A Critical Role for STING Signaling in Limiting Pathogenesis of Chikungunya Virus

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The stimulator of interferon gene (STING) pathway controls both DNA and RNA virus infection. STING is essential for induction of innate immune responses during DNA virus infection, while its mechanism against RNA virus remains largely elusive. We show that STING signaling is crucial for restricting chikungunya virus infection and arthritis pathogenesis. Sting-deficient mice (*Sting*^{gt/gt}) had elevated viremia throughout the viremic stage and viral burden in feet transiently, with a normal type I IFN response. *Sting*^{gt/gt} mice presented much greater foot swelling, joint damage, and immune cell infiltration than wild-type mice. Intriguingly, expression of interferon- γ and *Cxcl10* was continuously upregulated by approximately 7 to 10-fold and further elevated in *Sting*^{gt/gt} mice synchronously with arthritis progression. However, expression of chemoattractants for and activators of neutrophils, *Cxcl5*, *Cxcl7*, and *Cxcr2* was suppressed in *Sting*^{gt/gt} joints. These results demonstrate that STING deficiency leads to an aberrant chemokine response that promotes pathogenesis of CHIKV arthritis.

Keywords. stimulator of interferon genes; STING; arthritogenic; alphavirus; chikungunya; viral; arthritis.

Arthritogenic alphaviruses are transmitted by mosquitoes and pose a public health threat in the tropical/subtropical regions. Chikungunya virus (CHIKV) is the causative agent of acute and chronic crippling arthralgia that reemerged on the Indian Ocean Islands in 2005–2006. Since late 2013, a new wave of CHIKV epidemics has taken place in the Americas, resulting in over 2.5 million infection cases in more than 40 countries (source, Pan America Health Organization) [1]. Approximately 50% of CHIKV-infected patients suffer from rheumatic manifestations that last 6 months to a year, whereas another approximately 5% of patients may have rheumatoid arthritis-like illness [2]. However, no licensed vaccines or specific antiviral therapeutics are available. This is partly due to an incomplete understanding of the protective and detrimental immune responses elicited by CHIKV. The type I interferon (IFN-I) system is clearly a critical early antiviral mechanism [3], while the antibody response is essential for viral clearance later [4]. However, the CHIKV viremia levels are not predictive of arthritis severity. For instance, even with heightened and prolonged viremia, B/T-cell deficient mice presented similar arthritic disease as wild type (WT) [5]. Of note, chronic CHIKV arthritis may progress without active viral replication [6].

The stimulator of interferon genes (STING) participates in innate immunity to both DNA and RNA viruses. During DNA virus infection, STING senses a second messenger cyclic GMP-AMP (cGAMP), which is synthesized by a viral DNA receptor, cGAMP synthase (cGAS) [7–9], leading to IFN-I expression. Our study with West Nile virus and other recent reports conclusively demonstrate that STING is also important for the control of RNA virus infection in mouse models [7, 10–12]; the underlying molecular mechanism, however, remains largely unclear. Several mechanisms of action have been proposed for STING during RNA virus infection. STING may cross-talk to retinoic acid-inducible gene I (RIG-I) [7] or phosphatidylinositol-3-OH kinase (PI3K) [13] to induce IFN-I. It may also sustain RIG-I signaling [14] at late stages of infection. Certain RNA virus infections, such as dengue virus, may damage mitochondria, which results in DNA leakage and activates the cGAS-STING pathway [15]. The cGAS-STING pathway may also be important to maintain a low basal level of interferon stimulated genes (ISGs) as a sentinel against invading viruses [16]. Alternatively, STING could initiate IFN-I-independent antiviral actions, such as the adaptive immune response in the central nervous system [17], induction of a specific set of chemokines through signal transducer and activator of transcription 6 (STAT6) [12], and inhibition of viral protein translation [18]. Here we study the physiological function of STING in the pathogenesis of CHIKV. Sting-deficient mice have high viral loads and exacerbated arthritis, along with a normal IFN-I response. These mice also have higher expression of IFN- γ and *Cxcl10* in the joints than WT.

