivity of CHIKV-LR was not significantly different in the presence or absence of GAGs, CHIKV-181/25 exhibited a high dependence, exhibiting a reduction in infectivity of ~90% on GAG-deficient cells ($p < 0.0001$; Fig. 7A), similar to that observed for HS binding SINV mutants (Klimstra et al., 1998). Furthermore, sodium chloride (NaCl) elution, which disrupts virion binding to HS on cell surfaces (Klimstra et al., 1998) indicated that the CHIKV-181/25 vaccine virus was effectively eluted by 350 mM salt, whereas the wild-type virus was not (Fig. 7B).

The genome sequences of CHIKV-LR, CHIKV-181/25 and its parent, CHIKV-15561 were downloaded, and the E2 protein genes were identified and translated (GenBank accession numbers DQ443544.2, EF452493.1 and L37661.3, respectively). Alignment of the E2 glycoprotein amino acid sequences revealed that, while there are 15 amino acid differences between wild-type CHIKV-LR and CHIKV-15561, the CHIKV-181/25 vaccine strain is identical to its parent at

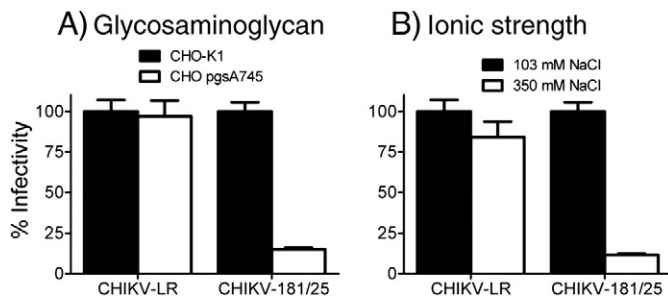


Fig. 7. Differential dependence of CHIKV infectivity upon ionic interaction and glycosaminoglycans for infectivity. Relative infectivity of CHIKV-LR and CHIKV-181/25 on CHO-K1 (black bars) and pgsA745 GAG-deficient (white bars) cells (A). Relative infectivity of CHIKV-LR and CHIKV-181/25 on CHO-K1 cells in 103 mM (black bars) or 350 mM (white bars) NaCl (B). Percent infection was normalized to infectivity on CHO-K1 cells in RPMI1640 without added salt (103 mM NaCl). Percent infection was normalized to infectivity on control cells. All datum points are $n = 3 \pm \text{SD}$.

all of these positions (Table 1). However, two amino acid substitutions have arisen during cell culture passage to make CHIKV-181/25 at E2 position 12 (T to I) and E2 position 82 (G to R), but are conserved between the wild-type strains. Notably, the E2R82 mutation increases the net positive-charge of the CHIKV-181/25 E2 glycoprotein, coincident with acquisition of GAG-dependent binding in vitro, reduced replication/dissemination in vivo, and amelioration of disease.

Discussion

The molecular and immunologic mechanisms underlying CHIKV pathogenesis, virulence and attenuation are not well understood, impeded by the lack of a robust small animal model of acute and persisting polyarthritides/polyarthralgia. However, careful analysis of adult C57BL/6 mice has produced a model for non-fatal CHIKV infection, where infectious virus is obtained from various tissues, musculoskeletal inflammatory disease with joint involvement is observed and proinflammatory mediators (IFN- α/β , IFN- γ , MCP-1, TNF- α and IL-6) are elevated in both acute-phase sera and footpad tissue (Gardner et al., 2010). Younger C57BL/6 animals (two-week-old) develop more severe inflammatory disease, with evidence of arthritis, tenosynovitis and myositis, and viral persistence (Morrison et al., 2011). Here, we have investigated the role of IFN responses in amelioration of wild-type CHIKV-induced musculoskeletal disease. We demonstrate for the first time that IFN-mediated antiviral responses, largely dependent upon STAT1-mediated signaling, control the replication of wild-type CHIKV in muscle and joint-associated tissues of mice, and coincidentally ameliorate clinical signs of polyarthralgia. Importantly, we have shown that the disease course in animals deficient in IFN responses when infected subcutaneously with wild-type CHIKV strains mimics key aspects of severe human disease, such as virus tropism for bone and joint tissues, edema and indications of arthralgia in multiple limbs, and associated tissue pathologies and inflammatory infiltrates. These findings suggest that IFN deficiencies in the human host and/or species-specific evasion/antagonism of IFN responses by the virus would likely exacerbate musculoskeletal disease.

