UBXN3B Controls Immunopathogenesis of Arthritogenic Alphaviruses by Maintaining Hematopoietic Homeostasis

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ABSTRACT Ubiquitin regulatory X domain-containing proteins (UBXN) might be involved in diverse cellular processes. However, their in vivo physiological functions remain largely elusive. We recently showed that UBXN3B positively regulated stimulator-of-interferon genes (STING)-mediated innate immune responses to DNA viruses. Herein, we reported the essential role of UBXN3B in the control of infection and immunopathogenesis of two arthritogenic RNA viruses, Chikungunya (CHIKV) and O’nyong’nyong (ONNV) viruses. Ubxn3b deficient (Ubxn3b−/−) mice presented higher viral loads, more severe foot swelling and immune infiltrates, and slower clearance of viruses and resolution of inflammation than the Ubxn3b+/− littermates. While the serum cytokine levels were intact, the virus-specific immunoglobulin G and neutralizing antibody levels were lower in the Ubxn3b−/− mice. Ubxn3b−/− mice had more neutrophils and macrophages, but much fewer B cells in the ipsilateral feet. Of note, this immune dysregulation was also observed in the spleens and blood of uninfected Ubxn3b−/− mice. UBXN3B restricted CHIKV replication in a cell-intrinsic manner but independent of type I IFN signaling. These results demonstrated an additional role of UBXN3B in the maintenance of immune homeostasis and control of RNA virus replication.

IMPORTANCE The human genome encodes 13 ubiquitin regulatory X (UBX) domain-containing proteins (UBXN) that might participate in diverse cellular processes. However, their in vivo physiological functions remain largely elusive. Herein, we reported an essential role of UBXN3B in the control of infection and immunopathogenesis of arthritogenic alphaviruses, including Chikungunya virus (CHIKV), which causes acute and chronic crippling arthralgia, long-term neurological disorders, and poses a significant public health problem in the tropical and subtropical regions worldwide. However, there are no approved vaccines or specific antiviral drugs. This was partly due to a poor understanding of the protective and detrimental immune responses elicited by CHIKV. We showed that UBXN3B was critical for the control of CHIKV replication in a cell-intrinsic manner in the acute phase and persistent immunopathogenesis in the post-viremic stage. Mechanistically, UBXN3B was essential for the maintenance of hematopoietic homeostasis during viral infection and in steady-state.

KEYWORDS UBXN, alphavirus, Chikungunya virus, arthritogenic, hematopoiesis

Alphavirus is a genus of the Togaviridae family of positive sense, single-stranded RNA viruses. These viruses are primarily mosquito-borne and pose a public health threat worldwide, particularly in tropical/subtropical regions. Several of them, including Chikungunya (CHIKV), O’nyong’nyong (ONNV), and Ross River viruses (RRV), are known to elicit arthritis in humans. Of note, CHIKV causes acute and chronic crippling arthralgia and long-term neurological disorders. CHIKV was first discovered in 1952, in Tanzania and was relatively silent for several decades. However, since 2005 CHIKV has re-emerged and caused several large outbreaks in Africa, Asia, and South America (1). CHIKV appeared on the Caribbean Islands in 2013 and has spread rapidly throughout Central and South America since then, resulting in ~3 million human infections and...
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An essential role of UBXN3B in the control of CHIKV/ONNV pathogenesis. We recently showed that UBXN3B controls DNA virus infection via STING (37), and STING is critical to the control of CHIKV infection and arthritis pathogenesis in mice (38). However, the UBXN3B function in RNA virus infection has not been studied in depth. To this end, we employed a tamoxifen-inducible Cre/LoxP model because UBXN3B was essential for embryonic development. We directly injected CHIKV into one footpad to induce swelling and acute arthritis in Ubxn3b+/+ (corn-oil treated Cre+) Ubxn3bfl/fllox/+ mice, Ubxn3b−/−mice (tamoxifen-treated Cre− Ubxn3bfl/fllox/+), and Ubxn3b+/− (tamoxifen-treated Cre negative Ubxn3bfl/fllox/+), a control to exclude any effect of tamoxifen on foot swelling/viral replication) littermates. Both the Ubxn3b+/+ and Ubxn3bfl/fllox/+ mice showed progressive footpad swelling, which peaked at 6 days postinfection (pi) and resolved rapidly. In contrast, the Ubxn3b−/− mice presented more severe and persistent foot swelling at 4 days pi through 14 days pi (Fig. 1A and B). Histopathological analyses by hematoxylin and eosin (H&E) staining confirmed more...
immune infiltrates in the muscle and synovial cavity of Ubxn3b−/− than those of Ubxn3b+/+ littermates at 8 and 16 days pi (Fig. 1C to E). Similar results were observed with infection of another arthritogenic alphavirus, O’nyong’nyong (ONNV) at 12 days pi (Fig. 1E and G). Moreover, the CHIKV viremia at 2 and 4 days pi, and viral loads in the ipsilateral feet of Ubxn3b−/− mice at 4 and 8 days pi were significantly higher than those in Ubxn3b+/+ and Ubxn3bfox/fox littermates (Fig. 2). Of note, the infectious virions were cleared from the blood by 4 days and the feet by 8 days pi in the Ubxn3b+/+ while it remained high in the Ubxn3b−/− mice (Fig. 2B and D), suggesting a critical role of UBXN3B in the resolution of viral infection.