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METHODS

Mice

Wild-type C57BL/6J (JAX Stock number 000664) and Sting mutant (*Sting^{glt}* on C57BL/6 background, JAX Stock number 017537) breeders were obtained from the Jackson Laboratory, sex and age (range 6–12 weeks)-matched mice were used for all the experiments. The animal protocol was approved and performed according to the guidelines of the Institutional Animal Care and Use Committee at the University of Connecticut Health Center and Yale University.

Reagents and Antibodies

The rabbit anti-GAPDH (catalogue number 5174), anti-STING (catalogue number 13647) antibodies were purchased from Cell Signaling Technology. The mouse anti-OAS1a (catalogue number sc-365357) and rabbit anti-ISG56 (catalogue number sc-134949) were obtained from Santa Cruz Biotechnology. The rat anti-CD45 (catalogue number 103101), AF488 rat anti-Ly6C (catalogue number 128021), and AF 488 rat anti-F4/80 (catalogue number 123119) were purchased from BioLegend.

Cells and Viruses

Vero cells (monkey kidney epithelial cells, catalogue number CCL-81) and L929 (mouse fibroblasts, catalogue number CCL-1) were purchased from American Type Culture Collection. These cells were grown at 37°C and 5% CO₂ in Dulbecco's modified Eagle medium (DMEM; Corning) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Corning). These cell lines are not listed in the database of commonly misidentified cell lines maintained by the International Cell Line Authentication Committee, and tested negative for mycoplasma contamination in our hands. To ensure cell culture mycoplasma free, we regularly treated cells with MycoZap (Lonza). Bone marrows were isolated from the tibia and femur bones and then differentiated into macrophages (BMDMs) in conditioned RPMI1640 (20% FBS, 30% L929-conditioned medium, antibiotics/antimycotics) in Petri dishes at 37°C for 5 days. The attached BMDMs were cultured in tissue-culture treated plastic wares in regular RPMI1640 medium (10% FBS and 1% penicillin-streptomycin) overnight before further treatment. The CHIKV French La Reunion strain LR2006-OPY1 was a kind gift of the Connecticut Agricultural Experiment Station, New Haven, CT.

Plaque-Forming Assay

Quantification of infectious viral particles in cell culture supernatants/mouse tissue homogenates/mouse sera was performed on a Vero cell monolayer in a 6-well plate following an established protocol [19]. A serial of 10-fold dilutions of viral samples was prepared in DMEM without fetal bovine serum. In a 6-well plate, 500 µL of diluted samples were added to a Vero monolayer. The plate was incubated at 37°C and 5% CO₂ for

2 hours. The inoculum was then removed and replaced with 2 mL of complete DMEM medium with 1% SeaPlaque agarose (Lonza). The plate was incubated at 37°C and 5% CO₂ for 3 days, and plaques were visualized by a Neutral Red exclusion assay. The viral loads from footpads were normalized by tissue mass (gram).

Mouse Infection and Disease Monitoring

CHIKV, 3×10^5 plaque-forming units (PFUs), was inoculated subcutaneously in the mouse hind footpad. Foot swelling as an indicator of inflammation was recorded over a period of 8 days after infection. The thickness and width of the perimetatarsal area of the hind foot were measured using a precision digital metric caliper in a blinded fashion. The foot dimension was calculated as width $\times$ thickness, and the results were expressed as fold increase in the foot dimension after infection compared to before infection (day 0 baseline). For histology, footpads/joints were fixed in 4% paraformaldehyde, decalcified, and processed for hematoxylin and eosin staining. Arbitrary arthritic disease scores (on a 1–5 scale with 1 being the lightest, 5 the worst) were assessed using a combination of histological parameters, including exudation of fibrin and inflammatory cells into the joints, alteration in the thickness of tendons or ligament sheaths, and hypertrophy and hyperlexia of the synovium [20] in a double-blinded manner. Slides were imaged using an AccuScope EXI-310 inverted microscope with Infinity Capture software.

