Antibodies against nonstructural protein 1 protect mice from dengue virus-induced mast cell activation

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Dengue virus (DENV) infection causes dengue fever, dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). DHF/DSS patients have been reported to have increased levels of urinary histamine, chymase, and tryptase, which are major granule-associated mediators from mast cells. Previous studies also showed that DENV-infected human mast cells induce production of proinflammatory cytokines and chemokines, suggesting a role played by mast cells in vascular perturbation as well as leukocyte recruitment. In this study, we show that DENV but not UV-inactivated DENV enhanced degranulation of mast cells and production of chemokines (MCP-1, RANTES, and IP-10) in a mouse model. We have previously shown that antibodies (Abs) against a modified DENV nonstructural protein 1 (NS1), designated DJ NS1, provide protection in mice against DENV challenge. In the present study, we investigate the effects of DJ NS1 Abs on mast cell-associated activities. We showed that administration of anti-DJ NS1 Abs into mice resulted in a reduction of mast cell degranulation and macrophage infiltration at local skin DENV infection sites. The production of DENV-induced chemokines (MCP-1, RANTES, and IP-10) and the percentages of tryptase-positive activated mast cells were also reduced by treatment with anti-DJ NS1 Abs. These results indicate that Abs against NS1 protein provide multiple therapeutic benefits, some of which involve modulating DENV-induced mast cell activation.

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Dengue virus (DENV) is a member of *Flavivirus* genus of *Flaviviridae* family and is transmitted to humans by *Aedes* mosquitoes. An increase in infection has been seen in recent years due to many factors, such as international travel and climate change. There are about 390 million dengue infections per year, including 96 million cases with apparent clinical manifestations.[1] Infection with any of the four DENV serotypes can cause a wide range of clinical syndromes from asymptomatic infection to a self-limiting febrile illness, dengue fever to severe dengue disease, ie, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).[2] Severe dengue diseases are characterized by fever, high levels of proinflammatory cytokines, thrombocytopenia, hemorrhagic manifestations, and increased vascular permeability with plasma leakage and hypotensive shock.[3–6] Although a dengue vaccine has been licensed in a few countries,[7] therapeutic drugs and more effective vaccines still need to be developed.

For many years, it has been speculated that mast cells may be involved in dengue pathogenesis. DHF patients exhibit increased levels of urinary and plasma histamine, which is a major granule-associated mediator from mast cells.[8,9] A recent report also showed that the production of mast cell-derived VEGF and proteases were increased in DSS patients.[10] Furthermore, mast cell-derived leukotrienes and chymase also promote vascular leakage in a DENV-infected mouse model.[11] *In vitro* studies indicated that antibody (Ab)-enhanced DENV infection of mast cells selectively induces production of chemokines, including CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES),[12] as well as cytokines, including IL-6, IL-1β, and TNF-α.[13] The TNF-α produced from Ab-enhanced DENV infection of mast cells as well as of monocytes can trigger endothelial cell activation.[14,15] These findings suggest that mast cells may have a role in vascular perturbation as well as leukocyte recruitment.

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For dengue vaccine development, DENV prM and E proteins are the important neutralization targets of Abs against DENV infection,[16] but such Abs against viral structural proteins may bind to FcγR-bearing cells and result in Ab-dependent enhancement (ADE) of infection.[17,18] To avoid ADE, nonstructural protein 1 (NS1) has been considered an alternative vaccine candidate.[19,20] Several studies showed that active immunization with NS1 protein or NS1 DNA vaccine as well as passive immunization with anti-NS1 Abs provided protection against DENV infection in the mice.[21–26] However, we previously found that the cross-reactivity of anti-DENV NS1 Abs with endothelial cells and platelets might cause endothelial cell apoptosis[27,28] and platelet dysfunction or depletion.[29,30] From proteomic analysis, we found that the C-terminal region of DENV NS1 might be responsible for the cross-reactivity with self-antigens due to sequence homology.[31] For safety concerns of vaccine development, the epitopes that generate cross-reactive Abs need to be deleted or modified. Therefore, we generated a chimeric DJ NS1 protein, which consisted of N-terminal DENV NS1 (aa 1–270) and C-terminal Japanese encephalitis virus (JEV) NS1 (aa 271–352). We found that anti-DJ NS1 Abs showed a lower binding activity to endothelial cells and platelets than that of anti-DENV NS1 Abs. Passive immunization with anti-DJ NS1 Abs could reduce DENV-induced prolonged mouse tail bleeding time, local skin hemorrhage, and viral NS3 antigen expression in DENV-infected mice.[32] In the present study, we used the mouse model to test the therapeutic effect of anti-DJ NS1 Abs on DENV-induced mast cell activation.

MATERIALS AND METHODS
Mice
C3H/HeN mice were obtained from Charles River Breeding Laboratories and maintained on standard laboratory food and water in the Laboratory Animal Center of National Cheng Kung University Medical College. Their 8-week-old progeny were used for the generation of Abs. $STAT1^{-/-}$ mice, which are more susceptible to DENV infection,[33] were obtained from Dr Chien-Kuo Lee, Graduate Institute of Immunology, National Taiwan University College of Medicine and maintained at National Laboratory Animal Center, Tainan facility. So far, there is no report about the effects of *STAT* knockout on mast cell production and degranulation. Their 8–10-week-old progeny were used for animal therapeutic models. The animal use protocol was reviewed, approved, and followed by the Institutional Animal Care and Use Committee.

Preparation of Recombinant Proteins and Abs
DENV2 NS1 (New Guinea C strain) cDNA was cloned into the pRSETb vector with a His-tag. DJ NS1 (aa 1–270 of DENV NS1 and aa 271–352 of JEV NS1) cDNA were cloned into the pET28a vector also with His-tag. The plasmids were introduced into *E. coli* BL21. The recombinant proteins were induced by 1 M isopropyl β-D-1-thiogalactopyranoside

(Calbiochem, San Diego, CA, USA) and purified in urea buffer (8 M urea, 500 mM NaCl, 20 mM Tris-HCl) with Ni^{2+} column (GE Healthcare Life Science, UK). After purification, proteins were examined using 10% SDS-PAGE. Proteins from SDS-PAGE were excised and homogenized in Freund's adjuvant to intraperitoneally (i.p.) immunize C3H/HeN mice at a dose of 25 μg for a total of five times. The first dose was administered in complete Freund's adjuvant (Sigma-Aldrich, St Louis, MO, USA) and the following four doses were given in incomplete Freund's adjuvant. Mouse sera were collected 3 days after the last immunization. The polyclonal Abs against DENV2 NS1 and DJ NS1 were purified with protein G agarose column (Millipore, Bedford, MA, USA) and recovered with HCl–glycine. The preparations were subjected to testing for endotoxin contamination using a Limulus amebocyte lysate assay (Pyrotell, Associates of Cape Cod, Falmouth, MA, USA), and the endotoxin concentrations of anti-DENV2 NS1 and anti-DJ NS1 were all <0.03 EU/ml.

Virus Culture
DENV2 16681 was propagated in C6/36 cells. Briefly, monolayers of C6/36 cells were inoculated with DENV at a multiplicity of infection of 0.01 and incubated at 28 °C in 5% CO_2 for 5 days. The culture medium was harvested and cell debris was removed by centrifugation at 2000 *g* for 5 min. The virus supernatant was collected and stored at − 70 °C until use in experiments. Virus titer was determined by plaque assay using the BHK-21 cell line.

Animal Model
Mice were intradermally (i.d.) inoculated with 200 μl DENV (10^7 or 10^8 PFU/mouse) at four sites (50 μl/site) on the upper back as previously described[34] and were killed on 2 or 3 days postinfection (d.p.i.). For therapeutic model, mice were i.p. inoculated with Abs (150 μg/mouse) on 1 and 2 d.p.i. and were killed on 3 d.p.i.