Acute CHIKV infection was associated with a systemic IFN and inflammatory response. Next, we measured the concentrations of serum innate immune molecules, including early antiviral type I IFNs (IFN-α) and type II IFN (IFN-γ), and those inflammatory mediators associated with severe arthritis in humans such as tumor necrosis factor
TNF-α), interleukin 6 (IL-6), IL-10, C-X-C motif chemokine ligand 10 (CXCL10), IL-1β, and granulocyte-macrophage colony-stimulating factor (GM-CSF). We noted that most cytokine levels were upregulated following CHIKV infection, though with different kinetics, except for IL-1β and GM-CSF (Fig. 3). The levels of CXCL10, TNF-α, IL-6, IL-10, CCL2, and CCL3 were higher in the $Ubxn3^{b−/−}$ than those in $Ubxn3^{+/+}$ mice at one or several time points even after viremia had been cleared at 8 days pi through 16 days pi (Fig. 3). The IFN-γ level peaked on day 6 in the $Ubxn3^{+/+}$ mice, and this peak shifted to day 8 in the $Ubxn3^{b−/−}$ animals, and the IFN-α level was similar between the $Ubxn3^{b−/−}$ and $Ubxn3^{+/+}$ littermates at several time points, and TNF-α at 12 days pi (Fig. 4). The differences between CHIKV and ONNV infection were likely due to different infection kinetics. We noted that ONNV infection caused more moderate foot swelling than CHIKV infection. Nonetheless, both the CHIKV and ONNV data demonstrated that cytokine responses were still intact or even higher in the $Ubxn3^{b−/−}$ mice.

An essential role of UBXN3B in the maintenance of immune cell homeostasis.