Real-Time Quantitative RT-PCR

Total RNA was isolated from blood samples and footpad tissues using a RNAasy mini-prep kit (Invitrogen) and converted into cDNA using a First Strand cDNA Synthesis Kit (Takara). Quantitative polymerase chain reaction (qPCR) was performed with gene-specific primers and iTaq Universal SYBR Green Supermix (Bio-Rad). The primers for CHIKV were forward primer (5'-GCGAATTTCGGCGCAGCACCAAGGACAACCTTCA-3') and reverse primer (5'-AATGCGGCCGCTAGCAGCATATTAGGCTAAGCAGG-3'). The housekeeping gene control used was β -actin (*Actb*). The results were calculated using the $-\Delta\Delta$ threshold cycle (Ct) method. The qPCR primers and probes for immune genes were reported in our previous studies [21].

PCR Array

Total RNA was extracted from white blood cells or skinned ankle joints, and reverse-transcribed using a cDNA synthesis kit (Bio-Rad). The cDNA was used for the real-time RT Profiler PCR Array (Qiagen) in combination with SYBR Green qPCR master mix. Delta Ct (threshold cycle of qPCR) was the difference between the gene of interest and an average of 5 reference genes (*Actb*, *B2m*, *Gapdh*, *Gusb*, and *Hsp90ab1*). Fold change was then calculated using formula $2^{\Delta\Delta Ct}$ [ΔCt (*Sting^{glt}*) – ΔCt (WT)].

RNASeq and Data Analysis

For sample preparation, 2×10^6 bone marrow-derived WT and *Sting*^{gt/gt} macrophages were infected with CHIKV at a multiplicity of infection of 5 for 12 hours or mock infected. RNA was extracted using the Qiagen RNeasy kit and quantitated using an Agilent 2100 Bioanalyzer. To construct a transcript library for sequencing, mRNA was purified with poly-T oligoconjugated magnetic beads. The purified mRNA was fragmented and primed with random hexamers for cDNA synthesis. cDNA fragments were adenylated and then ligated to indexing adapters. The cDNA fragments were purified and enriched by PCR, then sequenced on a BGISEG-500 using single-ended read and 50bp cycles. The total clean reads were 21.9 M, clean bases 1.1 Gb. The clean read ratio was >99.7%, total mapping ratio >96%, and unique mapping ratio 77.3%–79.3%. Sequences are available from the GEO (accession number GSE159450). SOAPnuke version 1.5.2 (<https://github.com/BGI-flexlab/SOAPnuke>) was used to analyze the RNA-sequence data. Bowtie2 version 2.2.5 and RSEM version 1.2.12 were used to map sequencing read to

mm10 genome and quantify reads. DESeq2 version 1.0.0 was applied to calculate the differential expression of genes. The genes with a ≥ 2 fold difference and false discovery rate ≤ 0.01 were analyzed for canonical functional pathways by the ingenuity pathway analysis.

Statistical Analysis

All data were analyzed with GraphPad Prism software by non-parametric Mann-Whitney test, 2-tailed Student *t* test, or multiple *t* tests, depending on the data distribution and the number of comparison groups. *P* values of less than .05 were considered statistically significant.

RESULTS

STING Signaling Restricts CHIKV Infection and Arthritis Pathogenesis

Our studies with West Nile virus and other recent studies conclusively demonstrated that STING is also important for the control of RNA virus infection in mouse models [7, 10–12]. This was also observed with CHIKV infection

Figure 1. Increased CHIKV infection in *Sting*^{gt/gt} mice. Age- and sex-matched, C57BL/6 (WT) and STING deficient (*Sting*^{gt/gt}) mice were infected with CHIKV. *A*, Quantitative RT-PCR quantification of CHIKV loads in whole-blood cells. *B*, Viremia of day 2 sera quantified by plaque forming assay (PFU/mL serum). *C*, Quantitative PCR analysis of CHIKV loads in the spleen. *D*, Footpads viral load in day 4, 6, and 8 and viral titers in day 4 post infection (PFU/g tissue). Each symbol represents 1 mouse; short horizontal line indicates the median. The data represent 2 independent experiments. *, *P* < .05; **, *P* < .01 (nonparametric Mann-Whitney *U* test). Abbreviations: CHIKV, chikungunya virus; PFU, plaque-forming unit; RT-PCR, reverse transcription polymerase chain reaction; STING, stimulator of interferon gene; WT, wild type.