Bleeding Time
Bleeding time was performed by a 3 mm tail-tip transection. Blood droplets were collected on filter paper every 30 s. Bleeding time was recorded when the blood spot was <0.1 mm in diameter.[35,36]

Mast Cell Staining
Skin sections were embedded in paraffin and sliced on slides. Slides were deparaffinized by using xylene and gradient alcohol (100, 95, 85, 70, and 50%). The deparaffinized sections were stained with 10% toluidine blue (pH 2.3) (Sigma-Aldrich) for 3 min at room temperature. Mast cells were counted in six × 400 microscopic fields from each mouse.

Immunohistochemistry Staining
Skin sections were embedded in paraffin and sliced on slides. Slides were deparaffinized using xylene and an alcohol
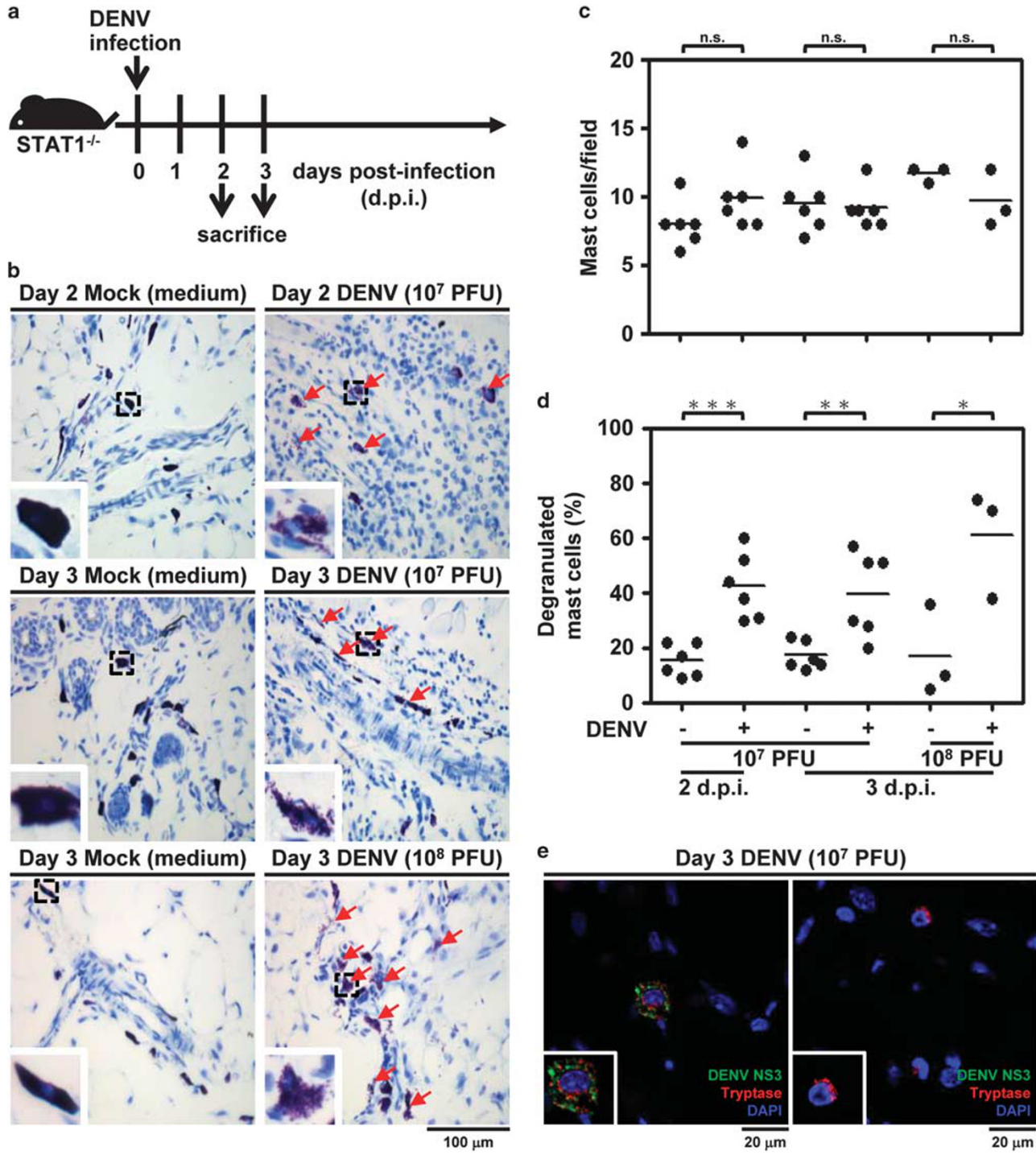
Figure 1 Mast cell degranulation in DENV-infected mice. (**a**) Experimental design of the DENV-induced hemorrhage model in *STAT1*$^{-/-}$ mice. (**b**) Mice were i.d. inoculated with medium (Mock) or DENV (10^7 or 10^8 PFU/mouse) at four sites on the upper back. On 2 or 3 d.p.i., the local skin sections were stained with toluidine blue (blue-purple). Red arrows indicate degranulated mast cells (magnification: ×400). (**c**) The positive-stained mast cells were counted in six (×400) microscopic fields per mouse and the average numbers of mast cells per field were calculated. (**d**) Degranulated mast cells are shown as the percentage of total mast cells. (**e**) Skin sections were stained with anti-DENV NS3 and tryptase Abs, followed by Alexa-488-conjugated donkey anti-rabbit IgG and Alexa-594-conjugated donkey anti-mouse IgG. Nuclei were stained with DAPI. The insets show representative mast cells (NS3 and tryptase double-positive in the left panel, and NS3-negative, tryptase-positive in the right panel). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. NS, nonsignificant. ($n = 6$ mice for the Mock and 10^7 PFU DENV groups, $n = 3$ mice for the 10^8 PFU DENV group).
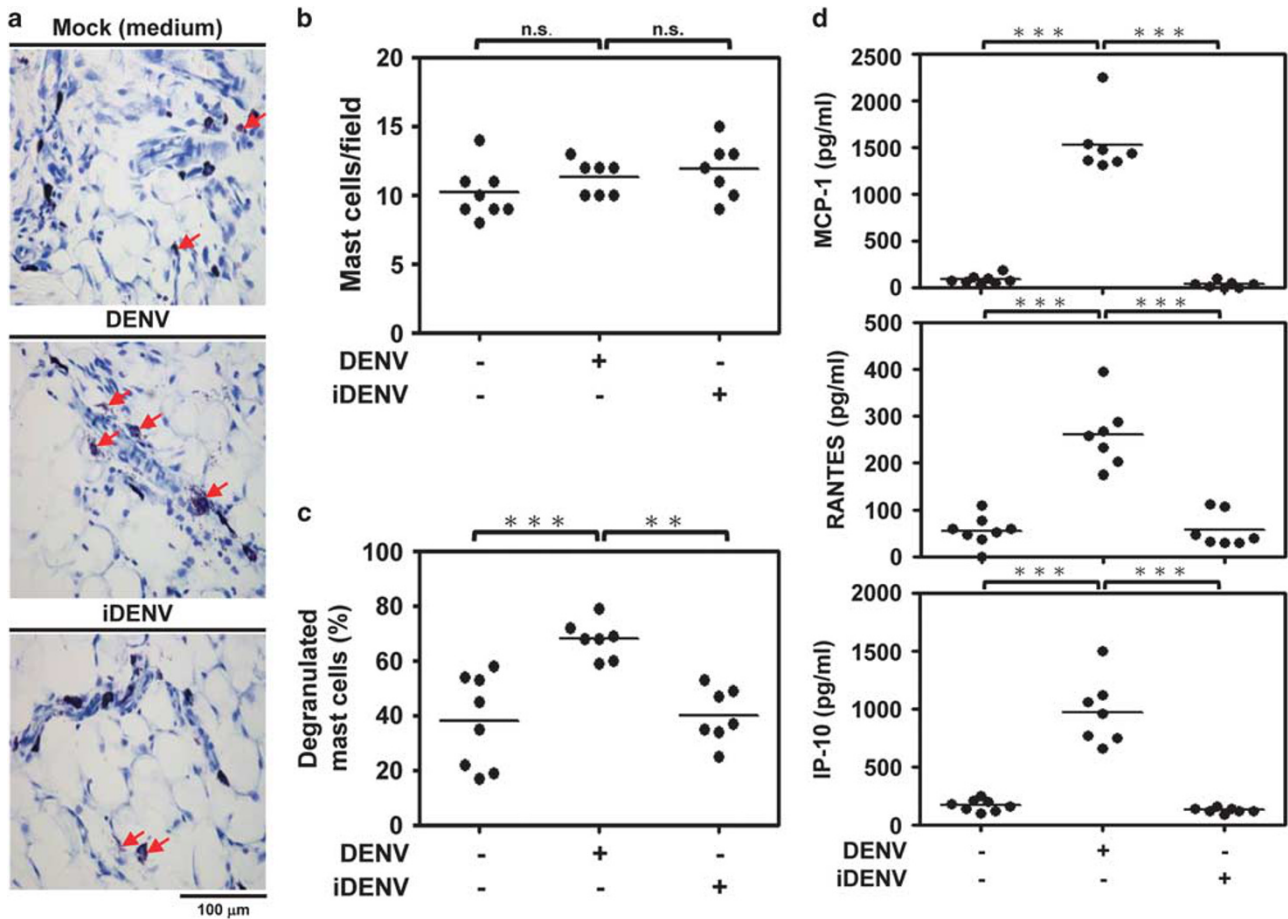
Figure 2 DENV but not UV-inactivated DENV enhances mast cell degranulation and chemokine production. *STAT1*$^{-/-}$ mice were i.d. inoculated with medium (Mock) or DENV (10^7 PFU/mouse) or UV-inactivated DENV (iDENV) at four sites on the upper back. On 3 d.p.i., the local skin sections were stained with toluidine blue. (**a**) The positive-stained mast cells are blue-purple. Red arrows indicate degranulated mast cells (magnification: ×400). (**b**) The positive-stained mast cells were counted in six (×400) microscopic fields per mouse and the average numbers of mast cells per field were calculated. (**c**) Degranulated mast cells are shown as the percentage of total mast cells. (**d**) Mouse sera were collected on 3 d.p.i. The chemokines were detected by ELISA. **$P < 0.01$, ***$P < 0.001$. NS, nonsignificant. ($n = 8$ mice for the Mock group, $n = 7$ mice for the 10^7 PFU DENV and iDENV groups).

