Supporting Information

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SI Methods

Mice and Reconstitution with Bone Marrow Mast Cells (MCs). Both MC-deficient mice (W^{sh}/W^{sh}; Sash) and control mice (+/+; WT on a C57BL/6 background) were originally purchased from Jackson Laboratories. Sash mice were repleted with in vitro–matured bone marrow–derived MCs from congenic controls to generate reconstituted Sash (Sash-R) mice as follows. Bone marrow was flushed from femurs and grown in RPMI medium containing 10% FBS, penicillin and streptomycin, Hepes, trypsin inhibitor, sodium pyruvate, recombinant IL-3 (5 ng/mL), and stem cell factor (5 ng/mL) for a period of 4 wk, at which time the resulting cell cultures were determined to consist of >98% MCs by toluidine blue staining. Cells (1×10^7) were injected into Sash mice via tail vein and allowed to mature for at least 6 wk. These mice were then used in experiments as repleted MC-deficient (Sash-R) mice, and the presence of MCs within the footpad tissue was confirmed in some mice by staining tissue sections. Additional C57BL/6 mice were purchased from the National Cancer Institute. All experiments were performed according to protocols approved by the Duke-National University of Singapore or Duke Division of Laboratory Animal Resources and their respective university Institutional Animal Care and Use Committees.

In Vivo Virus Quantitation. In vivo viral quantitation in the draining lymph nodes (DLNs) was undertaken after dengue virus (DENV) footpad challenge. Here, 2×10^5 DENV2 were injected into the rear footpads of MC-sufficient or -deficient mice. Popliteal DLNs were removed at 24 h postinfection and homogenized in tissue lysis buffer (Buffer RLT; Qiagen) with ceramic beads (Glen Mills) using a mechanical homogenizer. Total RNA then was isolated with an RNeasy kit (Qiagen) according to the manufacturer's protocol. Before quantitative RT-PCR, cDNA of total RNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad), with 0.5 μL of a forward-sense nonstructural protein 1 (NS1) primer added to the standard reaction to make cDNA of this viral product because the kit would otherwise only amplify RNA containing a poly(A) tail. Real-time PCR was performed with SYBR Green reagent and an iCycler machine (Bio-Rad). For quantification, a DENV standard curve was generated with serial dilutions of DENV genomic RNA. Readings were averaged from triplicates, before averaging the calculated pfu from multiple ($n \geq 6$) LNs. The NS1 primer sequences used were as follows: 5′-CCG CTG ACA TGA GTT TTG AGT-3′ and 5′-CAT GAC AGG AGA CAT CAA AGG A-3′.

Viral Strains and Growth Conditions. All four DENV serotypes used were Eden Singaporean clinical isolates (1) and were grown in *Aedes albopictus* C6/36 mosquito cells (CRL-1660; ATCC) maintained in RPMI medium 1640 (Gibco) supplemented with 5% FCS. Virus stocks were stored −80 °C. When necessary, UV inactivation of the virus was performed by exposure to UV light for 30 min while on ice.

In Vitro Virus Quantitation. Before their use, plaque assays were carried out to quantify the number of infectious viral particles for each strain, using baby hamster kidney BHK-21 (CCL-10; ATCC) cells. Briefly, BHK cells were cultured to ∼80% confluency in 24-well plates (Nunc). The virus stock was 10-fold serially diluted from 10^{-1} to 10^{-8} in RPMI medium 1640 (Gibco). BHK-21 monolayers were infected with 200 μL of each virus dilution. After incubation at 37 °C and 5% CO_2 for 1 h with rocking at 15-min intervals, the medium was decanted and 1 mL of 1% (wt/vol) carboxymethyl cellulose in RPMI supplemented with 2% FCS was added to each well. After 4 d of incubation, the cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet dissolved in 37% formaldehyde. After thorough rinsing with water, the plates were dried, and the plaques were scored visually.

MC Cell Lines and Culture Conditions. Rat basophilic leukemia-2H3 cells (RBLs) were cultured in α-MEM supplemented with 10% FBS and 100 U/mL penicillin–streptomycin in an incubator with 5% CO_2 at 37 °C. Adherent cells were subcultured with trypsin–EDTA. Human MC LAD2 cells were grown in StemPro medium (Gibco-Invitrogen) supplemented with 100 ng/mL stem cell factor, 2 mM L-glutamine, 100 IU/mL penicillin, and 50 μg/mL streptomycin and passaged by hemidepletion once a week.

