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γδ T Cells Play a Protective Role in Chikungunya Virus-Induced Disease

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ABSTRACT

Chikungunya virus (CHIKV) is an alphavirus responsible for causing epidemic outbreaks of polyarthralgia in humans. Because CHIKV is initially introduced via the skin, where γδ T cells are prevalent, we evaluated the response of these cells to CHIKV infection. CHIKV infection led to a significant increase in γδ T cells in the infected foot and draining lymph node that was associated with the production of proinflammatory cytokines and chemokines in C57BL/6J mice. γδ T cell$^{-/-}$ mice demonstrated exacerbated CHIKV disease characterized by less weight gain and greater foot swelling than occurred in wild-type mice, as well as a transient increase in monocytes and altered cytokine/chemokine expression in the foot. Histologically, γδ T cell$^{-/-}$ mice had increased inflammation-mediated oxidative damage in the ipsilateral foot and ankle joint compared to wild-type mice which was independent of differences in CHIKV replication. These results suggest that γδ T cells play a protective role in limiting the CHIKV-induced inflammatory response and subsequent tissue and joint damage.

IMPORTANCE

Recent epidemics, including the 2004 to 2007 outbreak and the spread of CHIKV to naive populations in the Caribbean and Central and South America with resultant cases imported into the United States, have highlighted the capacity of CHIKV to cause explosive epidemics where the virus can spread to millions of people and rapidly move into new areas. These studies identified γδ T cells as important to both recruitment of key inflammatory cell populations and dampening the tissue injury due to oxidative stress. Given the importance of these cells in the early response to CHIKV, this information may inform the development of CHIKV vaccines and therapeutics.

Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus belonging to the *Togaviridae* family and was first isolated in Tanzania in 1952 (1–3). It is responsible for epidemics of debilitating rheumatic disease associated with inflammation and destruction of musculoskeletal tissues in humans (4). Beginning in 2004, CHIKV reemerged, causing millions of infections in coastal Africa, islands of the Indian Ocean, and India (5–9). Infected visitors to these areas of CHIKV epidemics returned to Europe, Australia, the United Kingdom, Japan, Belgium, Canada, and the United States (10–15), and importation of human cases to northern Italy, New Caledonia, China, the French Riviera, and Saudi Arabia produced autochthonous outbreaks resulting from infection of naive mosquito populations (16–20). In late 2013, the first local transmission of CHIKV in the Americas was identified in Caribbean countries and territories, indicating that mosquito populations in these areas had become infected with the virus and are competent to transmit it to humans (21–23). Since that time, a total of approximately 1.2 million suspected and over 24,000 confirmed cases of CHIKV have been reported in 44 countries or territories in the Caribbean or South America (24), including hundreds of travelers returning to the United States, with subsequent localized transmission in Florida in 2014.

Analysis of the explosive 2004–2007 epidemic suggests that new disease manifestations may be associated with CHIKV that increase cause for concern. During the epidemic, CHIKV infection resulted in increased morbidity as well as mortality in adults (25, 26). Additionally, greater numbers of CHIKV-infected persons developed the more severe forms of the disease, including neurological complications and fulminant hepatitis, while maternal-fetal transmission associated with neonatal encephalopathy was also reported (25, 27–30). Of particular concern with this outbreak was the threat of CHIKV introduction and spread into new regions, in part through successful adaptation of the virus, allowing it to infect not only the classical vector *Aedes aegypti* but also the widely distributed mosquito vector *Aedes albopictus* (5, 31–35).

Symmetrical polyarthritis is the hallmark of CHIKV infection and is responsible for the severe arthralgia and inflammation associated with infection (36, 37). In addition to rheumatic disease, CHIKV infection is also associated with fever, headache, rigors, photophobia, myalgia, and a petechial or maculopapular rash (38, 39). The acute phase of CHIKV infection typically lasts from days to several weeks; however, multiple studies have reported chronic fatigue and chronic joint pain for a significant time postinfection,

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with one study demonstrating persistent joint pain in roughly 79% of patients at 27.5 months postinfection, with 5% of those infected with CHIKV meeting a modified version of the American College of Rheumatology criteria for rheumatoid arthritis (14, 37, 40–45). In both human cases and mouse models, CHIKV-induced immunopathology is implicated as the primary mediator of damage and persistent pain (46–50). Support for the immune-mediated pathogenesis of CHIKV comes from the observation that inflammation continues well after viral replication has ceased (51). Additionally, high levels of proinflammatory cytokines are observed both in humans and in mouse models of CHIKV disease. Finally, the contributions of immune mediators to pathology in closely related alphavirus infections have been well described (52–57).

The bulk of the work examining CHIKV-induced pathology and viral clearance has been centered on the innate immune response following infection (58–60). In particular the type I interferon (IFN) pathway is critical for controlling viral replication and pathogenesis during the early stages of CHIKV infection, though this response does not completely ablate CHIKV replication or disease (61–66). With respect to adaptive immunity, B cells and antibodies are known to be important, as CHIKV RNA persists in the serum of B cell-deficient mice for greater than a year and passive transfer of CHIKV-immune serum to IFNAR$^{-/-}$ mice protects against lethal infection (67). In human acute CHIKV infection, CD8$^+$ T cells predominate in the early stages of the disease, with CD4$^+$ T cells mediating the adaptive response at later times postinfection (60). Both CD4$^+$ and CD8$^+$ T cells have been shown to infiltrate CHIKV-infected tissues in mouse models of infection (66, 68). CD4$^+$ T cells were recently shown to contribute to pathogenesis during CHIKV infection in mice independent of changes in viral titer and IFN-γ production (69). CD4$^{-/-}$ mice had lower levels of anti-CHIKV antibody with reduced neutralizing activity, although this did not affect their ability to control CHIKV infection (67). Regulatory T cells are also important in the pathogenesis of CHIKV, as interleukin-2 (IL-2) antibody-mediated expansion of regulatory T cells results in inhibition of CHIKV-specific CD4$^+$ effector cells and abolishes joint pathology in mice (70). While these studies provided evidence that T cells contribute to CHIKV protection and pathogenesis, characterization of the different types of T cells responsible and the mechanism by which these cells contribute to CHIKV infection requires further study.