gradient (100, 95, 85, 70, and 50%). The sections were incubated in 2 N HCl solution for 20 min and then treated with 20 µg/ml proteinase K in TE buffer (50 mM Tris Base, 1 mM EDTA, and 0.5% Triton X-100, pH 8.0) for another 20 min at room temperature. The sections were incubated with 3.5% H$_2$O$_2$ in phosphate-buffered saline (PBS) for 15 min to inhibit endogenous peroxidase activity and blocked by 5% bovine serum albumin (BSA) in PBST for 30 min.

The primary and secondary Abs were appropriately diluted in Ab diluents (Dako Corporation, Carpentaria, CA, USA). Primary Abs, including goat anti-mouse CCL5 (RANTES; 1:10, R&D Systems, Minneapolis, MN, USA), rabbit anti-mouse CXCL10 (IP-10) (1:200, PeproTech, Rocky Hill, NJ, USA), rabbit anti-mouse CCL2 (MCP-1; 1:300, Abcam, Cambridge, MA, USA), and rat anti-mouse F4/80 (1:50, Serotec, Raleigh, NC, USA), were incubated overnight at 4 °C, followed by biotin-labeled donkey anti-goat IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA),

biotin-labeled donkey anti-rabbit IgG (Jackson Immunor-esearch Laboratories), and biotin-labeled donkey anti-rat IgG (Jackson Immunoresearch Laboratories), respectively, for 2 h at room temperature. After washing twice with PBST, the sections were incubated with HRP-conjugated streptavidin (Dako Corporation) for 15 min at room temperature. For activated mast cells, sections were stained with mouse anti-mouse tryptase Abs (Abcam) overnight at 4 °C. After blocking, endogenous mouse IgG in the skin section with the Mouse on Mouse kit (Vector Laboratories, Burlingame, CA, USA). After washing twice with PBST, the sections were incubated with HRP-conjugated goat anti-mouse IgG (Cal-biochem) for 1 h at room temperature. Following washing, the skin sections were developed with the Ab-Mediated Chromogen (AEC) Substrate Kit (Vector Laboratories) and nuclei were stained with hematoxylin for 3 min. The sections were analyzed using a TissueFAXS (TissueGnostics, Vienna, Austria) image cytometer. Cells were counted in 15 regions
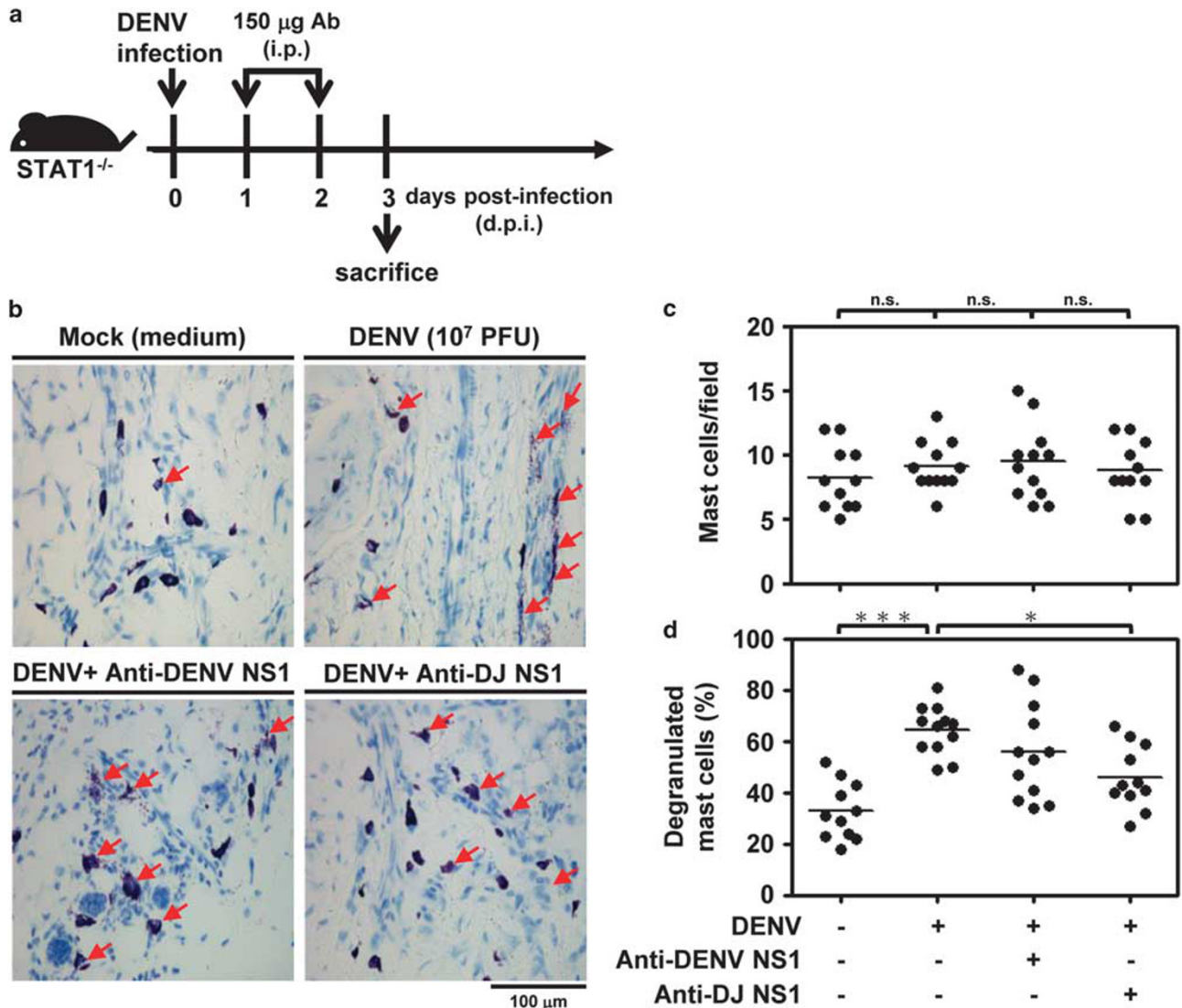
Figure 3 Anti-DJ NS1 Abs reduce mast cell degranulation in DENV-infected mice. (**a**) Experimental design of the therapeutic model in *STAT1*$^{-/-}$ mice. Mice were i.d. inoculated with DENV (10^7 PFU/mouse) at four sites on the upper back. Mice were i.p. inoculated with Abs (150 μg/mouse) on 1 and 2 d.p.i. (**b**) On 3 d.p.i., the local skin sections were stained with toluidine blue (blue-purple). Red arrows indicate degranulated mast cells (magnification: ×400). (**c**) The positive-stained mast cells were counted in six (×400) microscopic fields per mouse and the average numbers of mast cells per field were calculated. (**d**) Degranulated mast cells are shown as the percentage of total mast cells. *$P < 0.05$, ***$P < 0.001$. NS, nonsignificant. ($n = 11$ mice for the Mock and DENV+anti-DJ NS1 Abs groups, $n = 12$ mice for the other groups).

