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Dendritic Cell Immunoreceptor Regulates Chikungunya Virus Pathogenesis in Mice

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Chikungunya virus (CHIKV) is a mosquito-borne alphavirus responsible for recent epidemic outbreaks of debilitating disease in humans. Alphaviruses are known to interact with members of the C-type lectin receptor family of pattern recognition proteins, and given that the dendritic cell immunoreceptor (DCIR) is known to act as a negative regulator of the host inflammatory response and has previously been associated with rheumatoid arthritis, we evaluated DCIR's role in response to CHIKV infection. Although we observed an increase in the proportion of dendritic cells at the site of CHIKV infection at 24 to 36 h postinfection, these cells showed decreased cell surface DCIR, suggestive of DCIR triggering and internalization. *In vitro*, bone marrow-derived dendritic cells from DCIR-deficient (DCIR$^{-/-}$) mice exhibited altered cytokine expression following exposure to CHIKV. DCIR$^{-/-}$ mice exhibited more severe disease signs than wild-type C57BL6/J mice following CHIKV infection, including a more rapid and more severe onset of virus-induced edema and enhanced weight loss. Histological examination revealed that DCIR-deficient animals exhibited increased inflammation and damage in both the fascia of the inoculated foot and the ankle joint, and DCIR deficiency skewed the CHIKV-induced cytokine response at the site of infection at multiple times postinfection. Early differences in virus-induced disease between C57BL6/J and DCIR$^{-/-}$ mice were independent of viral replication, while extended viral replication correlated with enhanced foot swelling and tissue inflammation and damage in DCIR$^{-/-}$ compared to C57BL6/J mice at 6 to 7 days postinfection. These results suggest that DCIR plays a protective role in limiting the CHIKV-induced inflammatory response and subsequent tissue and joint damage.

Chikungunya virus (CHIKV) belongs to a group of mosquito-borne alphaviruses which also includes Ross River virus (RRV), Mayaro virus, and O'nyong-nyong virus. These viruses are responsible for explosive epidemics of debilitating rheumatic disease associated with inflammation and destruction of musculoskeletal tissues in humans (1). Previous epidemics included a 1979-1980 epidemic of RRV in the South Pacific which affected over 60,000 people (2) and an outbreak of O'nyong-nyong virus from 1959 to 1962 which was estimated to infect over 2 million people in Africa (3). Epidemic CHIKV disease was first described in East Africa in 1952 (4, 5), and for the next 50 years, various outbreaks were described in both Africa and Asia (6). Beginning in 2004, the largest documented outbreak of CHIKV began in coastal Africa and rapidly spread to islands of the Indian Ocean and India (7–11). Of particular concern with this outbreak was the threat of CHIKV introduction and spread into new regions (12–14), in part through its adaptation to the widely distributed mosquito vector *Aedes albopictus* (7, 15–18).

The name Chikungunya comes from the Kimakonde language of Tanzania, where the virus was first identified, and roughly translates to "that which bends up." The name reflects the stooped posture observed in individuals suffering from the severe joint pain that is the hallmark of CHIKV infection. The arthralgia associated with CHIKV infection is most often symmetrical, involves swelling, and affects multiple joints, including fingers, wrists, ankles, toes, and knees (19, 20). In addition to rheumatic disease, CHIKV infection is also associated with fever, headache, rigors, photophobia, myalgia, and a petechial or maculopapular rash (21, 22). The acute phase of CHIKV infection typically lasts from days to weeks; however, persistent joint pain and chronic fatigue can persist in about a third of patients for up to 4 months and in 12% of infected persons for 3 to 5 years (12, 20, 23–27).

In both human cases and mouse models of CHIKV pathogenesis, virally induced immunopathology has been implicated as the primary mediator of damage and persistent pain. Evidence for this comes from the fact that recruitment of inflammatory cells to sites of infection continues well after viral replication ceases. In humans, viral replication ends prior to a CHIKV-specific IgG or T cell response (28). Additionally, bindarit, an inhibitor of monocyte chemotactic proteins, significantly reduces CHIKV disease and inflammation in mice independently of viral titer (29). High levels of proinflammatory cytokines in human patients and mice further implicate immune cells and their products in driving the tissue and joint pathology of CHIKV disease (29–32). Finally, viral immunopathogenesis has been well characterized in other closely related alphaviruses, such as RRV (33–36).

Early interactions between virus and host determine the course of infection and are likely key to understanding viral pathogenesis and control. Alphavirus transmembrane glycoproteins are N-linked glycosylated, with the number of sites varying among viruses and the exact composition being dependent on the host species in which the virion was assembled. There is ample evidence that viral glycan interactions with host c-type lectin recep-

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tors (CLRs) modulate alphavirus infections. *In vitro*, mosquito cell-derived Sindbis virus (SINV) and Venezuelan equine encephalitis virus (VEE) have been shown to utilize dendritic cell (DC)-specific intercellular adhesion molecule-3-grabbing nonintegrin (SIGN) and liver-specific SIGN (L-SIGN) as attachment receptors on DCs; and RRV, Barmah Forest virus (BFV), and VEE differentially induced type I interferon (IFN) on the basis of the various glycosylation patterns between mosquito- and mammal-produced virus (37–40). *In vivo*, specific alterations in the glycosylation pattern of SINV increased replication and virulence in mice (41), and the soluble CLR mannose binding lectin (MBL) was recently shown to promote RRV disease in both mice and humans (36).

The dendritic cell immunoreceptor (DCIR) is a CLR expressed on monocytes, macrophages, and DCs, as well as B cells and neutrophils (42). DCIR contains an immunoreceptor tyrosine-based inhibitory motif (ITIM), and evidence from several studies supports its role as an inhibitory receptor (43–45). Recently, elevated DCIR expression on several cell types was implicated in the chronic inflammation associated with rheumatoid arthritis and myocardial infarction in humans (46) and with mouse models of rheumatoid arthritis (44). Given DCIR's role as a negative regulator of the host inflammatory response and its association with rheumatoid arthritis, we evaluated whether DCIR played a role in the response to CHIKV infection. Utilizing an established mouse model of CHIKV infection, we found that DCIR, but not two other C-type lectin receptors, mouse DC-SIGN (mDC-SIGN) and SIGNR3, played a role in limiting the CHIKV-induced inflammatory response. DCIR-deficient ($DCIR^{-/-}$) mice exhibited more severe disease signs than wild-type (wt) mice following CHIKV infection, including a faster and more severe onset of virus-induced edema at the site of inoculation and significantly enhanced weight loss. DCIR-deficient animals also exhibited increased inflammation and damage in both the fascia of the inoculated foot and the ankle joint, while DCIR deficiency skewed the CHIKV-induced cytokine response both *in vivo* and *in vitro*. These results suggest that DCIR plays a role in limiting CHIKV-induced inflammation during infection and indicates that DCIR may play a role in the pathogenesis of CHIKV or other virus-induced inflammatory diseases.

