Chikungunya Virus Arthritis in Adult Wild-Type Mice^{▽†}

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Chikungunya virus is a mosquito-borne arthrogenic alphavirus that has recently reemerged to produce the largest epidemic ever documented for this virus. Here we describe a new adult wild-type mouse model of chikungunya virus arthritis, which recapitulates the self-limiting arthritis, tenosynovitis, and myositis seen in humans. Rheumatic disease was associated with a prolific infiltrate of monocytes, macrophages, and NK cells and the production of monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor alpha (TNF- α), and gamma interferon (IFN- γ). Infection with a virus isolate from the recent Reunion Island epidemic induced significantly more mononuclear infiltrates, proinflammatory mediators, and foot swelling than did an Asian isolate from the 1960s. Primary mouse macrophages were shown to be productively infected with chikungunya virus; however, the depletion of macrophages ameliorated rheumatic disease and prolonged the viremia. Only 1 μ g of an unadjuvanted, inactivated, whole-virus vaccine derived from the Asian isolate completely protected against viremia and arthritis induced by the Reunion Island isolate, illustrating that protection is not strain specific and that low levels of immunity are sufficient to mediate protection. IFN- α treatment was able to prevent arthritis only if given before infection, suggesting that IFN- α is not a viable therapy. Prior infection with Ross River virus, a related arthrogenic alphavirus, and anti-Ross River virus antibodies protected mice against chikungunya virus disease, suggesting that individuals previously exposed to Ross River virus should be protected from chikungunya virus disease. This new mouse model of chikungunya virus disease thus provides insights into pathogenesis and a simple and convenient system to test potential new interventions.

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus that has caused periodic outbreaks of predominantly rheumatic disease in Africa and Asia (69). The disease usually involves weeks to months of arthralgia/arthritis and can involve myalgia, fever, and/or a rash (6). During 2004 to 2007 the largest documented outbreak of CHIKV disease occurred in Indian Ocean islands and India. Over 260,000 cases (about one-third of the population) were reported in Reunion Island (France) (56), with 1.39 million cases in India (42) and a small outbreak of ~200 cases also occurring in Italy (56, 74). The recent outbreak was associated with the emergence of a new clade of CHIKV viruses within the large East, Central, and South African phylogroup, which is distinct from the more distantly related Asian phylogroup (52, 55, 62). A key mutation in the E1 gene (A226V) is believed to have allowed efficient CHIKV transmission by *Aedes albopictus* mosquitoes (13, 76, 80), which were the main vector in the outbreak in Reunion Island and in some parts of India (31). The recent epidemic was associated with a low level of asymptomatic infections and appeared to result in an increase in disease severity compared with that of previous epidemics (8, 51). A small percentage of

cases resulted in death (42, 72), although in such cases other underlying medical conditions may have contributed to mortality (14). CHIKV has been declared a high-priority pathogen by the U.S. NIH (60). No licensed vaccine or particularly effective drug is available for human use for any alphavirus (60), although analgesics and nonsteroidal anti-inflammatory drug treatment can provide relief from rheumatic symptoms (48, 68).

The development and testing of new interventions are greatly facilitated by the use of mouse models, which can also provide insights into disease pathogenesis (59). Mouse models of CHIKV disease have recently been developed and involve lethal infections of neonatal mice (89) or adult mice defective in the alpha/beta interferon (IFN- α/β) receptor (9). Such models have been used to illustrate the potential utility of treatment with adoptively transferred anti-CHIKV antibodies (10). A third model used intranasal inoculation of CHIKV but showed no rheumatic signs or symptoms (82). All these models used lethality rather than rheumatic manifestations as disease measures. In humans, arthritis/arthralgia is the main manifestation of CHIKV disease, and disease is only rarely fatal (14). The requirement for young mice makes the testing of prophylactic vaccines difficult, as there is insufficient time for vaccination. Human infants also tend not to develop arthritic symptoms following CHIKV infection (78). The use of mice lacking IFN- α/β responses complicates the testing of vaccines and other immunological interventions, as the absence of IFN- α/β signaling can affect both vaccine (26, 77) and virus (60) behaviors.

Here we describe the behaviors of two virus isolates of

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CHIKV, an Asian isolate and a Reunion Island isolate, in a new adult wild-type mouse model of CHIKV arthritis. The Asian isolate was collected in the 1960s in Thailand, and the Reunion Island isolate was collected during the recent outbreak (55). The model produced a measurable self-limiting perimetatarsal foot swelling with clear histological signs of acute and persistent inflammatory disease. We also characterize the cells and inflammatory mediators associated with infection and disease and illustrate the use of the model for studying vaccines, IFN- α therapy, and cross-protection with Ross River virus (RRV), an Australasian arthrogenic alphavirus related to CHIKV (16, 69).

MATERIALS AND METHODS

Ethics statement. All animals were handled in strict accordance with good animal practice as defined by the National Health and Medical Research Council of Australia. All animal work was approved by the Queensland Institute of Medical Research animal ethics committee.

Virus isolates and preparation. The Asian CHIKV isolate, collected in Asia in the early 1960s, was inoculated into suckling mouse brain and then serially passaged nine times in adult C57BL/6 mice. Partial E1 sequencing (GenBank accession number FJ457921) showed it to be closely related to the Indian isolate IND-63-WB1 and the Thai isolate AF15561. The Reunion Island isolate (LR2006-OPY1) is a primary isolate from the recent outbreak in Reunion Island (55) and was passaged twice in C6/36 cells (ATCC CRL-1660). The Reunion Island isolate shows ~97% amino acid sequence identity with the Indian and Thai isolates. Virus was harvested 24 h after infection (multiplicity of infection [MOI] of 0.04 to 0.06) of C6/36 cells cultured in medium comprising RPMI 1640 medium supplemented with 5% fetal calf serum (FCS). RRV (T48) was prepared as described previously (38). The virus preparations had undetectable endotoxin and mycoplasma contamination as measured by sensitive bioassays (27, 33); titers were determined by a log₁₀ 50% cell culture infectivity dose (CCID₅₀) assay on Vero cells, as described previously (38), using eight replicates, and virus preparations were stored frozen in aliquots at -70°C.