per mouse field and the average numbers were calculated by the HistoQuest software (TissueGnostics). HistoQuest separated the AEC stain and the counterstain (hematoxylin). The positive cells were detected by signals of AEC in the nucleated cells. Results were displayed as dot plots with each dot representing a single cell in the tissue sample. The cutoff threshold was determined by the AEC signal intensity based on AEC-negative cells in the same section. The forward/backward gating tool of the HistoQuest software was used for quality control of measurements. By clicking on high or low AEC staining intensity cells in the image, the forward gating tool showed the individual staining intensities of selected cells in the scattergram. Backward gating was used to verify data by visual inspection on the original image.[37]

Immunofluorescence

Skin sections were embedded in paraffin and sliced on slides. Slides were deparaffinized using xylene and an alcohol gradient (100, 95, 85, 70, and 50%). The sections were incubated in 2 N HCl solution for 20 min and then treated with 20 μg/ml proteinase K in TE buffer (50 mM Tris Base, 1 mM EDTA, and 0.5% Triton X-100, pH 8.0) for another 20 min at room temperature. The sections were blocked with 5% BSA in PBST for 30 min and then using 1 M NH$_4$Cl to
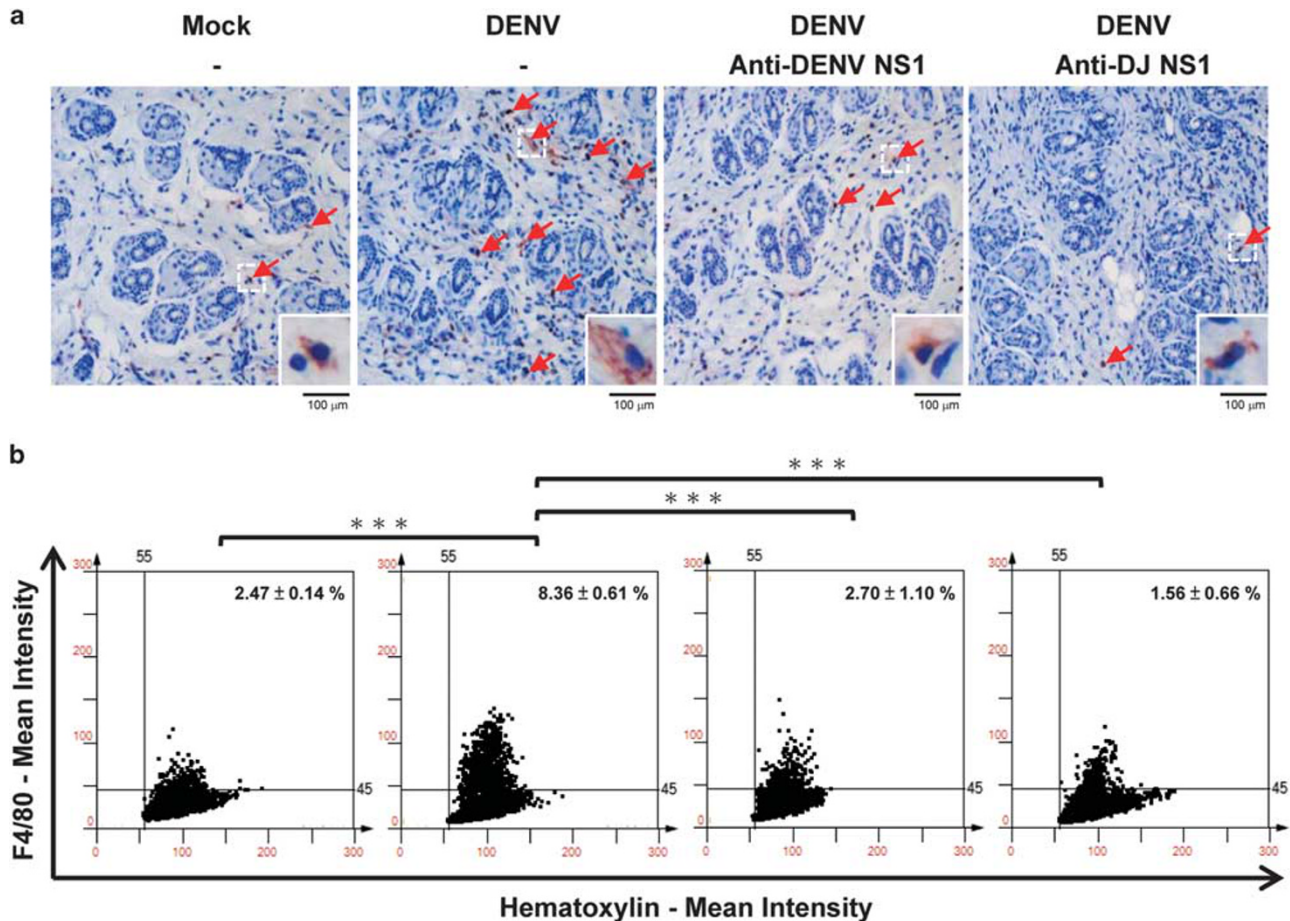
Figure 4 Anti-DJ NS1 Abs reduce macrophage infiltration and MCP-1 production in DENV-infected mice. $STAT1^{-/-}$ mice were i.d. inoculated with DENV (10^7 PFU/mouse) at four sites on the upper back. Mice were i.p. inoculated with Abs (150 μg/mouse) on 1 and 2 d.p.i. and were killed on 3 d.p.i. The local skin sections were stained with anti-F4/80 ($n = 3$) and anti-MCP-1 Abs ($n = 6$) and nuclei were stained with hematoxylin. (**a**) Red arrows indicate the F4/80-positive macrophages (magnification: $\times$200). (**b**) The F4/80-positive macrophages were counted in 15 regions per mouse field and the average numbers of macrophages were calculated by the HistoQuest software. (**c**) Red arrows indicate the MCP-1-positive cells (magnification: $\times$200). (**d**) Quantification of MCP-1 staining was performed on skin sections using the HistoQuest analysis software. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

reduce autofluorescence for 30 min. The skin sections were incubated with primary Abs rabbit anti-DENV2 NS3 (1:500, Genetex, Irvine, CA, USA) plus mouse anti-mouse tryptase (1:500, Abcam) overnight at 4 °C. After washing with PBST, the sections were incubated with secondary Abs Alexa-488-conjugated donkey anti-rabbit IgG (1:250, Invitrogen, Carlsbad, CA, USA) and Alexa-594-conjugated donkey anti-mouse IgG (1:250, Invitrogen) for 2 h. Cell nuclei were stained with DAPI (1:1500, Calbiochem) for 10 min. Fluorescence images were performed using a confocal microscope (Olympus BX-51).