Since arboviruses are delivered to the human host subcutaneously by feeding mosquitos, it stands to reason that resident skin immune cells would serve as an important first defense against these infections. Immune cell populations within the skin are comprised of a heterogeneous population, including numerous types of dendritic cells (DCs), monocytes, and lymphocytes, with γδ T cells being the most abundant resident T lymphocytes in the skin and mucosal surfaces (71). γδ T cells lack major histocompatibility complex (MHC) restriction such that they react to antigens without the need for conventional processing and have been shown to be key players in cytotoxicity, cytokine secretion, and enhancement of DC maturation and function (72, 73). Notably, increased inflammation and exaggerated tissue damage observed in γδ T cell-deficient mice infected with various pathogens identified a requirement for γδ T cells in the resolution of potentially damaging immune responses and prevention of immune-mediated damage (74–79). γδ T lymphocytes have been studied in several different arbovirus infections, where they have been shown to contribute to both the innate and adaptive immune response (80–84).

To determine if γδ T cells play a role in CHIKV infection, wild-type (wt) C57BL/6J mice and mice deficient for the γδ T cell receptor (γδ T$^{-/-}$) were infected and monitored for disease severity, viral replication, and joint and tissue pathology. Our results indicated that in the absence of γδ T cells, monocytic inflammatory cells and inflammatory cytokines increase at the site of CHIKV infection, and disease signs and tissue damage are exacerbated.

MATERIALS AND METHODS

Virus. The virus used in this study was produced from electroporation of baby hamster kidney (BHK-21) cells with *in vitro*-transcribed RNA (mMESSAGE mMACHINE SP6 transcription kit; Life Technologies) derived from plasmid pMH56.2, which is an infectious clone that carries the full-length genome of CHIKV isolate SL15649. pMH56.2 was produced by sequential ligation of commercially synthesized genome fragments (BioBasic) into a modified pSinRep5 plasmid (Invitrogen). Detailed sequences and cloning details are available from the authors upon request. The clinical isolate SL15649 has been previously described (68). All studies were performed in certified biological safety level 3 facilities in biological safety cabinets with protocols approved by the University of North Carolina at Chapel Hill Department of Environment, Health and Safety and the Institutional Biosafety Committee.

Mice. C57BL/6J and γδ T cell$^{-/-}$ mice on a C57BL/6J genetic background were obtained from the Jackson Laboratory. All mice were bred in-house as homozygous knockouts. Twenty-four-day-old mice were inoculated in the left rear footpad with 100 PFU of CHIKV in sterile phosphate-buffered saline (PBS) in a 10-μl volume. Control animals were inoculated with 10 μl of sterile PBS alone. Mice were weighed daily and monitored for clinical signs of disease, including swelling of the ipsilateral foot by using calipers. At indicated times postinfection, mice were sacrificed by isoflurane (Attane; Minrad, Inc.) overdose. Animal husbandry and experiments were performed in accordance with the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee guidelines and approval.

Virus titers. At various times postinfection, mice were sacrificed and perfused by intracardial injection with 1× PBS. Blood was collected prior to perfusion, removed to serum separator tubes, and centrifuged for 5 min at full speed to collect serum. Tissues were dissected, weighed, homogenized in PBS, and stored at −80°C. The amount of infectious CHIKV present was determined by standard plaque assay on Vero cells (ATCC CCL-81).

Histological analysis. Mice were sacrificed and perfused by intracardial injection with 4% paraformaldehyde (PFA), pH 7.3. Hind limb tissues were embedded in paraffin, and 5-μm sections were prepared. Tissues were stained with hematoxylin and eosin (H&E), and tissue sections were evaluated for the extent and severity of inflammation and musculoskeletal tissue damage. Photomicrographs were obtained using an Olympus BX43 model microscope with CellSens software.

Immunohistochemistry. Tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 μm, and affixed to positively charged glass slides. Slides were deparaffinized with a graded series of xylene and ethanol washes followed by microwaving for 20 min in 10 mM sodium citrate buffer (pH 6) for antigen retrieval. Specimens were blocked in 10% donkey serum (Jackson ImmunoResearch) and subsequently stained with antibody specific for nitrotyrosine (Millipore). Staining was detected with the use of biotinylated secondary antibodies followed by streptavidin–DyLight-594 conjugate (Jackson ImmunoResearch). Stained sections were mounted in ProLong Antifade Reagent Gold with 4′,6-diamidino-2-phenylindole (Invitrogen) and viewed with an Olympus BX60 fluores-

cence microscope; images were collected with the iVision software v.4.0.0 (BioVision Technologies).