Because immune infiltration into muscles and joints was a hallmark of CHIKV arthritis, by H&E staining we confirmed that there were much more immune cells in the CHIKV-inoculated $Ubxn3^{b−/−}$ than $Ubxn3^{+/+}$ feet (Fig. 1C). We then resolved the different immune compartments in the feet inoculated with CHIKV by flow cytometry. We observed higher ratios and numbers of neutrophils and macrophages, but fewer B and CD4 T cells in the $Ubxn3^{b−/−}$ than $Ubxn3^{+/+}$ feet on day 7 pi. Notably, the B cell counts were reduced by ~5-fold. These differences were observed in other CHIKV-target immune organs, e.g., blood and spleens. In the spleen, both CD4 and CD8 T cell populations were also reduced by ~3-fold (Fig. 5A and B). Of note, the neutrophil-to-lymphocyte (N/L) ratios were much higher in all the tissues of the $Ubxn3^{b−/−}$ mice (Fig. 5C). These data suggested that UBXN3B signaling was essential for not only viral clearance but also immune cell homeostasis. We then asked if this dysregulated immune system in the $Ubxn3^{b−/−}$ mice was induced by CHIKV or constitutive. To this end, we analyzed the steady-state immune compartments. The total immune cell numbers in the feet of both uninfected $Ubxn3^{b−/−}$ and $Ubxn3^{+/+}$ mice were low with macrophages (mostly resident macrophages) being the dominant immune population, and
there were no significant differences in either the myeloid or lymphoid compartments (Fig. 6). The ratios and counts of neutrophils and macrophages were much higher (4- to 7-fold), while B cells were much lower (3- to 10-fold) in the spleens and blood of Ubxn3b−/− than those in Ubxn3b+/+ animals (Fig. 6). The T cell counts were lower in the spleens of Ubxn3b−/− than those in Ubxn3b+/+ mice. After infection, the numbers of total immune cells and individual compartments in both Ubxn3b+/+ and Ubxn3b−/− feet increased significantly compared to the uninfected Ubxn3b+/+ and Ubxn3b−/− mice, respectively. While the changes (post/prior CHIKV) in neutrophil, B, and T cell counts were comparable between the Ubxn3b+/+ and Ubxn3b−/− mice, the change in macrophage in the Ubxn3b−/− mice was 2.5 times as many as in the Ubxn3b+/+ mice. Nonetheless, the absolute counts of neutrophils and macrophages were significantly greater, while the B cell number was significantly smaller in the infected-Ubxn3b−/− than infected-Ubxn3b+/+ mice (Fig. 5). These data demonstrated that UBXN3B was essential for immune cell homeostasis during steady-state and CHIKV infection.

Because B lymphocytes were significantly reduced in the Ubxn3b−/− mice, we asked if this deficiency influenced the production of virus-specific antibodies. Indeed, the concentrations of serum anti-CHIKV/ONNV immunoglobulin G (IgG) were lower in the Ubxn3b−/−
than those in Ubxn3b+/+ mice (Fig. 7A). Moreover, the CHIKV-neutralizing antibody levels in the Ubxn3b−/− sera were also reduced. The fold-dilution for neutralizing 50% infectious virions (NT50) was 7595 for the Ubxn3b+/+ sera and 1595 for Ubxn3b−/− sera (day 16 pi), suggesting that the titers of CHIKV-neutralizing antibodies in the Ubxn3b−/− sera were reduced by 3.7-fold (Fig. 7B).

A cell-intrinsic antiviral function of UBXN3B. The above-mentioned data demonstrated a deficiency in the B cell compartment and IgG production in the Ubxn3b−/− mice, which may underlie delayed viral clearance. However, the Ubxn3b−/− mice presented much higher CHIKV viremia as early as at 2 days pi with an intact type I IFN response (Fig. 2A and B) before the adaptive immune response was activated. We then asked if UBXN3B played a cell-intrinsic antiviral role. To this end, we infected bone marrow-derived dendritic cells (BM-DCs) over 48 h. Skin DCs were one of the early targets of mosquito-transmitted CHIKV in nature and may help CHIKV disseminate systemically. Although CHIKV replication was not productive in DCs, the viral RNA loads in the Ubxn3b−/− were ~2-fold higher than those in the Ubxn3b+/+ cells at 48 h pi (Fig. 8A). The mRNA levels of Ifna, Tnfa, and Oas1a (an interferon-induced gene) in the Ubxn3b−/− cells trended slightly higher than those in the Ubxn3b+/+ cells (Fig. 8B). We confirmed the critical role of UBXN3B in the control of CHIKV replication in a highly permissive cell type, primary mouse embryonic fibroblast (MEF) (Fig. 8C) (37). These results suggested that UBXN3B restricted CHIKV replication independently of type I IFNs. To corroborate this, we examined the role of UBXN3B in CHIKV infection when type I IFN signaling was absent. We used an anti-IFNAR1 antibody to block the type I IFN receptor signaling. We noted that the viral loads in the anti-IFNAR1 antibody-treated cells were higher than those in control IgG-treated cells, and induction of interferon-stimulated genes (Oas1a, lsg15) was completely abolished suggesting an efficient blockade of IFNAR1 (Fig. 8D). Nonetheless,
the viral loads in Ubxn3b<sup>−/−</sup> cells were still higher than those in Ubxn3b<sup>+/+</sup> cells in either situation (Fig. 8E).

