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Liver injury caused by antibodies against dengue virus nonstructural protein 1 in a murine model

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Clinical manifestations of severe dengue diseases include thrombocytopenia, vascular leakage, and liver damage. Evidence shows that hepatic injury is involved in the pathogenesis of dengue infection; however, the mechanisms are not fully resolved. Our previous *in vitro* studies suggested a mechanism of molecular mimicry in which antibodies directed against dengue virus (DV) nonstructural protein 1 (NS1) cross-reacted with endothelial cells and caused inflammatory activation and apoptosis. In this study, the pathogenic effects of anti-DV NS1 antibodies were further examined in a murine model. We found, in liver sections, that anti-DV NS1 antibodies bound to naive mouse vessel endothelium and the binding activity was inhibited by preabsorption of antibodies with DV NS1. Active immunization with DV NS1 resulted in antibody deposition to liver vessel endothelium, and also apoptotic cell death of liver endothelium. Liver tissue damage was observed in DV NS1-immunized mice by histological examination. The serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were increased in mice either actively immunized with DV NS1 protein or passively immunized with antibodies obtained from DV NS1-immunized mice. Furthermore, histological examination revealed mononuclear phagocyte infiltration and cell apoptosis in mice passively immunized with antibodies obtained from mice immunized with DV NS1. Increased AST and ALT levels were observed in mice passively immunized with purified immunoglobulin G (IgG) from dengue patients compared with normal control human IgG-immunized mice. The increased AST and ALT levels were inhibited when dengue patient serum IgG was preabsorbed with DV NS1. In conclusion, active immunization with DV NS1 protein causes immune-mediated liver injury in mice. Passive immunization provides additional evidence that anti-DV NS1 antibodies may play a role in liver damage, which is a pathologic manifestation in dengue virus disease.

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Dengue virus (DV) infection causes a spectrum of illness from mild dengue fever to severe dengue hemorrhagic fever (DHF) and dengue shock syndrome.[1,2] The global prevalence of dengue has grown dramatically in recent decades. The disease is now endemic in more than 100 countries. There are at least 50 million cases of dengue infection and several hundred thousand cases of DHF every year.[3,4] Several pathogenic mechanisms for DHF have been considered: antibody-dependent enhancement of infection,[5–10] viral serotype variation,[11–13] and abnormal immune activation.[14–19] There is no effective dengue vaccine, although several candidate vaccines are currently being evaluated.[4,20–26]

Hepatic injury leading to coagulopathy might be related to the progression of hemorrhage in DHF. Elevated serum levels of the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are signs of liver dysfunction in DHF patients.[14,15,27–29] A previous study[30] indicated that the severity of cytopathic effects and the increase in AST levels correlated with the virus replication rate in DV-infected liver cell lines. Further studies[31–33] showed cell activation and apoptosis in DV-infected liver cell lines. In addition to a direct viral cytotoxic effect, an indirect route of immunopathogenesis via cytokines, chemokines, and infiltrating leukocytes may also be involved.[15,17] In fact, hepatic

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injury caused by vessel endothelium disruption in the liver has been reported.[34,35] Therefore, both endothelial cell dysfunction and endothelial cell-derived inflammatory events may cause pathological effects that lead to liver injury.

Anti-DV nonstructural protein 1 (NS1) antibodies generated in mice have been shown to cross-react with host components, including blood clotting factors, integrin/adhesin proteins, and endothelial cells.[36] We found endothelial cell cross-reactive antibodies in dengue patients and described a mechanism of molecular mimicry between DV NS1 and endothelial cell antigen(s).[37] Cell apoptosis and inflammatory activation are the two major responses causing anti-DV NS1-induced endothelial cell damage.[38–41] To elucidate the effect of anti-DV NS1 *in vivo*, we used a murine model of active immunization with recombinant DV NS1 protein or passive immunization with antibodies obtained from mice immunized with DV NS1. We found liver damage in both active and passive immunization models, which supports a pathologic mechanism of liver injury mediated by anti-DV NS1 antibodies.

MATERIALS AND METHODS

Mice

C3H/HeN breeder mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and maintained on standard laboratory food and water *ad libitum* in our medical college laboratory animal center. Their male 8-week-old progeny were used for the experiments. Housing, breeding, and experimental use of the animals were performed in strict accordance with the Experimental Animal Committee in National Cheng Kung University.

Murine Model

The preparation of recombinant NS1 was described previously.[38] For the active immunization model, C3H/HeN mice were intraperitoneally injected with 20 μg of recombinant DV NS1, Japanese encephalitis virus (JEV) NS1, or bovine serum albumin (BSA) in complete Freund's adjuvant, followed by four weekly injections with 20 μg of protein in incomplete Freund's adjuvant. For the passive immunization model, mice were injected intravenously with anti-DV NS1, anti-JEV NS1, control immunoglobulin G (IgG), or patient serum IgG (500 μg) for 48 h. To induce hepatic injury as a positive control, mice were intraperitoneally injected with 700 mg/kg of galactosamine plus 5 μg/kg of lipopolysaccharide (LPS).

Anti-NS1 Antibodies Obtained from Dengue Patient Sera or NS1-Immunized Mice

Dengue patient sera were collected from two DHF and four dengue fever patients with DV3 infection during July and August 2006. DV infection was confirmed by the Center for Disease Control, Department of Health, Taiwan. Patient sera were collected on 1–11 days after fever onset. C3H/HeN mice were intraperitoneally injected with recombinant DV2 or JEV NS1 protein, as previously described.[38] Purified IgG was obtained using protein G-Sepharose affinity chromatography. Control IgG was purified from the sera of healthy donors or nonimmunized mice.

