

# Dengue Virus Infects Macrophages and Dendritic Cells in a Mouse Model of Infection

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**Dengue fever is a mosquito-borne viral illness caused by 4 dengue viruses (DENV-1–4). The cellular tropism of DENV has not been definitively determined, despite its importance for understanding viral pathogenesis and identifying therapeutic targets. To define DENV cellular tropism in a small animal model, 129/Pas mice lacking interferon- $\alpha/\beta$  and/or  $-\gamma$  receptors were infected with DENV via a subcutaneous route. During the first week after infection, virus was present in lymph nodes, spleen, bone marrow, and circulating white blood cells. F4/80<sup>+</sup>CD11b<sup>+</sup> macrophages and CD11c<sup>+</sup> dendritic cells were demonstrated to be targets for DENV-2 infection in the spleen by flow cytometry directed to structural and nonstructural DENV proteins and by magnetic bead separation followed by strand-specific reverse-transcriptase polymerase chain reaction. We find that the initial cellular tropism of DENV in mice is similar to that reported in humans, thereby paving the way for investigation of cellular tropism and pathogenesis of DENV in primary and secondary infections.**

Dengue is a mosquito-borne viral febrile illness caused by 4 dengue virus serotypes (DENV-1–4) and is currently the most prevalent arthropod-borne viral disease worldwide, with an estimated 100 million infections each year in tropical and subtropical regions. As the fever breaks, a percentage of symptomatic infections proceed to a more-severe, life-threatening disease characterized by increased vascular permeability, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) [1]. The molecular basis of DENV pathogenesis is not well understood, in part because of the lack of a representative animal model in which to test hypotheses generated by clinical and epidemiological observations.

Clinical and autopsy findings in humans, as well as studies involving nonhuman primates, have indicated that cells of the mononuclear phagocyte lineage are the primary cell targets. Human autopsy studies have described DENV antigen-positive cells to be primarily macrophages and lymphocytes located in the spleen, lymph nodes, peripheral blood, lungs, liver, kidney, and stomach [2–4], as well as endothelial cells [2, 4], hepatocytes [3, 5, 6], and cells of the central nervous system [3, 7, 8]. DENV antigens have been detected in human skin biopsy samples in mononuclear cells [9] and in CD1a<sup>+</sup> Langerhans (dendritic) cells from an individual who received a live attenuated tetravalent vaccine [10]. In the peripheral blood, monocytes have been identified as the principal targets of DENV infection by flow cytometry [11] (A. Durbin, A. Balmaseda, and E. Harris, unpublished data), and B cells have been identified by immunofluorescence [12] or flow cytometry [13] as minor targets as well. Although further studies of cellular tropism in humans are warranted, experimental questions linking cellular tropism to disease pathogenesis can only be fully explored using a small animal model of DENV infection.

Mouse models of DENV infection by peripheral inoculation have been reported to manifest some aspects of human disease, in particular hemostatic and/or he-

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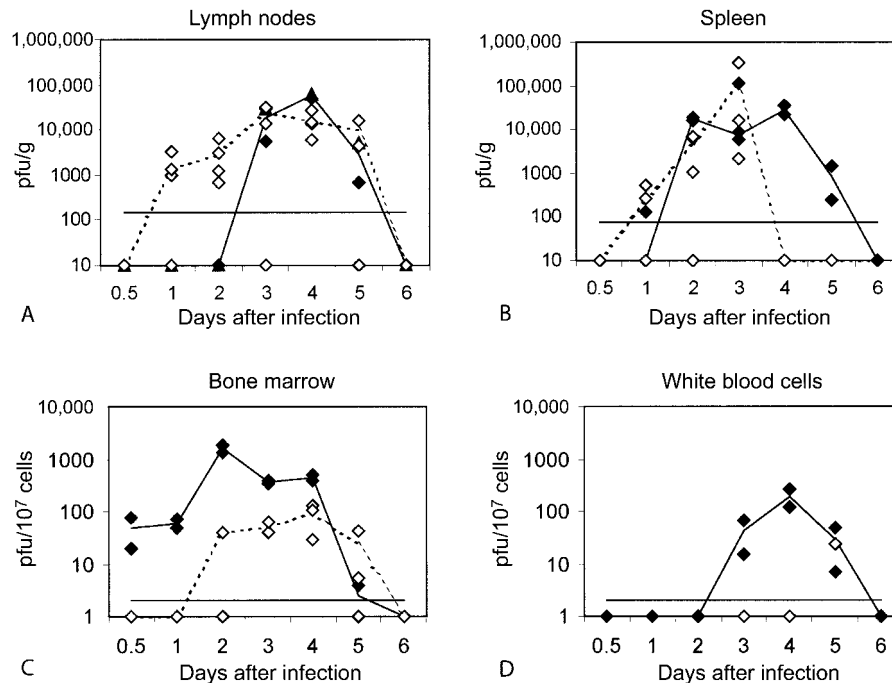
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**Figure 1.** Dengue virus-2 replication in hematopoietic tissues in AG129 mice after subcutaneous infection. Two mice were infected with  $10^7$  pfu of either PL046 ( $\diamond$ ) or D2S10 ( $\blacklozenge$ ), euthanized at each time point after infection, and perfused with ice-cold PBS. Plaque assays were performed on tissue from the spleen, lymph nodes, liver, kidneys, lungs, brain, and spinal cord and on cells from the bone marrow and peripheral blood. Virus was isolated from lymph nodes (A), spleen (B), bone marrow cells (C), and peripheral white blood cells (D). The dashed lines connect the average value for all PL046-infected mice at each time point, and the solid lines connect the average values for D2S10-infected mice. Horizontal lines represent the limit of detection of the assay.