Flow cytometry. Inoculated mice were sacrificed and perfused with 1× PBS. The skin was removed from the hind limbs and the leg was dissected just above the patella, ensuring that the bone marrow was not exposed. Following gentle separation of tissues from bone with a scalpel, the entire tissue sample was incubated for 1 h with vigorous shaking at 37°C in digestion buffer (RPMI 1640, 10% fetal bovine serum, 15 mM HEPES, 2.5 mg/ml collagenase A [Roche], 1.7 mg/ml DNase I [Sigma]). Following digestion, cells were passed through a 40-μm cell strainer and washed in wash buffer, and total viable cells were determined by trypan blue exclusion. Cells were stained in fluorescence-activated cell-sorting staining buffer (1× Hanks balanced salt solution, 1% fetal bovine serum, 0.01% sodium azide) with the following antibodies from eBioscience: fluorescein isothiocyanate (FITC)–F4/80, phycoerythrin cyanine (PE)-NK1.1, PE-Texas Red (PETR)–CD11c, peridinin chlorophyll (PerCP)-Ly6C, PE-cyanine 7 (Cy7)–lymphocyte common antigen (LCA), eF450-CD11b, allophycocyanin (APC)–MHC-IIc, APC-eF780–Ly6G, PE–γδ T cell receptor (TCR), PETR-CD4, and FITC-CD3. Cells were fixed in 4% paraformaldehyde overnight before being analyzed on a cyan cytometer (Dako Cytomation) using the Summit software (Beckman-Coulter).

Quantitative real-time PCR. Real-time PCR was performed using ABI Prism 7300 sequence detection system software (v14.0). For absolute quantification of viral RNA, a sequence-tagged CHIKV-specific reverse transcription (RT) primer in the reverse transcription reaction mixture, CHIKV sequence-specific forward reverse primers, and an internal TaqMan probe were used as previously described (50). Standard curves were derived from 15-fold dilutions of the CHIKV infectious clone plasmid, which contained nonstructural CHIKV coding sequences. Real-time PCR was run using ABI Prism 7300 sequence detection system software (v14.0).

Quantitative protein analysis. The treated ankle and foot from mock- or CHIKV-infected mice were harvested at the designated times and homogenized in sterile PBS. From the homogenates, protein levels of cytokines/chemokines were evaluated using multiplex bead array assays. All the antibodies and cytokine standards were purchased as antibody pairs from R&D Systems (Minneapolis, MN) or Peprotech (Rocky Hill, NJ). Individual Luminex bead sets (Luminex, Riverside, CA) were coupled to cytokine-specific capture antibodies according to the manufacturer's recommendations. Biotinylated antibodies were used at twice the concentrations recommended for a classical enzyme-linked immunosorbent assay (ELISA) according to the manufacturer. All assay procedures were performed in assay buffer containing PBS supplemented with 1% normal mouse serum (Gibco BRL), 1% normal goat serum (Gibco BRL), and 20 mM Tris-HCl (pH 7.4). The assays were run using 1,200 beads per set of each of the cytokines measured per well in a total volume of 50 μl. The plates were read on a Luminex MagPIX platform. For each bead set, >50 beads were collected. The median fluorescence intensities of the following cytokines/chemokines were recorded for each bead and used for analysis with the Milliplex software using a 5P regression algorithm: granulocyte-monocyte colony-stimulating factor (GM-CSF), IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-15, IL-17α, IL-22, CXCL2/macrophage inflammatory protein 2α (MIP-2α), CXCL10/IP-10, CXCL1/KC, CXCL5/LIX, CXCL9/MIG, CXCL12/SDF1α, CXCL20/MIP-3α, CCL-2/monocyte chemoattractant protein 1 (MCP-1), CCL3/MIP-1α, CCL-4/MIP-1β, CCL5/RANTES, CCL7/MCP-3, and tumor necrosis factor alpha (TNF-α).

Statistical analyses. All statistical analyses were performed within R (www.r-project.org). Data were transformed to fit normality (transformations are indicated in the descriptions of specific results) and analyzed within an analysis of variance (ANOVA) framework. Specifically, we used two-way ANOVA to examine the role of time postinfection and of different host genetics on various responses to infection. Within assay type, we utilized Bonferroni corrections to maintain stringency.

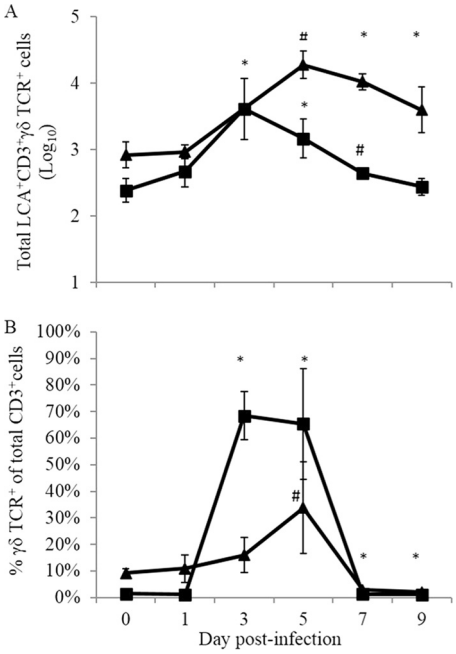

FIG 1 Numbers and proportions of γδ T cells following CHIKV infection. C57BL/6J mice were infected in the left footpad with 100 PFU CHIKV, and tissues were harvested and processed for flow cytometry analysis. (A) Numbers of γδ T cells identified in both the ipsilateral foot (▲) and draining lymph node (■) at the times indicated postinfection. (B) Proportions of γδ T cells in the same tissues. *, $P \leq 0.05$; #, $P \leq 0.01$ (compared to day 0 values for each tissue, using a two-tailed *t* test).

RESULTS

CHIKV infection increases activated γδ T cells at the site of infection and within the draining lymph nodes of C57B6/J mice. Although the contributions of αβ T cells to CHIKV clearance and pathogenesis have been examined (66, 68, 69, 85), to date no experiments have aimed at examining potential roles for γδ T cells in the disease process. To assess whether CHIKV infection of wild-type mice induced a γδ T cell response, infected feet and draining lymph nodes (DLNs) were harvested, and single-cell suspensions were assayed by flow cytometry. As shown in Fig. 1A, LCA^+ $CD3^+$ γδ TCR^+ T cells significantly increased in number over day 0 levels by 3 to 7 days postinfection (dpi) in both the ipsilateral foot/ankle and DLNs following CHIKV infection. In addition to increased numbers, the proportion of all $CD3^+$ γδ TCR^+ T cells increased in both the DLNs and ipsilateral foot throughout the course of infection, with the percentage of $CD3^+$ γδ TCR^+ T cells peaking in the DLNs at 3 to 5 dpi and in the infected foot at 5 dpi (Fig. 1B).