Assessment of MC Degranulation. For in vivo assessment of MC degranulation, either 1×10^5 or 2×10^5 pfu of DENV2 was injected into mouse footpads, and the cell media used to grow virus was injected into contralateral control footpads. Footpads were frozen in optimal cutting temperature compound and cut in 10-μm-thick sections. To examine for ex vivo degranulation of monkey (cynomolgus macaques) skin MCs, ∼1.5-cm^2-sized dermal tissues were obtained from euthanized animals and injected at multiple sites with a total of 5×10^6 pfu of DENV2. A β-hexosaminidase assay was used to assess in vitro MC degranulation. RBLs were exposed to DENV at a multiplicity of infection (MOI) of 1, followed by detection of β-hexosaminidase in supernatants and cell lysates solubilized in 0.1% Triton X-100. β-Hexosaminidase activity was measured with *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.1 M sodium citrate (pH 4.5) for 1 h at 37 °C. After adding 0.1 M carbonate buffer (pH 10.0), the product 4-*p*-nitrophenol was detected by absorbance at 405 nm. The percentage degranulation was calculated by dividing absorbance in the supernatant by the sum of absorbance in the supernatant and cell lysate.

MC Expression of mRNA for Viral Immunity and MC Activation Factors. At indicated time intervals, total RNA was isolated with the TRIzol extraction method from 1×10^6 RBLs after exposure to DENV2 at an MOI of 1. Then 1 μg of each total RNA was used for making cDNA, using oligo(dT) as primer, and NS1-specific primers were used for making DENV cDNA. A total of 50 ng of each cDNA was used for real-time amplification of specific genes using respective primers in a Bio-Rad iQ-5 real-time thermal cycler. The expression of viral immunity gene products, cytokines, and chemokines was normalized to 18s ribosomal RNA expression, followed by normalization to expression levels at unstimulated conditions. For knockdown (KD) of gene expression by siRNA, target-specific siRNAs were purchased from Ambion, and RBLs were transfected with the Jetprime reagent (Polyplus) according to the manufacturer's instructions. The primer sequences used are included below.

Microscopy. For SEM, RBL MCs were seeded overnight onto MEM with 10% FBS and then exposed to DENV2 at an MOI of 1. At the indicated time points, the coverslip was gently rinsed with PBS and fixed in 2% glutaraldehyde in PBS. Samples were then postfixed with OsO_4, rinsed, dehydrated in ascending concentrations of ethyl alcohol, critical-point dried, and examined under a scanning electron microscope. For bright-field microscopy, LAD2 cells were exposed to DENV2 at an MOI of 1 in tissue culture conditions, followed by cytospinning onto slides.

Cells were then fixed with methanol and stained with hematoxylin and eosin. For toluidine blue staining of tissues, sectioned tissues were fixed with Carnoy's fixative (60% ethanol, 30% chloroform, and 10% glacial acetic acid) before staining. To examine for the presence of natural killer (NK) cells and MCs at the site of DENV infections in the rear footpads, 10-μm frozen tissue sections were prepared, acetone-fixed, and blocked for 1 h at room temperature in blocking buffer (1% BSA in 1× PBS). Tissue sections (or cells) were stained by using PE-conjugated antibodies against NK1.1, rat antibodies against CD31 or CD3 (BD Biosciences), followed by an anti–rat-conjugated Cy5 (Jackson Laboratories), and the MC-specific probe, avidin, conjugated to FITC or TRITC (Invitrogen); for dsRNA by using antibody J2 (English & Scientific Consulting); and for NS3 by using a rabbit polyclonal antibody against dengue NS3 (laboratory source) followed by anti–rabbit-conjugated Cy5 (R&D Systems) or DENV envelope–specific antibody 4G2 (laboratory source) followed by anti–mouse-conjugated FITC. Samples were mounted with ProLong antifade reagent (Molecular Probes). Confocal images of stained footpad tissue sections were obtained with a three-laser Nikon confocal laser scanning instrument with a channel-series approach diminished spectral overlap. For staining monkey skin MCs, explants were incubated at 37 °C for 1 h, after which they were acetone-fixed, blocked overnight in PBS containing BSA, then incubated at 4 °C overnight in blocking buffer containing avidin–TRITC (Sigma). After washing with PBS, tissues were whole-mounted on a slide for visualization under epifluorescence illumination. Images were prepared for publication with ImageJ software.