Mouse infection and disease monitoring. Female C57BL/6 mice that were at least 6 weeks old were inoculated with CHIKV (10⁴ CCID₅₀ in 40 μ l RPMI 1640 medium supplemented with 2% FCS) subcutaneously (s.c.) into the ventral side of each hind foot, toward the ankle. The inoculation dose was confirmed by a CCID₅₀ assay (using residual material in the syringe) on Vero cells using eight replicates. The height and width of the perimetatarsal area of the hind feet were measured by using Kincrome digital vernier calipers. Infection with RRV was performed as described previously (38) with 500 CCID₅₀ administered intraperitoneally (i.p.) in 500 μ l phosphate-buffered saline (PBS) into 6- to 8-week-old female C57BL/6 mice. In animals of this age, RRV infection is asymptomatic. Animals were monitored daily, and no adverse events requiring euthanasia were reported during the experiments.

Measurements of virus titers. The indicated tissues were dissected, weighed, added to medium (~50 to 100 mg tissue/ml RPMI 1640 medium supplemented with 10% FCS), snap-frozen on dry ice, crushed and macerated while thawing using metal mesh and a 5-ml syringe barrel, and vortexed, followed by a 30-s spin with a microcentrifuge at top speed. Supernatants were then assayed for virus by 10-fold serial dilutions in 96-well plates in duplicate on C6/36 cells (2 $\times$ 10⁴ cells/well). After 3 days of incubation at 28°C, 25 μ l from each well was transferred into a parallel well of parallel 96-well plates containing Vero cells (10⁴ cells/well). After incubation for 4 days at 37°C the plates were stained with crystal violet to visualize cytopathic effects (CPE) (5). Viremia was measured by collecting ~40 μ l of blood from a tail vein into 0.8-ml MiniCollect serum separation tubes (Greiner Bio-One GmbH, Kremsmünster, Austria). The tubes were spun at 7,000 rpm for 2.5 min on a bench-top microcentrifuge. Serum was collected and diluted 1 in 10 in medium, snap-frozen, and assayed by serial dilution on C6/36 cells as described above. Viral titers were expressed as CCID₅₀ (38) per ml of serum or gram of tissue.

Infection of MEFs. Primary murine embryonic fibroblasts (MEFs) were trypsinized in low-endotoxin trypsin-EDTA (Gibco), seeded into 24-well (5 $\times$ 10⁴ cells/well) plates, and infected the next day at an MOI of 0.1 of each of the viral isolates in RPMI 1640 medium supplemented with 2% FCS. After five 5-min washes with medium, virus titers in the supernatant were assayed as described above. MEFs were seeded into 96-well plates (10⁴ cells/well) and

infected as described above, and CPE were determined by crystal violet staining of replicate plates on the indicated days.

Histology and immunohistochemistry. Tissues were fixed in 10% neutral buffered formalin, feet were decalcified, tissue was embedded in paraffin wax, and 6- μ m-thick sections were cut and stained with hematoxylin-eosin-safranin. For immunohistochemistry, joints were fixed for 24 h in formalin, decalcified with 15% EDTA in 0.1% phosphate buffer over 10 days, and embedded in paraffin wax. Proteinase K antigen recovery and staining were undertaken by using the IntelliPATH automated immunostaining machine (Biocare Medical, Concord, CA). Sections were stained with F4/80 (Abcam, Cambridge, MA) and rat probe polymer (Biocare Medical) as the secondary antibody, and color was developed by using DAB (3,3'-diaminobenzidine). Sections were counterstained with Mayer's hematoxylin.

Fluorescence-activated cell sorter (FACS) analysis. At the peak of foot swelling, feet were removed (n = 6 to 12 feet per group), tissue was scraped from bone, and the material was digested with collagenase-dispase (Roche, Dee Why, NSW, Australia) for 30 min at 37°C with occasional mixing. The mixture was spun for 1 min at 8 $\times$ g to remove debris, and the supernatant was layered onto Percoll (GE Healthcare, Sweden) and spun at 500 $\times$ g for 30 min. Cells at the interface were collected and washed once, red blood cells were lysed with ACK buffer (Sigma), and the cells were washed again. Cells were then stained with anti-CD45-phycoerythrin (PE) (30-F11) (Bio Legend, San Diego, CA) or anti-CD3-PE (145-2C11) (BD Pharmingen, Heidelberg, Germany) and anti-CD4 (GK1.5), anti-CD8 (YTS156.7.7), anti-CD19 (ID3), anti-CD11b (M1/70) (Bio Legend), anti-NK1.1 (PK136) (BD Pharmingen), anti-B220 (RA3682), anti-PDCA1 (927) (Bio Legend), and/or anti-F4/80 (CI:A3-1) (Serotec, Martinsried, Germany) labeled with fluorescein isothiocyanate (FITC) or allophycocyanin (APC). 2.4G2 was used to block Fc receptors. Isotype control antibodies (Bio Legend) were used to set appropriate gates. Cells were analyzed by using a FACSCalibur apparatus (Becton Dickinson, North Ryde, NSW, Australia). Events with low forward scatter and side scatter, previously identified as dead cells and/or cell debris, were excluded from the analysis.

Infection of splenocytes and macrophages. Splenocytes from three mice were pooled and infected for 2 h with the Reunion Island isolate expressing green fluorescent protein (GFP) (75) (MOI of 2). The cells were then cultured for 24 h cells, stained with anti-F4/80-PE antibody (Serotec), and analyzed by FACS. To infect macrophages, splenocytes were cultured overnight, and nonadherent cells were removed by extensive washing. Cells were cultured for 4 days in RPMI 1640 medium supplemented with FCS with undetectable endotoxin contamination (27), infected in triplicate (2 $\times$ 10⁴ cells/ml/well) with the Reunion Island isolate at the indicated MOI for 2 h, washed 10 times, and seeded into 24-well plates, and on days 0, 1, 2, and 3, 50 μ l of supernatant was then removed and assayed for viral titers. Cells in these cultures were >85% F4/80 positive (F4/80⁺).

Cytokine/chemokine analyses. Serum cytokine/chemokine protein levels were analyzed by using the BD Cytokine Bead Array Bioanalyzer system (Becton Dickinson, Franklin Lakes, NJ) according to the manufacturer's instructions.