This finding that γδ T cells rapidly accumulate at the site of infection and within the draining lymph nodes of CHIKV-infected C57BL/6J mice suggests that these cells are early responders to CHIKV infection in the mice and may affect the outcome of infection.

Mice lacking γδ T cells develop more severe CHIKV-induced disease than wild-type animals. Previous studies demonstrated that C57BL/6J mice infected with a clinical isolate of CHIKV developed disease characterized by decreased weight gain and edema/swelling of the inoculated foot and that these disease signs correlated with inflammation and damage of infected tissues (59,

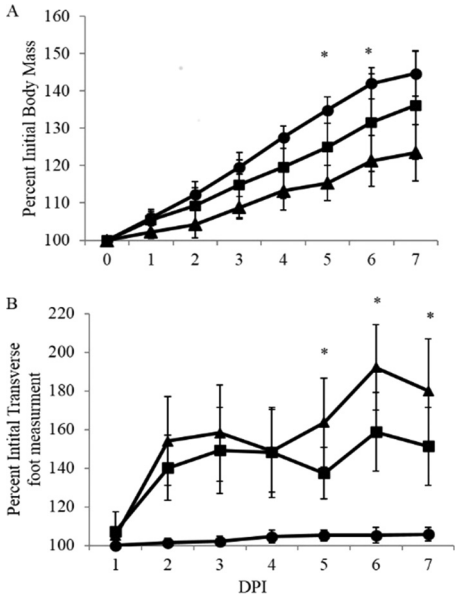

FIG 2 Mice lacking γδ T cells develop more severe CHIKV-induced clinical signs of disease than do wt C57BL/6J mice. Wild-type C57BL/6J (■), and γδ $T^{-/-}$ (▲) mice were infected with 100 PFU of CHIKV or mock infected (●) and monitored at 24-h intervals for signs of disease, including weight gain (A) and swelling of the ipsilateral foot, measured using calipers (B). Results are representative of four duplicate experiments ($n = 5$). Statistical significance was determined by a two-tailed *t* test: *, $P \leq 0.05$ (comparing C57BL/6J to γδ$T^{-/-}$ CHIKV-infected animals).

68). We directly assessed the role of γδ T cells in the pathogenesis of CHIKV infection by infecting 24-day-old mice deficient for the γδ TCR and wt mice with 100 PFU CHIKV in the left rear footpad. Mice were monitored daily for weight gain and swelling in the ipsilateral foot, measured using calipers. Clinically, CHIKV infection was more severe in γδ $T^{-/-}$ mice, evidenced by both their failure to gain weight at the same rate as infected wt mice (Fig. 2A) and more pronounced ipsilateral hind limb swelling measured at 5 to 7 dpi (Fig. 2B). These clinical disease differences correlated with the time of maximum γδ T cell presence at the site of infection, further supporting the idea that this T cell subset is a major player in the disease process and/or immune response to CHIKV infection.

CHIKV infection induces greater inflammatory tissue damage in γδ $T^{-/-}$ mice than in wt mice. Footpad inoculation of C57BL/6J mice with CHIKV results in inflammation and musculoskeletal pathology, with peak severity from 7 to 10 dpi (68). In these studies, H&E staining was used to analyze inflammation and tissue damage following CHIKV infection of wt and γδ $T^{-/-}$ mice. Mock-infected feet displayed no significant histopathological changes (Fig. 3A and D). Both CHIKV-infected C57BL/6J (Fig. 3H and K) and γδ $T^{-/-}$ (Fig. 3I and L) joint tissues displayed variable histopathological changes compared to mock-infected tissues (Fig. 3G and J), but no noteworthy histopathologic differences were observed between wild-type and γδ $T^{-/-}$ mice. In either strain, following CHIKV infection, the synovium was expanded to a thickness of 2 to 4 cells by hypertrophied (reactive) synovial cells and infiltrating inflammatory cells. Perisynovial tissue appeared variably expanded by edema and mixed inflammation. Joint spaces variably contained scattered sloughed cells and infiltrating mixed inflammatory cells. In contrast to synovial joints, CHIKV-induced myositis within the foot was more severe in γδ $T^{-/-}$ mice than in wt C57BL/6 mice. CHIKV infection of wild-type mice resulted in abundant inflammatory cells replacing nearly all pedal myocytes which had been lost to necrosis (Fig. 3B and E). Myocyte loss and inflammation were even more severe in CHIKV-infected γδ $T^{-/-}$ mice, with complete replacement of myocytes by inflammation and associated tendons remaining only partially intact (Fig. 3C and F). Based on a lack of remaining myocytes in the γδ $T^{-/-}$ mice, it appears that the absence of γδ T cells increased tissue damage in the CHIKV-infected foot by 7 dpi. To qualitatively assess the degree of tissue damage at the site of CHIKV infection and replication, immunofluorescence was performed on sections from mock- and CHIKV-infected mice to detect nitrotyrosine (NT), a well-established marker of protein damage caused by oxidative stress. Nitrotyrosine is a stable end product of peroxynitrite oxidation and is a marker for inflammation and NO-dependent damage *in vivo* (86, 87). The presence of nitrotyrosine has been detected in various inflammatory processes, including arthritis in humans (88) and rodents (89). As shown in Fig. 4, compared to mock-infected mice (Fig. 4A and D), NT was increased at sites of inflammation in the ipsilateral foot of both C57BL/6J (Fig. 4B and E) and γδ $T^{-/-}$ mice (Fig. 4C and F). However, the presence of NT staining was enhanced in the infected feet of CHIKV-infected γδ $T^{-/-}$ mice compared to feet of wt animals. These data taken together with the clinical data and histological evidence of increased inflammation suggest that γδ T cells have a role in limiting inflammatory cell-induced tissue damage during CHIKV infection.