Flow Cytometry. Footpads were isolated, and the tissue was digested into single-cell suspensions using collagenase (Invitrogen) before passing through a cell strainer (BD Biosciences). Cells were then stained with antibodies for NK1.1–PE or CD3–FITC (Invitrogen) before acquiring data with a FACSCalibur machine (BD Biosciences). Quantification of cells was determined by counting with a hemocytometer.

Primer Sequences. Virus-induced signaling adapter (VISA): 5′-CTC TTC TAC TGG ATC AGC TT-3′ and 5′-CAG TGG ACT TTG TAC TGA TG-3′; melanoma differentiation-associated gene 5 (MDA5): 5′-AAG CAG AAG TTG AAA AAC CA-3′ and 5′-TAA TGG TAA AGG CAT CGA GA-3′; retinoic acid inducible gene 1 (RIG-I): 5′-GTC ATA ACA CCA GCA AAC ACC-3′ and 5′-AGC ATC TCC AAC ACC AAC G-3′; Toll-like receptor 3 (TLR3): 5′-CAT CAC CTA CTG AAA GAC CCG-3′ and 5′-GCA GAG TGC ATG GTT TAG TTT G-3′; CCL5: 5′-AGT ATT TTT ACA CCA GCA GC-3′ and 5′-ATG GAG AGA CCC TCT ATT CT-3′; CXCL12: 5′-GGA CAA AGC CTT AAA CAA GA-3′ and 5′-TCA AAT GGT GAA AGT GTA GC-3′; CX3CL1: 5′-AGA CAA ACC CAG TTC ATA CT-3′ and 5′-ACA GGA GTG ATA AAC ACA CT-3′; TNF: 5′-ATT TGC CAT TTC ATA CCA GG-3′ and 5′-GAC TCC GTG ATG TCT AAG TA-3′; IFN-α: 5′-ACA CGA AAA TAC TTG AGA GC-3′ and 5′-TTT TGC TTT ACT GTT GCT GA-3′; 18s: 5′-TGT TCA AAG CAG GCC CGA G-3′ and 5′-CGG AAC TAC GAC GGT ATC TGA TC-3′; and NS1: 5′-CCG CTG ACA TGA GTT TTG AGT-3′ and 5′-CAT GAC AGG AGA CAT CAA AGG A-3′.

Statistical Analysis. Results were analyzed for statistical significance with the unpaired Student's *t* test or by one-way ANOVA, as appropriate, with Microsoft Excel or Prism software. Differences between groups were considered significant at $P \leq 0.05$. All error bars represent the SEM.

1. Low JG, et al. (2006) Early Dengue infection and outcome study (EDEN)–study design and preliminary findings. *Ann Acad Med Singapore* 35(11):783–789.

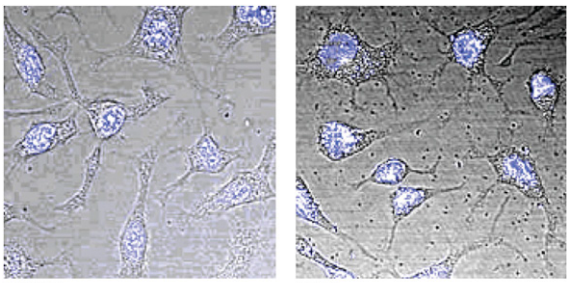

Fig. S1. DENV-induced MC degranulation. Images are of RBLs after exposure to control medium alone or DENV2, acquired in bright field with DAPI staining of nuclei. A large number of extracellular granules can be visualized surrounding MCs after exposure to DENV2.