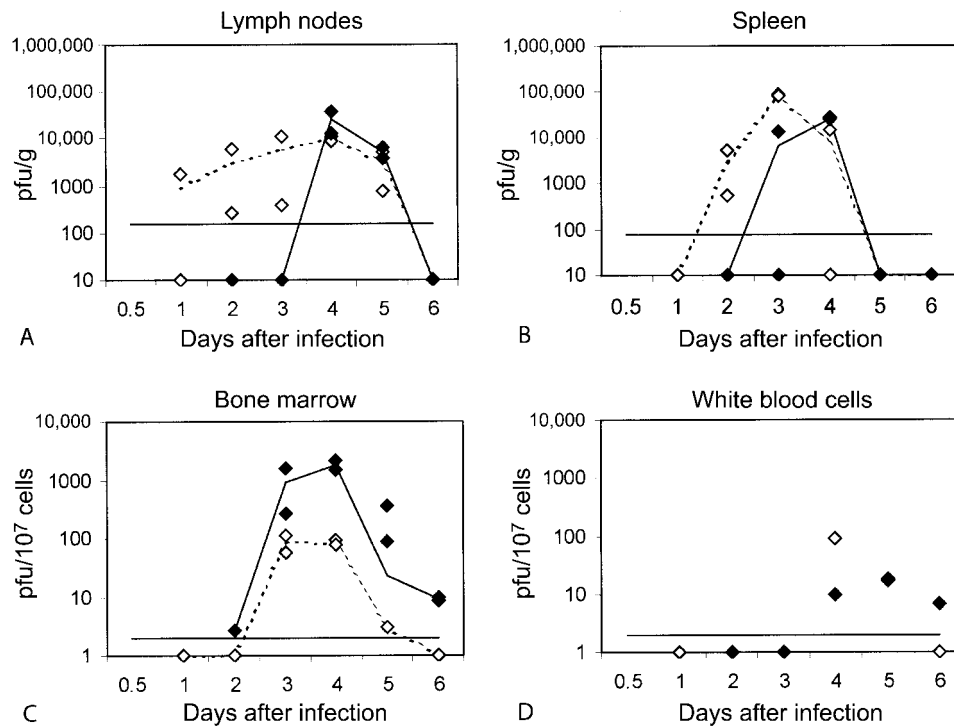
matopoietic abnormalities [14–16]. Other mouse models of DENV infection have documented liver involvement [17, 18] and have described a role for CD8<sup>+</sup> T cells in both protection and pathogenesis [19]. DENV infection in mice after peripheral inoculation usually results either in recovery or in a fatal encephalitic paralysis [14–24]. However, in one recent report, humanized NOD/SCID mice were found to develop fever, rash, and thrombocytopenia after DENV-2 infection [25], and, in another, increased vascular permeability was demonstrated in response to DENV-2 infection of interferon (IFN) receptor-deficient mice [26].

In the endeavor to establish a more complete murine model of DENV infection, it is necessary to identify the cell types infected *in vivo*. Utilizing DENV infection of IFN receptor-deficient mice, we find that the initial cellular tropism in humans and mice are the same, even though the ultimate outcome of infection in mice remains either recovery or fatal encephalitis. Using multiple techniques, including strand-specific reverse-transcriptase polymerase chain reaction (RT-PCR) and flow cytometry, we report here that DENV infects primarily macrophages and dendritic cells in the first 6 days after inoculation by a subcutaneous route in a mouse model of primary infection.

## MATERIALS AND METHODS

**Viruses and cell lines.** DENV was propagated in the *Aedes albopictus* cell line C6/36 (American Type Culture Collection [ATCC]) as described elsewhere [27]. DENV-2 strain PL046 is a Taiwanese isolate (obtained from H.-Y. Lei, National Cheng Kung University, Taiwan), and DENV-2 strain D2S10 was derived in our laboratory [26]. DENV-1 strain Mochizuki (mouse-passaged) was obtained from R. Tesh (University of Texas Medical Branch at Galveston), and DENV-4 strain 664 from Thailand was a gift of S. Kliks (Pediatric Dengue Vaccine Initiative). Virus titers were obtained by plaque assay on baby hamster kidney (BHK 21; clone 15) cells, as described elsewhere [27]. Before injection into mice, virus was diluted directly in PBS for low doses or centrifuged at 53,000 g for 2 h to concentrate the virus for high-dose inocula and then resuspended in PBS.

**Infection of AG129 and A129 mice.** 129/Pas mice lacking receptors for IFN- $\alpha/\beta$  and  $-\gamma$  are designated AG129, whereas A129 mice lack only the receptor for IFN- $\alpha/\beta$  [28, 29]. Mice originally obtained from M. Aguet (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland) were bred in the University of California (UC), Berkeley, animal facility. All



**Figure 2.** Maintenance of dengue virus (DENV) tropism in mice with lower infectious doses of DENV-2. Two mice were infected with  $10^5$  pfu of strain PL046 ( $\diamond$ ) or  $10^2$  pfu of strain D2S10 ( $\blacklozenge$ ), euthanized at each time point after infection, and perfused with ice-cold PBS. Plaque assays were performed on tissue from the spleen, lymph nodes, liver, kidneys, lungs, brain, and spinal cord and on cells from the bone marrow and peripheral blood. Virus was isolated from lymph nodes (A), spleen (B), bone marrow cells (C), and peripheral white blood cells (D). The dashed lines connect the average values for all PL046-infected mice at each time point, and the solid lines connect the average values for D2S10-infected mice. Horizontal lines represent the limit of detection of the assay.