ELISA of Chemokine Levels
The CCL2 (MCP-1) and CCL5 (RANTES) levels were measured using a sandwich ELISA technique with commercial ELISA development kits (R&D Systems). The 96-well plates were coated with diluted capture Abs in PBS and incubated overnight at room temperature. After washing

three times with wash buffer (0.05% Tween 20 in PBS) and blocking with blocking buffer (1% BSA in PBS) for 1 h, cell culture supernatants and standards were diluted with reagent diluents (1% BSA in PBS) and then added into plates for 2 h incubation at room temperature. After washing, the detection Abs in reagent diluents (1% BSA in PBS) were added and incubated for 2 h. After washing, streptavidin–HRP (1:200, R&D Systems) was added for 20 min. Finally, the substrate solution TMB (Clinical Science Products, MA, USA) was added and the reaction was stopped with 2 N H_2SO_4. The optical density was determined using a microplate reader set to 450 nm.

IP-10 concentration was measured using a sandwich ELISA technique with commercial ELISA development kits (Pepro-Tech). The 96-well plates were coated with diluted capture Abs in PBS and incubated overnight at room temperature. After washing three times with wash buffer (0.05% Tween 20 in PBS) and blocking with blocking buffer (1% BSA in PBS)
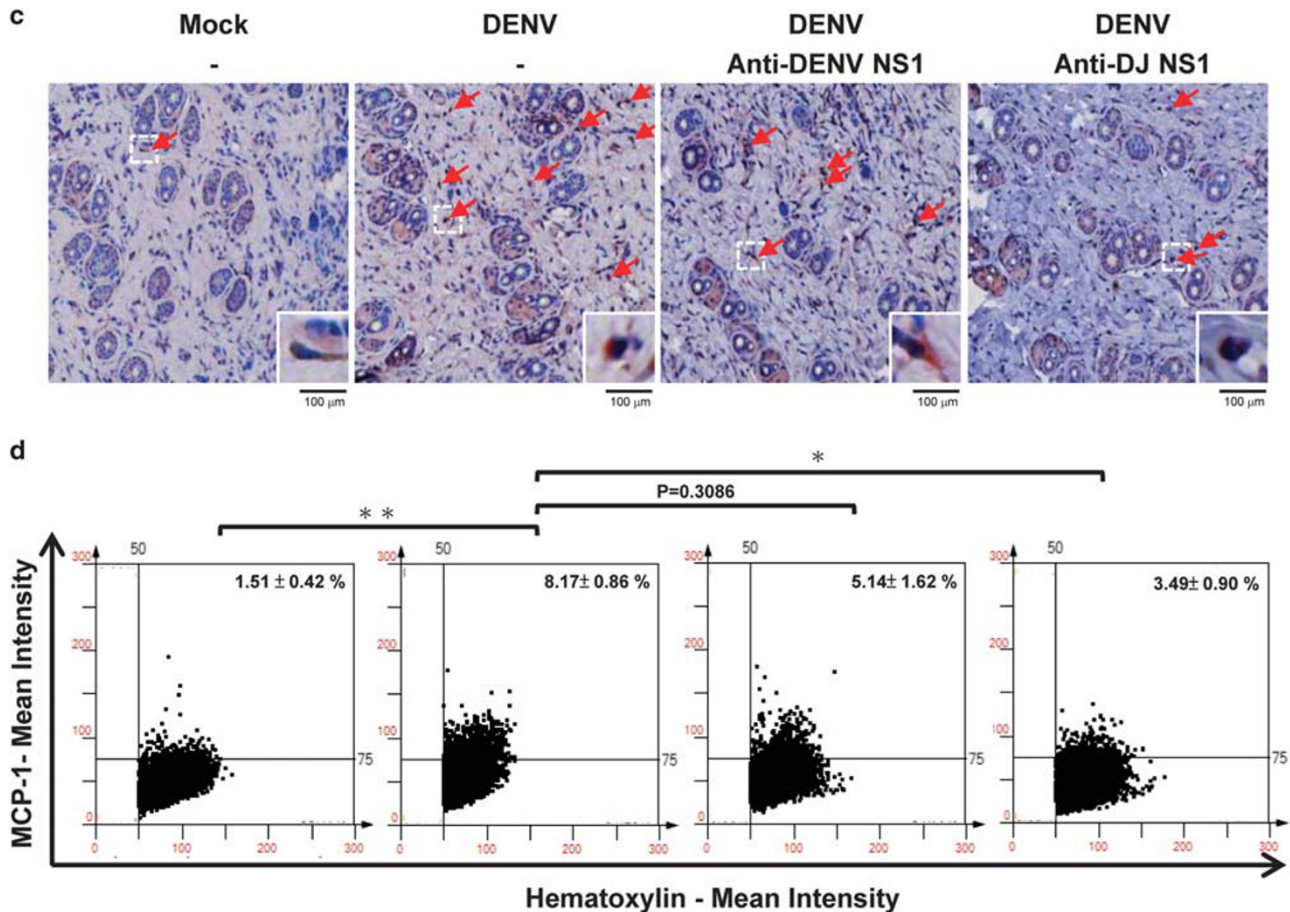
Figure 4 Continued.

for at least 1 h, mouse sera and standards were diluted with 0.05% Tween-20 and 0.1% BSA in PBS and then added into plate wells for 2 h incubation at room temperature. After washing, the detection Abs in 0.05% Tween-20 and 0.1% BSA in PBS were added and incubated for 2 h. After washing, streptavidin–HRP (1:2000, R&D Systems) was added for 30 min. Finally, the substrate solution ABTS (Sigma-Aldrich) was added and incubated at room temperature for color development with a microplate reader set to 405 nm.

Statistical Analysis

Two-group comparisons were performed using Student *t*-test with GraphPad Prism version 6.0. Statistical significance was set at $P < 0.05$. Comparisons between various treatments were assessed by one-way ANOVA, followed by Tukey's test with GraphPad Prism version 6.0.

RESULTS
Mast Cell Degranulation in Response to DENV Inoculation of Mice

A previous study demonstrated degranulation and histamine release from mouse mast cells sensitized with DENV-immune sera.[38] Another study reported that DENV induced

degranulation of the rat mast cell line RBL as well as mouse and monkey skin mast cells.[39] We first examined the response of mast cells in DENV-infected $STAT1^{-/-}$ mice. $STAT1^{-/-}$ mice were i.d. inoculated with medium or DENV (10^7 and 10^8 PFU/mouse) at four sites on the upper back (Figure 1a). The skin inoculation sites were harvested from mice on 2 or 3 d.p.i. and sectioned, followed by staining with mast cell-specific stain, toluidine blue. On both 2 and 3 d.p.i., the morphology of mast cells was dense or compacted in medium (mock)-injected $STAT1^{-/-}$ mice (Figure 1b, left panels). After DENV infection, mast cell degranulation was observed (Figure 1b, right panels). We further quantified the staining results and found that the average numbers of mast cells showed no significant difference between medium- and DENV-inoculated $STAT1^{-/-}$ mice (Figure 1c). However, the levels of mast cell degranulation were significantly increased in DENV-infected mice as compared with medium control mice (Figure 1d).