**DISCUSSION**

Although UBXN3B has been implicated in lipid metabolism (39–46), protein degradation (47, 48), mRNA stability (49), protein translocation (50), and stress granule disassembly (51), its in vivo physiological functions remain largely elusive. With a mouse model, we recently demonstrated that UBXN3B regulates STING-mediated innate immunity to a DNA virus (37). In this study, we discovered a critical role of UBXN3B in the control of RNA virus replication and pathogenesis. Similar to Sting deficiency (38), Ubxn3b deletion led to elevated viremia and exacerbated foot swelling during CHIKV infection.

**FIG 6** UBXN3B was essential for steady-state immune cell hemostasis. Various immune cell compartments in the foot, spleen, and blood were analyzed by flow cytometry. (A) The percentage relative to CD45<sup>+</sup> cells, and (B) counts of each immune cell type. Neu, neutrophil; Mac, macrophage; DC, dendritic cell. Each symbol is one mouse. Bar, mean ± SEM; N = 4. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 (two-tailed Student’s t test). The results are representative of 2 independent experiments.

**FIG 7** Virus-specific IgG production was impaired in Ubxn3b<sup>−/−</sup> mice. Sex-and-age matched littermates were administered 1 × 10<sup>5</sup> PFU of CHIKV or 5 × 10<sup>5</sup> of ONNV subcutaneously in one footpad. (A) The serum IgG levels were quantitated by ELISA and presented as optical density at 450 nm (OD<sub>450</sub>). Each symbol is one animal. The small horizontal line is the median of the result. (B) Percent plaque reduction of day 16 CHIKV sera over a preimmune serum. NT<sub>50</sub>, neutralizing titer of 50%. Preimmune sera assay for day 16 sera. *, P < 0.05; **, P < 0.001 (two-tailed Student’s t test). The results are representative of 2 independent experiments.
and ONNV infection. However, the cytokine expression remained largely intact or even higher in the Ubxn3b<sup>−/−</sup> mice and DCs. Moreover, viral replication was still enhanced in Ubxn3b<sup>−/−</sup> cells when type I IFN signaling was simultaneously blocked. These results suggested that UBXN3B signaling restricted CHIKV and ONNV replication in a cell-intrinsic manner but independently of type I IFNs. However, both STING and UBXN3B are known to contribute to the induction of type I IFNs by some RNA viruses, including vesicular stomatitis virus (VSV) and Sendai virus (SeV) (37, 52). This discrepancy is likely because VSV and SeV solely depend on RIG-I (53), which can crosstalk with STING to induce type I IFNs (54). CHIKV and ONNV are less reliant on RIG-I and can activate melanoma differentiation-associated protein 5 (MDA5) for a sufficient type I IFN response (55). Mechanistically, STING is known to restrict the translation of RNA viruses (56). Because both UBXN3B and STING localize to the endoplasmic reticulum (ER), it is reasonable to speculate that UBXN3B works together with STING to inhibit RNA virus translation.

Notably, Ubxn3b deficiency increased myeloid immune cell (neutrophil, macrophage) numbers, while a reduction in B lymphocytes in the feet, spleens, and blood during CHIKV infection. Neutrophil and B cell ratios were strikingly different between the Ubxn3b<sup>−/−</sup> and Ubxn3b<sup>+/+</sup> mice. Neutrophils infiltrate joints rapidly after CHIKV infection and seem to contribute to acute CHIKV arthritis pathogenesis (57), while B cells are essential for the control of CHIKV replication and arthritis (58). Macrophages constituted the largest immune cell population in feet following CHIKV infection, and its number was moderately higher in the Ubxn3b<sup>−/−</sup> mice. These activated macrophages could be the main cellular reservoir for CHIKV persistence during the late stages of infection and contribute to sustained inflammation (24, 59). On the other hand, heightened immunopathology and tissue damage persisted in the Ubxn3b<sup>−/−</sup> mice even after CHIKV clearance (16 days pi), suggesting that UBXN3B controls CHIKV immunopathogenesis separately from viral replication. Indeed, chronic CHIKV arthritis pathogenesis can progress without active viral replication (60). Thus, more myeloid, and fewer B cells may account for increased viral replication, delayed viral clearance, and exacerbated, and prolonged inflammation in the Ubxn3b<sup>−/−</sup> mice. Strikingly, this aberrant immune system was also observed in the uninfected Ubxn3b<sup>−/−</sup> mice, suggesting an important role of UBXN3B in constitutive hematopoietic homeostasis.
This new function is irrelevant to STING as Sting-deficient mice have normal immune cell compartments (61). Instead, UBXN3B deficiency might resemble the aging immune state, which is characteristic of low-grade inflammation mainly driven by macrophages and immunosenescence (62).