γδ TCR deficiency does not affect viral replication *in vivo*. To determine if the increased severity of disease in γδ $T^{-/-}$ mice correlated with differences in viral titers, infectious virus levels were quantified by standard plaque assay. Serum samples and ipsi- and contralateral foot/ankle tissues were harvested from wt or γδ $T^{-/-}$ mice at 24-h intervals following infection with 100 PFU CHIKV. Tissues were homogenized in PBS and assayed on Vero cell monolayers. CHIKV replicated in the serum (Fig. 5A) and the ipsilateral foot/ankle (Fig. 5B) with no titer differences observed between C57BL/6J and γδ $T^{-/-}$ mice. Further, no difference in titer was observed in contralateral feet (data not shown). As confirmation, we ran CHIKV-specific real-time PCR to assess viral RNA copies, and we saw no difference in the amount of CHIKV RNA in the ipsilateral foot/ankle at 1 to 7 dpi (Fig. 5C). These results demonstrate that the increased disease and damage associated with CHIKV infection in γδ $T^{-/-}$ mice are not due to increased viral replication, spread, or the inability to clear virus.

γδ T cell deficiency increases key populations of inflammatory cells in infected tissues. Based on the finding that γδ $T^{-/-}$ mice have increased inflammation and damage within infected tissues despite equivalent viral titers, it was of interest to assess the composition of the inflammatory cell infiltrates. To this end, leukocytes were isolated from the ipsilateral foot/ankle tissues at 5 and 7 days after inoculation with 100 PFU in CHIKV into wt and γδ $T^{-/-}$ mice. At 5 dpi, the proportion of total monocytes (LCA^+ $CD11b^+$ CDllc-$Gr1^-$), inflammatory $Ly6C^+$ monocytes, and regulatory $Ly6C^-$ monocytes were all significantly increased in the γδ $T^{-/-}$ mice (Fig. 6A to C). Similar increases were not seen until day 7 in the wt animals. Given that monocytes/macrophages have been shown to contribute to CHIKV-induced inflammatory pathology (66), the finding that these monocyte populations were