MATERIALS AND METHODS

Viruses and cells. Isolation and culture of the SL15649 CHIKV strain used in these studies have been previously described (47). 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. C57BL6/J wt mice were obtained from The Jackson Laboratory. Mice deficient for the C-type lectin receptors DC-SIGN, SIGNR3, and DCIR were obtained from the Consortium for Functional Glycomics (Scripps Research Institute, La Jolla, CA). All mice were bred in-house as homozygous knockouts. Fourteen-day-old mice were inoculated in the left rear footpad with 10^2 PFU of CHIKV diluted in sterile phosphate-buffered saline (PBS) supplemented with 1% donor calf serum, Mg^+, and Ca^+ in a 10-μl volume. Control animals were inoculated with diluent alone. All 14-day-old mice remained with their mothers throughout the course of the experiment. Mice were weighed daily and monitored for clinical signs of disease, including measurement of swelling in the inoculated versus uninoculated foot using calipers. As uninoculated feet do not show any swelling (47), this comparative measurement allowed us to account for differences in mouse size. At the 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 of 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, and stored at −80°C. The amount of infectious CHIKV present was determined by standard plaque assay on Vero cells (ATCC CCL-81).

Measurement of edema and vascular permeability. CHIKV- or mock-infected mice were injected intraperitoneally (i.p.) with 50 μl of a 1% (wt/vol) solution of Evans blue dye (EBD) in PBS at the indicated times postinfection. At 6 h after EBD administration, animals were euthanized and the left foot was removed just above the ankle. The ankle was weighed and homogenized in 1 ml dimethylformamide (DMF) using a mini-BeadBeater (BioSpec Products, Inc.), followed by rapid shaking at 50°C for 24 h. The absorbance of 0.2-ml samples of the supernatants was measured in triplicate at 630 nm using a FLUOstar Omega plate reader and Omega Analysis software, version 1.02 (BMG Labtech). The concentration of EBD present in the extracts was determined from a standard curve of the dye prepared in DMF and normalized to tissue weight.

Histological analysis. Mice were sacrificed and perfused by intracardial injection of 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) to evaluate inflammation and tissue damage. H&E-stained sections were evaluated for both the degree of inflammatory cell infiltration and the extent of tissue damage using a Nikon Motic BA400 model microscope. Photomicrographs were obtained using SPOT imaging software (version 4.7.0.26).

Measurement of tissue damage by EBD. Damaged muscle fibers in the skeletal muscle of CHIKV-infected mice were visualized by vital staining with EBD (48). Ten days after CHIKV infection, mice were inoculated i.p. with 0.1 ml EBD. Six hours later, mice were euthanized and tissues were fixed by immersion in 4% PFA. Foot/ankle tissues were decalcified, embedded in optimal-cutting-temperature compound (Tissue-Tek), and frozen overnight at −80°C. Cryosections (5 μm) were cut, mounted with ProLong Gold reagent with DAPI (4′,6-diamidino-2-phenylindole; Invitrogen), and analyzed by fluorescence microscopy. Damaged muscle fibers, which were visible as areas of blue macroscopically, could be seen as red fluorescent fibers microscopically.

Flow cytometry. Inoculated mice were sacrificed and perfused with 1× PBS. The tibia and fibula were disarticulated at the knee joint to ensure that bone marrow was not released. Following gentle separation of tissues from bone with a scalpel, the entire sample was incubated for 1 h with gentle 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 70-μm-sieve-size cell strainer. Total viable cells were determined by trypan blue exclusion for all single-cell suspensions. Cells were stained in fluorescence-activated cell sorting staining buffer (1× Hanks balanced salt solution, 1% fetal bovine serum, 0.1% sodium azide) with the following antibodies from eBioscience: leukocyte common antigen (LCA)-eFluor 450, CD80-fluorescein isothiocyanate (FITC), CD86-peridinin chlorophyll protein (Per-CP), CD11b-PE-cyanine 7 (Cy7), CD11c-PE-Texas Red (TR), and major histocompatibility complex class II (MHC-II)-allophycocyanin (APC). PE-conjugated mouse anti-DCIR and its isotype control were purchased from R&D Systems. Cells were fixed in 4% paraformaldehyde for a minimum of 2 h before being analyzed on a CyAn cytometer (Dako Cytomation) using Summit software (Beckman-Coulter).

BMDC cultures and virus infection. Murine bone marrow-derived dendritic cells (BMDCs) were generated from C57BL6/J and $DCIR^{-/-}$ mouse bone marrow as described previously (49, 50). At day 7 after bone marrow harvest, cells were infected with CHIKV at a multiplicity of infec-

tion (MOI) of 20 PFU for 1 h at 37°C in a total volume of 0.2 ml. Following infection, 0.3 ml of medium was added to the culture. At 20 h postinfection (hpi), cells were harvested and total RNA was extracted to prepare cDNA for analysis.

Extraction of total RNA and reverse transcription. Total RNA was extracted from tissues using the TRIzol RNA extraction protocol (Invitrogen). RNA was reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) per the manufacturer's protocol.

Quantitative real-time PCR. mRNA expression levels were analyzed on an ABI Prism 7300 system with sequence detection system software (v14.0) using ABI TaqMan gene expression assays specific for mouse genes of interest. The copy numbers of each mRNA were normalized to the quantity of 18S rRNA detected in each sample.

Quantitative Luminex analysis. The ankle and foot from mock- or CHIKV-infected mice were harvested at designated times and homogenized in sterile PBS. From the homogenate, cytokine concentrations for the following proteins were measured with the cytokine mouse 20-plex panel (Invitrogen) and a Luminex 100 analyzer: fibroblast growth factor (FGF) basic, granulocyte-macrophage colony-stimulating factor (GM-CSF), gamma interferon (IFN-γ), interleukin-1α (IL-1α), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p40/p70), IL-13, IL-17, IFN-γ-induced protein 10 (IP-10), keratinocyte chemoattractant (KC), monocyte chemotactic protein-1 (MCP-1), cell migration-inducing protein (MIG), macrophage inflammatory protein-1α (MIP-1α), tumor necrosis factor alpha (TNF-α), and vascular endothelial growth factor (VEGF).