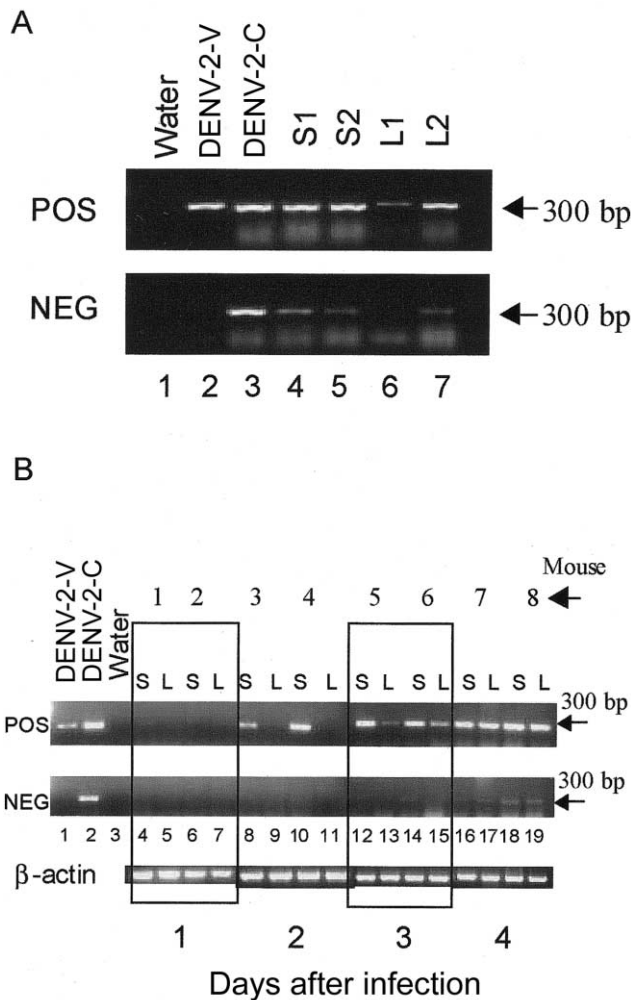
experimental procedures were preapproved and were performed according to the guidelines of the UC Berkeley Animal Care and Use Committee. Experiments were initiated with mice 5–7 weeks of age, and groups of 2–4 mice were used per time point. Subcutaneous injection was performed under the skin of the ventral hindlimbs, in a total volume of 200  $\mu$ L divided equally between both right and left limbs.

**Quantitation of virus in tissues by plaque assay.** Viral load was determined in samples obtained from the blood, lymph nodes, spleen, bone marrow, liver, brain, and spinal cord. Tissue samples were processed and quantitated as described elsewhere [27]. Bone marrow cells were collected from both femur and tibia bones directly into PBS. Whole blood was collected into EDTA-coated microtainer tubes (Becton Dickinson) after cardiac puncture. Red blood cells in both bone marrow and whole blood samples were lysed using red cell lysis buffer (eBioscience).

**RT-PCR.** Viral RNA from tissue was extracted using an RNeasy kit (Qiagen) [27], and the NS3 region of the DENV genome was targeted using a forward primer (5'-TTCCATACA-ATGTGGCATGTCAC-3') and a reverse primer (5'-GGAGAT-CCTGAGGTTCCAGGAG-3'), based on a previously published RT-PCR protocol [30]. Positive- or negative-sense genomic RNA from tissue or cells was detected by incorporating a non-

DENV specific “tag” sequence (5'-CATGGTGGCGAATAACA-TAATCG-3' for positive strand; 5'-TATCGTGGCGAATAATC-ATACTCC-3' for negative strand) into the first-strand cDNA synthesis, as described initially for hepatitis C virus [31]. First-strand synthesis and PCR were performed according to the manufacturer’s instructions with Superscript II and *Taq* polymerase (Invitrogen), except that 5 pmol of first-strand primer was used with a reaction temperature of 55°C, and 5  $\mu$ L of the resulting cDNA was used in the PCR.

**Flow cytometry.** Splensens from mice injected with either PBS or live or heat-inactivated DENV were injected with 2–3 mL of 2 mg/mL Collagenase D (Boehringer Mannheim) in Dulbecco’s PBS (Invitrogen), incubated for 30 min at 37°C, and then pressed through a 100- $\mu$ m cell filter. Red blood cells were lysed and cells were resuspended in FACS buffer consisting of PBS supplemented with 0.5% bovine serum albumin (BSA; Fisher Scientific) and 0.02% sodium azide (Sigma-Aldrich). Cells were blocked with 200  $\mu$ g/mL of mouse IgG (Caltag), stained with 4  $\mu$ g/mL of phycoerythrin (PE)-conjugated monoclonal rat anti-CD3 or anti-F4/80 antibodies (eBioscience), 4  $\mu$ g/mL of PE-conjugated monoclonal hamster anti-CD11c antibodies (eBioscience), or 1  $\mu$ g/mL of PE-Cy5-conjugated rat anti-CD11b or anti-CD19 antibodies, washed, and fixed in 4%



**Figure 3.** Demonstration of actively replicating dengue virus (DENV) by detection of antigenomic viral RNA. Mice were infected with either  $10^7$  (A) or  $10^2$  (B) pfu of DENV D2S10 and euthanized at each time point after infection, and then tissue samples were collected and stabilized in RNAlater (Qiagen). Strand-specific reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using a tagged amplification method with primers targeted to the NS3 region of the genome. For panel A, at day 2 after infection with  $10^7$  pfu of D2S10, strand-specific RT-PCR was performed on RNA extracted from spleen (S) tissue or lymph nodes (L) from 2 separate mice. Lane 1, water control; lane 2, DENV-2 virions (DENV-2-V); lane 3, DENV-2-infected C6/36 cells (DENV-2-C); lane 4, S tissue from mouse 1 (S1); lane 5, S tissue from mouse 2 (S2); lane 6, L tissue from mouse 1 (L1); lane 7, L tissue from mouse 2 (L2). For panel B, at days 1–4 after infection with  $10^2$  pfu of D2S10, strand-specific RT-PCR was performed on RNA extracted from S tissue or L. Lane 1, DENV-2 virions (DENV-2-V); lane 2, DENV-2-infected C6/36 cells (DENV-2-C); lane 3, water control; lanes 4–7, RNA from mice 1–2 at day 1 after infection; lanes 8–11, RNA from mice 3–4 at day 2 after infection; lanes 12–15, RNA from mice 5–6 at day 3 after infection; lanes 16–19, RNA from mice 7–8 at day 4 after infection. POS, positive strand; NEG, negative strand.

paraformaldehyde. Cells were permeabilized in FACS buffer plus 0.1% saponin and blocked with 300  $\mu\text{g}/\text{mL}$  mouse IgG. Mouse anti-DENV antibodies 3H5, 15F3 (ATCC), and 1H4 [32] (gift of B. Puffer, Integral Molecular, Philadelphia, PA) were biotinylated with EZ-Link Sulfo-NHS-Biotin (Pierce). 1H4, originally raised against West Nile virus (WNV) nonstructural protein 1 (NS1) [33], was confirmed by Western blot to recognize DENV-2 NS1. Biotinylated mouse antibodies were used at 50  $\mu\text{g}/\text{mL}$  and detected with 0.4  $\mu\text{g}/\text{mL}$  Alexa 488–conjugated streptavidin (Invitrogen). Cells were washed, fixed in PBS with 1% paraformaldehyde, and stored at 4°C until analysis on a Coulter EPICS XL (Beckman Coulter). Results were analyzed using Flow Jo software (version 7.1.2; TreeStar).