Bioactive IFN- α / β was measured by a CPE inhibition bioassay using Semliki Forest virus infection of L-929 cells (ATCC CCL1) (39). The assay was standardized by using recombinant mouse IFN- α A (Sigma-Aldrich, St. Louis, MO). Mouse serum was diluted 1 in 10 in RPMI 1640 medium supplemented with 2% FCS and exposed to 960 mW/cm² UV-C for 2 h to inactivate CHIKV before addition to L-929 cells (2-fold serial dilution in duplicate) (2 $\times$ 10⁴ cells/96 wells). After overnight incubation, Semliki Forest virus was added (100 CCID₅₀/well), and after 4 days, CPE were determined by crystal violet staining.

For real-time reverse transcription (RT)-PCR analysis, feet were cut lengthways, placed into RNAlater solution (Ambion, Austin, TX) for 24 h at 4°C, and frozen at -70°C. RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand total cDNA synthesis was performed with a 20- μ l reaction mixture containing 1 μ g of total RNA, 500 μ M deoxynucleoside triphosphates (dNTPs), 200 ng of random hexamer oligonucleotides, 1 $\times$ Superscript first-strand buffer, 10 mM dithiothreitol (DTT), and 200 U of Superscript III (Invitrogen). Real-time PCR analysis used the following nucleotide primers: 5'-CAGCCAGATGAGTTAACGC-3' and 5'-CAGACCTCTCTCTTGAGCTTGG-3' for monocyte chemoattractant protein 1 (MCP-1), 5'-AATTCGAGTGACAAGCCTGTAGC-3' and 5'-AGTA GACAAGGTACAACCCATCG-3' for tumor necrosis factor alpha (TNF- α), 5'-ACTGGCAAAGGATGGTGAC-3' and 5'-GCTGATGGCCTGATTGTC TT-3' for IFN- γ , and 5'-GAGGTCCGGTGAAGTACCA-3' and 5'-TGCAT CTTGGCCTTTTCTT-3' for RPL13A (45). The 20- μ l amplification reaction mixture contained 0.1 μ g of randomly primed cDNA, 0.5 μ M each primer pair, and 10 μ l of 2 $\times$ Platinum SYBR green qPCR Supermix-UDG (Invitrogen). Cycling conditions were as follows: one cycle of 50°C for 2 min and one cycle of 95°C followed by 45 cycles of 94°C for 5 s, 60°C for 10 s, and 72°C for 30 s. The

real-time PCR was performed by using a Rotor-Gene 3000 PCR machine (Corbett Research, Mortlake, Australia). The data were analyzed with Rotor-Gene real-time analysis software (Corbett Research, Sydney, Australia). Each sample was analyzed in duplicate and normalized to RPL13A mRNA, as mRNA expression for this housekeeping gene remains constant even under conditions of widespread gene induction (45).

Production of inactivated purified CHIKV antigen. CHIKV antigen was kindly provided by Inverness Medical Innovations Australia Pty. Ltd., Brisbane, Australia. Briefly, confluent monolayers of C6/36 cells grown in 2-stacker and 5-stacker cell factories (Corning Life Sciences Inc., Lowell, MA) were infected in a low volume with the CHIKV Asian isolate at an MOI of 0.04 to 0.06 CCID₅₀ for 90 min at 30°C with 5% CO₂. The cultures were topped up with medium consisting of RPMI 1640 medium supplemented with 2% FCS (SAFC Biosciences, Lenexa, KS), 25 mM HEPES (Invitrogen), and 100 U/ml penicillin–100 µg/ml streptomycin (Invitrogen). After incubation for 24 to 28 h, the supernatants were harvested and clarified by filtration using a 1-µm Polypure capsule (Pall Corporation, Ann Arbor, MI). The virus was inactivated by using 3 mM binary ethyleneimine (Sigma-Aldrich) for a period of 8 h at 37°C, as described previously (57), and shown to be inactive by three serial passages in C6/36 cells prior to a CPE assay on Vero cells. The virus was then purified by polyethylene glycol precipitation as described previously (58). The virus preparation was >90% pure by SDS-PAGE and contained 2.8 mg/ml of protein.

Mouse vaccination. The inactivated and purified CHIKV antigen described above was used as a vaccine. Mice were vaccinated once s.c. at the base of the tail with 50 µl of this preparation, which contained the indicated amount of antigen diluted in RPMI 1640 medium. Where indicated, Quil A (Iscotec AB, Lulea, Sweden) (10 µg/mouse) was mixed with the virus preparation prior to injection.

Macrophage depletion with clodronate. Mice were injected on day -1 intravenously (i.v.) with 200 µl of control liposomes or liposomes containing clodronate (79) or PBS. Spleen F4/80⁺ macrophages were depleted by >85% on day 0 (data not shown). Clodronate was a gift of Roche Diagnostics GmbH (Mannheim, Germany). Mice were infected with the Reunion Island isolate on day 0.

Adoptive transfer of antisera. Mice ($n = 5$ per group) were infected with RRV or the Reunion Island isolate as described above or were mock infected. After 4 weeks, serum was harvested and pooled for each group, and 300 µl was injected i.p. into naïve animals ($n = 3$ mice per group) 1 day before infection with the Reunion Island isolate.

ELISA and ELISPOT. Inactivated and purified CHIKV antigen (described above) was coated onto enzyme-linked immunosorbent assay (ELISA) plates (MaxiSorb; Nunc, Rochester, NY) at 2 µg/ml in carbonate buffer (pH 9) overnight, and the plates were blocked with 5% FCS–PBS. Mouse serum was added in 3-fold serial dilutions, and CHIKV-specific antibodies were detected with biotin-conjugated rat anti-mouse IgG2c (R19-15) and IgG1 (A85-1) (BD Pharmingen), streptavidin-horseradish peroxidase (HRP) (Biosource, Camarillo, CA), and ABTS [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)] substrate (Sigma-Aldrich, Castle Hill, NSW, Australia). An *ex vivo* IFN-γ enzyme-linked immunospot (ELISPOT) assay was performed as described previously (4) by using 10 µg/ml of the inactivated and purified virus described above.

Statistics. Statistical analysis was performed by using SPSS for Windows (version 15.0, 2007; SPSS Inc., Chicago, IL). For comparison of two samples, the *t* test was used if the difference in the variances was <4, skewness was >-2, and kurtosis was <2; otherwise, the nonparametric Mann-Whitney U test was used. A *P* value of <0.05 was deemed significant.