Statistical analyses. In order to determine differences in the composition and magnitude of the inflammatory cell infiltrate, we used analysis of variance to identify cell populations that were significantly different between treatments. For those populations that were significantly different, we used Tukey's honestly-significant-difference (HSD) test to identify which treatments were different from the others. For the Luminex data, where we had small samples sizes, we used the nonparametric Mann-Whitney U test to identify different levels of cytokines. All statistical analyses were conducted using the R statistical environment (51).

RESULTS

CHIKV infection decreases DCIR-positive (DCIR$^+$) dendritic cells at the site of infection and alters cytokine expression in cultures of BMDCs from DCIR$^{-/-}$ versus wt mice. There is strong evidence that the host inflammatory response plays a pathological role during infection with CHIKV and other arthritic alphaviruses (34, 36, 47, 52); however, the role that specific host factors play in modulating the CHIKV-induced inflammatory response is still poorly understood. A number of studies have found evidence that alphaviruses interact with C-type lectin receptors (CLRs) (36, 39, 40), and given that one of these receptors, DCIR, has been shown to regulate the severity of collagen-induced arthritis, we evaluated DCIR's role in the pathogenesis of CHIKV-induced disease.

Because DCIR has been shown to be internalized efficiently into DCs after triggering with DCIR-specific antibody (53, 54), following DC activation by exogenous cytokine signaling (42), and following attachment by HIV (55), we were interested in examining the cell surface expression of DCIR on DCs at the site of CHIKV infection. At 24 and 36 h following CHIKV or mock infection in the rear footpad, C57BL6/J mice were euthanized, the inoculated foot/ankle was removed, and tissues were dissociated, as described in Materials and Methods. While overall viable cell yields were consistent between samples, the proportion of DCs (LCA$^+$ CD11b$^+$ CD11c$^+$) in the foot/ankle was significantly increased in the tissues of CHIKV-infected mice by 36 hpi (6.4%) compared with that in the tissues of mock-infected animals (5.1%) (Table 1). However, the increase in DCs negatively correlated with the mean fluorescence intensity (MFI) of DCIR$^+$ DCs in infected tissues following CHIKV infection compared to the correlation for mock-infected animals. To address the possibility that the loss of DCIR cell surface expression resulted from virus-induced changes at the mRNA level, real-time PCR was used to determine if there were quantitative differences in the amount of DCIR mRNA following CHIKV infection. We found that the amount of DCIR mRNA (normalized to the amount of 18S rRNA) increased slightly over the course of the experiment by about 1.75-fold at 36 hpi (data not shown), which confirmed that decreased cell surface expression of DCIR did not result from a decrease in mRNA production following CHIKV infection. Further, there was no difference in expression of the activation marker MHC-II, CD80, or CD86 when mock-infected and CHIKV-infected tissues were compared (data not shown), which is consistent with findings that DCIR triggering does not influence expression of these costimulatory molecules (44, 54). Taken together, these results suggest that following CHIKV infection, CD11c$^+$ CD11b$^+$ DCs increase at the site of inoculation and that although DCIR mRNA levels show a slight increase, there is decreased expression of DCIR on the surface of these DCs by 36 hpi. While it is not clear whether the modulation of DCIR cell surface expression is due to direct triggering of DCIR or indirect effects on the DCs leading to DCIR downregulation, these results clearly indicate that DCIR expression on DCs is altered following *in vivo* CHIKV infection.

TABLE 1 Summary of flow cytometry data for total DCs and DCIR$^+$ DCs in the foot/ankle following CHIKV infection

Sample	% CD11b$^+$ CD11c$^+$ DCs[a]	MFI	
		DCIR MFI[b]	Isotype MFI[c]
Mock infected	5.1 ± 0.86	783.6 ± 27.5	344.6 ± 16.6
Sample collected at:			
24 hpi	5.1 ± 1.95	707.6 ± 68.0	328.7 ± 92.8
36 hpi	6.4 ± 0.30[d]	650.8 ± 41.3[d]	328.6 ± 23.9

[a] Mean percentage ± standard deviation.
[b] MFI ± standard deviation of DCs staining positive for PE-conjugated DCIR.
[c] MFI ± standard deviation of DCs staining positive for PE-conjugated isotype control antibody.
[d] $P \leq 0.01$.

The decreased cell surface expression of DCIR on DCs following CHIKV infection *in vivo* led us to ask whether the presence or absence of DCIR modulated DC function in response to CHIKV *in vitro*. Though previous studies have suggested that dendritic cells are not a major site of active CHIKV replication, due to their central immunoregulatory role, DCs are likely to shape the virus-induced inflammatory response even in the absence of productive viral replication (56). Therefore, cultures of wt and DCIR$^{-/-}$ BMDCs were infected with CHIKV and then assayed by quantitative real-time PCR for the expression of key proinflammatory cytokines. Consistent with previous reports (56), BMDCs were not productively infected by CHIKV (data not shown); however, distinct cytokine profiles were induced in the cultures of BMDCs from wt versus DCIR$^{-/-}$ mice following infection. Importantly, at 24 hpi the CHIKV-induced expression of IL-10 and IL-6 was significantly increased in DCIR$^{-/-}$ BMDC cultures over that in wt cell cultures, while IL-12 expression was significantly higher in cells from wt mice (Fig. 1). These data indicate that DCIR defi-

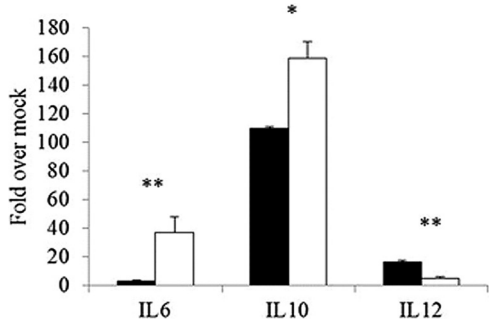

FIG 1 CHIKV infection of DCIR$^{-/-}$ BMDCs alters cytokine expression compared to infection of DCIR^{+} dendritic cells. BMDCs were harvested from C57BL6/J (■) or DCIR$^{-/-}$ (□) mice and cultured *in vitro* for 7 days with GM-CSF and IL-4 before 2×10^5 cells/well were infected at an MOI of 20 PFU/cell or mock infected for 24 h. Following TRIzol RNA extraction, cDNA was prepared using MMLV reverse transcriptase, and mRNA levels were measured using TaqMan real-time primer/probe sets for specific cytokines. Data are presented as the fold induction over mock-infected cells and normalized to the data for 18S rRNA. Data shown are the mean and standard deviation for samples ($n = 3$) and are representative of two independent experiments. *, $P \leq 0.05$ determined by two-tailed *t* test; **, $P \leq 0.01$ determined by two-tailed *t* test.