In order to directly combat antiviral activity or to subvert the transition from innate to adaptive immunity, pathogenic alphaviruses have evolved to evade and/or antagonize the IFN- α/β responses (Burke et al., 2009; Fros et al., 2010; Gardner et al., 2008; Simmons et al., 2009; Yin et al., 2009) and resistance of a given alphavirus to the IFN response appears to be a critical virulence determinant (Couderc et al., 2008; Fragkoudis et al., 2007; Gardner et al., 2009; Ryman et al., 2000; Schilte et al., 2010; White et al., 2001). Central to this study is the finding that sensitivity of CHIKV to murine IFN-mediated antiviral responses is sufficient to protect against disease, while deficiency in the IFN response leads to severe polyarthralgia with similarities to human CHIK fever. In contrast, virulent human infections presumably occur in individuals with properly functioning IFN responses. The apparent difference in efficacy between human and murine STAT1-dependent IFN- α/β responses against CHIKV infection implies species-specificity, such that human IFN responses are significantly less effective than murine in ameliorating disease, at least in symptomatic infections. Alternatively, CHIKV may have evolved mechanisms to circumvent/antagonize IFN responses in natural primate hosts which are less effective in mice. Interestingly, CHIKV nsP2 has been shown to inhibit JAK-STAT signaling-dependent type I and II IFN responses in primate-derived Vero cells (Fros et al., 2010), presumably allowing the virus to overcome or circumvent STAT1-dependent responses in vivo, which may contribute to enhanced virulence in primates over rodents if this ability proves to be species-specific. Furthermore, CHIKV globally inhibits translation of cellular mRNAs in highly permissive primate-derived cells, blunting IRF3-

Table 1

Amino acid differences in the E2 glycoprotein sequences of wild-type and live-attenuated CHIKV strains.

Virus strain	E2 amino acid position (numbered from N-terminus)															
	2	12	82	118	149	157	164	194	205	255	312	317	318	375	377	384
CHIKV-LR	T	T	G	S	K	V	T	G	G	I	M	V	V	T	I	M
CHIKV-15561	I	T	G	G	R	A	A	S	D	V	T	I	R	S	V	V
CHIKV-181/25	I	I	R	G	R	A	A	S	D	V	T	I	R	S	V	V

dependent innate immune activation (White et al., 2011). Other possible antagonism/evasion strategies have not yet been investigated.

In the absence of STAT1-dependent IFN responses, wild-type CHIKV infection led to rapid viral dissemination, most likely by infecting and commandeering migratory DCs, as described for other alphaviruses (Ryman et al., 2000, 2005). CHIKV-LR amplified in the DLN, seeded a serum viremia and spread to other tissues, notably exhibiting a strong tropism for muscle and joint-associated tissues. In particular, muscle, bone epiphyse, and bone marrow were sites of replication in STAT129 mice and exhibit extensive pathology, generally consistent with previously published studies (Gardner et al., 2010; Morrison et al., 2011), with two notable exceptions. First, myositis was greatly reduced in our studies although edema was far more severe, presumably due to differences in mouse strain, age (two weeks older), or time-point (5 d p.i. versus 7 d p.i.). However, myositis is infrequently described in CHIKV-infected humans (Ozden et al., 2007). Second, striking deposition of protein in the dermis, hypodermis, and underlying tissues was associated with inflammatory cell infiltration and hemorrhage in CHIKV-LR-infected STAT129 mice.

Subcutaneous infection of A129 and STAT129 mice readily distinguishes virulent wild-type CHIKV infection from the live-attenuated CHIKV-181/25 vaccine strain, with “vaccination” providing protection against fatality following wild-type challenge, consistent with the findings of Partidos et al. (2011) and also against swelling of the limbs. Attenuated CHIKV-181/25 vaccine virus infection of STAT129 and A129 animals was correlated with partial to complete attenuation compared to wild-type CHIKV-LR, with reduced joint-associated replication and pathology, although inflammatory cell infiltration was comparable or possibly superior to wild-type infected mice. Thus, the pathogenesis of CHIKV-181/25 was dramatically restricted compared with the wild-type virus even in the absence of STAT1-dependent IFN activity, from which we infer that the primary mechanism underlying CHIKV-181/25 attenuation is not increased sensitivity to the IFN response, although IFN- γ responses may contribute. In keeping with this interpretation, the sensitivity of the wild-type and vaccine strain to STAT1-dependent IFN responses in primary DCs was indistinguishable.