As DENV can enhance mast cell degranulation, we further investigated the expression of DENV antigen NS3, a marker for viral replication, and tryptase, an activation marker of mast cells, to ascertain whether degranulated mast cells were infected by DENV. The results showed first that mast cells are
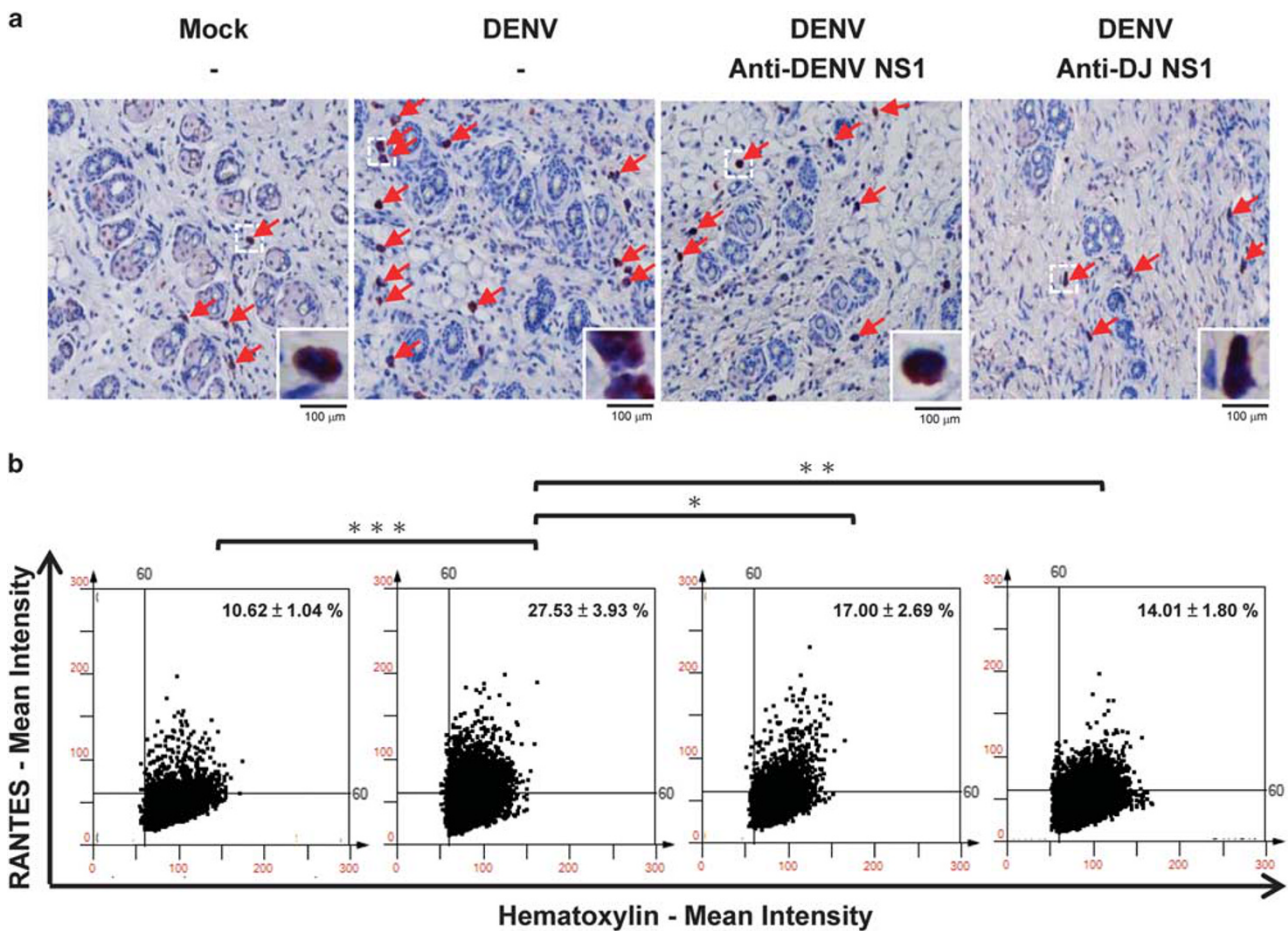
Figure 5 Anti-DJ NS1 Abs reduce RANTES and IP-10 production in DENV-infected mice. $STAT1^{-/-}$ mice were i.d. inoculated with DENV (10^7 PFU/mouse) at four sites on the upper back. Mice were i.p. inoculated with Abs (150 μg/mouse) on 1 and 2 d.p.i. and were killed on 3 d.p.i. The local skin sections were stained with anti-RANTES ($n = 6$) and anti-IP-10 Abs ($n = 4$ mice for Mock, $n = 5$ mice for DENV, and $n = 6$ mice for the other groups) and nuclei were stained with hematoxylin. (**a**) Red arrows indicate RANTES-positive cells (magnification: ×200). (**b**) The RANTES-positive cells were counted in 15 regions per mouse field and the average numbers of the RANTES-positive cells were calculated by the HistoQuest software. (**c**) Red arrows indicate IP-10-positive cells (magnification: ×200). (**d**) Quantification of IP-10 staining was performed on skin sections using the HistoQuest software. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

infectable by DENV following *in vivo* inoculation of mice (Figure 1e, left panel, green) and second that the tryptase staining (red) in NS3-positive cell (Figure 1e, left panel) was not as dense as in NS3-negative cell (Figure 1e, right panel). The results indicate that DENV infection might affect mast cell degranulation and tryptase distribution.

We showed further that live DENV was required to induce mast cell degranulation. UV-inactivated DENV-infected mouse skin sites showed fewer numbers of degranulated mast cells (Figure 2a). The quantified results showed that the average numbers of mast cells still showed no significant difference between live DENV and UV-inactivated DENV-infected groups, showing similar levels as the Mock group (Figure 2b). However, the levels of mast cell degranulation were significantly lower in UV-inactivated DENV-infected mice as compared with DENV-infected mice (Figure 2c). We

further determined the production of chemokines (MCP-1, RNATES, and IP-10) in mouse sera. The results showed that DENV but not UV-inactivated DENV caused enhanced chemokine production (Figure 2d). These results suggest that DENV replication is required for both mast cell degranulation and chemokine production.

Anti-DJ NS1 Abs Reduce DENV-Induced Prolonged Bleeding Time and Mast Cell Degranulation in Mice

Our previous studies showed that Abs against modified NS1 protein (DJ NS1) can provide therapeutic benefits in DENV-infected mice. Here we investigate the effects of these Abs on DENV-induced mast cell degranulation. We i.d. inoculated $STAT1^{-/-}$ mice with 10^7 PFU/mouse of DENV at four sites on the upper back. Mice were i.p. inoculated with 150 μg/mouse of Abs on 1 and 2 d.p.i. and were killed on 3 d.p.i. (Figure 3a).
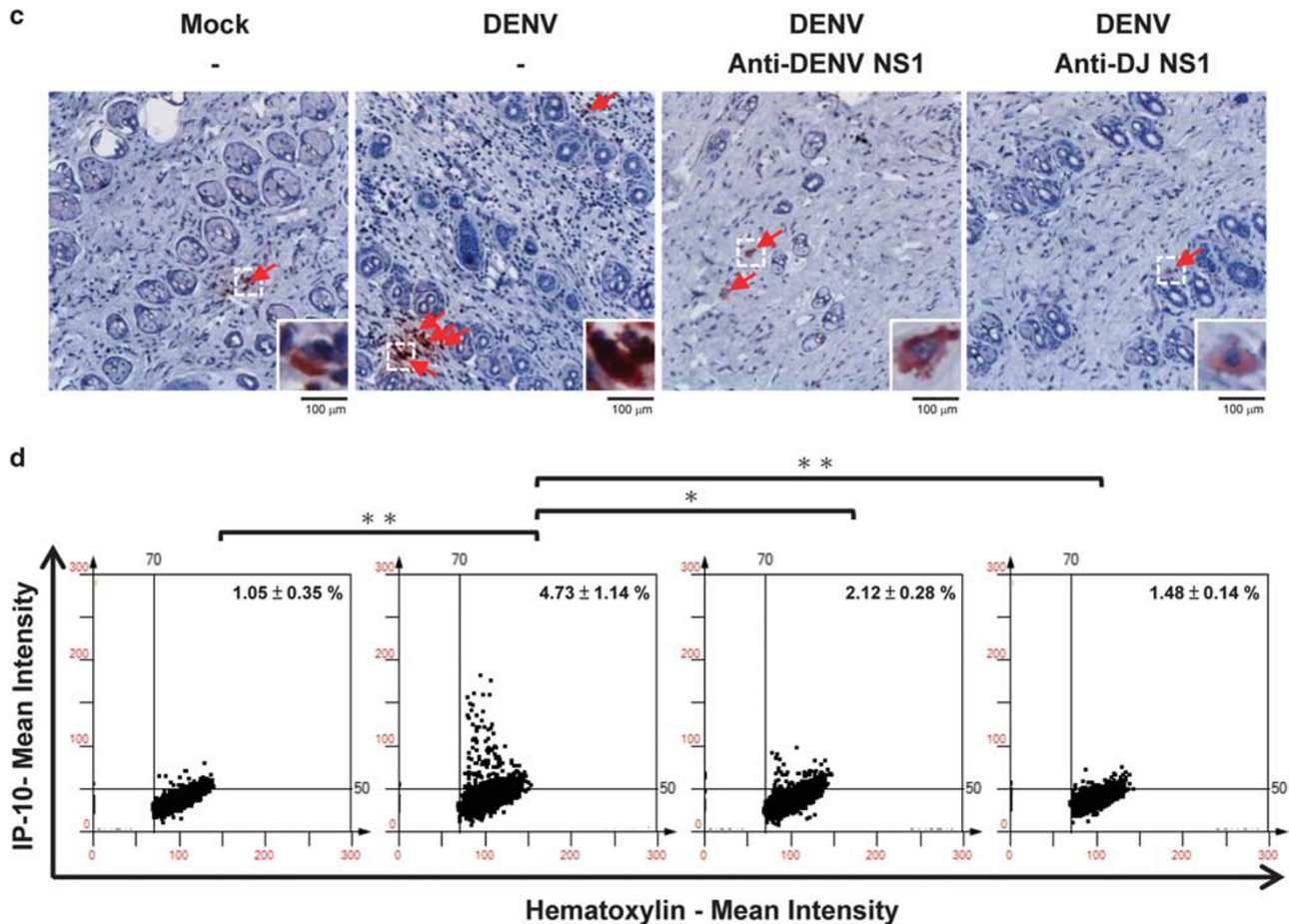
Figure 5 Continued.