Cell Culture

Human microvascular endothelial cell line-1 (HMEC-1) were grown in culture plates containing endothelial cell growth medium (EGM; Clonetics, Walkersville, MD, USA) composed of 2% fetal bovine serum, 1 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor, and antibiotics as previously described.[37]

DV NS1 Preabsorption and ELISA

For preabsorption of antibodies, 5 μg of DV NS1, JEV NS1, or BSA in coating buffer (0.1 M $Na_2CO_3H_2O$ and 0.1 M $NaHCO_3$, pH 9.6) were coated in wells of a 96-well plate. Following overnight blocking with 5% skim milk, 5 μg of anti-DV NS1 IgG, control, or dengue patient IgG were added and incubated for 1 h at 4°C. Thereafter, the supernatant was collected and used for experiments. To confirm the efficiency of absorption, the binding activity of preabsorbed antibodies to NS1 was detected using ELISA. Briefly, 0.1 μg of DV NS1, JEV NS1, or BSA in coating buffer was coated in wells of a 96-well plate. Following blocking, test samples were added and incubated for 1 h at room temperature. After washed with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T), 1 μl of 1 mg/ml horseradish peroxidase (HRP)-conjugated goat anti-human or mouse IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) was added to each well and incubated for 1 h at room temperature. After three washes, 100 μl per well of 2, 2′azinobis 3-ethylbenzthiaoline sulfonic acid (Sigma-Aldrich, St Louis, MO, USA) was added and analyzed using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm.

Detection of Endothelial Cell Binding Activity

Normal liver and kidney specimens were obtained from naive C3H/HeN mice. Following deparaffinization, the tissue sections were washed briefly in PBS, and then fixed with 1% formaldehyde in PBS for 10 min at room temperature. Tissue sections were washed twice with PBS and then incubated with 0.3% normal horse serum to block nonspecific binding. Mouse anti-DV NS1, anti-JEV NS1, or control IgG was then added and the tissue was incubated for 1 h at 4°C. After three washes in PBS, the samples were incubated with 1 μl of 1 mg/ml HRP-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories Inc.) for 1 h at 4°C. For vessel endothelial cell staining, tissue sections were stained with goat anti-mouse CD31 and then with HRP-labeled anti-goat antibodies (Jackson Immunoresearch Laboratories Inc.).

To detect the cell-binding ability of antibodies in DV NS1-immunized and anti-DV NS1-treated mice *in vivo*, mouse liver and kidney tissue slices were washed in PBS, and then incubated with 1 μl of 1 mg/ml HRP- or fluorescein

isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories Inc.) for 1 h at 4°C. Mice immunized with PBS in adjuvant were the controls. The tissue sections were viewed using a light microscope or a fluorescence microscope. For light microscopic observation, the specimens were counterstained with hematoxylin for the nuclear staining.

TUNEL Assay

To detect apoptotic cells in formalin-fixed, frozen sections of liver and kidney tissues, we used a terminal transferase dUTP nick-end labeling (TUNEL) reaction and an apoptosis detection kit (*In situ* Cell Death Detection Kit; Integrin, Indianapolis, IN, USA) according to the manufacturer's instructions. The apoptotic cells were visualized using colorimetric development and a substrate kit (AEC; Zymed, South San Francisco, CA, USA) and viewed through a light microscope or a fluorescence microscope.

Detection of Serum AST, ALT, and BUN

Mouse sera were collected and the enzyme levels including AST and ALT were measured using serum multiple biochemical analyzers including an Ektachem DTSCII Analyzer (Eastman Kodak, Rochester, NY, USA) and a HITACHI 7150 autoanalyzer (Japan). The serum levels of blood urea nitrogen (BUN) were measured using an Ektachem DT60II Analyzer (Eastman Kodak) according to the manufacturer's instructions.

Histopathology and Immunohistochemical Staining for Leukocyte Markers

Liver and kidney tissues were prepared in tissue blocks and sliced. For histopathology, the tissue slices were fixed in 10% neutral-buffered formalin and embedded in paraffin wax, and 5-μm sections were stained with hematoxylin-eosin (Sigma-Aldrich). For immunohistochemical analysis, 3-μm frozen sections were fixed with 1% formaldehyde in PBS. After the sections had been washed in PBS, they were incubated for 1 h at 4°C with 1 μl of 0.1 mg/ml FITC-conjugated anti-mouse F4/80 (Serotec, Oxford, UK) or anti-mouse CD3 and PE-conjugated anti-mouse CD16 antibodies (BD Biosciences, San Jose, CA, USA). They were viewed using fluorescence microscopy.

Statistical Analysis

Comparisons between various groups were performed using Student's *t*-test with SigmaPlot for Windows (version 8.0). Statistical significance was set at $P<0.05$.

RESULTS

Anti-DV NS1 Antibodies Bind to Endothelial Cells in Mouse Liver

We previously reported that antibodies generated against DV NS1 cross-reacted with HMEC-1 endothelial cells, but anti-JEV NS1 antibodies did not.[37,38] In this study, we investigated the effects of anti-DV NS1 antibodies in a mouse model. First, we found that anti-DV NS1 antibodies bound to vessel endothelium in the portal and central veins of naive mouse liver (Figure 1a). Neither anti-JEV NS1 nor control IgG showed any binding to liver vessels. The specificity of anti-DV NS1 on endothelial cell binding was confirmed using a DV NS1 preabsorption experiment (Figure 1b). The efficiency of DV NS1 absorption was shown by ELISA (Figure 1b, left panel). Our results showed that the binding of anti-DV NS1 antibodies to endothelial cells was inhibited by preabsorption of antibodies with DV NS1 protein, whereas BSA preabsorption had no effect.