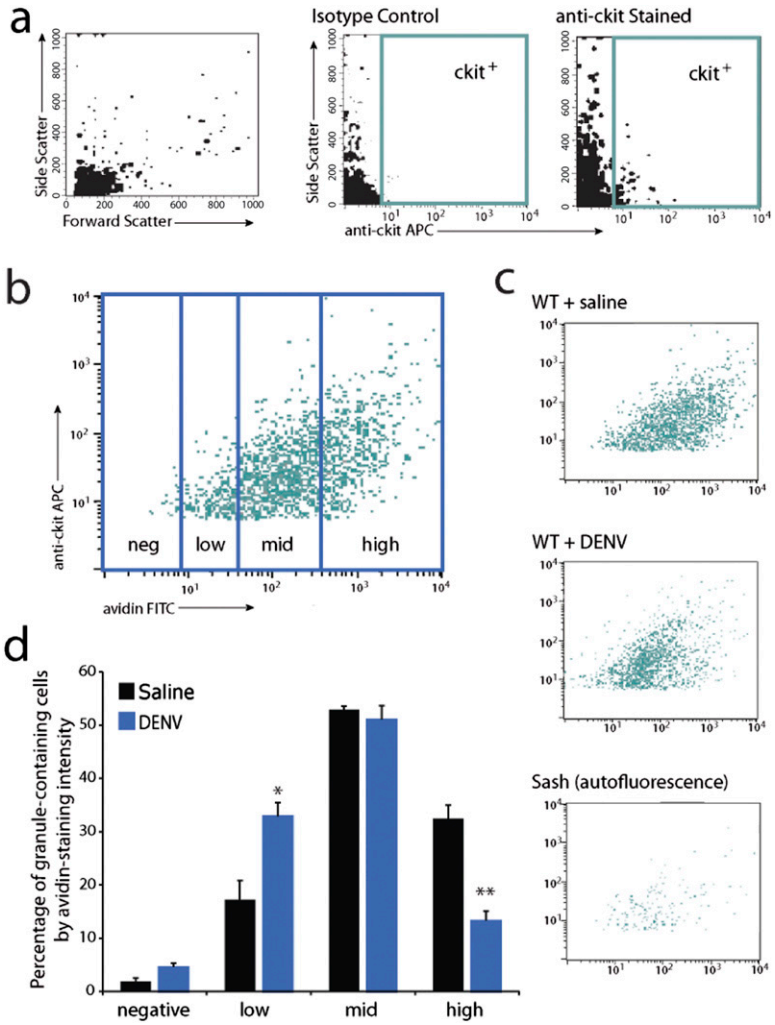

Fig. S2. Quantification of MC degranulation responses to DENV by flow cytometry. Mouse footpads were infected with DENV for 24 h, after which footpad tissue was harvested and prepared in single-cell suspensions for flow cytometry. Cells were surface-stained for the MC marker ckit, followed by intracellular staining for MC granules using the granule-specific probe avidin. (*A*) The flow cytometry plot depicts the gating strategy for ckit$^+$ cells. (*B*) The plot illustrates the gating strategy for avidin–FITC$^+$ cells for a control (saline-injected footpad) sample, after gating on ckit$^+$ cells. Avidin–FITC staining is represented along the *x* axis, and the blue boxes correlate to gated levels of granule-specific avidin staining, from negative to high. (*C*) A decrease in the intensity of avidin staining for granules within MCs is observed in DENV-infected tissue, compared with saline-injected tissues in WT mice having normal MCs. Because footpad tissue contains autofluorescent cells, this staining with MC-deficient Sash mice is presented to demonstrate the specificity of the staining protocol for MCs and the small degree of auto fluorescence. (*D*) Using $n \geq 4$, the percentage of ckit$^+$ MCs falling into each granule-staining category presented in *A* was quantified. The percentage of MCs containing high levels of granules significantly decreased (**), and the level of MCs containing low levels of granules significantly increased (*) during DENV infection, compared with saline-injected controls ($P < 0.05$ for each).