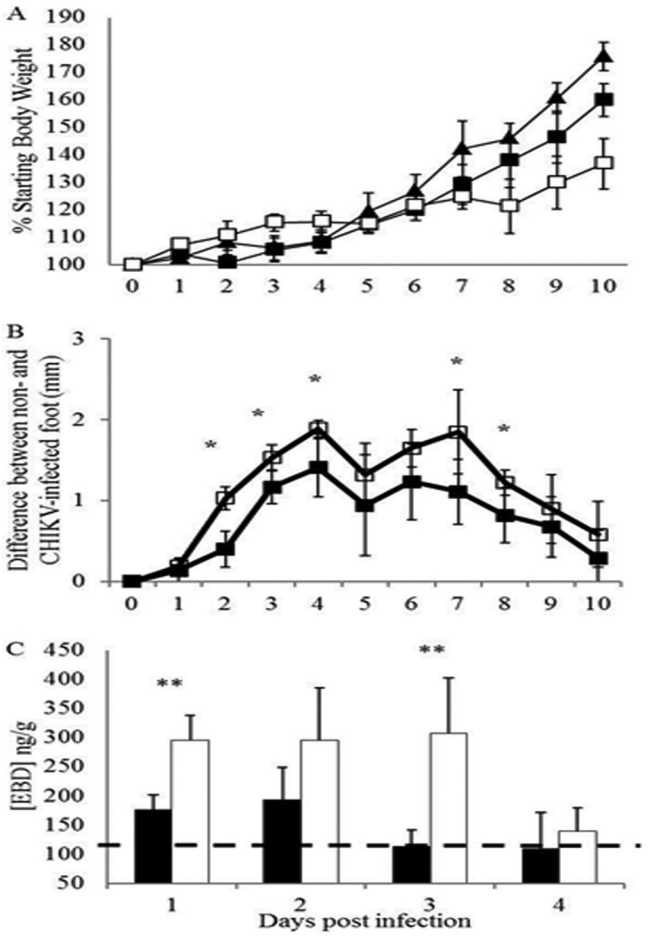

FIG 2 DCIR$^{-/-}$ mice exhibit increased CHIKV-induced disease compared to C57BL6/J mice. Wild-type C57BL6/J (■) and DCIR$^{-/-}$ (□) mice were infected with 100 PFU of CHIKV or mock infected (▲) and monitored at 24-h intervals for signs of disease, including weight gain (A) and difference in size between infected and uninfected rear feet of mock- or CHIKV-infected mice measured using calipers (B). Data shown are the average difference in thickness between the infected and uninfected footpad for an individual animal. Results are representative of three duplicate experiments ($n = 5$). (C) CHIKV-infected animals were given 50 μl of EBD i.p. at the indicated times postinfection. Six hours later, animals were sacrificed and the ipsilateral foot and ankle were removed. Tissues were weighed and homogenized in 1 ml DMF, followed by rapid shaking at 50°C for 24 h. The absorbance of 0.2 ml of the supernatant was measured in triplicate at 630 nm to calculate the concentration of EBD present. A standard curve of EBD in DMF was prepared and used in conjunction with tissue weights to determine the concentration of EBD (ng) per gram of tissue. Dashed line, average concentration of EBD for mock-infected animals ($n = 3$ mice/group). *, $P \leq 0.05$ determined by two-tailed *t* test; **, $P \leq 0.01$ determined by two-tailed *t* test.

ciency in BMDC cultures results in altered cytokine production, even in the absence of detectable productive viral replication, and suggest that DCIR may modulate the virus-induced inflammatory response during CHIKV infection.

DCIR deficiency results in more severe CHIKV-induced disease. Given that CHIKV infection modulates DCIR expression *in vivo* and that the presence or absence of DCIR altered DC responses to CHIKV *in vitro* (Fig. 1), we sought to directly test DCIR's role in the pathogenesis of CHIKV infection by infecting DCIR$^{-/-}$ mice. Fourteen-day-old wt and DCIR$^{-/-}$ mice were infected with 100 PFU of CHIKV and monitored daily for weight gain and clinical signs of infection. CHIKV-infected DCIR$^{-/-}$ mice failed to gain weight at the same rate as wt mice from days 8 to 10 postinfection (Fig. 2A). We and others have shown that CHIKV infection results in biphasic swelling of the foot and ankle joint of the ipsilateral foot, where the initial swelling is apparent at 48 h postinfection and peaks by 72 h postinfection, with a second round of swelling occurring on days 6 to 8 postinfection (47, 52, 57). In contrast, no swelling in the contralateral foot is noted at any time (47); thus, swelling in the inoculated foot can be compared to the baseline measurement of the uninoculated foot to give a precise measurement on an individual basis and account for any differences in mouse size. Beginning at the time of infection, the CHIKV-infected foot and uninfected foot of each mouse were measured daily using calipers. As shown in Fig. 2B, swelling was more severe in DCIR$^{-/-}$ mice than wt mice. No difference in the mock-infected versus uninfected hind limbs of mock-infected animals was measured (data not shown). The severity and early onset of CHIKV-induced swelling suggested that CHIKV infection might initiate an edematous process in the feet/ankles of infected mice. Therefore, quantitative evaluation of the edematous swelling of the inoculated foot following CHIKV was done using EBD, an azo dye that binds with serum albumin to form a dye-protein complex whose passage into tissues indicates vascular permeability (58). CHIKV infection of DCIR$^{-/-}$ mice resulted in increased extravasation of EBD into tissues of the inoculated foot and ankle compared to that in wt mice at 24 to 72 hpi (Fig. 2C). At between 96 hpi and 10 days postinoculation, the quantity of EBD recovered from tissues of both DCIR$^{-/-}$ and C57BL6/J mice was comparable to that recovered from mock-infected animals. No EBD extravasation was noted in the contralateral limb at any time postinfection (data not shown). Continued swelling in the infected feet over the course of the experiment with no detection of EBD extravasation suggests that while edema is certainly a component of the early swelling observed in CHIKV-infected mice, inflammation of the foot after 3 dpi likely shifts to an inflammatory process.

These results indicate that DCIR plays a role in limiting the severity of CHIKV-induced edema and inflammatory processes.