**Magnetic bead separation.** Single-cell suspensions from spleens were prepared as described for flow cytometry and from lymph nodes by crushing the nodes between 2 glass slides. All cells were resuspended in PBS with 0.5% BSA and 2 mmol/L EDTA. CD11c<sup>+</sup> cells were isolated according to the manufacturer's protocol using anti-CD11c–conjugated microbeads (Miltenyi Biotec). F4/80<sup>+</sup> cells were isolated by labeling cells with rat anti-F4/80 antibodies (Serotec), followed by magnetic cell sorting using goat anti–rat IgG microbeads (Miltenyi Biotec). CD11c<sup>+</sup> fractions were found by flow cytometry to be >98% CD11c<sup>+</sup>, and F4/80<sup>+</sup> fractions were enriched to >86% F4/80<sup>+</sup>.

**Primary cell cultures.** For in vitro infections, peritoneal lavage cells were collected from unstimulated mice in 5 mL of Dulbecco's MEM (DMEM; Invitrogen) supplemented with 10% FBS and 10 mmol/L HEPES and cultured in the same medium after red cell lysis. Nonadherent cells were removed after 3–4 days, and adherent cells were observed to have a macrophage-like morphology. Primary bone marrow–derived macrophages (BMDMs) were derived by incubation of bone marrow cells for 7 days in DMEM plus 15% L cell–conditioned medium, 10% FBS, 10 mmol/L HEPES, and 2 mmol/L glutamax (Invitrogen). The BMDMs were confirmed by flow cytometry to be >99% CD11b<sup>+</sup> and >99% F4/80<sup>+</sup>.

**Immunostaining.** DENV antigens were detected using anti-DENV monoclonal antibodies described above (at 1  $\mu\text{g}/\text{mL}$ ) or polyclonal rabbit antibodies (1:1000) generated in our laboratory against E and NS3 proteins, followed by either biotinylated goat anti-mouse antibody (3  $\mu\text{g}/\text{mL}$ ; Vector Labs), an alkaline phosphatase–conjugated avidin-biotin complex (ABC method, visualized using Vector Red substrate; Vector Labs), or by Alexa 488–conjugated goat anti-rabbit antibody (0.4  $\mu\text{g}/\text{mL}$ ; Invitrogen). Nuclei were stained with Hoechst (Sigma-Aldrich) or Harris hematoxylin (Fisher Scientific). Cells were phenotyped using monoclonal rat anti-F4/80 antibody (Serotec) followed by biotinylated goat anti-rat antibody (3  $\mu\text{g}/\text{mL}$ ; Vector Labs) and the ABC method described above.