In humans, severe CHIKV arthritis is associated with elevated serum cytokine/chemoattractant levels, such as IL-1β, IL-6, TNF-α, CXCL10, etc. (63). Indeed, we noted that the serum CXCL10, IL-6 and TNF-α levels were higher in the Ubxn3b<sup>−/−</sup> than those in Ubxn3b<sup>+/+</sup> mice, consistent with more severe arthritic pathology in the Ubxn3b<sup>−/−</sup> mice. Notably, the CXCL10 levels remained higher even after the viremia clearance at 8 to 16 days after CHIKV or ONNV infection, which is correlated with the prolonged foot inflammation in the Ubxn3b<sup>−/−</sup> mice. CXCL10 is a chemoattractant for monocytes/macrophages/T cells/natural killer cells/DCs and is associated with severe CHIKV arthritis in humans (64). CXCL10 may contribute to alphaviral arthritis pathogenesis by recruiting monocytes/macrophages to the alphavirus-infected mouse feet (65). IL-1β is another established biomarker of CHIKV arthritis severity in humans (66, 67). We also observed an increase in the serum CCL2 and CCL3 levels in the Ubxn3b<sup>−/−</sup> mice. CCL2 and CCL3 are chemoattractants expressed primarily by monocytes and macrophages and essential regulators of neutrophil and macrophage recruitment and function. These data agree with the increase of neutrophil and macrophage populations in the Ubxn3b<sup>−/−</sup> mice. However, we did not detect a significant increase in the serum IL-1β levels throughout CHIKV infection (1 to 16 days) compared with prior infection in all the mice. This might be not surprising because CHIKV infection is much less productive in adult wild-type mice than in humans. However, some studies have demonstrated the induction of IL-1β expression by CHIKV in mice. One study showed that the il1b mRNA expression was upregulated in the ipsilateral feet of adult mice following CHIKV infection (~10⁴ PFU). Another study with 4-week-old mice infected with 10⁴ PFU of CHIKV showed that the IL-1β protein levels in the ipsilateral feet were upregulated too (68). In neonatal mice (8 to 9 day-old), the serum IL-1β levels were upregulated following CHIKV infection (1 × 10⁴ to 2 × 10⁵ PFU) (69, 70). In contrast, no significant serum IL-1β protein was detected during CHIKV infection (1 × 10⁴ PFU) in adult mice (71). These studies demonstrated that, although CHIKV infection in mice upregulated IL-1β expression locally in the ipsilateral feet, whether it can increase the systemic (serum) IL-1β protein level was probably reliant on the animal models and infection conditions of choice.

In summary, our studies suggested that UBXN3B restricted CHIKV/ONNV infection and immunopathogenesis by dual mechanisms, including STING signaling and immune cell homeostasis. The mouse UBXN3B function may be potentially recapitulated in humans because the UBXN3B protein is highly conserved in mammals (98% identity between human and rodent protein). Immediate future work will pinpoint the role of UBXN3B in hematopoietic homeostasis, particularly B cell development.