CHIKV infection in the absence of DCIR results in an increased lymphocyte response and tissue damage. Footpad inoculation of 14-day-old C57BL6/J mice with CHIKV results in inflammation and musculoskeletal pathology which peaks at 7 to 10 dpi (47). In these studies, inflammation in the inoculated foot tissues became evident by 4 dpi in both wt and DCIR$^{-/-}$ mice, and neither the levels of inflammation nor the levels of tissue destruction were different between wt and DCIR$^{-/-}$ animals at between 4 and 8 dpi (data not shown). Upon histological examination of H&E-stained foot and ankle tissues at 10 dpi, it was noted that DCIR$^{-/-}$ animals had increased inflammatory damage characterized by tendonitis, arthritis in the talocrural joint (ankle), and fasciitis in the ipsilateral plantar fascia and musculature of the foot compared to the characteristics of wt animals (Fig. 3A). EBD was used to semiquantitatively assess damaged skeletal myofibers within the infected foot at 10 dpi (Fig. 3B) (59). The increased damage noted both in H&E-stained sections and in the EBD assay confirmed that damage within tissues of the inoculated foot of DCIR$^{-/-}$ mice was more severe during the CHIKV-induced inflammatory response than what was observed for wt mice.

To further evaluate DCIR's impact on CHIKV-induced pathology, we conducted flow cytometric analysis of the immune cell infiltrate in both the infected foot and the draining popliteal lymph node at 6, 8, and 10 days after CHIKV infection of C57BL6/J and DCIR$^{-/-}$ animals. Infection of both C57BL6/J and DCIR$^{-/-}$ animals with CHIKV resulted in an increase in the overall number of lymphocytes, macrophages, neutrophils, and DCs within the foot compared to that in mock-infected animals. However, while DAPI staining (Fig. 3B) indicated that there might be an increase in the overall cellularity within the joint tissues of CHIKV-infected DCIR$^{-/-}$ compared to wild-type mice, with the exception of lymphocytes, which were increased in CHIKV-infected DCIR$^{-/-}$ mice compared to infected wt mice at day 10 postinfection ($\log_{10}$ number of cells [mean ± standard deviation], 3.83 ± 0.21 for C57BL6/J mice and 4.34 ± 0.35 for DCIR$^{-/-}$ mice; $P = 0.036$), we observed no significant differences in macrophage, neutrophil, or DC populations (data not shown) at any time point between the two strains.

CHIKV infection initiates distinct cytokine profiles in the presence or absence of DCIR *in vivo*. In light of the increased edema and swelling in DCIR$^{-/-}$ mice compared to wt mice following CHIKV infection, further investigation into the immune environment at the site of infection was examined. Mice were infected with CHIKV or mock infected and their feet/ankles were harvested daily at 24 to 72 hpi. Tissues were homogenized in PBS, and the cell homogenates were analyzed using the Invitrogen cytokine mouse 20-plex panel for the Luminex platform to quantitate the levels of 20 different mouse cytokines within the infected foot. We did not detect induction of a small subset of cytokines (GM-CSF, IL-4, IL-17, KC, or MIP-α) in either wt or DCIR$^{-/-}$ mice following CHIKV infection, while the induction of FGF basic, IL-1α, IL-1β, IL-2, IL-5, IL-10, TNF-α, and VEGF was equivalent between the two strains. However, we did observe an increase in IL-6, IP-10, IL-12, IFN-γ, MIG, IL-13 and MCP-1 within DCIR$^{-/-}$ mice compared to wt mice (Fig. 4A), where differences in IL-6 (24 h postinfection), as well as IL-12 and MCP-1 (24 and 48 h postinfection), were statistically significant. By 72 hpi, the levels of all cytokines measured either had fallen below the limit of detection or were not different between wt and DCIR$^{-/-}$ mice.

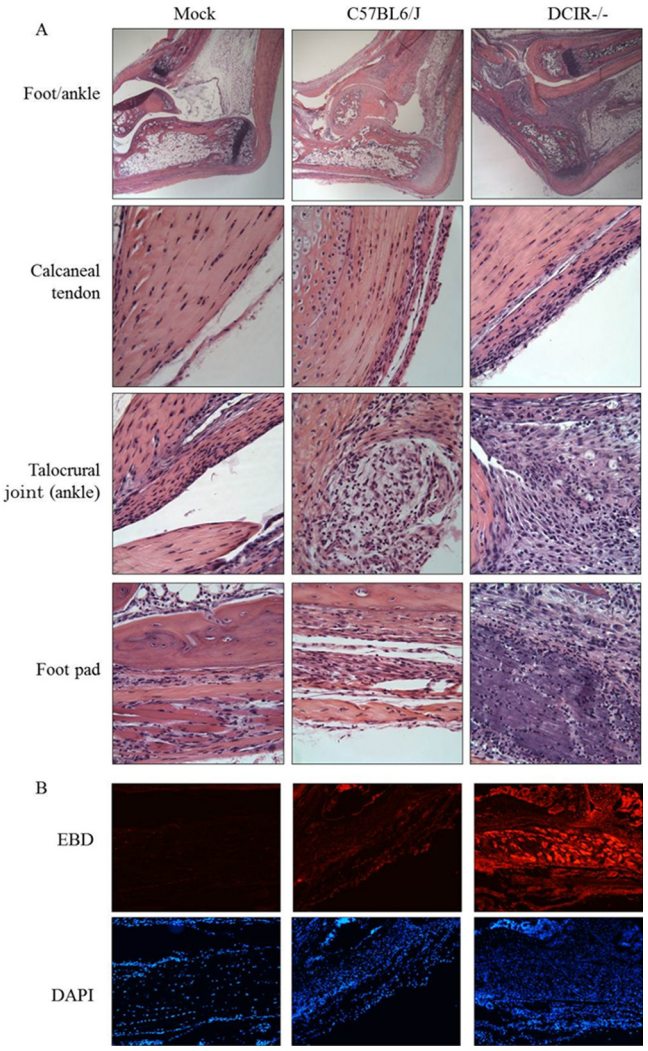

FIG 3 DCIR deficiency increases inflammation and tissue damage compared to those in wt mice. Fourteen-day-old C57BL6/J or DCIR$^{-/-}$ mice ($n = 6$) were inoculated with diluent only (mock) or 100 PFU of CHIKV in the left rear footpad. At 10 dpi, mice were sacrificed and perfused by intracardial injection with 4% paraformaldehyde. (A) Tissue sections of the foot and ankle were stained with H&E and assessed for evidence of inflammation and damage in the calcaneal tendon, ankle joint, and footpad. (B) Additional mice ($n = 7$) were given 0.05 ml of 1% EBD into the peritoneal cavity. Six hours later mice were euthanized and perfused with 4% PFA. Cryosections were prepared, mounted with ProLong Gold reagent with DAPI (Invitrogen), and examined microscopically to identify areas of EBD in the tissues as a measure of tissue damage (red) and DAPI expression as a marker for increased cell numbers indicative of inflammation (blue). Magnifications, ×40 or ×200 (A) and ×100 (B).