Liver Injury in DV NS1-Immunized Mice

To characterize the binding of anti-DV NS1 antibodies generated *in vivo*, mice were immunized with DV NS1, JEV NS1, or PBS in adjuvant. We found that only DV NS1-immunized mice showed antibody deposition on vessel endothelium in paraffin-embedded liver sections (Figure 2a). In contrast, IgG from DV NS1-immunized mice did not bind to kidney tissue (data not shown). We found apoptotic cells surrounding the vessels in the liver portal vein (Figure 2b) and central vein (data not shown) but not in kidney tissues in DV NS1-immunized mice. Also, there were no apoptotic cells in the liver or kidney tissues of JEV NS1- or PBS-immunized mice (Figure 2b). Mice treated with galactosamine + LPS were used as the positive control showing the apoptotic hepatocytes by TUNEL staining.

Histological examination of the liver tissues of DV NS1-immunized mice revealed typical pathologic changes: hepatic fibrosis (Figure 3Ad), fatty liver (Figure 3Ae), cell infiltration (Figure 3Af), necrotic body (Figure 3Ag), and vesicle formation (Figure 3Ah). These changes were not found in the liver tissues of normal (Figure 3Aa), PBS-immunized (Figure 3Ab), or JEV NS1-immunized (Figure 3Ac) mice. We found no marked histopathology in the kidney tissues from any groups (Figure 3B). Increased AST and ALT levels in the sera of DV NS1-immunized mice, but not in JEV NS1- or PBS-immunized mice, confirmed the pathogenic effects of anti-DV NS1 (Figure 4). There were, however, no changes in the serum levels of BUN.

Liver Injury in DV NS1-Immunized Mouse Sera IgG-Treated Mice

To further confirm that anti-DV NS1 antibodies cause hepatic injury, we passively immunized mice with IgG purified from mice immunized with DV NS1. At 2 days later, serum levels of AST and ALT were higher in mice administered anti-DV NS1 IgG, but not anti-JEV NS1 or control IgG (Table 1). Again, serum levels of BUN did not change. Mice treated with galactosamine + LPS were used as the positive control for liver damage.

Hepatic inflammation can be caused by the recruitment of immune cells.[42,43] Therefore, to characterize anti-DV NS1-induced liver injury, we examined the infiltration of immune