RESULTS

Infection of adult C57BL/6 mice with Asian and Reunion Island isolates of CHIKV resulted in virus replication and rheumatic disease. To develop an adult wild-type mouse model of CHIKV arthritis, two CHIKV isolates were tested for their abilities to cause viremia and rheumatic disease. An Asian isolate initially produced inconsistent viremias and was thus serially passaged in C57BL/6 mice nine times before consistent viremias and disease could be obtained by using this virus (data not shown). The Reunion Island isolate behaved consistently without the requirement for mouse adaptation.

Infection of adult C57BL/6 mice with the Asian or a Reunion Island CHIKV isolate resulted in the development of clearly visible foot swelling (Fig. 1A). The swelling peaked 6 to

FIG. 1. Disease and virus replication. (A) Pictures of feet at the time (day 7) of peak swelling after inoculation of medium (control) or the Asian or the Reunion isolate of CHIKV. (B) Perimetatarsal foot swelling over time after inoculations described above (A) ($n = 6$ to 12 feet per group). Peak swelling (day 7 for Reunion isolates and day 8 for the Asian isolate) is indicated (*) and was ~75% higher for the Reunion Island isolate ($P = 0.024$). (C) Peripheral blood viremia ($n = 6$). (D) Virus titers in the foot ($n = 6$ to 10 mice per group). (E) Virus titers in quadriceps muscle, spleen, inguinal lymph nodes, and liver ($n = 3$ to 6 mice per group). (F) Replication (*, $P = 0.03$) and induction of CPE (*, $P = 0.021$ and 0.029) following infection of MEFs by the two CHIKV isolates (MOI of 0.1) (means of data from four replicates).

8 days after virus inoculation, with the Reunion Island isolate inducing significantly more swelling (Fig. 1B). The injection of inactivated CHIKV into naïve mice failed to produce significant foot swelling (data not shown).

The foot swellings were preceded by peripheral blood viremias that lasted 4 to 5 days and were similar for the two viral isolates (Fig. 1C). Viral titers in the feet peaked on day 1 after infection with the Reunion Island isolate and were on average higher than those for the Asian isolate at this time (Fig. 1D). On day 2, Asian isolate-infected mice had foot titers that were an average of 0.7 logs higher than those of Reunion Island isolate-infected animals (Fig. 1D). Replication-competent virus was detected in the feet until day 9 for both isolates (Fig. 1D). Virus was also prominent in muscle, spleen, lymph nodes, and liver but was detected only until day 3 in liver and day 5 in the remaining tissues (Fig. 1E). No virus was detected in brain tissues (data not shown).

Replication and cytopathicity in MEFs. Both the Asian and Reunion Island isolates replicated and induced CPE in primary murine embryonic fibroblasts (MEFs) (Fig. 1F). The Reunion Island isolate produced a higher titer on day 1 (Fig. 1F, left) and more cytopathicity on days 2 and 3 postinfection (Fig. 1F, right).

Arthritis was associated with a prolific infiltrate of mononuclear lymphocytes. A histological examination of feet of CHIKV-infected animals during the time of peak swelling showed a prodigious and generalized infiltrate of mononuclear cells, marked subcutaneous edema (Fig. 2), and large foci of cellular infiltrates in muscle tissues (Fig. 2). Image analysis showed that the overall level of infiltrate in the feet of mice infected with the Reunion Island isolate was significantly higher than that seen for mice infected with the Asian isolate (see Fig. S1A and S1B in the supplemental material). Clear signs of arthritis were evident for both isolates with marked mononuclear cellular infiltrates in and around the synovial membranes, with synovial membrane architecture also being disrupted (Fig. 2). Higher magnifications show a disruption of the synovial membranes, with normal cohesive cells (Fig. 2) lining the synovial membranes absent in infected animals (Fig. 2), although basement membranes were still visible (Fig. 2). Mononuclear cell infiltrates were present in the underlying connective tissues, and also evident in the articular spaces were fibrinous exudates, which were prominent in Reunion Island isolate-infected mice (Fig. 2). Fibrinous exudates were noted previously for reactive arthritis models (20, 30). Tenosynovitis was clearly evident with marked inflammatory cell infiltration of the connective tissues surrounding the tendons, with inflammatory cells also present in the capsules and sometimes in the distended space between the capsules and the tendons (Fig. 2). The tendons appeared normal. Skeletal muscle was severely affected. In Asian isolate-inoculated mice, some interstitial inflammatory cells were observed to form hypercellular foci of ~300 μm , which surrounded groups of necrotic muscle fibers. In Reunion Island isolate-inoculated mice, inflammatory foci of ~1 mm were observed, with the infiltrate severely distending endomysial and perimysial tissues, and the muscle fibers showed extensive necrosis (Fig. 2).

Other tissues exhibited milder lesions, with no differences apparent between the CHIKV isolates. Lymph nodes were mildly hypertrophic with mononuclear cell infiltration of the

cortex. Red pulp of the spleen was also infiltrated with numerous mononuclear cells, and white pulp exhibited some moderate hyperplasia of the T-cell-dependent areas (data not shown).

Long-term persistence of mononuclear infiltrates in feet. As CHIKV infection has been associated with prolonged illness (35), we undertook a histological examination of tissues from Reunion Island isolate-infected mice at several time points after foot swelling had subsided. Although tibial skeletal muscle, liver, spleen, and lymph node histology had returned to normal by day 14, mononuclear cell infiltrates were observed in subcutaneous and peritendinous connective tissues from days 14 to 21 (see Fig. S2A and S2B in the supplemental material). Small focal infiltrates were also observed in the muscle tissue of the feet (Fig. S2C and S2D), although these had decreased significantly from those seen on day 7. Although synovial membrane architecture had returned largely to normal by day 14, perisynovial tissues were mildly infiltrated by mononuclear cells, and some mild edema was also observed (Fig. S2E). Although occasionally present, these persistent infiltrates had largely resolved by days 30 to 40 (data not shown).

Arthritic infiltrates showed a predominance of monocytes, macrophages, and NK cells. Staining of the infected feet on day 7 with mouse macrophage-specific monoclonal antibody F4/80 showed a widespread infiltration of macrophages into synovial and surrounding connective tissues (Fig. 2). The staining was slightly more marked in feet of mice infected with the Reunion Island isolate, although this did not reach significance (see Fig. S1C in the supplemental material).