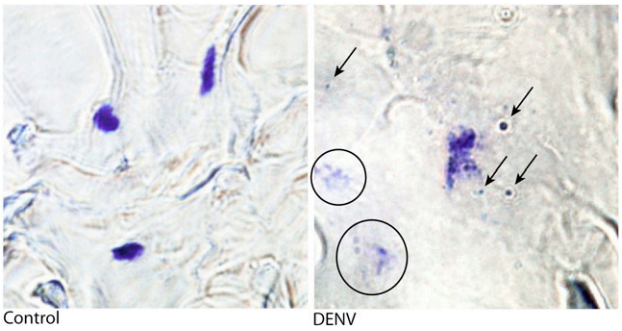

Fig. S3. DENV activation of monkey MCs, visualized by toluidine blue staining. Sections from control or DENV-treated monkey skin explants that were incubated ex vivo for 5 h were stained with toluidine blue to reveal activated MCs in DENV-injected tissues but quiescent MCs in control tissues. Some extracellular granules are denoted by arrows. In DENV-treated tissues, some clusters of loosely associated metachromatic staining are highlighted with circles. These areas are consistent with the staining patterns of partially degranulated cells and may also be activated MCs.

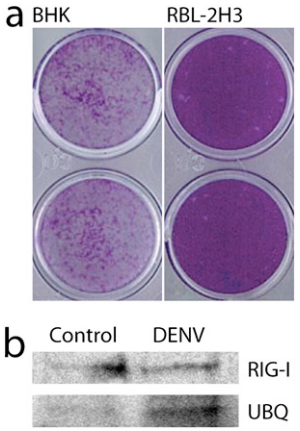

Fig. S4. DENV replication in MCs triggers pattern-recognition receptors. (*A*) RBLs and BHK cells were infected with DENV for 24 h, followed by trypsinization, washing, and replating of these cells at various densities on a monolayer of BHK cells. A standard plaque-forming assay was then performed to determine the percentage of original RBL and BHK cells that were infected. In the image, plaques can be observed in the wells seeded with DENV-exposed RBLs, demonstrating that RBLs can produce intact and infective virus. Based on comparison with BHKs, of which 100% of cells become infected with DENV, only 2.5% of RBLs were calculated to produce infective viral particles. (*B*) Ubiquitination of RIG-I occurs during DENV infection of MCs. Cell lysates were isolated from RBLs infected with DENV for 24 h at an MOI of 1 or from control cells. Proteins were then separated by SDS/PAGE and probed with specific antibodies to determine the total RIG-I in the cell lysate and ubiquitinated (UBQ) RIG-I. Ubiquitination of RIG-I appears to increase with DENV treatment.