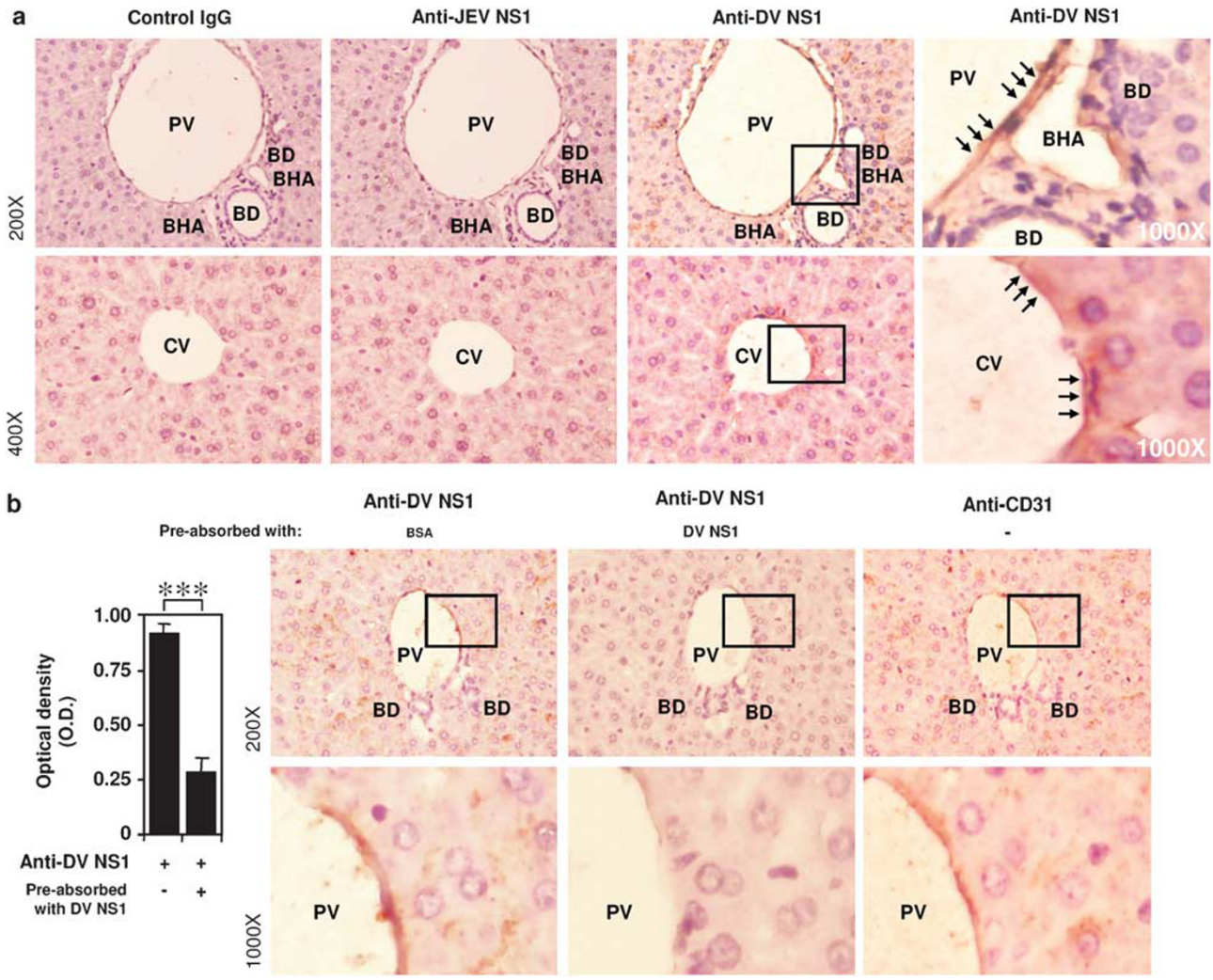

Figure 1 Anti-DV NS1 antibodies cross-react with liver vessel endothelium. (**a**) Cross-reactivity of anti-DV NS1 antibodies with mouse liver vessel endothelium including portal vein (upper panel, magnification $\times 200$ and $\times 1000$) and central vein (lower panel, magnification $\times 400$ and $\times 1000$). The continuous sections of specimens were viewed using a light microscope. Arrows indicate positive staining. (**b**) The endothelial cell binding ability of anti-DV NS1 in portal vein after bovine serum albumin (BSA) or DV NS1 preabsorption (magnification $\times 200$ and $\times 1000$). The efficiency of DV NS1 absorption was determined using ELISA and the optical density (OD) is shown. The endothelial cells in mouse liver portal vein are also visualized using anti-CD31 staining. PV, portal vein; BHA, branch of hepatic artery; BD, bile duct; CV, central vein. $^{***}P<0.001$.

cells using immunostaining with specific antibodies against mononuclear phagocytes, T cells, and NK cells. First, liver tissue sections from mice passively immunized with anti-DV NS1 showed antibody deposition in vessel endothelium (Figure 5a). Staining with anti-F4/80, anti-CD3, or anti-CD16 of liver sections showed an increase of infiltrated mononuclear phagocytes in anti-DV NS1-treated mice, but T and NK cells were not detectable (Figure 5b). We found no infiltrated cells in the liver tissues of anti-JEV NS1-treated mice. Using a TUNEL assay, we found apoptotic cells in the liver tissue of anti-DV NS1-treated but not anti-JEV NS1-treated mice (Figure 5c). Galactosamine + LPS-treated mice, the positive controls, showed acute hepatic injury with mononuclear phagocyte, T-cell and NK-cell infiltration, and cell apoptosis (Figure 5b and c).

Liver Injury in Dengue Patient Sera IgG-Treated Mice

We next determined whether sera derived from dengue patients can also induce liver damage in mice. The presence of anti-DV NS1 IgG in dengue patient sera was confirmed by ELISA (data not shown). Using IgG purified from dengue patient sera to immunize mice (500 μg per mouse), the increase in AST and ALT but not BUN was shown after 48 h in dengue patient IgG-immunized mice as compared with normal control IgG-immunized mice (Figure 6a and b). The increased AST levels could not be observed by 96 h (Figure 6b). Liver injury induced by IgG derived from dengue patient sera was inhibited by preabsorption with DV NS1 (Figure 6a). We also confirmed the efficiency of DV NS1 absorption by ELISA, showing that DV NS1 but not JEV NS1 absorbed anti-DV NS1 antibodies (Figure 6c). The AST concentration