**MATERIALS AND METHODS**

**Mouse models.** The mouse line with the exon 1 of Ubxn3b flanked by two LoxP sites (Ubxn3b<sup>fl/fl</sup>) was generated via homologous recombination by Fujimoto at Nagoya University (43). The homozygous Ubxn3b<sup>fl/fl</sup> was then crossed with homozygous tamoxifen-inducible Cre recombinase-estrogen receptor T2 mice (Jackson Laboratories, stock number 008463) to generate Cre<sup>+</sup> Ubxn3b<sup>fl/fl</sup> littermates. To induce Ubxn3b deletion, 6-8-weeks old mice were injected with 100 µL of tamoxifen (10 mg/mL in corn oil) (Sigma, number T5648) via intraperitoneal (IP) every 2 days for a total duration of 8 days (4 doses). Successful deletion of Ubxn3b was confirmed in our recent study (32). Half of Cre<sup>+</sup> Ubxn3b<sup>fl/fl</sup> litters were treated with tamoxifen and designated Ubxn3b<sup>−/−</sup>. The other half were treated with corn oil only and designated Ubxn3b<sup>+/+</sup>. Mice were allowed to purge tamoxifen for at least 4 weeks before any infection or analyses were performed. The animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Connecticut Health Center and Yale University.

**Antibodies, cell lines, and viruses.** The goat anti-mouse IFNAR1 (number AF30369) was purchased from R&D Systems (Minneapolis, MN 55413, USA), rabbit anti-GAPDH (Clone D16H11, number 5174), anti-β-Actin (Clone 13E5, number 4970) and STING (Clone D2P2F, number 13647) were from Cell Signaling Technology (Danvers, MA, USA). Human embryonic kidney 293 cells transformed with T antigen of SV40 (HEK293T, number CRL-3216) and Vero cells (monkey kidney epithelial cells, number CCL-81) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). These cell lines...
were not listed in the database of commonly misidentified cell lines maintained by ICLAC. Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics/antimycotics (Life Technologies, Grand Island, NY, USA). We routinely added MycoZAP (Lonza Group, Basel, Switzerland) to cell cultures to prevent mycoplasma contamination.

The CHIKV French La Reunion strain LR2006-OPY1 was a kind gift of the Connecticut Agricultural Experiment Station in New Haven, CT, USA. The CHIKV vaccine strain 181, clone 25 was recovered from a plasmid and used for in vitro infection and enzyme-linked immunosorbent assay (ELISA) (72, 73). The ONNV UgMP30 strain (NR-51661) was provided by BEI Resources. All these viruses were propagated in Vero cells.

**Differentiation of bone marrow DCs, isolation and treatment of embryonic fibroblasts with 4-OH tamoxifen.** Bone marrow-derived DCs were induced from bone marrow cells with 10 ng/mL murine GM-CSF (PeproTech) in RPMI 1640 medium containing 10% (volume/volume [vol/vol]) FBS (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) for 6 to 8 days at 37°C and 5% CO₂ (37). Mature BM-DCs were cultured in RPMI 1640 medium overnight and then washed once with the prewarmed fresh medium before use.

Pregnant Cre<sup>+</sup> Ubxn3b<sup>fl/fl</sup> females were euthanized on days 12 to 14 of gestation. Embryos were decapitated and eviscerated and then digested with trypsin for 10 min at 37°C rotating. Fibroblasts were filtered through a 100 μm filter, cultured in RPMI 1640 medium (Life Technologies, NY, USA), and supplemented with 10% FBS and antibiotics/antimycotics. One-half of the Cre<sup>+</sup> Ubxn3b<sup>fl/fl</sup> embryonic fibroblasts (MEFs) were treated with 2 μM 4-hydroxy (OH) tamoxifen for 4 to 5 days to generate the Ubxn3b<sup>−/−</sup> cells. The other half was treated with solvent (dimethyl sulfoxide, DMSO), resulting in Ubxn3b<sup>+/−</sup>. After induction, the cells were further passaged three times in RPMI medium without tamoxifen.

**Plaque-forming assay.** Quantification of infectious viral particles in sera, cell culture supernatants, and homogenized tissues were performed on Vero cell monolayers (74). Briefly, viral samples (appropriately diluted) were incubated with confluent Vero cells (6-well plate) at 37°C for 2 h. The inoculum was then removed and replaced with 2 mL of DMEM complete medium with 1% SeaPlaque agarose (catalog number 570100, Lonza). The cells were incubated at 37°C, 5% CO₂, for 3 days, and on the fourth day, the cells were stained with Neutral Red (Sigma-Aldrich) overnight.