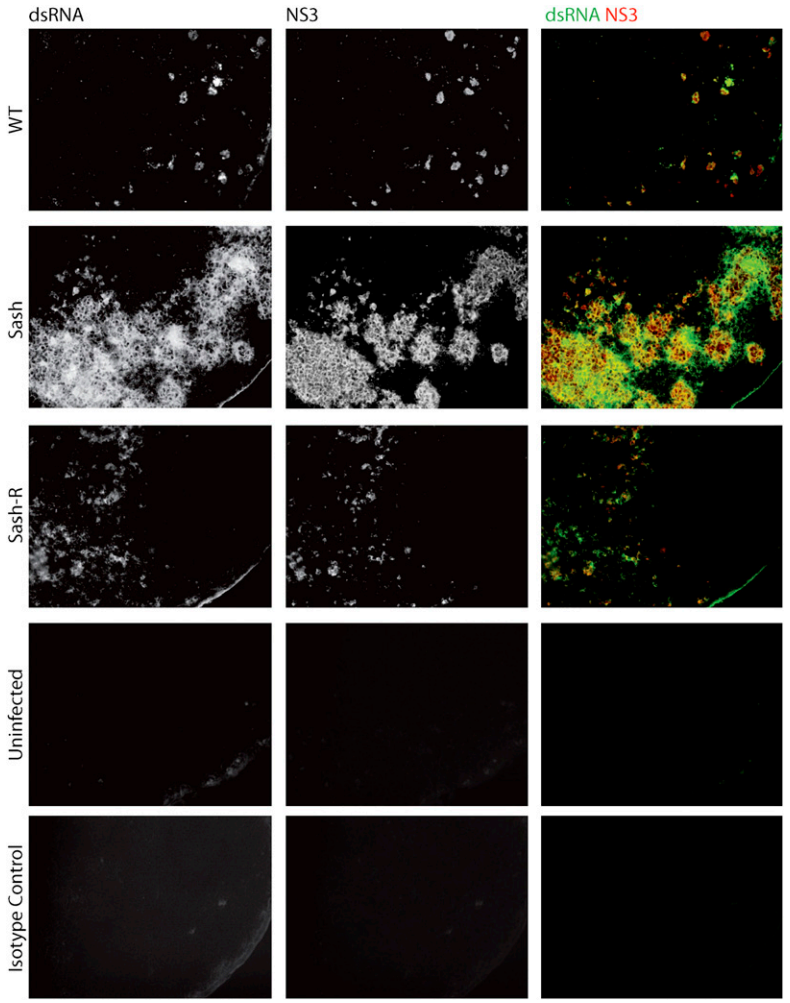

Fig. S5. Channel-series staining of LNs for products of DENV replication. The channel-series images corresponding to Fig. 4*B* are presented, with additional uninfected and isotype controls.