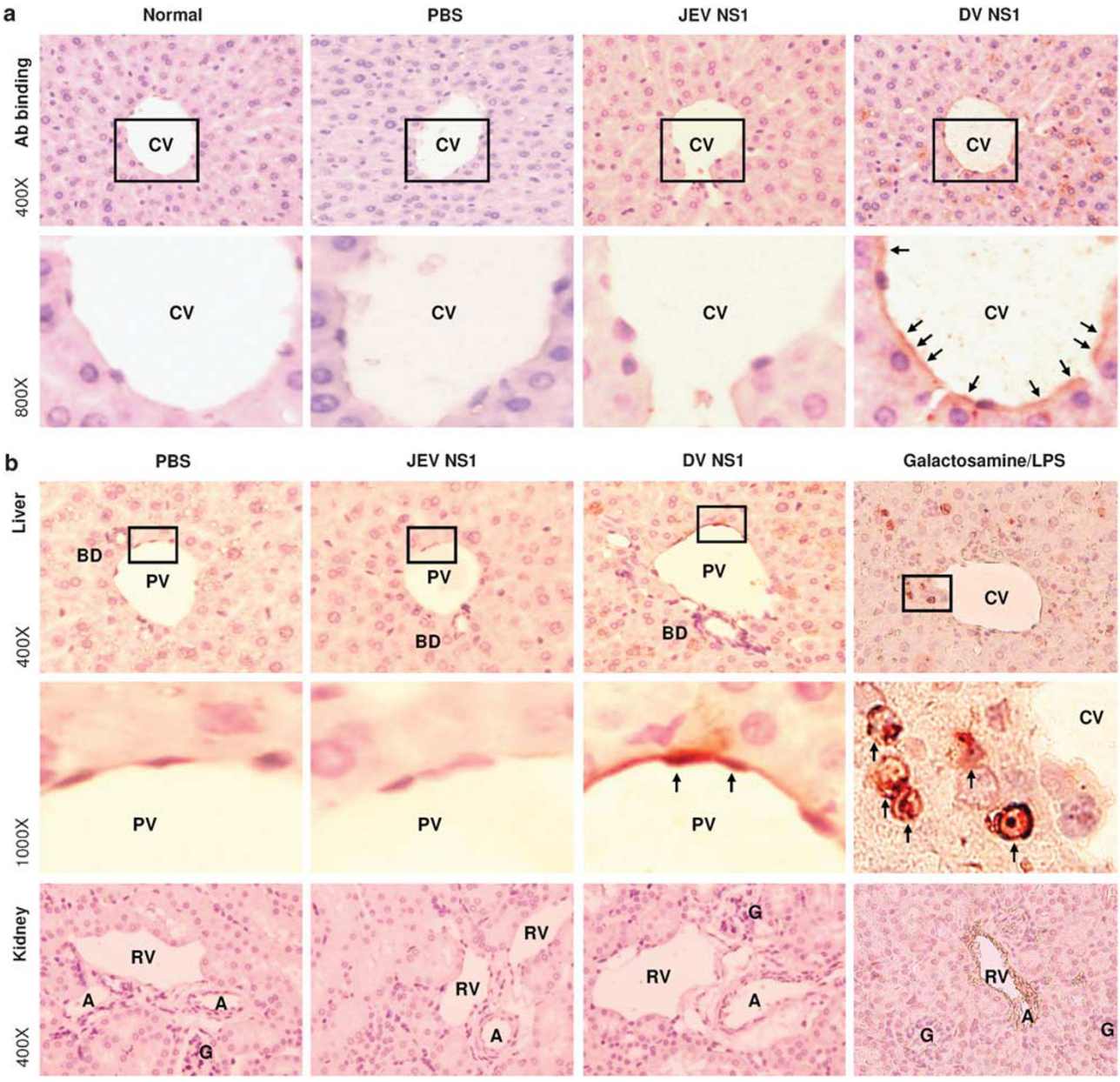

Figure 2 Antibody deposition and cell apoptosis in liver vessel endothelium from mice immunized with DV NS1. (**a**) Deposits of antibodies on liver vessel endothelium in DV NS1-immunized mice but not in PBS- or JEV NS1-immunized or normal mice. Specimens were viewed using a light microscope (magnification $\times 400$ and $\times 800$). Arrows indicate positive staining. (**b**) Liver and kidney sections were prepared from PBS-, JEV NS1-, and DV NS1-immunized mice, and cell apoptosis (arrows) was detected using a TUNEL assay (magnification $\times 400$ and $\times 1000$). Mice treated with galactosamine + LPS were used as the positive control. CV, central vein; PV, portal vein; BD, bile duct; RV, renal vein; A, artery; G, glomerulus.

was significantly reduced after NS1 preabsorption, and the ALT concentration showed a trend of reduction, although not statistically significant, by NS1 preabsorption (Figure 6a). We previously demonstrated the involvement of nitric oxide (NO) in anti-DV NS1-induced endothelial cell apoptosis.[38,39] Using NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) to investigate its protective effect in liver injury, we found that dengue patient IgG-induced mouse serum AST increase was partially inhibited by L-NAME (Figure 6b).

DISCUSSION

In the present study, we found that anti-DV NS1 antibodies cause liver injury in mice, which resembles the clinical manifestations seen in DHF patients.[28,29,44–47] In contrast to the liver, other organs such as kidney are not affected in our mouse model. Cases of dengue-induced acute kidney injury have been reported, as summarized by Lima *et al*.[48] In this study, we were unable to observe BUN increase or renal pathological changes in DV NS1-immunized mice. Also, IgG from DV NS1-immunized mice did not bind to kidney tissue.