(Figure 1). When inoculated directly into a mouse footpad, CHIKV elicits brief viremia that lasts approximately 5 days with overt arthritic symptoms, including the first peak of foot swelling characteristic of edema during 2–3 days, followed by a second peak at 6–8 days post infection (dpi) [22] with massive infiltration of immune cells into infected feet [23, 24]. Sting-deficient mice (*Sting*^{gt/gt}) [25] presented a significant increase in viremia from 12 through 96 hours post infection (hpi) compared to WT mice (Figure 1A and 1B), which accompanied higher viral loads in the spleen of *Sting*^{gt/gt} than WT mice (Figure 1C). The viral loads in the feet were elevated at 4 dpi in *Sting*^{gt/gt} compared to WT mice; however, this difference disappeared by 6–8 dpi. (Figure 1D). Moreover, *Sting*^{gt/gt} mice showed much greater footpad swelling from 4 to 8 dpi compared to WT mice (Figure 2A). Hematoxylin and eosin and immunofluorescence staining for CD45 demonstrated a significant increase of immune cells in the muscle/synovial cavity/tendon of *Sting*^{gt/gt} compared to

WT mice (Figure 2B–2D). Interestingly, although the viral loads in *Sting*^{gt/gt} joints were the same as WT at 6 and 8 dpi (Figure 1D), the foot and joint disease manifestations were much more severe in *Sting*^{gt/gt} (Figure 2).

STING Signaling Is Dispensable for the IFN-I Response During CHIKV Infection

The aforementioned results clearly demonstrate the importance of STING in the control of CHIKV infection at the very early stage of infection and disease pathogenesis in vivo. Next, we evaluated the IFN-I response. The mRNA expression of *Ifna* by leukocytes was barely induced at the peaks of viremia (days 2–4) in both WT and *Sting*^{gt/gt} mice, consistent with a dominant role of nonhematopoietic cells in IFN-I production [3]. While *Ifit1* and *Ifit2* ISGs were both upregulated by CHIKV infection and increased in *Sting*^{gt/gt} mice on 4 and 2 dpi, respectively (Figure 3A). We further analyzed the expression of 84 genes related to the IFN-I response by PCR array in WT and *Sting*^{gt/gt}

Figure 2. Exacerbated joint inflammation in *Sting*^{gt/gt} mice. **A**, Fold changes in the footpad dimensions of infected (at days 0.5, 1, 4, 6, and 8 post infection) over the uninfected (day 0). Bars indicate mean + SEM. **B**, Representative micrographs of staining H&E from mock- and CHIKV-infected joints at day 8 post infection (× 40). The boxed areas indicate the regions with severe immune cell infiltration and tissue damage (× 200). **C**, Arbitrary H&E scores for the CHIKV-infected footpads/ankle joints at day 8 post infection using a scale of 1 to 5, with 5 representing the worst disease presentation. Each symbol represents 1 mouse; short horizontal line indicates the median. n = 6/genotype. *, *P* < .05; **, *P* < .01 (2-tailed unpaired Student *t* test). The data represent 2 independent experiments. **D**, Confocal microscopic images of immunofluorescence staining for a pan-immune cell marker CD45 (× 200). Abbreviations: B, bone; CHIKV, chikungunya virus; H&E, hematoxylin and eosin; M, muscle; STING, stimulator of interferon gene; T, tendon; WT, wild type.