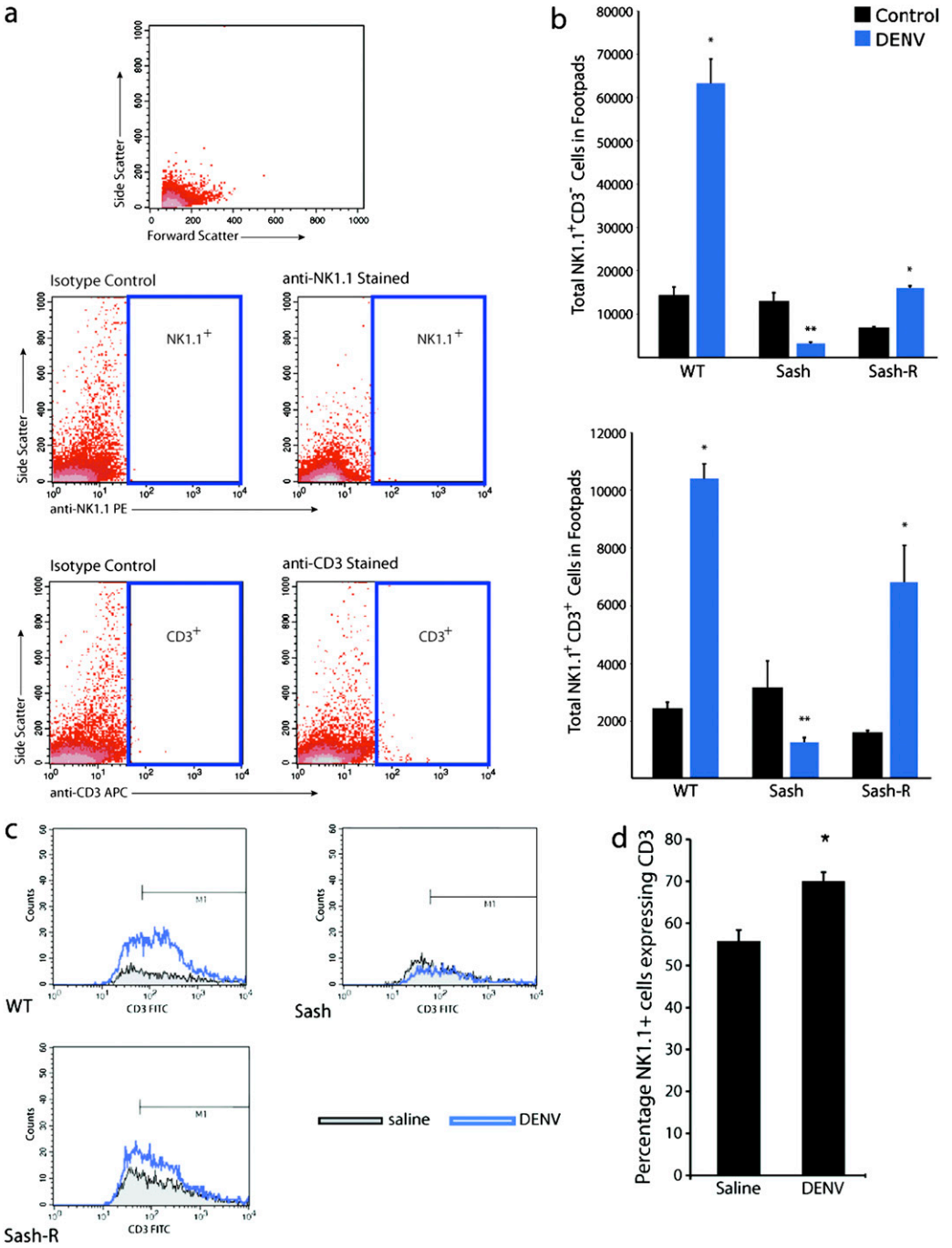

Fig. S6. MC-dependent influx of NK and NKT cells into DENV-infected tissue. (*A*) The gating strategy to determine NK1.1$^+$ and CD3$^+$ cells in footpad tissue with and without s.c. DENV infection in WT, Sash, and Sash-R mice. Blue boxes indicate the gates for positive populations. (*B*) Total numbers of NK1.1$^+$CD3$^-$ and NK1.1$^+$CD3$^+$ cells were calculated for each sample based on the gating strategy defined in *A* and the total cell numbers, as determined by counting. Both CD3$^-$ and CD3$^+$ subsets of NK1.1-staining cells increase after infection with DENV compared with saline controls in MC-competent mice (WT and Sash-R), designated by *. (*C*) Footpads from WT, Sash, and Sash-R mice were removed 24 h after either saline injection (gray histogram) or DENV infection (blue histogram) and prepared for flow cytometry. Single-cell suspensions were stained for NK1.1 and CD3. Histograms depict cells that are gated as NK1.1$^+$, with the intensity of staining for CD3 along the *x* axis. The M1 marker defines the CD3$^+$ population. Both CD3$^-$ and CD3$^+$ subsets of NK1.1-staining cells increase after infection with DENV compared with saline controls in WT and Sash-R but not Sash mice. (*D*) This graph represents the percentage of NK1.1$^+$ cells within footpads (and the associated vasculature) that also express CD3 (n = 8), showing that NK1.1$^+$CD3$^+$ cells are enriched compared with NK1.1$^+$CD3$^-$ cells during DENV infection, although both of these populations are augmented in *C*. ** designates a significant decrease compared with controls for Sash mice. For all bars, $P < 0.05$ and $n \geq 2$; error bars indicate the SEM.

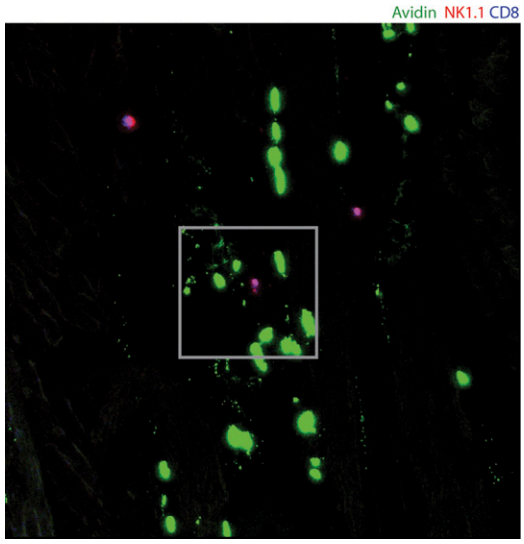

Fig. S7. Microscopy of a footpad section 24 h after injection of 2×10^5 pfu of DENV2, revealing many activated MCs (green) and recruited NK1.1$^+$ cells (red) that also express CD8 (blue). The region enlarged in Fig. 5*C* is boxed in grey.

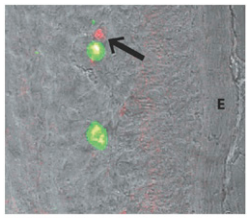

Fig. S8. An MC visualized interacting with an NK1.1$^+$ cell in vivo during DENV infection. Footpads were infected for 24 h with DENV before harvesting, sectioning, and staining for MCs (avidin–FITC; green) and the NK cell marker NK1.1 (red). The arrow points to a cell expressing NK1.1 that is physically associated with an MC. The MC here appears to be undergoing degranulation, as evidenced by the nearby extracellular granules staining for avidin. In bright field, it can be determined that this interaction occurred just under the epidermis (designated by the letter E).