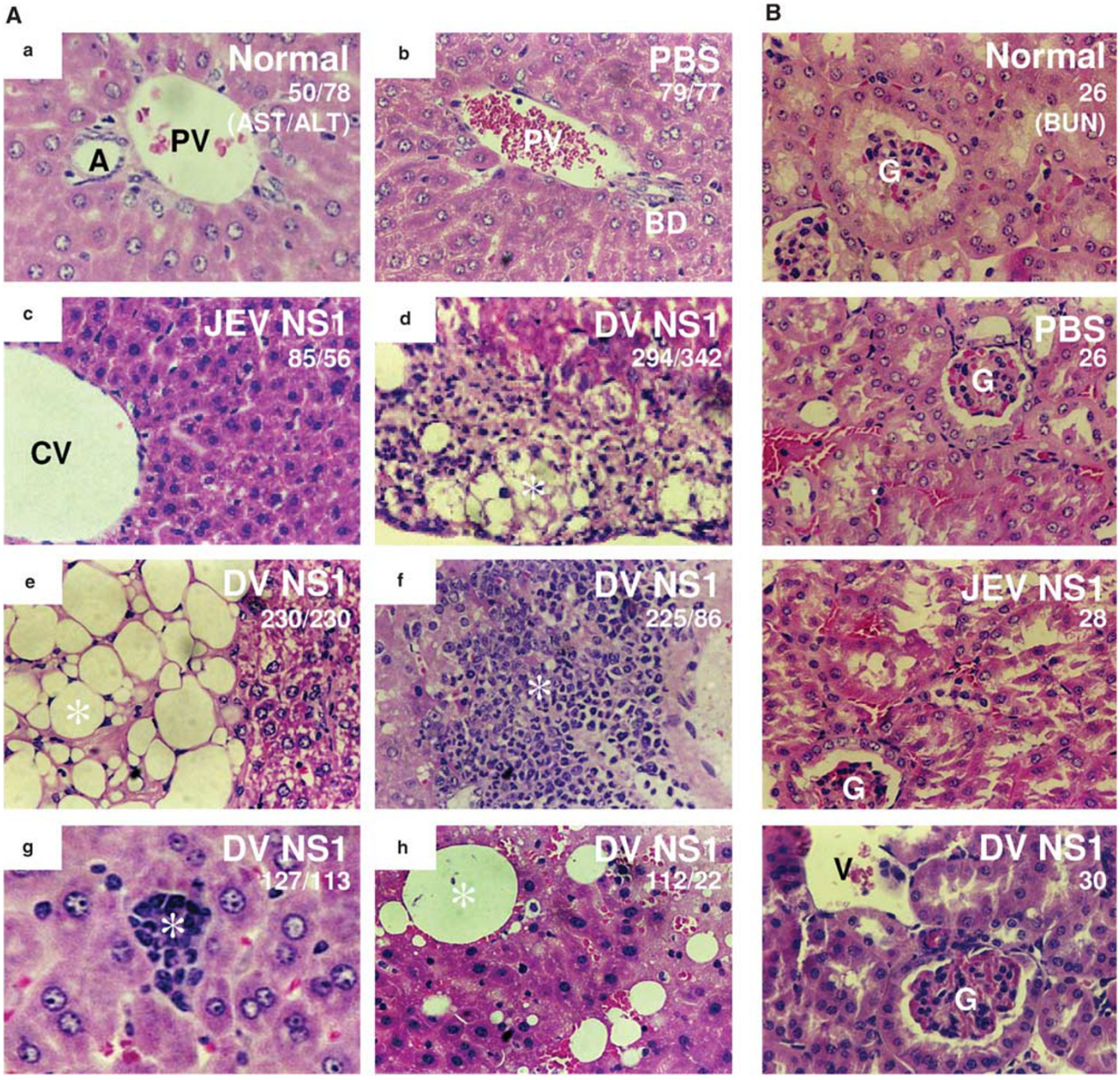

Figure 3 Histopathologic changes in the liver (**A**) and kidney (**B**) tissues of actively immunized mice. Using hematoxylin and eosin staining, the pathological changes and localization of infiltrated cells are marked with asterisks. (**a**): Normal; (**b**): PBS-immunized; (**c**): JEV NS1-immunized; (**d–h**): DV NS1-immunized. PV, portal vein; A, artery; BD, bile duct; CV, central vein; G, glomerulus. The AST, ALT, and BUN levels in mouse sera are shown.

We found apoptotic cells surrounding the vessels in the liver but not in kidney tissues in DV NS1-immunized mice. Therefore, we speculate that unlike liver damage in DV infection, kidney injury is independent of anti-DV NS1 effect. The basis of this organ specificity is a puzzle to be further investigated. Our preliminary results show several candidate target proteins on endothelial cells recognized by anti-NS1 antibodies. The possible differential expression of autoantigens between liver and kidney endothelium requires further investigation.

We demonstrate that anti-DV NS1 antibodies cause endothelial cell damage and mononuclear phagocyte infiltration in mouse liver after immunization with anti-DV NS1 antibodies. Our previous *in vitro* studies showed that anti-DV NS1 antibodies increased cytokine, chemokine, and cell adhesion molecule expression, as well as peripheral blood mononuclear cell adherence to endothelial cells.[40] Whether cytokine, chemokine, and cell adhesion molecule expression are induced in anti-NS1-treated mice remains to be determined. It has been reported that antibody-enhanced DV infection of monocytes triggers cytokine-mediated endothelial cell activation.[49] Therefore, both DV-infected monocytes and anti-NS1-mediated monocyte infiltration might be involved in endothelial cell activation and inflammatory responses in DV infection.

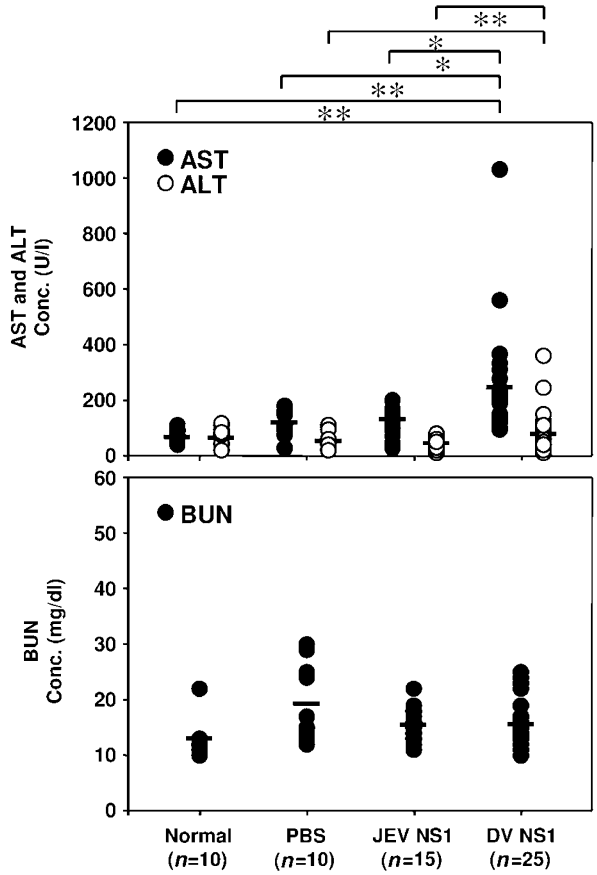

Figure 4 Serum AST and ALT, but not BUN, levels increase in mice immunized with DV NS1. The averages of each group are shown by the short horizontal lines in each column. *$P<0.05$; **$P<0.01$.

The causes of liver damage in DHF are multiple. DV can infect liver cells and cause damage.[30–33] Liver histopathology in fatal cases of DHF indicated that hepatocytes and Kupffer cells may be the target cells for viral replication and that an apoptotic mechanism was involved.[50] The autoantibodies present in dengue patient sera,[37] however, showed no cross-reactivity with liver cell lines tested, including Chang liver, HA22T, and Huh7 cells. In the present study, we showed that autoantibody-induced endothelial dysfunction may contribute to hepatic inflammation; whether mediated by Kupffer cells and infiltrated immune cells remains to be investigated. Although we showed that endothelial damage occurred mostly in portal and central veins, the possibility that anti-DV NS1 also caused sinusoid endothelial damage has not been excluded. Several autoimmune diseases associated with hepatic injury have been related to the generation of autoantibodies.[51,52] Liver damage in autoimmune diseases involves both cytotoxicity and inflammation facilitated directly or indirectly by autoantibodies. We previously showed that anti-DV NS1 antibodies induced endothelial cell apoptosis by a NO-regulated pathway.[38,39] Other studies have reported the involvement of NO in hepatotoxicity,[34,53–55] and that NO causes hepatocyte injury primarily by inducing apoptosis.[55,56] We found that dengue patient IgG-induced serum AST increase in mice was partially inhibited by NO synthase inhibitor L-NAME. Whether NO-induced endothelial cell dysfunction also plays a role in anti-DV NS1-mediated liver damage, as detected in our mouse model, requires further investigation.