We and others have shown previously that: i) wild-type Old World alphaviruses including wild-type strains of CHIKV-LR do not bind to cells via HS (Gardner et al., 2011; Heil et al., 2001; Klimstra et al., 1998; unpublished data); ii) HS binding capability is rapidly acquired by these viruses as a cell culture-adaptive mechanism during in vitro passage by the accumulation of positively charged amino acid substitutions in the E2 envelope glycoprotein (Heil et al., 2001; Klimstra et al., 1998; Smit et al., 2002; Zhang et al., 2005); and iii) this typically results in attenuation in vivo due to sequestration of virus in tissues (Byrnes and Griffin, 1998, 2000; Klimstra et al., 1999; Ryman et al., 2000). E2 amino acid sequence comparison revealed that in vitro passage of wild-type CHIKV to create the CHIKV-181/25 vaccine resulted in two amino acid substitutions in the E2 attachment protein, one of which increases the net positive-charge (E2R82). Moreover, we showed that the vaccine strain is highly dependent upon GAGs for infectivity in vitro. We propose that GAG (most likely HS) binding may be an attenuating mechanism in the CHIKV-181/25 strain, mediated by the E2R82 mutation, restricting viremic potential and dissemination beyond the DLN, and attenuating disease. Consistent with this idea, we have demonstrated previously

that an equivalent HS-binding SINV mutant remains attenuated even in the absence of IFN- α/β responses (Ryman et al., 2000). The contribution to attenuation of individual CHIKV-181/25 mutations in E2 and other viral proteins is currently being investigated.

Overall, we propose that CHIKV infection of IFN-deficient mice provides a valuable small animal model, exhibiting key features of severe human CHIKV infection, in which to evaluate contributions of virus and host genetics to virulence/attenuation and protection, and in which to test candidate vaccines and therapeutics without limitations of host age. We are exploring the possibility that wild-type CHIKV evades/antagonizes IFN responses by species-specific mechanisms to further refine this model.

Materials and methods

Mice

Wild-type 129 Sv/Ev (WT129) and STAT1 deficient (STAT129) mice were purchased from Taconic laboratories. Mice deficient in receptors for IFN- α/β (A129), IFN- γ (G129) or both (AG129) were bred in-house. All mice were housed under specific pathogen-free conditions, and used at 8 weeks of age. Experiments were conducted in accordance with Institutional Animal Care and Use Committee policies at Louisiana State University Health Sciences Center at Shreveport and the University of Pittsburgh.

Cell lines and primary cell cultures

Baby hamster kidney cells (BHK-21; ATCC) were maintained in alpha minimum essential medium (AMEM) supplemented with 10% donor bovine serum (DBS) and 10% tryptose phosphate broth. Murine RAW264.7 monocyte/macrophages were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Chinese hamster ovary cells (CHO-K1) and CHO-K1 cells deficient in all glycosaminoglycans including HS (pgsA-745) were maintained in Ham's F12 supplemented with 10% FBS. All media for cell lines also contained penicillin (100 U/mL), streptomycin (0.05 mg/mL) and L-glutamine (0.05 mg/mL).

mDCs and macrophages were generated from mouse bone marrow. mDCs were cultured as previously described (Gardner et al., 2008). Briefly, bone marrow aspirated from femurs and tibias was plated for 1 h (37 °C; 5% CO₂) to allow contaminating macrophages to adhere, after which the non-adherent mDC progenitors were removed and cultured in RPMI1640 medium supplemented with 10% FBS, 1% nonessential amino acids, 100 U/mL penicillin, 0.05 mg/mL streptomycin, 0.05 mg/mL L-glutamine, 1 mM sodium pyruvate, 5 mM HEPES buffer, 50 μ M β -mercaptoethanol, 10 ng/mL GM-CSF and 10 ng/mL IL-4 (Peprotech, Rocky Hill, NJ). After 7 d, non-adherent mDCs were pelleted, counted and resuspended in supplemented RPMI1640 medium for infection.

Adherent macrophages from the bone marrow were cultured for 3 d (immature) or 7 d (mature) in DMEM supplemented with 10% FBS, 20% L929-conditioned supernatant, 10% FBS, 100 U/mL penicillin, 0.05 mg/mL streptomycin and L-glutamine. Cells were harvested by scraping, pelleted, and seeded for infection.