Skin sections from medium-, DENV-, and DENV plus Ab-injected mice were stained with toluidine blue (Figure 3b). We then quantified the images and the results showed that the average numbers of mast cells were not significantly different between the DENV-infected and Ab-treated groups (Figure 3c). However, treatment with anti-DJ NS1 Abs significantly reduced mast cell degranulation (Figure 3d). We also measured the granule-associated mediator release, such as histamine and serotonin, in the mouse sera. Results showed that anti-DJ NS1 Abs significantly reduced histamine and serotonin release, suggesting an inhibition on DENV-induced mast cell activation (Supplementary Figure S1).

Anti-DJ NS1 Abs Reduce DENV-Induced Macrophage Infiltration and Chemokine Production in Mice

A previous study reported that, in areas of DENV-infected endothelial cells, macrophage infiltration into the vicinity of the endothelium was observed.[40] Our previous studies also showed that DENV infection caused the infiltration of macrophages and treatment with the Abs against modified NS1 can reduce macrophage infiltration in DENV-infected

mice.[32] In this study, macrophage infiltration in the dermis layer was also observed after DENV infection in *STAT1*^{−/−} mice. Fewer infiltrating macrophages were observed in mice treated with anti-DENV NS1 and anti-DJ NS1 Abs (Figure 4a). We further quantified the images using the HistoQuest analysis software and found that treatment with anti-DJ NS1 Abs as well as anti-DENV NS1 Abs showed significant reduction of infiltrating macrophages (Figure 4b). CCL2 (MCP-1) is a highly expressed chemokine in DHF/DSS patients that may act on endothelial cells, causing permeability change and contributing to plasma leakage.[41–43] MCP-1 also contributes to the migration and infiltration of monocytes/macrophages, memory T cells, and NK cells,[42] which are involved in dengue disease pathogenesis. Therefore, we detected the expression of MCP-1 at the skin inoculation site by immunohistochemical staining. Results showed that DENV infection can increase the expression of MCP-1 in local skin section. After treatment with anti-DJ NS1 Abs, the expression of MCP-1 was decreased (Figure 4c). Immunohistochemical staining images were further quantified by HistoQuest analysis, and the levels of MCP-1 were significantly decreased in the anti-DJ NS1 Ab-treated
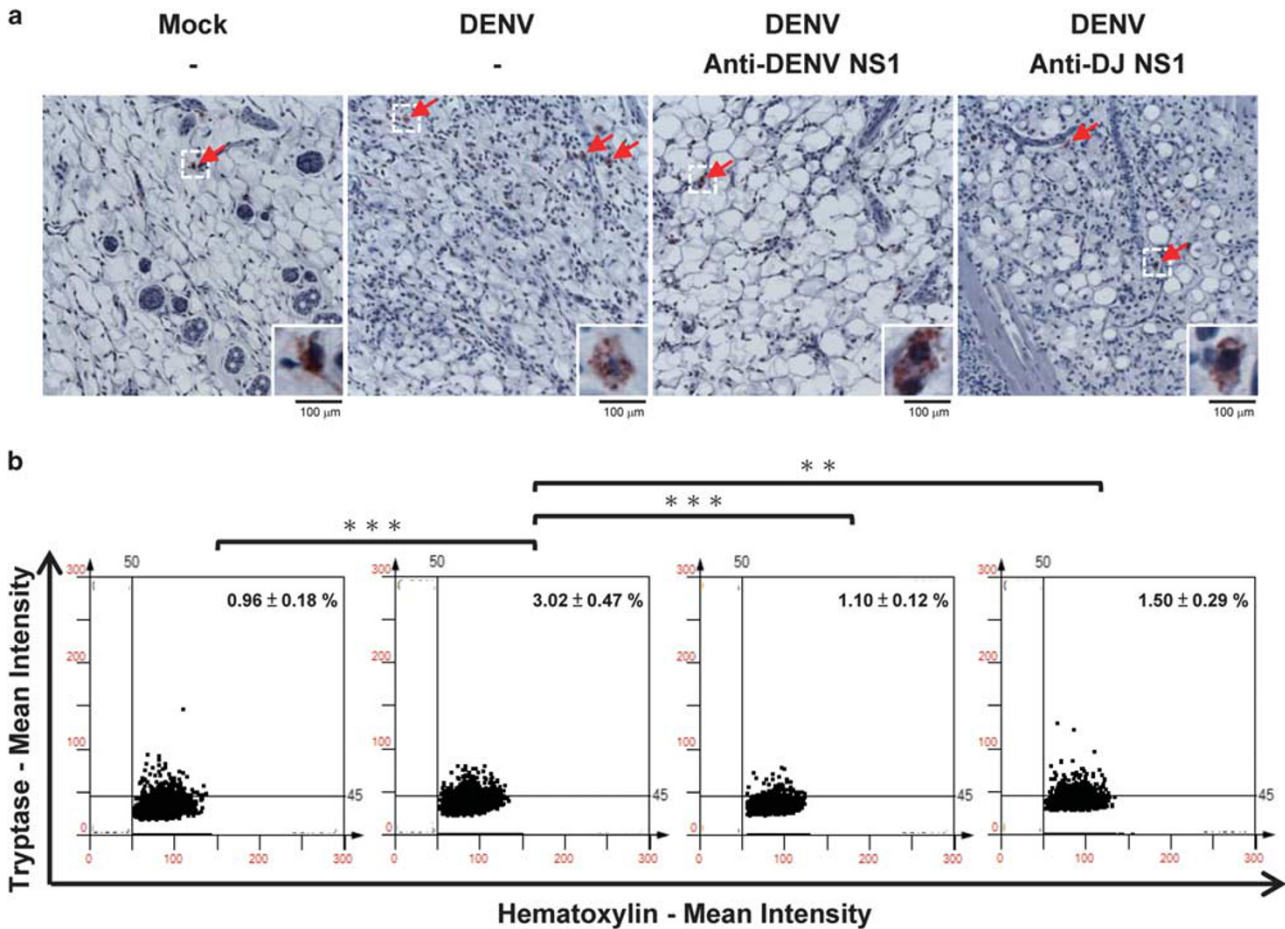
Figure 6 Anti-DJ NS1 Abs reduce tryptase production in DENV-infected mice. $STAT1^{-/-}$ mice were i.d. inoculated with DENV (10^7 PFU/mouse) at four sites on the upper back ($n = 9$). Mice were i.p. inoculated with Abs (150 μg/mouse) on 1 and 2 d.p.i. and were killed on 3 d.p.i. The local skin sections were stained with anti-tryptase Abs and nuclei were stained with hematoxylin. (**a**) Red arrows indicate tryptase-positive cells (magnification: ×200). (**b**) The tryptase-positive cells were counted in 15 regions per mouse field and the average numbers of the tryptase-positive cells were calculated by the HistoQuest software. **$P < 0.01$, ***$P < 0.001$.

groups as compared with the DENV alone group (Figure 4d). There was a trend, although not statistically significant, of reduced levels of MCP-1 by treatment with anti-DENV NS1 Abs.