Table 1 Serum levels of AST, ALT, and BUN in mice after passive administration with antibodies

Time	Untreated	Control IgG	Anti-JEV NS1	Anti-DV NS1	Galactosamine+LPS
AST (U/l)					
Day 0	65 ± 4.7	68 ± 5.1	68 ± 2.1	72 ± 3.6	86 ± 5.8
Day 1	57 ± 4.4	47 ± 3.6	74 ± 4.5	57 ± 5.6	> 30 ×
Day 2	84 ± 5.2	80 ± 6.6	76 ± 12.7	210 ± 8.3***	ND
ALT (U/l)					
Day 0	54 ± 4.5	70 ± 3.9	53 ± 5.6	51 ± 4.4	54 ± 4.5
Day 1	50 ± 3.1	58 ± 7.4	40 ± 3.5	57 ± 7.6	> 30 ×
Day 2	50 ± 4.9	76 ± 8.3	57 ± 10.1	146 ± 6.7***	ND
BUN (mg per 100 ml)					
Day 0	21 ± 1.4	23 ± 1.8	15 ± 1.5	20 ± 1.2	23 ± 2.9
Day 1	22 ± 1.1	25 ± 1.0	17 ± 0.9	17 ± 1.3	55 ± 3.9
Day 2	25 ± 1.9	26 ± 2.1	18 ± 1.2	20 ± 1.5	ND

Abbreviations: IgG, immunoglobulin G; LPS, lipopolysaccharide; JEV, Japanese encephalitis virus; DV, dengue virus; NS1, nonstructural protein 1; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen.
The AST, ALT, and BUN were measured using EKTACHEM DT60II (for BUN) and DTSCII (for AST and ALT) analyzers. The galactosamine plus LPS was used for positive control. Three individual experiments were performed in each group and the averages of values are expressed as mean ± s.d. ***$P<0.001$ as compared to untreated, control IgG, and anti-JEV NS1 groups.