Viruses

Stocks of CHIKV-LR and CHIKV-37797 viruses were generated from genome-length cDNA clones (Tsetsarkin et al., 2006; Vanlandingham et al., 2005). Low passage strain CHIKV-37997 was isolated from Senegalese mosquitoes in 1983. The CHIKV-LR strain was isolated on La Réunion Island during the ongoing epidemic. Briefly, linearized cDNA templates were used to generate capped infectious viral RNA by *in vitro* transcription (mMessage mMachine, Ambion). BHK-21 cells were electroporated with the infectious RNA, and virus particles were harvested from the supernatant when cytopathic effect (CPE) was observed, 20–24 h later. The biological CHIKV-181/25 vaccine strain, also called TSI-GSD-218 (Levitt et al., 1986), was generously provided by Dr. Scott Weaver (University of Texas Medical Branch, Galveston, TX) and passed once on BHK-21 cells. For all virus stocks, cell supernatants were clarified by centrifugation and single-use aliquots were stored at -80°C . Titers of virus stocks were determined by standard plaque assay on BHK-21 cells, expressed as pfu/mL.

Virus growth curves

All cells were infected at a multiplicity of infection (MOI) of 0.1 pfu/cell. Adherent bone marrow-derived and RAW264.7 macrophages were infected in a 24 well plate. After 1 h incubation, cells were washed with PBS-1% DBS, and appropriate medium was replaced before cells were incubated (37°C ; 5% CO_2). Nonadherent cells were infected in a 96 well plate 1 h at 37°C , and then washed in PBS-1% DBS by pelleting and resuspension. Cells were resuspended in appropriate medium and seeded into 24 well plates and incubated (37°C ; 5% CO_2). Supernatant was harvested at time zero and subsequent times p.i. for titration by plaque assay.

Morbidity/mortality and pathogenesis studies

Mice were injected subcutaneously in the hind footpad using a 27-gauge needle and 100 μL gas-tight Hamilton syringe with 10 μL of inoculum containing 10^3 pfu of virus in phosphate-buffered saline (PBS) containing 1% DBS. Mock-infected mice received 10 μL of PBS-1% DBS by the same route. Mice were scored and weighed daily, and footpad width and height in the metatarsal region were measured with calipers where appropriate to calculate area (width $\times$ height) and fold-increase over pre-infection area. AST and percent mortality were calculated and statistically analyzed.

At predetermined times post-infection (p.i.), groups of three mice were euthanized under isoflurane anesthesia and blood was collected by cardiac puncture. Sera were separated from whole blood using Microtainer tubes (Becton-Dickinson), aliquoted and stored at -80°C . Mice were then perfused with PBS-1% DBS for 10 min at 7 mL/min to flush blood-borne virus. Perfused tissues were homogenized in PBS-1% DBS after one freeze–thaw by mechanical disruption and sedimentation of cell debris. Serum and supernatants from homogenized tissue were assayed for virus by a standard plaque assay on BHK-21 cells and titers were expressed as pfu/DLN, mL (sera and bone marrow aspirate) or g (all other tissues).

Histopathology

Mice were sacrificed 5 d p.i., briefly perfused with PBS-1% DBS to exsanguinate and then with 4% paraformaldehyde (PFA) for 10 min at 7 mL/min. Hindlimbs were decalcified in 4% PFA containing 10% EDTA for at least 3 wk before paraffin embedding. Five μm thick H&E-stained sections were viewed on a light microscope (Olympus CKX41) and pictures were taken with an Olympus DP72 camera using Olympus CellSens Standard software.

Glycosaminoglycan-dependency of infectivity

Virus was diluted in either RPMI1640 or RPMI1640 containing 350 mM NaCl and then used to infect CHO-K1 or GAG-deficient pgsA745 cells. Cells were incubated for 1 h (37°C ; 5% CO_2) before overlaying with immunodiffusion agarose. At 48 h p.i., cells were fixed overnight with 4% PFA, after which PFA and overlay were removed and cells were stained for CHIKV antigen. Briefly, cells were blocked with PBS buffer containing albumin, non-fat milk, Triton X-100, and normal donkey serum for 1 h at room temperature. Cells were then stained with CHIKV polyclonal antibody (ATCC) diluted in supplemented PBS buffer (1 h, room temperature). Primary antibody binding was detected by incubation with a fluorescently conjugated secondary antibody (donkey anti mouse DyLight 594; Jackson ImmunoResearch) diluted in supplemented PBS buffer for 1 h at room temperature in the dark. Infectious foci were then quantified on an Olympus CKX41 inverted fluorescent microscope.

Statistical analyses

Statistical significance for mortality curves was determined by Mantel–Cox log-rank test (GraphPad PRISM software) and for other experiments a two-tailed Student's *t* test with two-sample equal variance was used (Microsoft Excel software).

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