Figure 3. A normal type I IFN response in *Sting*^{g/gt} mice during CHIKV infection. **A**, qPCR quantification of immune gene expression in whole-blood cells. Each symbol represents 1 mouse; short horizontal line indicates the median. The data represent 2 independent experiments. *, $P < .05$ (nonparametric Mann-Whitney U test). **B**, PCR array of differentially expressed type I IFN response-related genes in the white blood cells of WT and *Sting*^{g/gt} mice at 2 and 4 days post-CHIKV infection (samples pooled from 8 mice, respectively). The results represent fold changes of *Sting*^{g/gt} over WT mice. A negative value (highlighted in blue) indicates downregulation; a positive (highlighted in red) denotes upregulation. N/A indicates expression below detection in either *Sting*^{g/gt} or WT. The cutoff is arbitrarily set at 3-fold. Abbreviations: CHIKV, chikungunya virus; IFN, interferon; qPCR, quantitative polymerase chain reaction; STING, stimulator of interferon gene; WT, wild type.

mice. Considering the larger variations among individual mice, we set an arbitrary cutoff of >3 fold. We observed no genes with a >3-fold difference at both time points (Figure 3B). In

vitro, the intracellular CHIKV load was increased in bone-marrow-derived *Sting*^{g/gt} macrophages compared to WT cells (Figure 4A). The mRNA expression of *Ifnb1* and ISGs (*Isg15*,

Figure 4. An intact type I IFN response in *Sting^{gt/gt}* macrophages during CHIKV infection. qPCR quantification of the (A) cellular viral loads and (B) select immune genes in WT and *Sting^{gt/gt}* BMDMs infected with CHIKV at a multiplicity of infection of 2 ($n = 4$ replicates). C, Immunoblots of the indicated interferon stimulated genes in BMDMs. Bars indicate mean + SEM. *, $P < .05$; **, $P < .01$ (2-tailed unpaired Student t test). The data shown are representative of 2 independent reproducible experiments. Abbreviations: BMDM, bone-marrow-derived macrophage; CHIKV, chikungunya virus; IFN, interferon; qPCR, quantitative polymerase chain reaction; STING, stimulator of interferon gene; WT, wild type.

Isg56, and *Oas1a*) was slightly higher in *Sting^{gt/gt}* than WT cells after infection (Figure 4B), as was *Isg56* (48 hpi) and *Oas1a* (24 hpi) protein expressions, while *Oas1a* was modestly lower in *Sting^{gt/gt}* (48 hpi) (Figure 4C). *Sting* protein expression was also upregulated throughout infection (Figure 4C), suggesting that CHIKV-induced IFN-I signaling is largely intact in *Sting^{gt/gt}*. To unbiasedly examine if the IFN-I response or other immune signaling pathways were deficient in *Sting^{gt/gt}* cells during CHIKV infection, we performed RNAseq analysis of whole-genome transcripts in bone-marrow-derived macrophages following mock or CHIKV infection. We compared the global gene expression profile between WT and *Sting^{gt/gt}* without infection (mock) or 12 hours after infection (CHIKV), between mock- and CHIKV-infected WT, and between mock- and CHIKV-infected *Sting^{gt/gt}* cells. We identified differentially expressed genes (Supplementary Table 1) and the biological pathways that were significantly altered between 2 groups using the differentially expressed genes with a $\log_2$ -fold change ≤ -1 (downregulated) or ≥ 1 (upregulated) and $Q \leq .01$ (Figure 5). The expression levels of 909 genes in *Sting^{gt/gt}* were different from those in WT without infection, and 677 were altered

after CHIKV infection. Compared to the uninfected mock, 2754 genes in WT cells and 3541 genes in *Sting^{gt/gt}* were either up- or downregulated after CHIKV infection (Supplementary Table 1). The top pathways that were repressed by CHIKV infection in both WT and *Sting^{gt/gt}* cells were cardiac hypertrophy signaling, cell cycle control of chromosomal replication, and xenobiotic metabolism signaling (Figure 5A). Compared to WT, hepatitis fibrosis, GP6, and cardiac hypertrophy signaling pathways were attenuated in *Sting^{gt/gt}* cells without (mock) or with CHIKV infection. Not surprisingly, the primary pathways activated by CHIKV were immune related, including IFN, pathogen pattern recognition receptors, cross-talk between dendritic cells and natural killer cells, and systemic lupus erythematosus. The activation Z scores for these pathways were similar between WT and *Sting^{gt/gt}* (Figure 5B). A specific examination of the IFN-I signaling pathway revealed that *Ifna/b*, *Ifit1*, *Ifit3*, and *G1p2* were upregulated most significantly and similarly between WT and *Sting^{gt/gt}* (Figure 5C). These results are in agreement with the aforementioned PCR array and qPCR results, suggesting that STING is nonessential for the IFN-I response during CHIKV infection.