**Mouse infection and monitoring.** About 1 x 10<sup>5</sup> to 5 x 10<sup>5</sup> plaque-forming units (PFU) of CHIKV LR2006-OPY1 or ONNV were inoculated at the ventral side of a hind foot subcutaneously. The thickness and width of the perimetaatarsal area of the hind foot (inoculated with the virus) were measured using a precision metric caliper. The foot dimension was calculated as width x thickness, and the results were expressed as the percent increase in the foot dimension after infection (D<sub>n</sub>) over its baseline before infection (D<sub>0</sub>), using the formula: 100% x (D<sub>n</sub>-D<sub>0</sub>) / D<sub>0</sub>.

**Tissue histology.** Tissue were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, cut into 4 μM-thick sections, immobilized to glass slides, decaffied, and processed for hematoxylin and eosin staining. Arbitrary arthritic disease scores (on a 1 to 5 scale with 1 being the slightest and 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 in a double-blinded manner (75).

**Reverse transcription and quantitative PCR.** Up to 1 x 10<sup>5</sup> cells or 10 mg of tissue was collected in 350 μL of lysis buffer (Qiagen RNeasy Minikit). RNA was extracted following the Qiagen RNeasy manufacturer’s instructions. Reverse transcription of RNA into complementary DNA (cDNA) was performed using the Bio-Rad iScript cDNA Synthesis kit. Quantitative PCR (qPCR) was performed with gene-specific primers and SYBR green PCR master mix. Results were calculated using the −ΔΔCt method and a housekeeping gene, beta-actin, as an internal control. The qPCR primers were reported in our previous studies (38, 65).

**Fluorescence-activated cell sorting.** FACS was performed according to our published study (65). Mouse tissues were minced with a fine scissor and digested in 4 mL of digestion medium (20 mg/mL collagenase IV [Sigma-Aldrich, St. Louis, MO, USA], 5 U/mL dispase [StemCell, Cambridge, MA, USA], and 50 mg/mL DNase I mix [Qiagen, Germantown, MD, USA]) in complete RPMI 1640 medium) at 37°C for 4 h. The lysate was filtered with a 40 μm cell strainer. Cells were then pelleted down by centrifugation at 500 x g for 5 min. The red blood cells in the cell pellet were lysed three times with a lysis buffer (catalog number 4029301 from Biolegend, San Diego, CA, USA). Cells were suspended in FACS buffer and stained for 30 min at 4°C with the desired antibody cocktails (Biolegend, San Diego, CA, US) of APC-Fire 750-anti CD11b (catalog number 101261, clone M1/70), Alexa Fluor 700-anti Ly-6G (catalog number 127621, clone 1A8), Brilliant Violet 421-anti CD45R (B220) (catalog number 103239, clone RA3-6B2), and Alexa Fluor 700-anti CD19 (catalog number 108735, clone PK136), Zombie UV (catalog number 423107), PE-Cy7-anti CD45 (catalog number 107625, clone M5/114.15.2), PE-anti Tetherin (PCDA1) (catalog number 101313, clone 20-F11), TruStain FcX-anti CD16/32 (catalog number 101319, clone 93), Brilliant Violet 421-anti CD45R (B220) (catalog number 103239, clone RA3-6B2), and Alexa Fluor 700-anti CD19 (catalog number 115527, clone 6D5). After staining and washing, the cells were fixed with 4% PFA and analyzed on a Becton, Dickinson FACS ARIA II, CyAn advanced digital processor (ADP). Data were analyzed using the FlowJo software. Among CD45<sup>+</sup> cells, CD11b<sup>+</sup> Ly6G<sup>−</sup> cells were classified as neutrophils, Ly6G<sup>−</sup>CD11b<sup>+</sup> F4/80<sup>+</sup> as macrophages, Ly6G<sup>−</sup>CD11b<sup>+</sup>CD115<sup>+</sup> as monocytes, Ly6G<sup>−</sup>CD11c<sup>−</sup> MHC II<sup>+</sup> as...
dendritic cells (DC), CD3⁺ as total T cells, CD3⁺ CD4⁺ as CD4 T cells, CD3⁺ CD8⁺ as CD8 T cells, CD19⁺ as B cells.