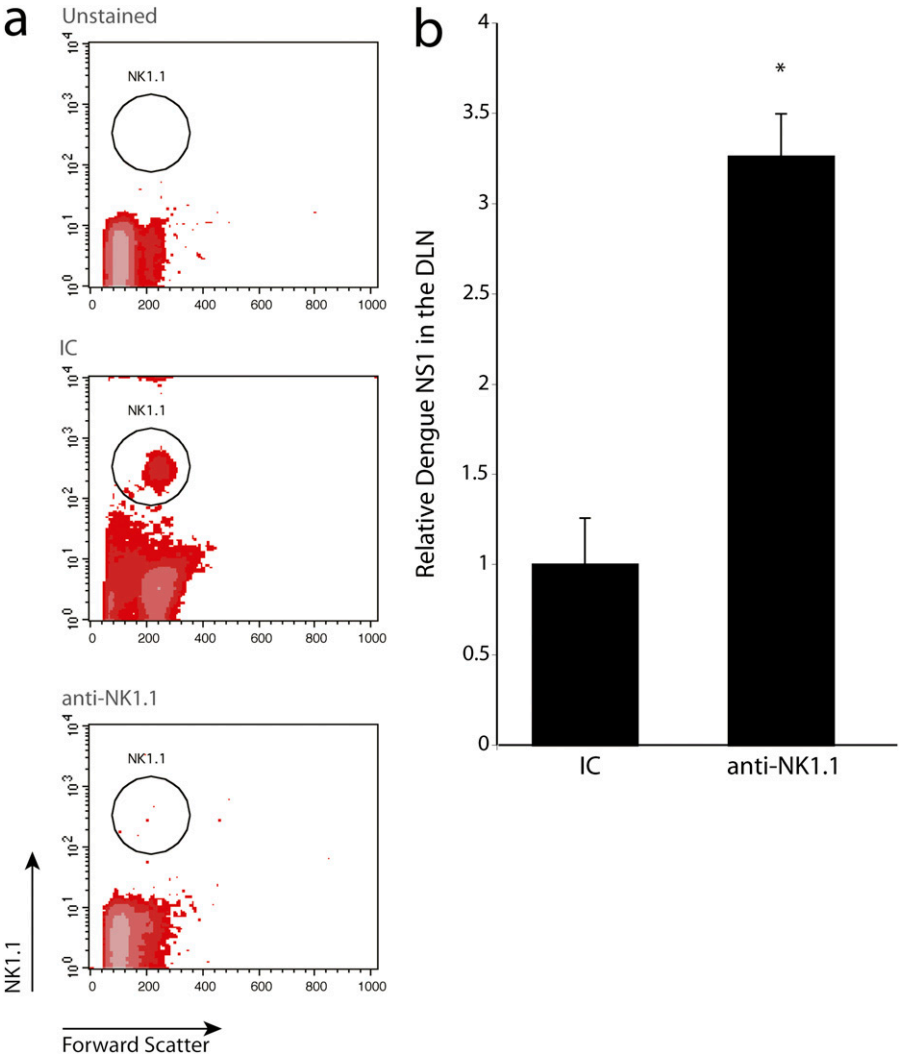

Fig. S9. NK1.1$^+$ cells are consequential to early DENV clearance in vivo. NK1.1$^+$ cells were depleted from mice by monoclonal antibody injection. (*A*) After 24 h, the peripheral blood mononuclear cells were obtained and stained to verify the drastic reduction of NK1.1$^+$ cells compared with isotype control-injected mice. (*B*) These mice were infected with 1×10^5 pfu of DENV. At 24 h postinfection, DLNs were harvested, RNA was isolated, and the viral burden in infected LNs was determined by real-time PCR. NK1.1$^+$ cell-depleted mice had significantly increased (more than threefold) viral burdens compared with isotype control-treated mice, demonstrating the importance of NK1.1$^+$ cells to limiting early viral progression to LNs in the host.