To determine if the cytokine profile differences observed in DCIR-deficient animals compared with those in wt animals were limited to the early immune response to CHIKV infection, we evaluated the expression of inflammatory cell markers within CHIKV-infected tissues at later times postinfection. At 5 and 7 dpi, the levels of mRNAs for both IL-10 and IL-12 were increased in DCIR$^{-/-}$ mice over those in wt-infected animals (Fig. 4B). The calgranulins S100A8 and S100A9 are endogenously expressed in

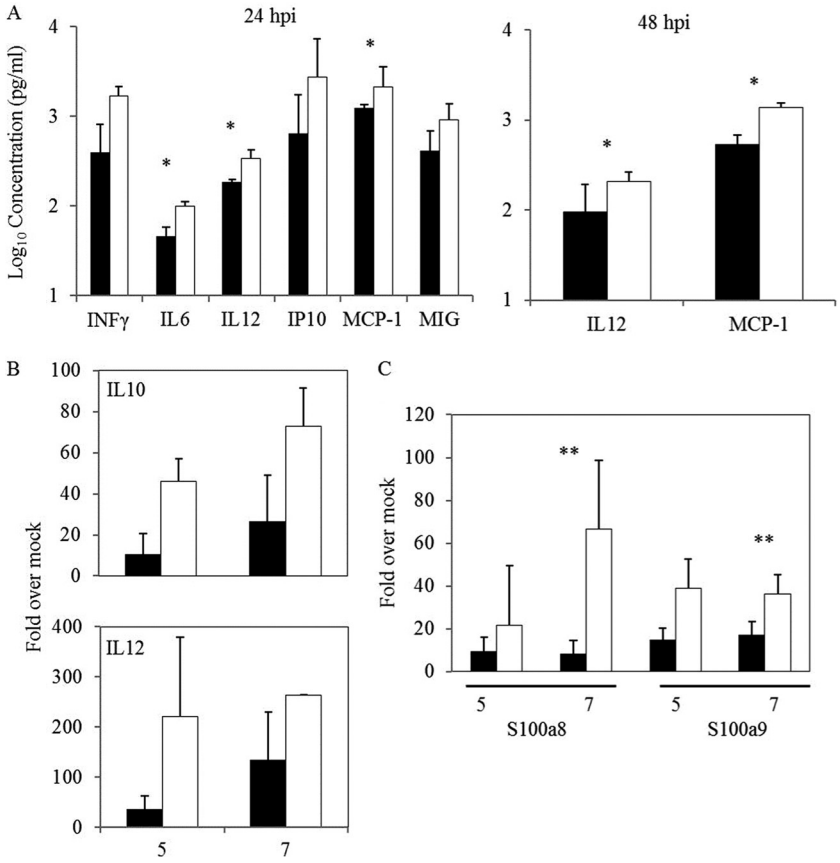

FIG 4 DCIR deficiency alters expression of key cytokines and immune mediators throughout the course of CHIKV infection. Fourteen-day-old C57BL6/J (■) or DCIR$^{-/-}$ (□) mice were infected with 10^2 PFU CHIKV or mock infected, and feet/ankles were harvested daily from 24 to 72 hpi. Tissues were homogenized in PBS, and cell homogenates were analyzed using the Invitrogen cytokine mouse 20-plex panel for the Luminex platform to quantitate the levels of 20 different cytokines within the infected foot. (A) Cytokine levels increased at the site of infection at 24 and 48 h after CHIKV infection. *, $P \leq 0.05$ determined by Mann-Whitney 1-tailed U test at 24 h ($u = 6$) and 48 h ($u = 9$ [IL-12] and $u = 6$ [MCP-1]) (71). (B) At 5 and 7 dpi, CHIKV- or mock-infected foot/ankle tissues were harvested and dissociated in TRIzol (Invitrogen) for RNA isolation. MMLV reverse transcriptase (Invitrogen) was used to prepare cDNA to measure gene expression using TaqMan real-time primer/probe sets for the specific cytokines IL-10 and IL-12 (C) and calgranulins S100A8 and S100A9 (D). Data are presented as the fold induction over mock-treated tissues and normalized to the data for 18S rRNA. Data shown are the mean and standard deviation for samples ($n = 5$) and are representative of two independent experiments. **, $P \leq 0.01$ determined by two-tailed t test.

neutrophils, macrophages, DCs, and monocytes and have been associated with pathogenesis in numerous inflammatory diseases; as such, they serve as powerful biomarkers of generalized or localized inflammation. At 5 to 7 days postinfection, real-time PCR revealed that the levels of expression of both S100A8 and S100A9 measured within the ankles/feet of DCIR$^{-/-}$ animals were increased over those measured in wt mice (Fig. 4C). The increased expression of these key mediators of inflammation both at early times after CHIKV infection and later, during the time of maximal inflammation and tissue damage, further supports a role for DCIR in limiting the CHIKV-induced inflammatory response.

DCIR-deficient mice exhibit delayed CHIKV clearance. Given the major impact of DCIR deficiency on CHIKV-induced disease, we wanted to determine whether the enhanced swelling and inflammatory tissue damage was due to increased levels of viral replication in DCIR$^{-/-}$ animals. Therefore, viral titers within the sera, as well as the ipsilateral and contralateral foot/ankle, were determined by plaque assay at 24-h intervals postinfection. No differences in viral titer were observed between wild-type and DCIR$^{-/-}$ mice between days 1 and 5 postinfection (Fig. 5A and B), which suggests that the enhanced swelling/edema within the feet and ankles of DCIR$^{-/-}$ mice was not due to increased levels of viral replication. However, at later times postinfection (days 6 and 7 postinfection), DCIR$^{-/-}$ mice did exhibit increased viral titers in the infected foot/ankle, suggesting that viral clearance may be delayed in DCIR$^{-/-}$ mice, which could contribute to the increased inflammatory damage (Fig. 3) and lymphocyte recruitment at late times postinfection. No differences in viral titer were noted for wt versus DCIR$^{-/-}$ mice in the contralateral foot/ankle at any time point examined.