FACS analysis of leukocytes isolated from the swollen feet at the time of peak swelling illustrated a predominance of monocytes, macrophages, and NK cells with minor populations of B cells, T cells, and dendritic cells (Table 1). Results from control mice are also shown (Table 1), with 31-fold $\pm$ 7.7-fold-more CD45⁺ cells isolated from feet of mice infected with the Reunion Island isolate than from control feet.

Depletion of macrophages ameliorated disease. Macrophage infiltrates are prominent in primates infected with CHIKV (32), and their depletion ameliorated rheumatic disease in a mouse model of RRV infection (37). To investigate their role in the current model, mice were treated with clodronate liposomes to deplete macrophages and were infected the next day with the Reunion Island isolate. Foot swelling was significantly reduced in mice treated with clodronate compared with mice treated with control liposomes or PBS (Fig. 3A). These data support the view that macrophages are important drivers of the rheumatic disease induced by alphaviral infections (32, 37) and are consistent with data from F4/80 staining (Fig. 2) and FACS analyses (Table 1). Clodronate treatment also significantly prolonged viremia (Fig. 3B), suggesting that macrophages are also required for the clearance of virus.

Macrophages were productively infected with CHIKV. Macrophages appear to be important targets for CHIKV infection and replication in primates and humans (32, 66). To ascertain whether adult wild-type mouse macrophages could also be productively infected, a Reunion Island isolate virus encoding GFP (75) was used to infect splenocytes *ex vivo*. The infected cells were analyzed by FACS, and a population of GFP-positive (GFP⁺) F4/80⁺ cells that was not present in uninfected cultures was clearly identified (Fig. 4A). After inoculation of

FIG. 2. Histology and immunocytochemistry of feet from control mice or mice infected with the Asian or Reunion Island CHIKV isolate day 7 postinfection. The row labeled "Foot" shows subcutaneous edema (*), and foci of inflammatory cell infiltrates in muscle tissue (arrows) are indicated (bars, 1 mm). B, bone; M, muscle. The row labeled "Joint" shows that synovial membranes (arrowheads) were disrupted and contained mononuclear cell infiltrates (bars, 100 μ m). SS, synovial space. The row labeled "Synovium" shows that the normal cohesive cells lining the synovial membrane (control) (arrows) were absent in infected mice, sometimes replaced by moderate amounts of fibrinous exudate (columns labeled "Asian" and "Reunion") (arrows). The basement membrane was always visible (arrowheads). Numerous inflammatory cells, composed mainly of large mononuclear cells, have infiltrated the underlying connective tissue in infected mice (bars, 10 μ m). The row labeled "Tendon" shows marked tenosynovitis in CHIKV-infected mice, with inflammatory cells present in the tendon capsule and sometimes in the distended space (arrowhead) between the capsule and the tendon (bars, 100 μ m). T, tendon. In the row labeled "Muscle," extensive inflammatory infiltrate can be seen in CHIKV-infected mice, which was more pronounced in mice infected with the Reunion Island isolate (bars, 100 μ m). The row labeled "F4/80" shows metatarsal areas stained with the F4/80 macrophage-specific antibody (bars, 1 mm).

TABLE 1. Leukocyte (CD45⁺) populations isolated from the feet of control mice and the swollen feet of mice infected with the Reunion Island isolate 7 days previously^a

Cell type	FACS marker(s)	Mean % of cells in control mice $\pm$ SE	Mean % of cells 7 days postinfection $\pm$ SE
Monocytes	CD11b ⁺ F4/80 ^{lo/-}	69 $\pm$ 15.5	45 $\pm$ 9.9
Macrophages	F4/80 ⁺ CD11b ⁺	22.3 $\pm$ 2.6	21 $\pm$ 4
NK cells	NK1.1 ⁺	2.3 $\pm$ 2	16.3 $\pm$ 6
CD4 T cells	CD3 ⁺ CD4 ⁺	1.3 $\pm$ 2.1	5.2 $\pm$ 1.3
CD8 T cells	CD3 ⁺ CD8 ⁺	0.2 $\pm$ 1.5	1.2 $\pm$ 0.2
B cells	CD19 ⁺	3.5 $\pm$ 2.5	4.6 $\pm$ 2
Conventional DC	CD11c ⁺ F4/80 ⁻ NK1.1 ⁻	0.9 $\pm$ 0.1	6.4 $\pm$ 1.1
Plasmacytoid DC	B220 ⁻ PDCA1 ⁻	<0.1	3.8 $\pm$ 2.1

^a Control mice were injected with PBS 7 days previously. Feet from virus-infected animals contained, on average, 31-fold $\pm$ 7.7-fold-more CD45⁺ cells than feet from control animals. Data represent the means of data from two to three experiments with cells from three to five animals pooled. DC, dendritic cells.

this virus into mice, only virus not expressing GFP could be recovered (data not shown), suggesting that GFP expression is not stable.

Infection of cultured adherent splenic cells (>85% F4/80⁺ macrophages) with wild-type virus also resulted in the very efficient production of infectious virus (Fig. 4B) and widespread CPE (data not shown). These analyses indicate that wild-type macrophages from adult mice can be productively infected with CHIKV.

CHIKV infection was associated with induction of TNF- α , MCP-1, IFN- γ , IL-6, and IFN- α/β . Proinflammatory media-

FIG. 4. Infection of macrophages. (A) Splenocytes were infected with CHIKV carrying GFP (MOI of 2) for 24 h, stained with anti-F4/80-PE, and analyzed by FACS. The area of GFP⁺ F4/80⁺ cells is indicated by a box with dotted lines. (B) Cultured adherent cells from spleen (2×10^4 cells/well) (>85% F4/80⁺) were infected with the indicated MOIs of the Reunion Island isolate. Cells were then washed extensively, and supernatants were then assayed for viral titers at 0, 1, 2, and 3 days. The means of data from three replicates are shown.

tors, including TNF- α , MCP-1 (CCL2), IFN- γ , interleukin-6 (IL-6), and IFN- α/β , have been identified in primates and humans infected with CHIKV and in other alphaviral arthritides (32, 51, 71). The serum levels of several proinflammatory mediators were measured during viremia and disease in the current mouse model, and elevated levels of TNF- α , MCP-1, IFN- γ , IL-6, and IFN- α/β were observed (Fig. 5A). No signif-

FIG. 3. Clodronate depletion of macrophages. Mice ($n = 3$ to 4 per group) were treated with clodronate liposomes, control liposomes, or PBS and were infected on the next day with the Reunion Island isolate of CHIKV. (A) Foot swelling showing significant differences on the indicated (*) days (day 6, $P = 0.002$; day 7, $P = 0.01$; day 8, $P = 0.045$). (B) Mean viremia in the same mice described above (A). Viremia was significantly different between the clodronate liposome and control liposome groups on day 4 (*) ($P = 0.037$).