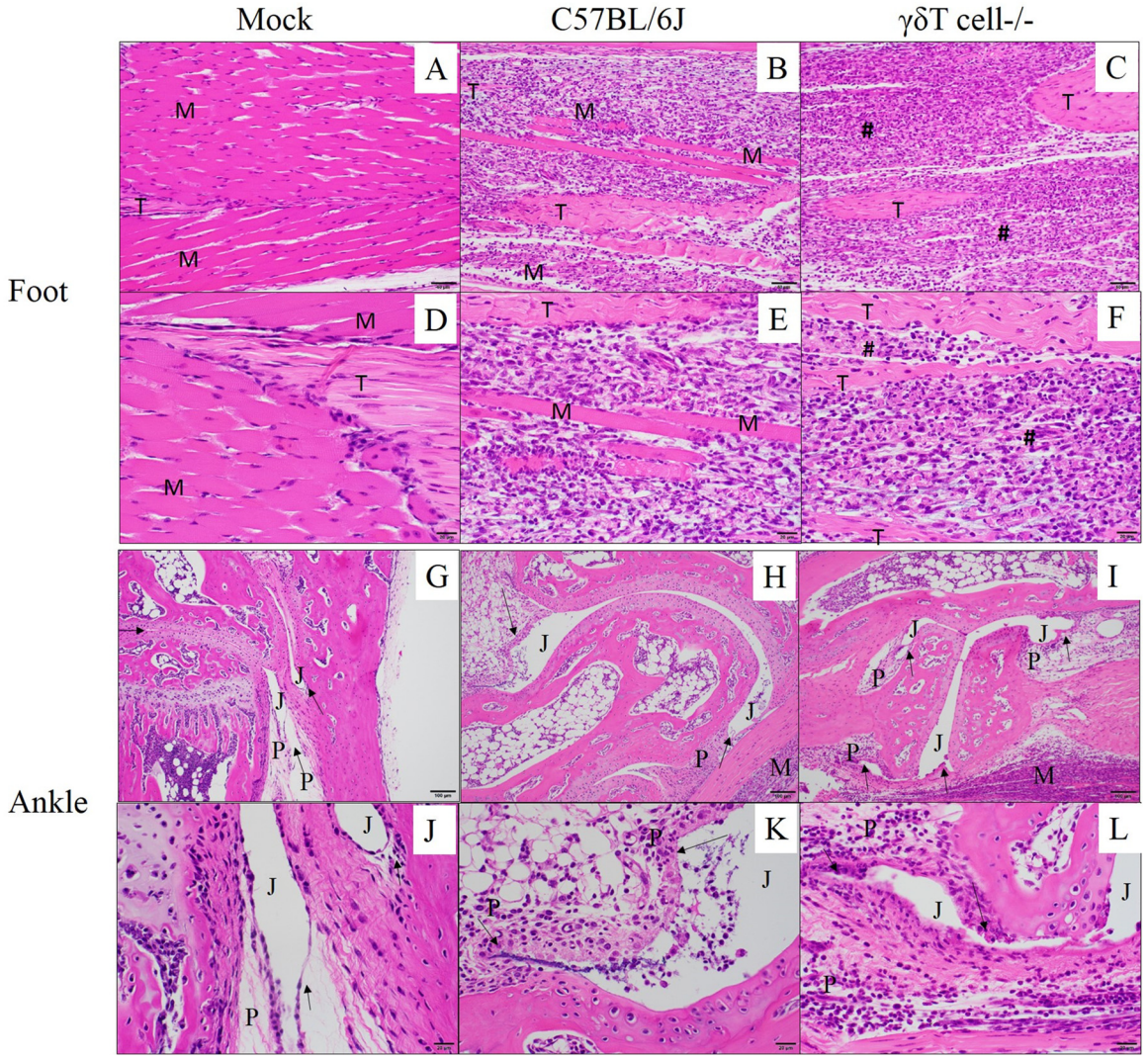

FIG 3 Inflammatory cell infiltrate increases in CHIKV-infected tissues in the absence of γδ T cells. Twenty-four-day-old C57BL/6J or γδ $T^{-/-}$ mice ($n = 5$) were inoculated with diluent only (mock) or 100 PFU of CHIKV in the left rear footpad. At 7 dpi, mice were sacrificed and perfused by intracardial injection with 4% paraformaldehyde. Tissue sections of the ipsilateral foot (A to F) or ankle joint (G to L) were stained with H&E to determine the degree of inflammation and tissue damage at the various sites. M, muscle tissue; #, area of complete myocyte loss with replacement by inflammation; T, tendon; P, periarticular tissue; J, joint space; arrows, synovial lining. Photomicrographs were taken at 200× (A, B, C, G, H, and I) and 400× magnification (D, E, F, J, K, and L).

increased in the γδ $T^{-/-}$ but not wt mice at early times in the disease process suggests that γδ T cells may decrease early monocyte influx and help limit tissue damage caused by these cells.

Lack of γδ T cells alters the levels of key mediators of inflammation at sites of CHIKV infection. Based on histological and flow cytometry data indicating increased monocyte populations in the feet/ankles of γδ T cell-deficient versus wt mice following CHIKV infection, we investigated the impact of γδ T cell deficiency on production of various inflammatory cytokines and chemokines following CHIKV infection by using a multiplex ELISA for 24 unique cytokines and chemokines. C57BL/6J and γδ $T^{-/-}$ mice were infected, and foot/ankle tissues were harvested at 5 and 7 dpi. No differences in the levels of cytokines/chemokines were observed in mock-infected C57BL/6J or mock-infected γδ $T^{-/-}$ animals at any time postinfection (data not shown). Chemokine/cytokine responses could be grouped into three categories: a group in which levels were unchanged between mock- and CHIKV-infected animals at the time points analyzed, a group in which CHIKV infection led to increased expression but with no differences between the two mouse strains, and a third category in which wild-type and γδ $T^{-/-}$ mice exhibited significant differences in expression following CHIKV infection. In the first group, IL-1β, IL-4, IL-15, IL-17α, IL-22, and GM-CSF showed no detectable induction over mock-infected animals at the time points tested in wild-type or γδ $T^{-/-}$ mice (data not shown). Within the second group, comprising IL-6, TNF-α, IL-10, IL12p70, and CXCL5/LIX, all showed induction following CHIKV infection, but no differences in expression were detected between the two mouse strains (data not shown). The last group was comprised of chemokines which showed enhanced expression in γδ $T^{-/-}$ compared to wild-type mice at day 7 postinfection: CCL2/MCP1, CCL4/MIP1β, CCL7/MCP3, CXCL2/MIP2, and CXCL9/MIG

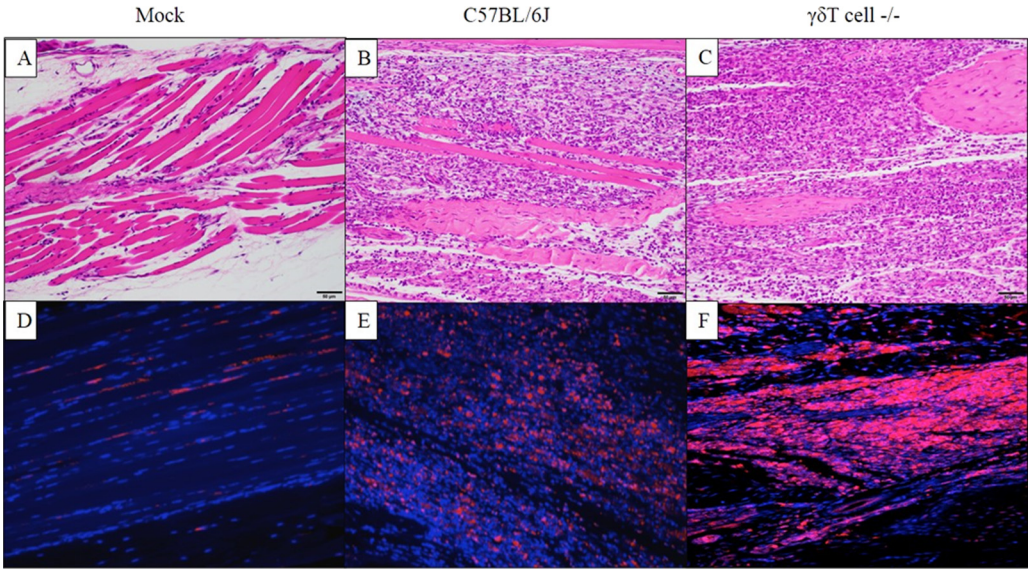

FIG 4 Inflammatory cell infiltrate is associated with increased tissue damage in CHIKV-infected tissues in the absence of γδ T cells. Twenty-four-day-old C57BL/6J or γδ $T^{-/-}$ mice ($n = 5$) were inoculated with diluent only (mock) or 100 PFU of CHIKV in the left rear footpad. At 7 dpi, mice were sacrificed and perfused by intracardial injection with 4% paraformaldehyde. Tissue sections of the ipsilateral ankle joint and footpad were stained with H&E (A to C), and immunofluorescence was done to detect nitrotyrosine within sites of inflammation in the ipsilateral foot (D to F). Panels A to C are representative H&E images of the respective tissues labeled in panels D to F.