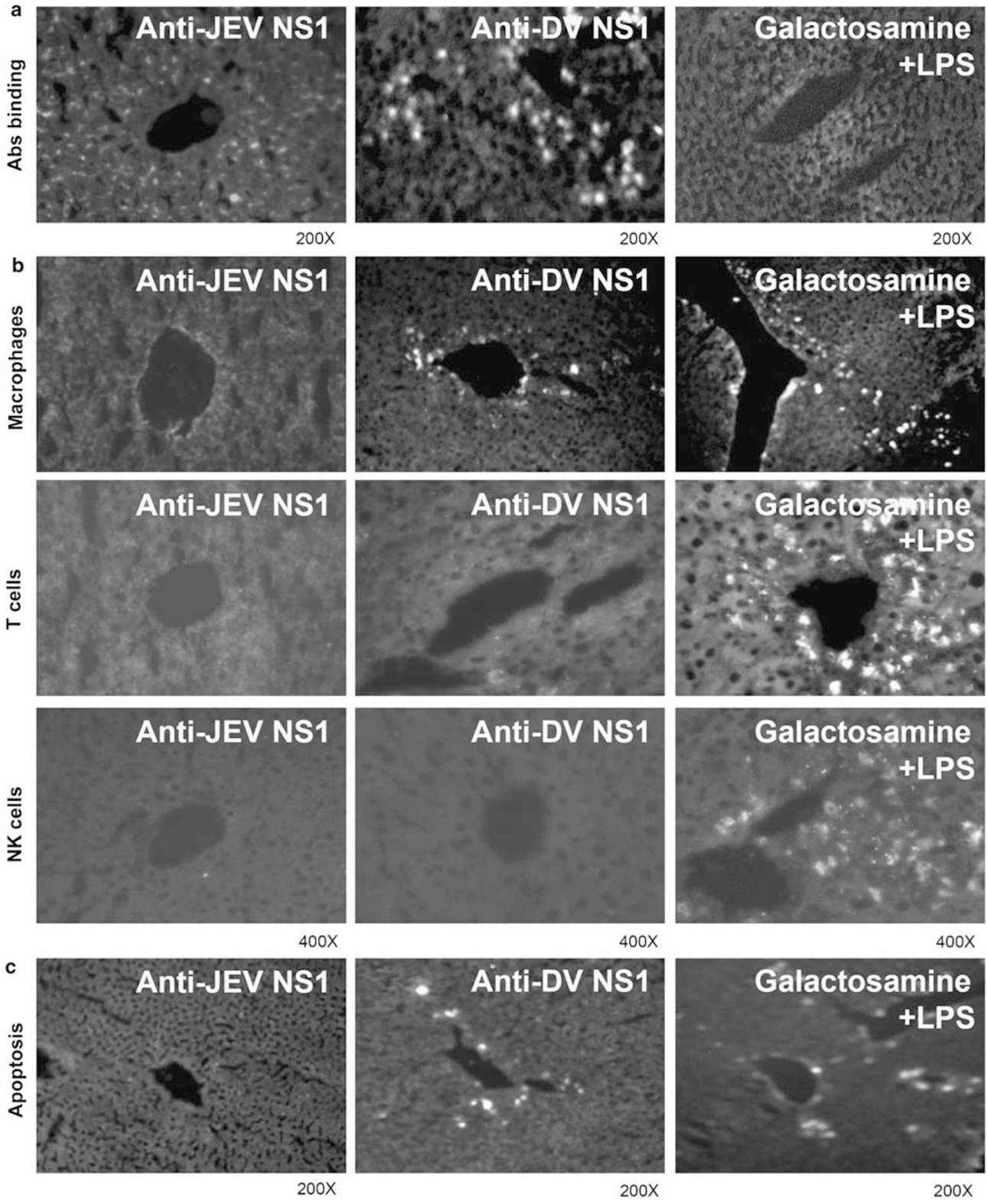

Figure 5 Cell infiltration and apoptosis in the liver of mice after passive immunization with anti-DV NS1 antibodies. (**a**) The cell binding activity of antibodies was shown by staining with FITC-conjugated anti-mouse IgG (magnification $\times 200$). (**b**) Immune cell infiltration of mononuclear phagocytes, T cells, and NK cells was detected using FITC-conjugated anti-F4/80 or anti-CD3 and PE-conjugated anti-CD16 antibodies (magnification $\times 400$). (**c**) Using TUNEL assay, cell apoptosis was detected in the liver tissue of anti-JEV NS1-, anti-DV NS1-, and galactosamine + LPS-treated mice. Galactosamine + LPS was the positive control (magnification $\times 200$).

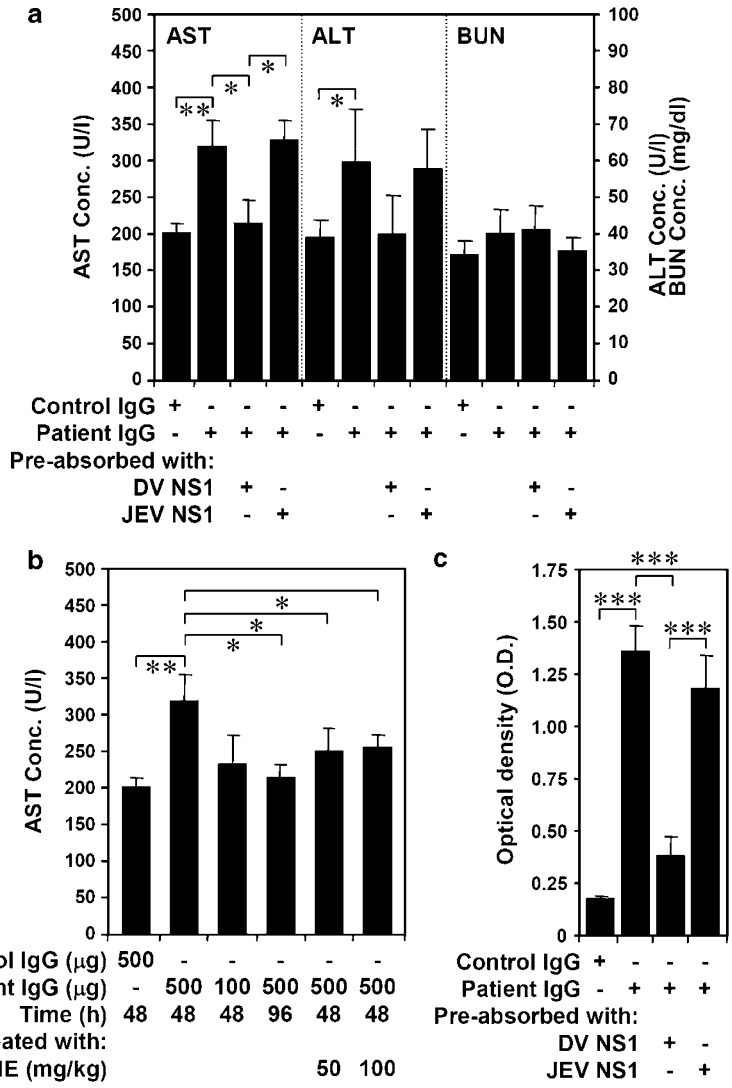

Figure 6 Serum AST and ALT levels increase in mice injected with IgG derived from dengue patient sera, but not in the group with DV NS1 protein preabsorption and NO synthesis inhibition. (**a**) Mice ($n=3$ per group) were intravenously injected with 500 μg IgG purified from the sera of healthy donors or dengue patients with or without DV NS1 or JEV NS1 preabsorption. After 48 h, the levels of serum AST, ALT, and BUN were determined using an autoanalyzer. The averages of values are shown as mean ± s.d. (**b**) Mice ($n=3$ per group) were intravenously injected with 100 or 500 μg of IgG purified from the sera of healthy donor or dengue patients for different time periods as indicated. In some groups, mice were injected intraperitoneally with different doses of NOS inhibitor L-NAME to investigate the effects of NO. The levels of serum AST were determined using an autoanalyzer and shown as mean ± s.d. (**c**) The efficiency of NS1 absorption was determined using ELISA and the optical density (OD) is shown. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

We previously reported that the levels of anti-endothelial cell antibodies were similar in patients infected by different DV serotypes.[57] These findings suggested that there is no serotype specificity for anti-NS1 autoantibody production. We also showed that the cross-reactivity of DV3-infected patient sera to endothelial cells, which led to induction of endothelial cell apoptosis, could be inhibited by DV2 NS1 preabsorption.[37] In this study, we showed that IgG purified from DV3-infected patient sera caused liver injury in mice, as evidenced by increased AST and ALT levels in mouse sera. Liver injury induced by IgG derived from patient sera was inhibited by preabsorption with DV2 NS1. Thus, consistent with our previous findings, anti-NS1 antibody-induced hepatic injury shows no dengue serotype specificity.

Although nonprimates are not natural hosts for DV, several DV-infected murine models have been established showing pathological effects resembling clinical symptoms in humans, including fever, rash, thrombocytopenia, and liver injury.[58–63] The present study demonstrates the pathogenic role of anti-DV NS1 antibodies in mice with manifestation of liver injury. This is the first reported animal model that, in the absence of virus itself, demonstrates an immune-mediated pathologic effect induced by DV protein. It provides important information for NS1 peptide-based vaccine

development, in which the generation of autoantibodies must be avoided.

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