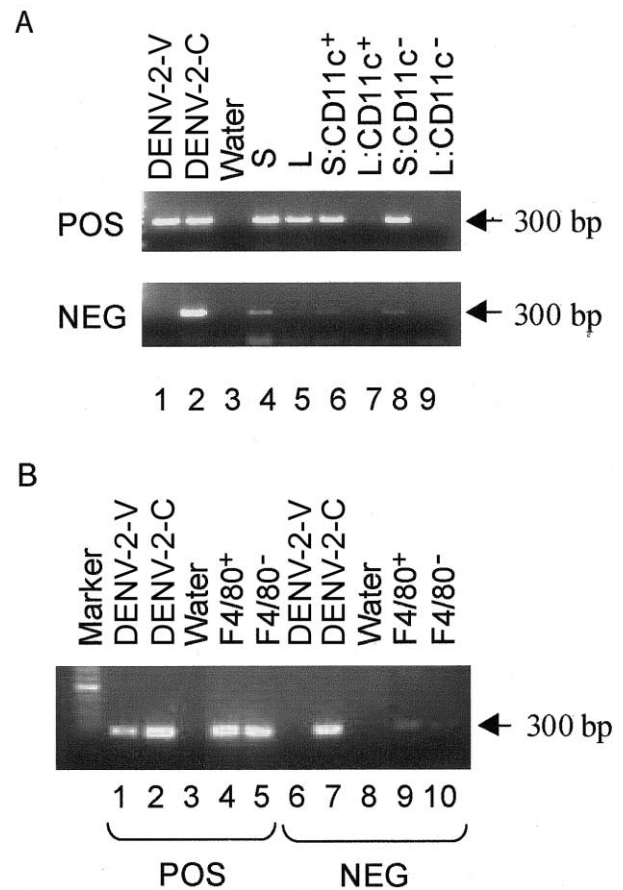
## RESULTS

### *Presence of DENV in hematopoietic tissues in AG129 mice.*

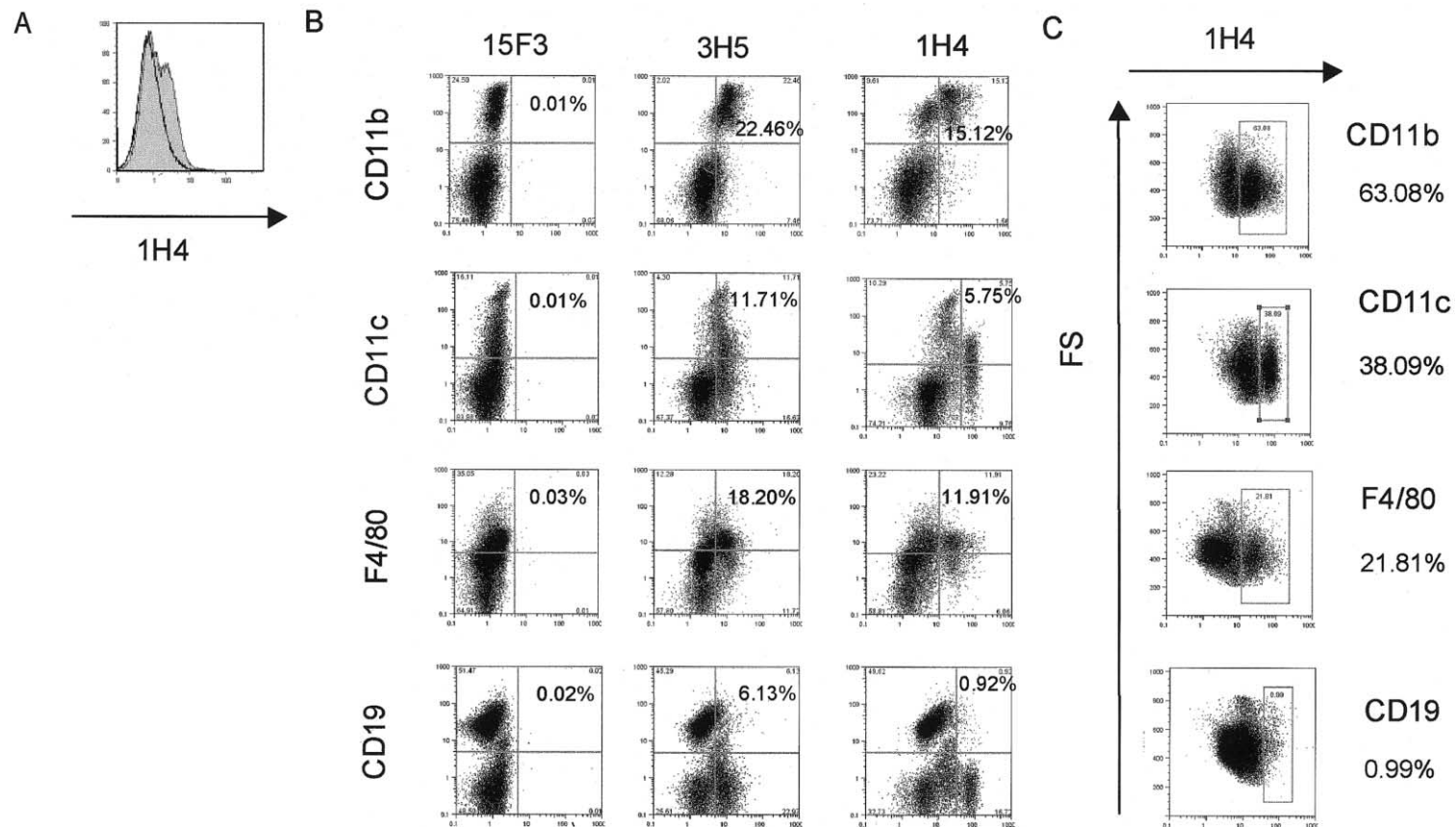
DENV-2 strain PL046 has been shown to replicate to high titers in A/J, AG129, and SCID-K562 mice [16, 21, 24, 27]. Recently, strain D2S10 was derived from PL046 and demonstrated an early death phenotype with increased vascular permeability in AG129 mice after an intravenous injection of  $10^7$  pfu [26]. Using these 2 viral strains, we characterized the kinetics and dissemination of DENV-2 in AG129 mice, which are highly susceptible to fatal DENV infection [23, 24] and are currently used for vaccine-testing studies [34, 35]. To approximate the route of introduction of DENV from the bite of a mosquito, mice were infected subcutaneously. After a dose of  $10^7$  pfu of either PL046 or D2S10, we tested multiple tissues during the first 6 days after infection by plaque assay and found that DENV-2 spreads to and replicates primarily in hematopoietic tissues during the first week after infection (figure 1). Viral titers in the lymph nodes and spleen reached similar peak levels in both PL046- and D2S10-infected mice. Viral titers in the bone marrow and white blood cells (WBCs) were 10-fold higher in D2S10-infected mice than in PL046-infected mice. At days 1–3 after infection, infectious virus was detected by plaque assay in muscle tissue near the site of injection (data not shown); however, DENV was not detected in the liver and was found only occasionally just above the limit of detection in the lung or kidney.

**Replication of lower doses of DENV-2 and multiple serotypes to similar levels in hematopoietic tissues.** Having determined the early tissue tropism of DENV-2, we next tested lower, more relevant doses of multiple serotypes of DENV. First, we tested the viral load and kinetics in AG129 mice after infection with DENV-2 doses ranging from  $10^2$  to  $10^5$  pfu. In comparison, the dose of the related flavivirus WNV received after a mosquito bite has been determined to be  $10^3$ – $10^5$  pfu [36]. After subcutaneous infection with  $10^2$  pfu of D2S10 or  $10^5$  pfu of PL046 (figure 2), viral titers in the lymph node and spleen reached similar levels as those observed after infection with  $10^7$  pfu of either strain (figure 1), although greater variability in viral titers in the spleen was seen after the lower dose of strain PL046. After subcutaneous infection with  $10^2$ ,  $10^4$ , or  $10^7$  pfu of D2S10 (figure 2 and data not shown), viral production reached similar titers in spleen, lymph nodes, and bone marrow, although peak viral titers in the bone marrow were reached 2 days later after infection with  $10^2$  pfu than with  $10^7$  pfu of D2S10. Similar tissue tropism results were obtained after mice were injected subcutaneously with  $10^4$  pfu of a mouse-adapted DENV-1 strain (Mochizuki), with peak titers in the lymph nodes reaching  $2.3 \times 10^4$  pfu/g on day 3 after infection, and, in the bone marrow, a peak titer of 23 pfu/ $10^7$  cells was detected on day 4 after infection. In contrast to the DENV-2