Figure 5. Analysis of altered canonical functional pathways in *Sting^{Gt/Gt}* macrophages. Bone-marrow-derived macrophages from 3 individual WT/*Sting^{Gt/Gt}* mice were infected by CHIKV at a multiplicity of infection of 5 for 12 hours or not infected (mock). The whole-genome transcriptome was analyzed by RNAseq. The genes with a ≥ 2 fold difference and $Q \leq .01$ were included for analysis of canonical functional pathways by ingenuity pathway analysis: (1) *Sting^{Gt/Gt}* versus WT cells without CHIKV (mock), (2) WT and *Sting^{Gt/Gt}* cells CHIKV-infected, (3) CHIKV-infected versus mock-infected WT cells, and (4) CHIKV-infected versus mock-infected *Sting^{Gt/Gt}* cells. The ranking of activation (orange) or suppression (blue) of a pathway is indicated by Z-score. **A**, The top activated/suppressed canonical pathways. **B**, The immune pathways. **C**, The type I IFN pathway. The individual genes highlighted in red/green were up- or downregulated in CHIKV-infected compared to uninfected cells. The genes in gray were not significantly different after infection (≥ 2 -fold difference and false discovery rate ≤ 0.01). Genes in white were not present in the dataset. WT and *Sting^{Gt/Gt}* are presented separately. The color intensity correlates with expression level: the darker the color, the higher the gene expression. Abbreviations: CHIKV, chikungunya virus; IFN, interferon; STING, stimulator of interferon gene; WT, wild type.

Aberrant Chemokine Expression in the *Sting^{Gt/Gt}* Joints During CHIKV Arthritis Progression

Because immune cell infiltration into joints and muscles is a hallmark of CHIKV arthritis, we examined chemoattractant expression in mouse ankle joints. A chemokine PCR array was performed with joint samples at the peak of disease (8 dpi). Two genes, *Ifng* and *Cxcl10* (also known as IFN- γ inducible gene-IP10, a chemoattractant for T cells, monocytes/macrophages), were upregulated by 4 to 5-fold and 3 genes, *Cxcl5*, *Cxcr2*, and *Cxcl7* (known to participate in neutrophil recruitment and activation) were downregulated by over 3-fold in *Sting^{Gt/Gt}* compared to WT mice (Figure 6A). We next validated the PCR array results and investigated the kinetics of

chemokine expression during the course of CHIKV infection. In WT mouse joints, *Ifng* and *Cxcl10* mRNA expression was continuously upregulated by approximately 7 to 10-fold, and further elevated in *Sting^{Gt/Gt}* mice at 4–8 dpi (Figure 6B), synchronously with arthritis progression. *Cxcl7* expression was equally upregulated in both WT and *Sting^{Gt/Gt}* mice at 4 dpi, but rapidly decreased at 8 dpi in *Sting^{Gt/Gt}* mice (Figure 6B). *Ccl5* (a chemoattractant for T cells, eosinophils, and basophils), *Ccl1* (chemoattractant for monocytes), and *Ccl20* (strongly chemotactic for lymphocytes) were transiently upregulated at 4 dpi and downregulated at 8 dpi similarly in both WT and *Sting^{Gt/Gt}* mice (Figure 6B). In agreement with increased expression of *Cxcl10*, the numbers of monocytes (Ly6C⁺) and macrophages