FIG. 5. Inflammatory mediator profiles. (A) Mice were infected ($n = 4$ to 8 per group) with Reunion Island or Asian CHIKV isolates, and on the indicated days, serum was analyzed for the indicated cytokines/chemokine. An asterisk indicates where levels were significantly different ($P = 0.007$ for TNF- α ; $P = 0.001$, 0.006, 0.004, and 0.004 for days 1, 2, 4, and 7, respectively for MCP-1; $P = 0.007$ for IFN- γ ; and $P = 0.015$ for IFN- α/β). (B) Feet ($n = 4$) taken 6 days postinfection were analyzed for cytokine/chemokine mRNA levels using real-time RT-PCR. mRNA levels for Reunion Island and Asian isolate-infected mice were significantly different for MCP-1 ($P = 0.019$) and IFN- γ ($P = 0.017$).

icant amount of IL-10 or IL-1 β was detected (data not shown). Compared with the Asian isolate, the Reunion Island isolate induced significantly more serum MCP-1, IFN- γ , and IFN- α/β during the viremic period and more TNF- α and MCP-1 during the disease period (i.e., period of foot swelling) (Fig. 5A). The significantly higher levels of IFN- α/β induced by the Reunion Island isolate on day 2 than those induced by the Asian isolate (Fig. 5A) were not associated with lower viremia, as might be expected (53, 60), but did correlate with lower Reunion Island virus titers in the feet on that day (Fig. 1D).

Analysis of the feet by real-time RT-PCR also showed that during the disease period, significantly more IFN- γ and MCP-1 mRNA was present in the feet of Reunion Island-infected mice than in the feet of Asian isolate-infected mice (Fig. 5B). Elevated TNF- α levels were also seen, although these levels were not significantly different between isolates. No significant IFN- $\alpha 4$ or IFN- β mRNA levels above those seen in mock-infected animals were detected (data not shown), suggesting that ongoing elevated levels of IFN- α/β are not required for the rheumatic inflammatory response (83). The increased levels of IFN- γ and MCP-1 mRNA induced by infection with the Reunion Island isolate were consistent with the increased rheumatic disease seen with this isolate (Fig. 1A and B). IFN- γ and MCP-1 are known to be involved in macrophage activation and recruitment as well as inflammation (19, 59, 71).

CHIKV infection induced high levels of CHIKV-specific IgG2c responses. The level of CHIKV-specific IgG1 and IgG2c antibodies induced after CHIKV infection was determined by standard ELISA. Infection produced high levels of CHIKV-specific IgG2c responses, with the Reunion Island isolate producing significantly higher levels than the Asian isolate ($P = 0.004$). The levels of IgG1 induced were very low and not significantly different for the two isolates (Fig. 6A). These data illustrate that CHIKV infection induces strongly biased Th1 responses.

An inactivated CHIKV vaccine protected against viremia and disease. A number of vaccines have been developed for CHIKV (2, 15, 47, 73, 82). However, it was previously not possible to evaluate the ability of CHIKV vaccines to prevent arthritis in an animal model. To illustrate the utility of the current model for testing prophylactic vaccines, mice were immunized once s.c. with a whole-virus, binary ethyleneimine-inactivated (7), Asian isolate CHIKV vaccine. A similar strategy was used previously for the generation of an experimental RRV vaccine (1). Ten micrograms of the CHIKV vaccine induced strong IgG2c and some IgG1 responses, with lower doses inducing much weaker responses. The addition of the adjuvant Quil A significantly increased the antibody responses; data are shown for the 0.1- μ g dose ($P < 0.001$) (Fig. 6A).

After challenge with the Reunion Island isolate, mice vaccinated with 0.01 μ g of vaccine had a significantly reduced viremia, and mice that received 0.1 to 10 μ g of vaccine showed no detectable viremia (Fig. 6B). Animals receiving the Quil A vaccine also showed no detectable viremia (data not shown). The same mice were assessed for foot swelling, with all vaccines significantly reducing the level of swelling, although a hint of swelling was still evident at the 0.01- μ g vaccine dose. This experiment illustrated not only that a vaccine derived from an old Asian isolate can protect against a virus isolate from the recent epidemic but also that vaccinated animals with

FIG. 6. Vaccination and CHIKV challenge. (A) Fifty percent end-point IgG1 and IgG2c titers 5 weeks after infection ($n = 6$) with the indicated virus isolate or vaccination with different doses of purified inactivated CHIKV vaccine ($n = 3$ per group). (B) Five weeks after vaccination, mice were challenged with the Reunion Island isolate, and the level of viremia was determined over 6 days. Mock vaccination was done with medium. (C) Foot swelling of the animals shown in B ($n = 6$ feet per group).

relatively low levels of antibody (compared with infected mice) were protected against viremia and disease.

IFN- α treatment before, but not after, infection prevented disease. IFN- α is used to treat a number of chronic viral infections, particularly hepatitis C (63), and has been proposed as a potential treatment for encephalitic alphaviruses (40, 84) and CHIKV (68). Like other alphaviruses (5, 60), CHIKV is sensitive to IFN- α/β (66). To determine whether IFN- α might have an application in preventing CHIKV arthritis, mice were injected with 10^3 IU IFN- α i.v., an IU/kg dose similar to that given per injection to humans with hepatitis C (43). The next day, the mice were challenged with the Reunion Island isolate. The mean peak viremia was significantly reduced by ~ 2.5 logs and delayed by 2 days (see Fig. S3A in the supplemental material), and disease was abolished (Fig. S3B). However, when IFN- α treatment was delayed until day 3, there was no significant effect on either viral titers in the feet or foot swelling (data not shown), suggesting that IFN- α treatment may have limited value as a therapeutic intervention.