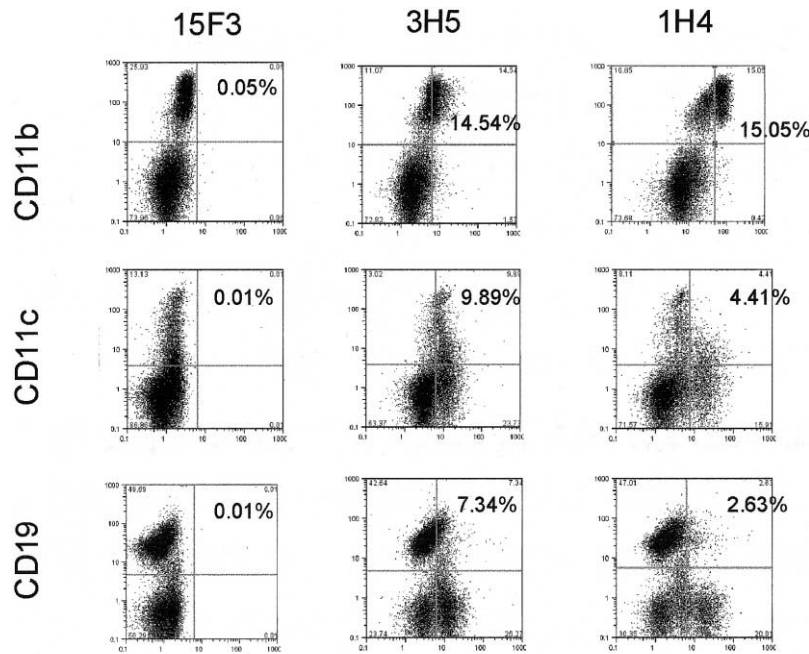
strains, very low levels of DENV-1 were isolated from the spleen. A clinical DENV-4 isolate (strain 664) was also observed to replicate in the spleen, lymph nodes, bone marrow, and WBCs after subcutaneous infection (S. Balsitis and E. Harris,



**Figure 4.** Dengue virus (DENV)–2 replication in CD11c<sup>+</sup> and F4/80<sup>+</sup> cells in AG129 mice after subcutaneous infection. Mice were infected with  $10^5$  pfu of DENV D2S10 and euthanized at day 3 after infection, and then tissue samples were collected and processed for magnetic bead separation. Strand-specific reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using a tagged method with primers targeted to the NS3 region of the genome. For panel A, spleen (S) and lymph nodes (L) cells were collected from 1 mouse, and CD11c<sup>+</sup> and CD11c<sup>-</sup> populations were separated using antibodies against CD11c conjugated to magnetic beads. Lane 1, DENV-2 virions (DENV-2-V); lane 2, DENV-2–infected C6/36 cells (DENV-2-C); lane 3, water control; lane 4, unseparated (total) S cells; lane 5, unseparated (total) L cells; lane 6, CD11c<sup>+</sup> S cells; lane 7, CD11c<sup>+</sup> L cells; lane 8, CD11c<sup>-</sup> S cells; lane 9, CD11c<sup>-</sup> L cells. For panel B, S tissues were collected and pooled from 2 mice, and F4/80<sup>+</sup> and F4/80<sup>-</sup> cell populations were separated using anti-F4/80 primary antibody followed by goat anti-rat–conjugated magnetic beads. Lanes 1–5 contain RT-PCR products from positive-strand (POS) conditions, and lanes 6–10 contain RT-PCR products from negative-strand (NEG) conditions. Lanes 1 and 6, DENV-2 virions; lanes 2 and 7, DENV-2–infected C6/36 cells; lanes 3 and 8, water control; lanes 4 and 9, F4/80<sup>+</sup> S cells; lanes 5 and 10, F4/80<sup>-</sup> S cells.



**Figure 5.** Dengue virus (DENV)-2 infection of CD11b<sup>+</sup>, CD11c<sup>+</sup>, F4/80<sup>+</sup>, and CD19<sup>+</sup> cells in AG129 mice after subcutaneous infection. Mice were infected with 10<sup>5</sup> pfu of DENV-2 D2S10 and euthanized at day 2 after infection, and then spleen cells were analyzed by flow cytometry. *A*, Representative histogram showing DENV-specific staining with 1H4 antibody. The histogram outlined in black shows staining without permeabilization, and the gray histogram shows staining with 0.1% saponin permeabilization. *B*, Representative flow cytometry dot plots from 1 of 3 independent experiments. Streptavidin-conjugated Alexa 488 (Invitrogen) was used to detect biotinylated monoclonal antibodies (isotype control DENV-1-specific envelope protein [15F3], DENV-2-specific envelope protein [3H5], or DENV nonstructural protein 1 specific [1H4]) and is shown along the x axis. The cell-specific markers anti-CD11b-phycoerythrin (PE) Cy5 (macrophages/granulocytes), anti-CD11c-PE Cy5 (dendritic cells), anti-PE F4/80 (macrophages), or anti-CD19-PE Cy5 (B cells) are shown along the y axis. For panel *C*, CD11b<sup>+</sup>, CD11c<sup>+</sup>, F4/80<sup>+</sup>, or CD19<sup>+</sup>-gated cells are displayed on dot plots demonstrating Alexa 488 (1H4) staining versus forward scatter (FS).



**Figure 6.** Dengue virus (DENV)-2 replication in CD11b<sup>+</sup>, CD11c<sup>+</sup>, and CD19<sup>+</sup> cells in A129 mice after subcutaneous infection. Mice were infected with 10<sup>5</sup> pfu DENV-2 D2S10 and euthanized at day 2 after infection, and then spleen cells were analyzed by flow cytometry. Representative flow cytometry dot plots are shown from 1 of 2 independent experiments. Streptavidin-conjugated Alexa 488 (Invitrogen) was used to detect biotinylated monoclonal antibodies (isotype control DENV-1-specific envelope protein [15F3], DENV-2-specific envelope protein [3H5], or DENV nonstructural protein 1 specific [1H4]) and is shown along the x axis. The cell-specific markers anti-CD11b-phycoerythrin (PE) Cy5 (macrophages/granulocytes), anti-CD11c-PE Cy5 (dendritic cells), or anti-CD19-PE Cy5 (B cells) are shown along the y axis.

unpublished results), thereby demonstrating that at least 3 of the 4 serotypes of DENV are capable of replication and dissemination in this murine model. Taken together, these results demonstrate that a physiologically relevant dose of DENV can be used to determine tissue and cell tropism *in vivo* in a murine model.