Increased pathology following CHIKV infection is specific to DCIR deficiency. To confirm that the differences observed between DCIR$^{-/-}$ mice and wt mice with CHIKV infection were specific to DCIR rather than a general CLR mechanism, we also evaluated mice that were deficient in two other CLRs, DC-SIGN and SIGNR3. Fourteen-day-old wt, DC-SIGN$^{-/-}$, and SIGNR3$^{-/-}$ mice were infected with 100 PFU of CHIKV and monitored daily for weight gain and swelling of the inoculated foot. Wild-type, SIGNR3$^{-/-}$, and DC-SIGN$^{-/-}$ mice were indistinguishable in terms of body weight (Fig. 6A), and no differences in foot swelling were noted over the course of the experiment. To further confirm the results of the subjective swelling scored, an

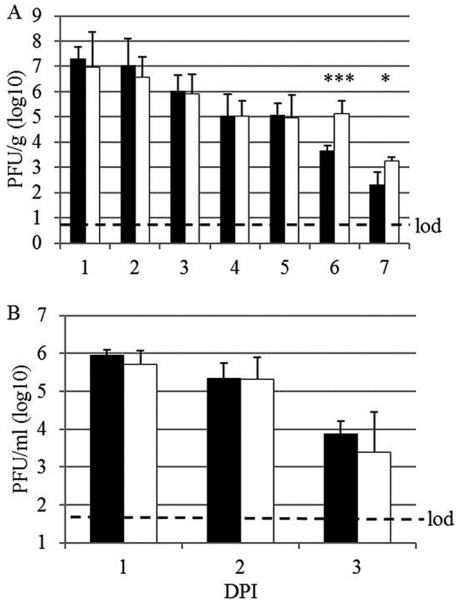

FIG 5 DCIR deficiency allows sustained CHIKV replication *in vivo*. Fourteen-day-old C57BL6/J (■) or DCIR$^{-/-}$ (□) mice were infected with 100 PFU CHIKV in the left rear footpad. At the indicated times postinfection, ipsilateral foot and ankle tissues (A) and sera (B) were harvested following intracardial perfusion with sterile PBS. Ankle/foot tissues were homogenized in PBS, and all samples were assayed on Vero cell monolayers by standard plaque assay. Data indicate mean viral titers and standard deviations and are representative of four independent experiments. *, $P \leq 0.05$ by two-tailed *t* test; ***, $P \leq 0.001$ by two-tailed *t* test. Dashed lines, limit of detection (lod).

additional set of wild-type and SIGNR3$^{-/-}$ mice was evaluated using caliper measurements, and consistent with the subjective scores and in contrast to our results in DCIR-deficient animals, no differences between CHIKV-infected wild-type and SIGNR3$^{-/-}$ mice were observed (Fig. 6B). At 10 dpi, mice were euthanized and tissues were processed for histological examination. In contrast to DCIR$^{-/-}$ mice, the inflammatory pathology observed in DC-SIGN$^{-/-}$ or SIGNR3$^{-/-}$ mice was indistinguishable from that observed in wt mice (Fig. 6B). These results demonstrate that the role for DCIR in CHIKV infection is specific to the effects of DCIR during infection rather than a more generalized glycan-receptor-induced response.

DISCUSSION

In both human cases and mouse models of Chikungunya virus infection, disease is characterized by swelling and other inflammatory markers, which suggests that virus-induced immune pathology contributes to the pathogenesis of the disease (29–32). Further, bindarit, an inhibitor of monocyte chemotactic proteins, significantly reduced CHIKV disease and inflammation in mice but did not reduce viral titers (29). However, while the field has also made significant progress in identifying specific host sensing pathways in the detection of CHIKV and subsequent induction of protective interferon responses (28, 56, 60, 61), the role of other host molecules in the pathogenesis of CHIKV-induced disease is still relatively poorly understood. Given that host CLRs likely interact with alphaviruses and since DCIR has been associated with other arthritic diseases (44, 46, 62), we tested whether DCIR played any role in the pathogenesis of CHIKV-induced disease. These studies demonstrated that CHIKV infection leads to DCIR-modulated DC responses to CHIKV *in vivo* and *in vitro* and that the absence of DCIR leads to significantly enhanced CHIKV-induced pathology at the site of infection. The role for DCIR in regulating the host response to CHIKV infection demonstrated in these studies suggests that targeting of the inflammatory pathways that DCIR modulates may have therapeutic benefit in the treatment of CHIKV or other alphavirus-induced inflammatory diseases.

CLRs have been shown to enhance alphavirus and flavivirus infection of dendritic cells (18, 36), and recent studies have implicated the CLR CLEC5A in promoting inflammatory pathology during both dengue virus and Japanese encephalitis virus infection (63, 64). Furthermore, another lectin binding protein, mannose binding lectin, has been shown to play a major role in promoting virus-induced inflammatory disease during Ross River virus-induced myositis (65). Therefore, the finding that DCIR plays a major role in limiting CHIKV-induced inflammatory disease represents the first demonstration that a CLR plays a major role in the pathogenesis of alphavirus-induced arthritis. This result is consistent with prior studies which have shown that DCIR polymorphisms are associated with susceptibility to rheumatoid arthritis in humans (46, 66, 67) and modulate the severity of collagen-induced arthritis in mice (44, 62). It is interesting that these studies showed that increased DCIR expression correlates with rheumatoid arthritis occurrence and severity. One explanation is that these studies are looking at a chronic, autoimmune, inflammatory environment which may be very different from what occurs in an acute infectious disease scenario, such as that occurring during CHIKV infection. Further, we demonstrated differences in DCIR expression on dendritic cells only, whereas other researchers examined DCIR cell surface expression on multiple cell types, including T cells and NK cells found in the rheumatic milieu. Our results suggest that DCIR may play a major role in regulating inflammatory responses within joint-associated tissues during the early, acute stages of disease. Further work must be done to look at the effect of DCIR during chronic CHIKV infection.

Though results presented in this report demonstrate that DCIR deficiency both *in vitro* and *in vivo* results in altered proinflammatory cytokine responses by DCs following exposure to CHIKV, it is unclear whether DCIR's effects on CHIKV-induced disease are due to direct modulation of DC function. Since DCIR is expressed on multiple cell types, including monocytes, macrophages, and DCs, as well as B cells and neutrophils (42), it is possible that DCIR modulates CHIKV-induced disease by affecting cell types other than DCs. However, during our flow cytometric analysis of inflammatory infiltrates at 24 and 36 hpi, we did not observe quantitative differences in DCIR expression on CD11b^{+} CD11c^{-} macrophages between mock- and CHIKV-infected mice (data not shown). Therefore, additional studies are needed to determine which cell type or types are regulated by DCIR and how these cell types affect the virus-induced disease process.