(Fig. 7A). The expression of IL-2 and IFN-γ, two key cytokines associated with T cell activation and function, were also differentially expressed in wild-type versus γδ $T^{-/-}$ mice (Fig. 7B), indicating that the absence of γδ T cells leads to altered cytokine/chemokine expression in response to CHIKV infection.

DISCUSSION

Chikungunya virus infection in both humans and mouse models is characterized by inflammatory responses and histopathology that implicates immune-mediated pathology and modulation as drivers of CHIKV-induce disease (46–48, 50, 90, 91). Although some host immune pathways have been implicated in the recognition of CHIKV and subsequent induction of protective and pathogenic responses (59, 62, 67, 69, 82, 92–94), the precise mediators and mechanisms of CHIKV pathogenesis are still relatively poorly understood. Given the prevalence of γδ T cells in the skin, the primary site of CHIKV infection, and evidence that this T cell subset plays a role in the pathogenesis of other arboviruses (95–98), we tested whether γδ T cells played any role in the pathogenesis of CHIKV-induced disease. These studies demonstrated that CHIKV infection leads to significant increases in the prevalence of γδ T cells in both the foot and draining popliteal lymph node and that the absence of these cells leads to enhanced clinical signs of disease as well as CHIKV-induced histopathologic changes. Regulation of the host response to CHIKV by γδ T cells, evidenced by altered inflammatory cytokine expression and increased monocyte populations present at the site of infection, suggests that targeting of the inflammatory pathways that this T cell subset modulates may have therapeutic benefit in the treatment of CHIKV-induced and other alphavirus-induced inflammatory diseases.

An important role for γδ T cells is the production of proinflammatory cytokines and chemokines that bias the inflammatory milieu toward a Th1-like environment as a response to infection or host cell dysregulation (99–101). In response to various bacterial and viral infections, γδ T cells can rapidly produce cytokines, such as IFN-γ, TNF-α, and IL-17 (82, 102–105). However, mechanisms by which these pathogens elicit cytokine responses in γδ T cells are poorly understood. West Nile virus replication in γδ T cells induces proinflammatory cytokines, including IFN-γ and TNF-α, as well as IL-17 and transforming growth factor β (80, 96); following West Nile virus infection of mice, γδ T cells expanded quickly and produced significant amounts of IFN-γ (82). While the impact of IFN-γ on the pathogenesis of CHIKV disease is somewhat controversial, with one study group finding that IFN-γ limited viral replication but not joint swelling (69) while another group found that IFN-γ promotes virus-induced swelling (85), our finding that mice lacking γδ T cells had increased IFN-γ production (Fig. 7B) suggests that it will be important to further analyze IFN-γ's role in the context of CHIKV infection.

In addition to cytokine production, γδ T cells have been described as a bridge between the innate and acquired immune responses by providing for the early and rapid movement and functions of key effector cells, such as neutrophils, macrophages, and NK cells, to the site of infection (74–76, 106, 107) and then downregulating the immune response after the danger has passed, to minimize potential immune-mediated injury (31, 72, 73, 96, 97). Monocytes have been shown to play a pathogenic role during CHIKV infection (66), and therefore the finding that monocyte numbers were increased in the foot/ankle of γδ $T^{-/-}$ mice suggests that γδ T cells may limit monocytic influx at day 5 and thereby decreasing the damage these cells cause within muscle tissue of the foot (Fig. 3 and 4).

In summary, these results suggest that γδ T cells may play a major role in regulating inflammatory responses within joint-associated tissues during the acute stages of CHIKV disease and that this occurs independently of changes in viral replication. These

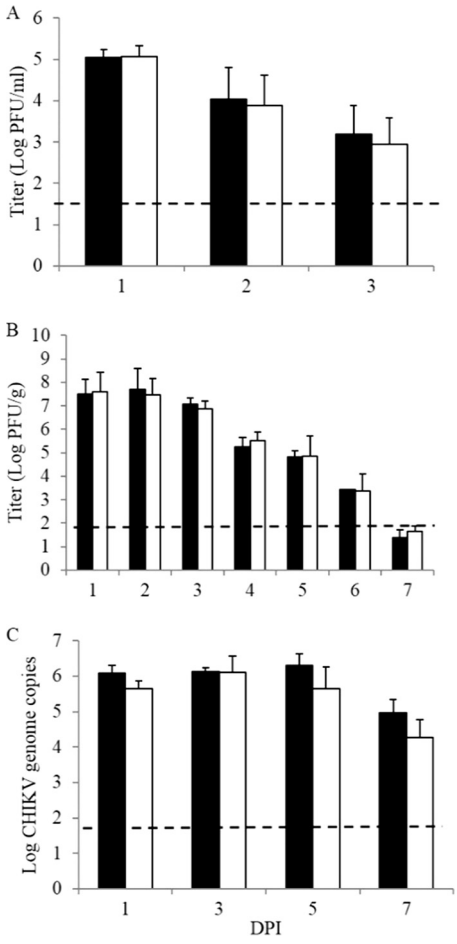

FIG 5 Absence of γδ T cells does not affect viral replication or clearance during acute CHIKV infection. (A and B) Twenty-four-day-old C57BL/6J (black bars) and γδ $T^{-/-}$ (white bars) mice were infected with 100 PFU CHIKV in the left rear footpad. Serum (A) and ipsilateral foot/ankle tissues (B) were harvested at the indicated times postinfection. Ankle/foot tissues were homogenized in PBS, and all samples were assayed on Vero cell monolayers in a standard plaque assay. (C) Total RNA was extracted from foot/ankle tissues of CHIKV- or mock-infected C57BL/6J and γδ $T^{-/-}$ mice by using the TRIzol RNA extraction protocol followed by reverse transcription using the SuperScript III reverse transcriptase system. CHIKV-specific real-time PCR was performed. Data represent mean viral titers and standard deviations and are representative of 3 independent experiments. Dashed lines indicate the limit of detection for each assay.