**DENV infection and replication in CD11b<sup>+</sup>, CD11c<sup>+</sup>, and F4/80<sup>+</sup> cells at early time points after infection.** To confirm the presence of actively replicating DENV, we developed a strand-specific RT-PCR assay to detect anti-genomic RNA. After infection with 10<sup>7</sup> pfu of D2S10, we detected positive-strand RNA in the spleen and lymph nodes of AG129 mice from day 0.5 to 6 after infection and negative-strand (antigenomic) RNA on days 1–3 after infection (figure 3A, for results from day 2 after infection). After infection with 10<sup>2</sup> pfu of D2S10, we detected positive-strand RNA on days 1–4 after infection and negative-strand RNA on days 3 and 4 after infection (figure 3B). Using a magnetic bead separation technique followed by strand-specific RT-PCR, we detected negative-strand viral RNA in both the CD11c<sup>+</sup> (dendritic cell [37]) and CD11c<sup>-</sup> cell fractions of spleen and lymph node cells at day 3 after infection with 10<sup>5</sup> pfu of D2S10 (figure 4A). Negative-strand DENV RNA was also detected in both the F4/80<sup>+</sup> (macrophage [38]) and F4/80<sup>-</sup> cell fractions of spleen cells tested under the same con-

ditions (figure 4B). Thus, replicating virus was found primarily in mononuclear phagocytic populations but also potentially in other cell types.

As a quantitative method to identify DENV-infected cell types, flow cytometric analysis was performed on spleen cells from AG129 mice after infection with 10<sup>5</sup> pfu of D2S10. A gate including all viable mononuclear cells was used for all analyses, and total percent positive cells was determined by subtracting background percent positive cells in control samples (either from mice infected with heat-killed virus or cells stained using an isotype control antibody). At day 2 after infection, 22%–27% of spleen cells were positive for DENV-2-specific antigen using a biotinylated monoclonal antibody (3H5) against the envelope (E) protein [39], and 18%–28% of spleen cells were positive for 1H4 [32], an antibody that recognizes DENV NS1 and thereby confirms viral replication (data not shown). Intracellular localization of the NS1 protein, which can also exist on the cell surface, was confirmed by parallel staining of cells with no permeabilization (figure 5A). The majority of 1H4-positive cells were CD11b<sup>+</sup> (average, 14.8%; range, 12.9%–17.0%), CD11c<sup>+</sup> (average, 6.8%; range, 5.8%–8.3%), or F4/80<sup>+</sup> (average, 5.1%; range, 2.1%–10.5%) (figure 5B). A small number of CD19<sup>+</sup> (B cells) were found to be positive at day 2 (average, 1.5%; range, 0.4%–3.2%). Less than 1% of total cells

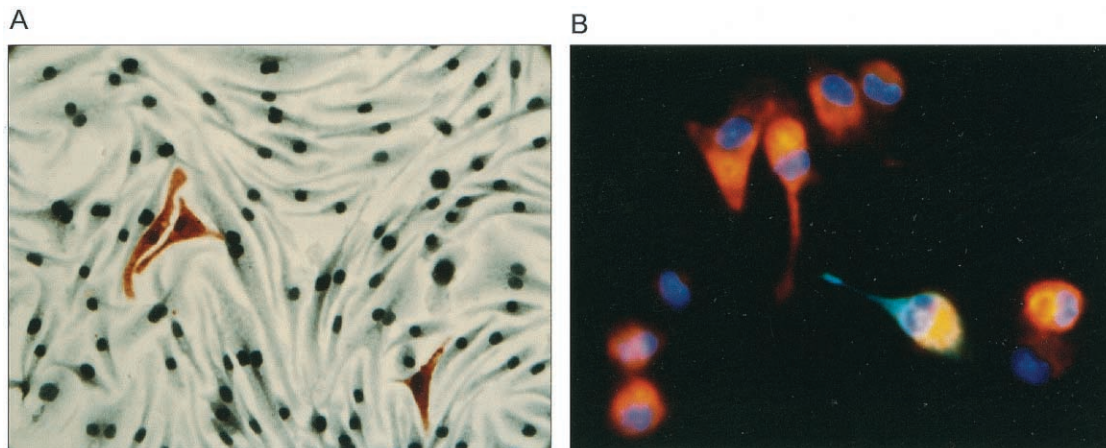
were positive for CD3 (T cells) and 1H4. At day 2 after infection, the majority of CD11b<sup>+</sup> cells were infected, as well as high percentages of CD11c<sup>+</sup> and F4/80<sup>+</sup> cells (figure 5C). At day 1 after infection, DENV-specific staining of spleen cells using monoclonal antibodies 3H5 (E) and 1H4 (NS1) was <5%. At day 3 after infection, DENV-positive staining with the 3H5 (25%–35%) and 1H4 (22%–24%) antibodies remained similar to that on day 2 after infection. Thus, on days 2–3 after infection, DENV-infected cells are primarily mononuclear phagocytic cells, such as macrophages and dendritic cells.