**Multiplex enzyme-linked immunosorbent assay.** We used a LEGENDPlex (Biolegend, San Diego, CA 92121, USA) bead-based immunoassay to quantify serum cytokine concentrations. The procedures were the same as in the product manual. Briefly, the supernatants or standards were mixed with antibody-coated microbeads in a 96-bottom microplate and incubated at room temperature for 2h with vigorous shaking at 500 rpm. After the removal of unbound analytes and two washes, 25 µL of detection antibodies were added to each well, and the plate was incubated at room temperature for 1 h with vigorous shaking at 500 rpm. Next, 25 µL of SA-PE reagent was then added directly to each well, and the plate was incubated at room temperature for 30 min with vigorous shaking at 500 rpm. The beads were washed twice with wash buffer and then transferred to a microcentrifuge tube. The beads were fixed with 4% PFA for 15 min and resuspended in the assay buffer. The beads were run through a BIORAD ZE5 and the concentrations of analytes were calculated with the standards included in the assay kit using the LEGENDPlex software. The CCL2 (number 88-7391-22) and CCL3 (number 88-56013-22) ELISA kits were purchased from Invitrogen and performed according to the product manual exactly (Waltham, MA, USA).

**Quantification of IgG by ELISA.** Next, $1 \times 10^4$ viral particles in coating buffer (0.05 M carbonate-bicarbonate, pH 9.6) were coated to a 96-well microplate at 4°C overnight. The plate was washed once with wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0), and blocked with 4% bovine serum albumin at room temperature for 2 h. Next, 100 µL of each diluted serum (500-fold) was added to a well and incubated at room temperature for 1 h, then washed three times. Then, 100 µL of diluted horseradish peroxidase-conjugated goat anti-mouse IgG was added to each well and incubated at room temperature for 1 h. After stringency wash, 100 µL of substrate 3,3',5,5' tetramethylbenzidine (TMB) was added to each well and incubated at room temperature for 5 to 30 min for color development and terminated by 100 µL of 0.16 M sulfuric acid. The absorption at wavelength 450 nm ($A_{450}$) was read on a Cytation 1 plate reader (BioTek, Winooski, VT, USA).

**Plaque neutralizing assay.** The preimmune and anti-CHIKV mouse sera were heated at 56°C for 20 min and serially diluted in phosphate-buffered saline (PBS). One hundred 20 PFU of CHIKV were mixed with 100 µL of diluted sera, and the mix was incubated at 37°C for 1 h. The mix and fetal bovine serum (FBS)-free DMEM medium was added to Vero cells (see description of the plaque-forming assay for preparation of Vero cells) in a 6-well plate and incubated at 37°C for 4 h. The inoculum was then removed and replaced with 2 mL of DMEM complete medium with 1% SeaPlaque agarose (catalog number 50100, Lonza). The cells were incubated at 37°C, 5% CO₂, for 3 days, and on the fourth day, the cells were stained with Neutral Red (Sigma-Aldrich) overnight. The dilutions and percent plaque reduction (over control serum) were plotted for polynomial equations, which were used to calculate the neutralizing titer at which viral plaques were reduced by 50% (NT₅₀).

**Data availability.** The sample sizes chosen for our animal experiments in this study were estimated according to our prior experience in similar sets of experiments and power analysis calculations (http://isogenic.info/html/power_analysis.html). All animals were included, and no method of randomization was applied. All data were analyzed with GraphPad Prism software by nonparametric or nonparametric two-tailed Student’s t test depending on the data distribution. $p \leq 0.05$ was considered statistically significant.

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T.G. designed and performed most of the experimental procedures and data analyses. D.Y. and T.L. contributed to some of the experimental procedures and/or provided technical support. P.W. conceived and oversaw the study. J.G.C. helped to improve the writing. T.G. and P.W. wrote the paper and all the authors reviewed and/or modified the manuscript.

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