RRV infection and anti-RRV antiserum protected against CHIKV. Cross-protection involving alphaviruses that cause lethal encephalitic disease in mice has been extensively studied

FIG. 7. Cross-protection with RRV. (A) Mice were infected with RRV or mock infected ($n = 6$ per group) and 4.5 months later were infected with the Reunion Isolate of CHIKV, and CHIKV viraemia was measured (an asterisk indicates significant differences [$P = 0.029$ and 0.005]). (B) CHIKV-induced foot swelling of mice that had received serum from naïve mice or mice previously infected with RRV or the Reunion Island isolate of CHIKV. (C) ELISA using serum from the RRV-infected mice described above (A) prior to CHIKV infection, with titers determined on purified inactivated CHIKV. (D) IFN- γ ELISPOT assay using splenocytes from a separate group of mice infected 4.5 weeks previously with the indicated virus and using purified inactivated CHIKV as an antigen.

(36, 81, 85). However, cross-protection against arthritis between arthrogenic alphaviruses has not been reported. To investigate this phenomenon, mice were mock infected or infected with RRV and were challenged with the Reunion Island isolate 4.5 months later. Mice previously infected with RRV had a ~3- to 4-log-lower mean peak viraemia after infection with CHIKV (Fig. 7A) and were protected against CHIKV disease (data not shown). To investigate the role of antibody in this cross-protection, antiserum from RRV-infected mice was adoptively transferred into naïve mice. These mice were then infected with CHIKV and showed a >3-log reduction in peak viraemia (data not shown) and were protected against CHIKV disease (Fig. 7B). As reported previously (10), antisera from CHIKV-infected mice protected against viraemia (data not shown) and disease, whereas sera from naïve mice had no effect (Fig. 7B). CHIKV and RRV both belong to the Semliki Forest virus antigenic complex (16). Therefore, as expected, serum from RRV-infected mice (prior to CHIKV challenge) cross-reacted with CHIKV (Fig. 7C) and showed CHIKV-

specific IgG1 and IgG2c antibody titers comparable to those seen after vaccination with 1 μ g of the inactivated CHIKV vaccine (Fig. 6A). T cells from RRV-infected mice also showed significant cross-reactivity with CHIKV virus, giving responses comparable to those seen after CHIKV infection (Fig. 7D).

DISCUSSION

Here we report a simple mouse model of CHIKV arthritis in wild-type adult mice. The model recapitulates the viraemia (55) and, importantly, the rheumatic symptoms of CHIKV infection of humans (6, 28, 54, 55), with clear signs of self-limiting foot swelling and histological evidence of acute and persistent arthritis, tenosynovitis, and myositis.

In establishing this model of CHIKV infection and disease, we observed that CHIKV obtained from mice infected Vero and BHK cells inconsistently (data not shown), for reasons which are currently unclear. To measure virus titers, we thus adopted a method of determining titers of serum and tissue extracts on C6/36 cells, followed by subculture onto parallel plates of Vero cells to determine which of the individual C6/36 wells contained virus. We also found that low levels of endotoxin or mycoplasma contamination can prevent efficient alphavirus infection *in vivo*, and we have used sensitive bioassays (27, 33) to ensure the absence of these potential contaminants in CHIKV preparations. Although s.c. and intradermal (i.d.) injections of CHIKV at the base of the tail produced viraemia, they produced no foot swelling (data not shown). The injection of one hind foot also did not result in significant swelling of the other hind foot (data not shown). We speculate that the delayed and/or lower CHIKV replication in the noninjected feet in the latter settings precludes the development of significant overt disease. These features probably represent a weakness of this mouse model, as mosquito-mediated infection in humans often results in the swelling of multiple joints (6, 50).

The cellular infiltrates seen in this mouse model of CHIKV disease correlate well with data from other studies of alphaviral arthritides. Pronounced monocyte/macrophage infiltrates in the synovial fluid of RRV-infected patients (18, 65) and in a monkey model of CHIKV infection (32) have been reported. The presence of high numbers of monocytes, compared to macrophages, has not previously been enumerated. Nevertheless, the abundance of monocytes/macrophages in the swollen feet of CHIKV-infected mice and the ability of macrophage depletion to ameliorate disease reaffirm the importance of these cells in the rheumatic disease induced by alphaviral infections (37, 46, 59, 71). NK cells were the next most abundant cell type in the swollen feet of CHIKV-infected mice (Table 1), and these cells were identified in the synovial fluid of RRV-infected patients (25) and in muscle tissues in a mouse model of RRV myositis (46). NK cells were shown previously to contribute to alphaviral encephalitis (3) and may thus also contribute to the immunopathogenesis of alphaviral arthritides (71). The low levels of B and T cells (Table 1) support the view that these cells do not play a major role in the immunopathogenesis in mouse models of alphaviral rheumatic disease (46). CD8 T cells have also been shown to have no protective effect against viraemia in a mouse model of RRV infection (38). Whether there is a role for T cells in human disease, where T cells in synovial effusions seem to be slightly more abundant

(65), remains unclear. The presence of dendritic cells and plasmacytoid dendritic cells is consistent with other studies showing the recruitment of these cells into the sites of virus replication (12, 23).

Macrophages have been shown to be important targets of infection in a primate model of CHIKV infection (32), and human macrophages have also been shown to be productively infected with CHIKV *in vitro* (66). Here we show that primary adult wild-type macrophages are also productively infected with CHIKV (Fig. 4C and D). However, clodronate depletion of macrophages significantly prolonged viremia, indicating that macrophages and/or their inflammatory products (e.g., TNF- α) (71) are also involved in promoting the clearance of virus. Clodronate treatment was recently reported to increase the viremia associated with another macrophage-tropic virus, dengue virus (17). Macrophages are thus a target for infection, promote viral clearance, and are important for the development of rheumatic disease. Given the phenotypic diversity, functional plasticity, and widespread distribution of macrophages, different types and/or sources of macrophages may be responsible for each of these activities. Long-term persistent infection of macrophages was reported previously for primates (32), and such infections may give rise to the chronic disease seen for some patients (35). Whether the persistent lesions seen in the feet in the current model are also due to low-level persistent infection of macrophages or are a result of an ongoing resolution of tissue damage remains to be established.