**Similar cellular tropism in A129 mice and DENV-2 infection of macrophages in vitro.** In addition to AG129 mice, the A129 mouse, which lacks only the IFN- $\alpha/\beta$  response, may be useful for studies in which viral tropism and not fatal encephalitis is the end-point measurement. Infection of A129 mice with 10<sup>5</sup> pfu of strain PL046 resulted in similar titers as seen in AG129 mice on day 2 after infection (between 1 × 10<sup>3</sup> and 5 × 10<sup>3</sup> pfu/g in spleen and lymph node tissues) and somewhat lower levels than in AG129 mice on day 4 after infection (between 0.5 × 10<sup>3</sup> and 1.5 × 10<sup>3</sup> pfu/g in spleen and lymph nodes). Flow cytometric staining of A129 spleen cells for DENV antigens on day 2 after infection demonstrated similar levels of DENV-positive (1H4) cells and cell-specific staining as AG129 mice (figure 6). Finally, we confirmed that macrophages from A129 and AG129 mice were susceptible to DENV in an in vitro assay. Both BMDMs from AG129 mice (figure 7A) and peritoneal macrophages from A129 mice (figure 7B) were infected with either PL046 or D2S10 and demonstrated the presence of DENV antigens.

## DISCUSSION

Our characterization of the initial tropism of DENV injected subcutaneously into 129/Pas mice lacking IFN- $\alpha/\beta$  and - $\gamma$  receptors or the IFN- $\alpha/\beta$  receptor alone revealed that DENV-2 primarily infects macrophages and dendritic cells during the first 6 days after infection. Using a combination of flow cytometry and magnetic bead separation plus strand-specific RT-PCR, we identified DENV antigen-positive cells as primarily CD11b<sup>+</sup> (granulocyte/macrophage), CD11c<sup>+</sup> (dendritic cell), F4/80<sup>+</sup> (macrophage), and, to a lesser extent, CD19<sup>+</sup> or B220<sup>+</sup> (B cell) populations at days 1–3 after infection. The detection of DENV NS1 antigen, a viral protein not present in the virion itself, confirmed the presence of replicating virus in these cell types. Similar results for tissue tropism were observed using 3 serotypes of DENV with relevant doses of virus. These results demonstrate that, early in infection, DENV targets similar cell types in our mouse model as in humans.

To our knowledge, these results are the most comprehensive characterization of early DENV cellular tropism in a mouse model published to date. Most previous models have not documented extraneural viral cellular tropism, with some exceptions. After intraperitoneal infection, DENV-2 New Guinea C strain viral antigen was detected in Kupffer cells (but not hepatocytes) in the liver and in mononuclear cells of lymph nodes of mice [40]. Two other reports identified DENV-2 antigen in draining lymph nodes after an intradermal infection [41] and in hepatocytes after an intraperitoneal injection [18]. Unlike some other mouse models [15, 17, 21], we did not find evidence of viral infection in the liver of AG129 mice, although it is



**Figure 7.** Dengue virus (DENV)-2 infection in peritoneal macrophages from A129 mice (B) and bone marrow-derived macrophages (BMDMs) from AG129 mice (A). Peritoneal or bone marrow cells were collected from mice. BMDMs were derived by incubation with supernatant from L929 cells. For panel A, BMDMs derived from AG129 mice were infected with D2S10 (MOI of 10), fixed and stained 5 days after infection with mouse anti-DENV-2 envelope antibody (red), and counterstained with hematoxylin. For panel B, peritoneal macrophages were collected from A129 mice, adhered to coverslips, infected in vitro with PL046 (MOI of 1), and then fixed and stained 4 days after infection with rabbit anti-DENV nonstructural protein 3 (green), rat anti-F4/80 (red), and Hoechst nuclear stain (blue).

possible that detection of DENV present only in Kupffer cells would fall below the level of detection of our assays. However, the available data from human autopsy studies are equivocal, with some researchers identifying hematopoietic cells as the primary viral targets [2, 4] and others additionally finding hepatocytes and/or neuronal cells to be targets of DENV infection [5–7]. A possible explanation for this discrepancy is that hepatocytes may or may not be initial sites of DENV replication and may represent one of the manifestations of a more severe DENV infection. Nevertheless, the identification of mononuclear phagocytes as the primary targets of DENV infection in A129 and AG129 mice signifies that these mouse models will be useful tools both to study DENV receptors in vivo and to define cellular mechanisms of immune protection or enhancement by antibodies or T cells in a model of secondary, heterologous DENV infection.

IFN- $\alpha/\beta$  has previously been shown to control early viral spread for other RNA viruses [42–47]. However, increased replication and dissemination of DENV in the absence of an IFN- $\alpha/\beta$  response alone does not lead to increased mortality nor to an indiscriminate change in tissue tropism [24], as with other viruses. Additionally, although the absence of either the IFN- $\alpha/\beta$  receptor alone or of both IFN receptors leads to increased DENV replication, this replication still occurs primarily in bone marrow-derived cells. We propose that the ability to respond to IFN- $\alpha/\beta$  is responsible for early control of viral replication in target cells but not for a change in the cellular tropism of the virus. In comparison, infection of A129 mice with WNV also led to increased detection of viral antigen in spleen cells, in particular of F4/80<sup>+</sup> macrophages, although in contrast to DENV, infection of mice lacking the IFN- $\alpha/\beta$  receptor with WNV led to a much broader dissemination across multiple tissues, compared with wild-type mice [42]. Although the immunocompromised nature of A129 and AG129 mice remains a limitation of this mouse model, there is increasing evidence that DENV, along with other flaviviruses, encodes 1 or more proteins that are capable of modulating the host IFN response. It is possible that, as is the case with paramyxovirus SV5 [48], DENV proteins encoded to subvert the human IFN response may not be as effective against the mouse IFN system, resulting in the increased resistance of mice to DENV infection.

By defining early cellular tropism in a mouse model of DENV infection, we have identified a potentially more relevant end point of DENV disease in mice than fatal encephalitis by demonstrating that early cellular tropism of DENV in mice is similar to that seen in humans. This end point will be useful both for testing the protection provided by experimental vaccines and therapeutics at the cellular level and for studying the protective versus pathogenic nature of DENV infection. In humans, infection by a second heterologous serotype is an important risk factor for DHF/DSS, and this mouse model will allow us to

confirm and extend in vitro and clinical observations regarding the poorly understood immunopathogenesis of a secondary DENV infection.

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