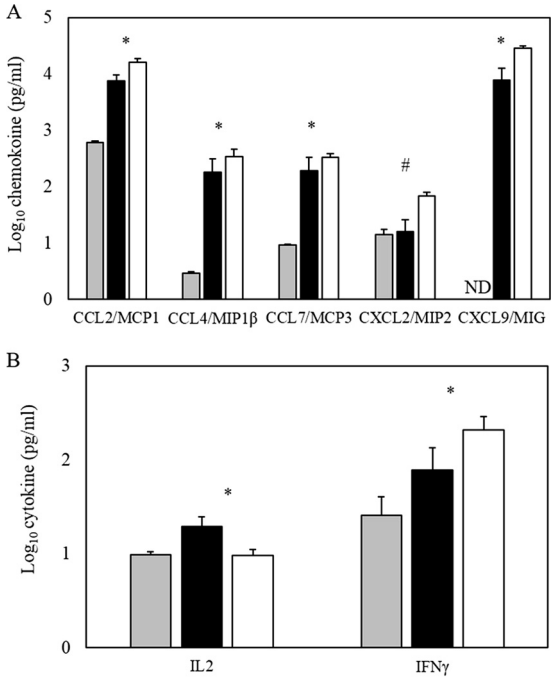

FIG 7 Absence of γδ T cells alters CHIKV-induced cytokine expression 7 days following infection. The ankle and foot from CHIKV-infected C57BL/6J (black bars) or γδ $T^{-/-}$ (white bars) mice or mock-infected mice (gray bars) were harvested at 7 dpi and homogenized in sterile PBS. From the homogenates, cytokine concentrations were measured by multiplex ELISA using the Luminex MAGPIX platform. The average expression for each chemokine (A) and T cell-associated cytokine (B) are shown. Data represent mean amounts of total protein detected (in picograms per milliliter) ($n = 5$) and the error bars indicate standard deviations. ND, not detected. These data are representative of two independent experiments. Values for mock-treated C57BL/6J and γδ $T^{-/-}$ mice were not different, and the data shown are the averages of all mock animals of both genotypes. Statistical significance: *, $P \leq 0.05$; #, $P \leq 0.01$ (determined by Student's *t* test for C57BL/6J versus γδ $T^{-/-}$ animals).

results suggest that further investigation of the role of γδ T cells in CHIKV pathogenesis may provide novel insights into how the host response modulates CHIKV-induced disease and lead to the identification of pathways which may be exploited for therapeutic or prophylactic therapies.

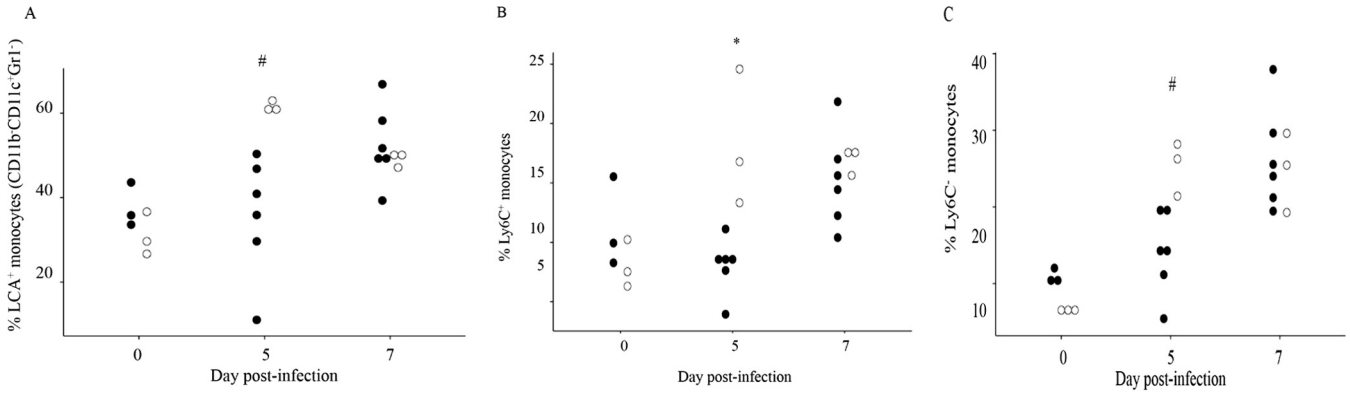

FIG 6 Absence of γδ T cells increases monocyte recruitment to the site of CHIKV infection at 5 dpi. Total cells were isolated from the foot/ankle tissues of C57BL/6J (■) ($n = 6$) and γδ $T^{-/-}$ mice (□) ($n = 3$) at 7 or 10 days postinfection. Viable cells were quantified by flow cytometry using the cell surface markers described in the text to determine the proportion of all LCA^+ cells that were $CD11b^+$ $CD11c^-$ $Gr1^-$ monocytes (A) and the proportions of monocytes that were $Ly6C^+$ (B) or $Ly6C^-$ (C). *, $P \leq 0.05$; #, $P \leq 0.01$ by ANOVA.

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