In addition to CCL2 (MCP-1), previous studies also showed that several chemokines (MIP-1α, MIP-1β, RANTES, IP-10) were increased in the plasma of dengue patients.[44,45] In our study, we found that RANTES was expressed in the dermis layer (Figure 5a). Immunohistochemical staining images were further quantified by HistoQuest analysis, and the results showed that the percentage of RANTES-positive cells was significantly decreased in the anti-DJ NS1 and anti-DENV NS1 Ab-treated groups as compared with the DENV alone group (Figure 5b). For CXCL10 (IP-10) production, we also found that IP-10 was expressed in the dermis layer (Figure 5c). Furthermore, the quantitative data showed that the percentage of IP-10-positive cells was significantly decreased in the anti-DJ NS1 and anti-DENV NS1 Ab-treated groups as compared with the DENV alone

group (Figure 5d). To further identify which cells were contributing to the chemokine production, the skin sections were double stained with macrophage marker (F4/80) or mast cell marker (c-kit) plus anti-MCP-1 or anti-RANTES Abs. Immunohistochemical staining images showed that the macrophages were one of the important sources to produce MCP-1 but not RANTES. On the other hand, mast cells were one of the important sources to produce RANTES but not MCP-1 (Supplementary Figure S2). However, we could not exclude the possibility of other sources to produce these chemokines.

We next measured the chemokine levels (MCP-1, RANTES, and IP-10) in the mouse sera. The amount of MCP-1 was significantly lower in the anti-DJ NS1-treated group than in the DENV alone group. There was a trend, although not statistically significant, of reduced levels of RANTES and IP-10 in the anti-DJ NS1-treated groups as compared with those in the DENV alone groups (Supplementary Figure S3).

Anti-DJ NS1 Abs Reduce DENV-Induced Expression of Tryptase from Mast Cells in Mice

A previous study reported that mast cell activation and mast cell-derived mediators (ie, VEGF, chymase, and tryptase) participate in the development of DHF/DSS.[10] In this study, we detected the expression of tryptase at the skin inoculation site by immunohistochemical staining. Tryptase was expressed in the dermis layer (Figure 6a). Immunohistochemical staining images were further quantified by HistoQuest analysis, and the results showed that the percentage of tryptase-positive cells was significantly decreased in the anti-DJ NS1 and anti-DENV NS1 Ab-treated groups as compared with the DENV alone group (Figure 6b). Therefore, the results demonstrate that tryptase expression was reduced by the treatment with anti-DJ NS1 and anti-DENV NS1 Abs.

DISCUSSION

Previous studies reported the elevation of plasma and urinary histamine in DHF patients.[8,9] A further study showed that sera from DENV-immunized mice caused mast cell degranulation and the production of histamine.[38] Recent studies also showed that DENV causes degranulation of mouse and monkey mast cells[39] and mast cell-derived VEGF and proteases are elevated in DSS patients.[10] Therefore, mast cells may have an important role in DENV infection. In this study, we examined the protective effects of Abs targeting on DENV NS1 by inhibiting mast cell activation in the mouse model.

First, we found that DENV-induced degranulation of mast cells required infectious DENV, as UV-inactivated DENV was ineffective. Mast cells contain three main classes of mediators, ie, preformed granule-associated mediators, newly generated lipid mediators, and a wide variety of cytokines and chemokines. The granule-associated mediators include histamine, serotonin, tryptase, chymase, carboxypeptidase, TNF, and VEGF.[46] Elevated levels of granule-associated mediators, including VEGF, tryptase, and chymase, were observed in severe dengue patients.[10] In an animal model, mast cell-derived mediator, chymase, also contributed to DENV-induced vascular leakage.[10] In addition, mast cells produced a variety of cytokines (IL-1β, IL-6, TNF-α) and chemokines (MIP-1α, MIP-1β, RANTES) in response to DENV.[12–14,47] In this study, we found that the production of tryptase, CCL5 (RANTES), and CXCL-10 (IP-10) were significantly increased in DENV-infected *STAT1*$^{-/-}$ mice. Taken together, considerable evidence suggests that DENV infection results in stimulation of mast cells to release granule-associated mediators and cytokines/chemokines, which may contribute to disease development.

It was previously reported that DENV-infected, *in vitro*-differentiated macrophages secrete multiple cytokines and chemokines, including TNF-α, IFN-α, IL-1β, IL-6, IL-8, IL-12, MIP-1α, and RANTES. MIP-1α and MIP-1β are also induced by infection with DENV in a myelomonocytic cell line as well as in peripheral blood mononuclear cells isolated from dengue patients.[48] The production of IP-10, RANTES, IL-6, IL-7, and IL-8 has been observed in DENV-infected endothelial cells.[49] MCP-1 can be presented on high endothelial venules for recruitment of monocytes.[50] In this study, we found that the levels of macrophage infiltration and MCP-1 expression were significantly increased in DENV-infected mice. A previous study demonstrated that co-culturing IgE-sensitized, antigen-stimulated mast cells with macrophages produced higher concentration of inflammatory cytokines than either cell population alone.[51] It is therefore likely that the interaction between macrophages and mast cells may amplify cytokine/chemokine production and contribute to endothelial cell damage or activation in DENV infection. However, the mechanism remains unknown and is worthy of further investigation.

DENV vaccine development is challenging due to the as yet incompletely understood pathogenic mechanisms of dengue disease. Recently, the first dengue vaccine has been approved in a few countries, although there are still limitations in its effectiveness.[7,52,53] Therefore, efforts to develop more effective vaccines or antiviral drugs are still needed. Our previous studies indicated that anti-DENV NS1 Abs cross-reacted with endothelial cells and platelets and induced endothelial cell apoptosis and platelet dysfunction.[27–30,54,55] In addition, we found liver damage, which is a pathological manifestation in DENV disease, in both active immunization with DENV NS1 protein or passive immunization with anti-NS1 Abs in mouse models.[56] As a potential vaccine candidate, we replaced the C-terminal region with JEV NS1 protein (DJ NS1), because the C-terminal region of DENV NS1 protein contains homologous sequences with host self-antigens. We showed that anti-DJ NS1 Abs can provide protection in DENV-infected mice.[32] In this study, we also found that treatment with anti-DJ NS1 Abs reduce mast cell degranulation; the production of MCP-1, RANTES, IP-10, and tryptase; and macrophage infiltration. The precise mechanism of action underlying the reduction of mast cell activation in DENV infection is unknown. As DENV NS1 has recently been reported to bind cell surface expressed TLR4[57] or TLR2 and TLR6,[58] Abs targeting NS1 may, therefore, bind to the free form of NS1 and block its multifaceted harmful effects.

Taken together, our findings indicate that anti-DJ NS1 Abs can reduce DENV-induced pathological effects at least in part by inhibiting mast cell activation. The results, along with our previous reports,[32,35] suggests that Abs against C-terminus-modified NS1 may provide multiple benefits as a therapeutic and vaccine strategy for dengue.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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