Figure 6. Differential chemokine expression in the joints of *Sting^{gt/gt}* following CHIKV infection. **A**, PCR array of differentially expressed chemokine genes in the ankle joints of WT and *Sting^{gt/gt}* mice at day 8 post infection (samples pooled from 8 mice per strain). The results represent fold changes of *Sting^{gt/gt}* over WT mice. A negative value (highlighted in blue) indicates downregulation; a positive (highlighted in red) denotes upregulation. The cutoff is arbitrarily set at 3-fold. **B**, qPCR validation of PCR array results. Each symbol represents 1 mouse; short horizontal line indicates the median. The data represent 2 independent experiments. *, $P < .05$; **, $P < .01$; ***, $P < .001$ (nonparametric Mann-Whitney U test). **C** and **D**, Representative microscopic images of immunofluorescence staining for Ly6C and F4/80 in the footpads at day 8 post CHIKV infection (objective lens $\times 20$). Abbreviations: CHIKV, chikungunya virus; qPCR, quantitative polymerase chain reaction; STING, stimulator of interferon gene; WT, wild type.

(F4/80⁺) were increased at 8 dpi in *Sting^{gt/gt}* relative to WT footpads (Figure 6C and 6D). These data demonstrate that IFN- γ and CXCL10 expression kinetics are in line with arthritis progression and suggest a role for IFN- γ and CXCL10 in disease pathogenesis.

DISCUSSION

The function and regulation of STING signaling has been well established in the context of cytosolic DNA stimulation of either self or foreign origin [26]. However, its anti-RNA virus mechanisms remain largely elusive [26]. The IFN-I expression in *Sting^{-/-}* cells and/or mice was mildly decreased during influenza virus [27], vesicular stomatitis virus (VSV), and Sendai virus (SeV), but not encephalomyocarditis virus [7, 8, 25]. We, however, observed no significant differences in the IFN-I response elicited by CHIKV in *Sting^{gt/gt}* macrophages and mice by multiple approaches (Figure 3, Figure 4, and Figure 5). One possible explanation for these discrepancies is that CHIKV activates both RIG-I and MDA5 for a full IFN-I response [28, 29], while VSV, SeV, and influenza

viruses rely predominantly on RIG-I [30, 31], which may promote cross-talk with STING to induce IFN-I [8]. Of note, several ISGs, *Ifit1/Ifit2*, were slightly higher in *Sting^{gt/gt}* mice. This may be a result of increased viremia that activates STING-independent pathways such as RIG-I, MDA5, and Toll-like receptors (TLR) to produce more IFN-I and ISGs. Alternatively, ISG transcription can be induced directly by transcription factors nuclear factor- κ B (NF- κ B), interferon regulatory factor 1 (IRF1), IRF2, and IRF3 [32], which could be more active due to elevated viremia in *Sting^{gt/gt}* than WT mice. Altogether, our results suggest that STING could initiate IFN-I-independent antiviral actions. Indeed, STING could activate STAT6 to induce a specific set of chemokines to RNA virus infection [12] and inhibit viral protein translation [18]. Additionally, STING could activate autophagy [33] to limit CHIKV infection in mice [34]. Our RNAseq analyses of whole-genome transcripts in CHIKV-infected macrophages revealed 2 upregulated signaling pathways, peroxisomal proliferator activated receptor (PPAR) and liver X receptor (LXR)/retinoid X receptor (RXR), and a number

of downregulated genes in *Sting*^{gt/gt} (Figure 5). Although the role of top downregulated genes, hepatitis fibrosis, GP6, and cardiac hypertrophy signaling pathways, in CHIKV infection is not clear, many others are immune related, such as NF-κB, dendritic cell activation, p38 MAPK, interleukin-6 (IL-6), etc. that may contribute to antiviral immune responses during CHIKV infection. Of note, PPAR and LXR are nuclear receptors that suppress the activity of transcription factors like NF-κB, STAT, and AP-1 [35], potentially leading to weakened antiviral immune responses.