DCIR contains an inhibitory ITIM motif, and once engaged, DCIR undergoes tyrosine phosphorylation by Src kinases, leading to recruitment and activation of protein tyrosine phosphatases SHP-1 and SHP-2 and the dephosphorylation of substrates which prevent cellular activation (68, 69). While the host pathways which are responsible for the induction of CHIKV-induced inflammatory disease are currently not known, DCIR has been shown to inhibit specific subsets of Toll-like receptors (53, 54) and

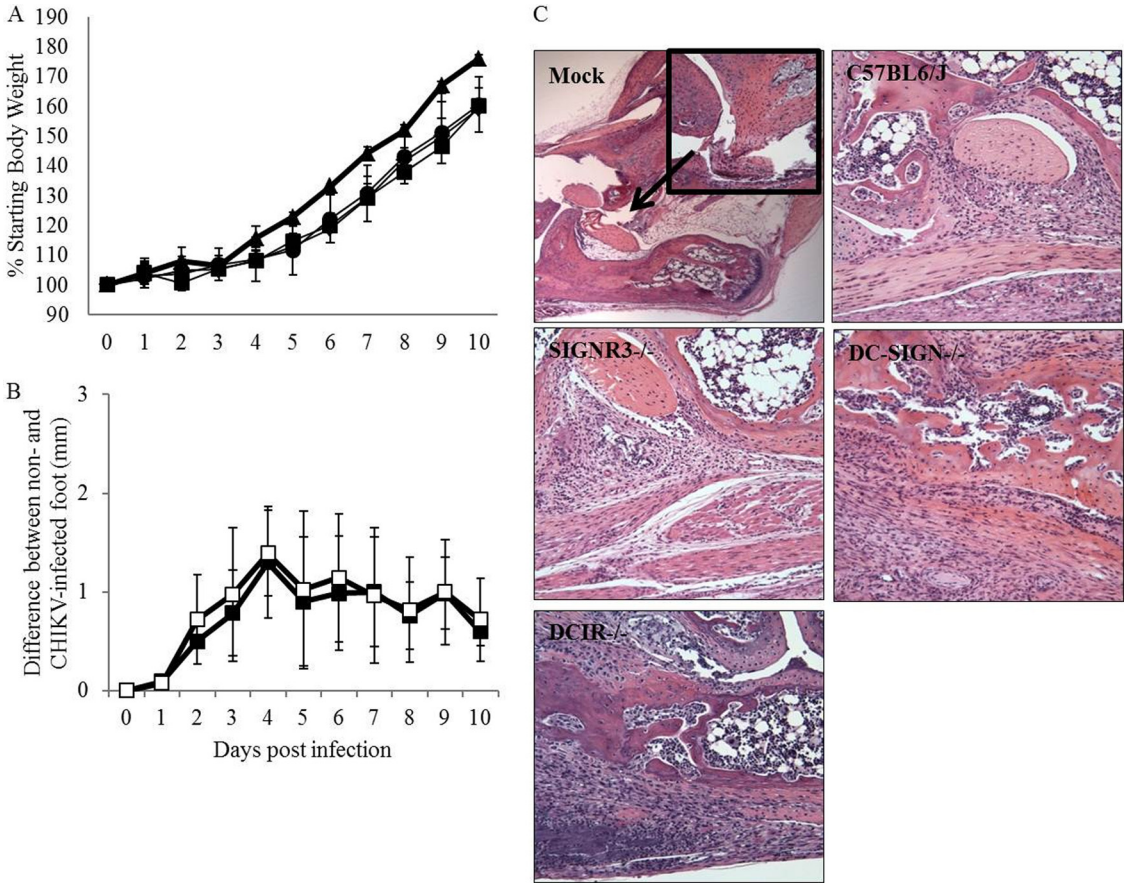

FIG 6 DCIR$^{-/-}$ but not DC-SIGN or SIGNR3$^{-/-}$ mice exhibit increased CHIKV-induced tissue pathology compared to C57BL6/J mice. Fourteen-day-old mice were infected with 100 PFU CHIKV or mock infected (▲). (A) Every 24 h, C57BL6/J mice (■), SIGNR3$^{-/-}$ mice (●), and DC-SIGN (◆) mice were weighed to assess weight gain. (B) The CHIKV-infected and noninfected feet of C57BL6/J mice (■) and SIGNR3$^{-/-}$ (□) mice were measured daily to monitor foot swelling. (C) Mice were euthanized at 10 dpi and perfused with 4% paraformaldehyde via intracardial injection. Whole ipsilateral leg tissues were paraffin embedded, cut to a thickness of 5 μm, and stained with H&E to visualize inflammatory cell infiltration and tissue damage. Images are of the ankle joint, and a micrograph from a CHIKV-infected DCIR$^{-/-}$ mouse is included for comparison. Magnifications ×40 (top left) and ×200 (inset at top left and all remaining panels). Images are representative of three to six mice per group and two independent experiments.

is thought to be paired with an activating CLR (DCAR) (70). Therefore, further investigation of the impact that DCIR has on CHIKV-induced inflammatory activation may provide new insights into the specific host signaling pathways that drive CHIKV-induced disease, thereby enhancing the field's understanding of CHIKV disease pathogenesis and identifying potential targets for therapeutic intervention in the treatment of CHIKV and other alphavirus-induced arthridites.

The natural ligand of DCIR is unidentified, though DCIR has been shown to mediate enhancement of HIV capture and infection by DCs (55), suggesting that DCIR could directly interact with CHIKV. Other C-type lectin receptors, including DC-SIGN and L-SIGN, have been shown to recognize N-linked glycans on a range of viruses, including alphaviruses (39), and it is therefore possible that DCIR is interacting with N-linked glycans or other structures on the virion. However, to date we have no evidence for direct interactions between DCIR and CHIKV. Furthermore, other CLRs, including DC-SIGN, interact with a number of cellular ligands to maintain homeostasis within the host or mediate immune signaling. Therefore, given that DCIR can also modulate non-virus-induced inflammatory states, it is very possible that DCIR is mediating its effects on CHIKV-induced inflammation independently of interactions with CHIKV or CHIKV-infected cells; however, these possibilities require further investigation.

In summary, these results demonstrate that DCIR plays an important role in limiting CHIKV-induced inflammatory disease that is largely independent of major effects on viral replication, which suggests that DCIR is regulating the activation state and pathological potential of the inflammatory cells entering the virally infected tissue. These results suggest that further investigation of DCIR's role in CHIKV pathogenesis may provide novel insights into the regulation of CHIKV-induced disease and lead to the identification of the host pathways which are modulated by DCIR and in its absence promote CHIKV-induced disease.

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