The *in vivo* cytokine/chemokine profiles associated with alphaviral arthritides have not been extensively studied (37, 51, 69). Here we show that CHIKV infection increased serum levels of MCP-1, IFN- α/β , IFN- γ , IL-6, and TNF- α and that the serum levels of these mediators peaked during the viremic period. Peripheral blood of monkeys infected with CHIKV also showed elevated levels of MCP-1, IFN- α/β , IFN- γ , IL-6, and TNF- α (32). A recent study of patients with CHIKV disease identified serum IL-1 β and IL-6 as biomarkers of CHIKV disease severity, with IFN- α , IL-5, IL-7, IL-10, and IL-15 also being detected (51). However, serum MCP-1, IFN- γ , and TNF- α levels were no different in CHIKV-infected patients and healthy controls (51). The current study may provide some insight into this apparent discrepancy. The peak in serum proinflammatory mediator levels (Fig. 5A) clearly preceded the onset of foot swelling (Fig. 1B), suggesting that once patients present with arthritis/arthralgia, the viremia, and the associated peripheral inflammatory response, may have abated (51). Importantly, in the arthritic feet of infected mice, the virus continues to replicate (Fig. 1D), and the levels of expression of MCP-1, IFN- γ , and TNF- α continue to be elevated (Fig. 5B). The levels of these inflammatory mediators are also elevated in a number of viral arthritides and are also key players in rheumatoid arthritis (71). Increased levels of MCP-1, IFN- γ , and TNF- α were also detected in synovial effusions in RRV-infected patients with polyarthritis (37), with RRV also reported to persist in the joints of these patients (65). These observations support the view that CHIKV arthritis/arthralgia is an inflammatory disease (56).

Preclinical testing of vaccines and immunotherapies for CHIKV-induced arthritis has previously not been possible. Here we illustrate the utility of the new mouse model by evaluating a simple inactivated CHIKV vaccine and IFN- α

treatment. Inactivated alphavirus vaccines have been developed for veterinary use (44) and were described previously for RRV (29). A simple, unadjuvanted, and inactivated CHIKV vaccine derived from the Asian isolate was shown herein to be immunogenic and protected mice against rheumatic disease induced by the Reunion Island isolate. Antibodies appear to be the main mediators of protection against alphaviruses (22, 44), including CHIKV (10) (Fig. 7B), and mice with only low levels of CHIKV-specific antibodies were protected against disease (see Fig. 6A and C at the 1- μ g/mouse dose). This finding is consistent with previous data showing that mice with only low levels of anti-RRV antibodies were protected against RRV viremia (87). Taken together, these data suggest that vaccines against arthrogenic alphaviruses may not need to induce high levels of immunity in order to mediate effective protection.

In contrast to alum (73), which was unable to improve the antibody responses induced by an inactivated RRV vaccine (87), Quil A was very effective at increasing antibody responses to the inactivated CHIKV vaccine. This adjuvant was shown previously to increase antibody responses to several inactivated-virus vaccines (67, 86). Quil A also tends to promote Th1 responses, whereas alum biases responses toward Th2 (73). Natural CHIKV infection induced an extraordinarily Th1-biased response, and Th2 bias is generally considered to promote virus replication (61). Th1-promoting adjuvants are therefore likely to be more appropriate for CHIKV vaccines.

IFN- α was proposed previously to be a potential treatment for encephalitic alphaviruses and CHIKV (40, 68, 84). We show here that IFN- α treatment given before infection reduced viremia and prevented rheumatic disease. However, if given on day 3 postinfection, there was no significant effect on the levels of virus recovered from the feet or any effect on disease. This parallels other previous reports that showed that IFN- α treatment is less effective against alphaviral encephalitic disease when given later in infection (21, 53). The activation of suppressors of cytokine signaling proteins by the inflammatory response (11) and/or disruption of IFN- α/β signaling by viral proteins (64) may be involved in limiting the effectiveness of IFN- α treatment in an established infection. The value of IFN- α as a therapy for CHIKV disease is thus probably limited.

RRV infection and anti-RRV antibodies were able significantly to reduce CHIKV viremia and to protect mice against CHIKV disease. Although an antibody-dependent enhancement of infection was shown previously for RRV (41, 70), there was no indication that anti-RRV antibodies enhanced CHIKV infection. The adoptive-transfer experiments (Fig. 7B) also confirmed that antibodies mediate effective protection against CHIKV (10). CD4 T cells may also play a role (36), as CD4 T cells were shown previously to protect mice against other alphaviruses (88). The high level of cross-reactivity seen for B- and T-cell responses (Fig. 7C and D) may reflect the >75% sequence homology (>60% sequence identity) between RRV and CHIKV in the structural polyprotein. The putative receptor binding domains of arthrogenic alphavirus E2 glycoproteins are also highly conserved (34). The C-terminal two-thirds of the capsid proteins of RRV and CHIKV also show >95% sequence homology (>90% identity), with this region containing a previously identified T-cell epitope (38). These results

suggest that individuals with prior exposure to RRV (mostly Australasians [24]) would be protected from CHIKV disease.

A number of differences between the behaviors of the Asian and Reunion Island isolates were evident, which may shed some light on pathogenesis. The Reunion Island isolate replicated faster in feet (Fig. 1D); showed a more pronounced mononuclear infiltrate (see Fig. S1 in the supplemental material); induced significantly more serum TNF- α , MCP-1, IFN- γ , and IFN- α/β (Fig. 5A) and more MCP-1 and IFN- γ mRNA in the feet (Fig. 5B); generated more virus-specific IgG2c responses (Fig. 6A); and replicated faster and induced more CPE in MEFs *in vitro* (Fig. 1F). One might speculate that faster initial replication (Fig. 1D) and, perhaps, more CPE (49) induce more early MCP-1, IFN- α/β , and IFN- γ , with IFN- γ likely derived from activated NK cells (3, 25, 46). The increased levels of these proinflammatory mediators may lead to more infiltration and activation of macrophages and MCP-1 and IFN- γ production, ultimately resulting in increased rheumatic inflammation (19, 37, 59, 71).

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