Intriguingly, exacerbated pathology progressed in *Sting*^{gt/gt} mouse feet even when viral loads were reduced to a similarly low level between WT and *Sting*^{gt/gt} mice at 6 and 8 dpi, respectively (Figure 1D and Figure 2). These results are in agreement with previous observations showing that CHIKV arthritis severity is not always positively correlated with viral loads [36], but is rather a primary consequence of dysregulated inflammatory responses. Indeed, immune cell infiltration is a hallmark of acute CHIKV infection, primarily including macrophages and monocytes, but also neutrophils, dendritic cells, NK cells, and lymphocytes [37]. Of note, although CD4⁺ T cells constitute only a small fraction of immune infiltrates in CHIKV-infected joints [38], they contribute significantly to arthritis pathogenesis [36]. In agreement with this, expression of CXCL10, a chemoattractant primarily for monocytes, macrophages, and T cells, was highly upregulated synchronously with arthritis progression, along with further elevation of monocytes and macrophages in *Sting*^{gt/gt} joints (Figure 6). In particular, the severity of CHIKV arthritis in humans is associated with a high level of serum CXCL10 and CXCL9 [39, 40]. Interestingly, we also noted elevated *Ifng* expression in *Sting*^{gt/gt} compared to WT joints, and its kinetics was coincident with disease progression (Figure 6B). This is in agreement with previous studies showing an association between elevated IFN-γ expression and CHIKV arthritis [40, 41]. IFN-γ could be predominantly expressed by infiltrating NK during the early stage of CHIKV infection (Figure 6B, day 4), and by CD4⁺ Th1 and CD8⁺ T cells once antigen-specific immunity develops (Figure 6B, day 8) [37, 42]. Thus, elevated *Ifng* mRNA expression in *Sting*^{gt/gt} joints suggests increased infiltration of these cells. In contrast, CXCL10 can be produced by many infiltrating immune cells including monocytes, macrophages, and neutrophils, etc. In particular, synovial macrophages could be a source of CHIKV persistence and CXCL10 [23, 43]. Moreover, CXCL10 is expressed by many tissue cells where synovial fibroblast hyperplasia is a signature of CHIKV arthritis [23]. These cells are also a primary target of CHIKV [44], and may express abundant CXCL10 through pathogen pattern recognition receptors and IFN-γ signaling pathways [45]. Skeletal muscle cells are a primary target of CHIKV and may also contribute to CXCL10 production [46]. Thus, our data suggest that STING signaling may not only limit viral replication at the early stage, but also keep aberrant inflammatory

responses in check to avoid immunopathology at the late stage of infection. Indeed, STING signaling can induce expression of negative regulators of innate immune signaling, such as suppressor of cytokine signaling, to control TLR-mediated hyperinflammatory immune responses in a lupus mouse model [47]. In particular, TLR7 (a viral RNA sensor) signaling could contribute to immunopathology as plasmacytoid dendritic cells express a very high level of TLR7 and were the most rapidly and highly expanded immune infiltrate in CHIKV-infected mouse footpads [48].

Neutrophils infiltrate the joints rapidly after CHIKV infection and seem to contribute to CHIKV arthritis pathogenesis [49]. Surprisingly, 3 genes related to neutrophil recruitment and activation (*Cxcr2* and its ligands *Cxcl5* and *Cxcl7*) [50] were down-regulated in *Sting*^{gt/gt} joints at 8 dpi (Figure 6B), which could result from elevated expression of IFN-γ, a suppressor of CXCL5 and CXCL7 [50]. These data suggest that increased arthritis severity in *Sting*^{gt/gt} mice is a result of accumulation of immune cells other than neutrophils. An abnormally high level of IFN-γ/CXCL10 could drive a robust Th1 response that exacerbates arthritis [49] in *Sting*^{gt/gt} joints. In the future, it would therefore be interesting to examine the kinetics of each immune population in the joints during the course of CHIKV infection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. T. G. designed and performed the majority of the experimental procedures and data analyses. T. L., A. G. H., and D. Y. contributed to experiments and/or provided technical support. A. T. V. and E. F. helped with data interpretations and writing. P. W. conceived and oversaw the study. T. G. and P. W. wrote the paper and all the authors reviewed and/or modified the manuscript.

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