JOURNAL OF VIROLOGY, June 2011, p. 5374–5383 Vol. 85, No. 11
0022-538X/11/$12.00 doi:10.1128/JVI.01915-10

Dengue Virus Type 3 Isolated from a Fatal Case with Visceral Complications Induces Enhanced Proinflammatory Responses and Apoptosis of Human Dendritic Cells▽†

Guilherme F. Silveira,[1] Florencia Meyer,[1]‡ Adriana Delfraro,[1,2] Ana Luiza P. Mosimann,[1] Norma Coluchi,[3] Cyntia Vasquez,[3] Christian M. Probst,[4] André Báfica,[5] Juliano Bordignon,[1]* and Claudia N. Duarte dos Santos[1]*

Laboratório de Virologia Molecular[1] and Laboratório de Genômica Funcional,[4] Instituto Carlos Chagas, FIOCRUZ, Curitiba, PR, Brazil; Sección Virologia, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay[2]; Laboratorio Central de Salud Publica, Servicio de Pediatria, Hospital Central, Instituto de Previsión Social, Asuncion, Paraguay[3]; and Laboratório de Imunofarmacologia e Doenças Infecciosas, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil[5]

Received 9 September 2010/Accepted 16 March 2011

A recent (2007 to 2009) dengue outbreak caused by dengue virus (DENV) in Paraguay presented unusual severe clinical outcomes associated with 50% mortality rates. Although it has been reported that inflammatory responses influence the severity of dengue virus infection (T. Pang, M. J. Cardosa, and M. G. Guzman, Immunol. Cell Biol. 85:43–45, 2007), there remains a paucity of information on virus-innate immunity interactions influencing clinical outcome. Using human dendritic cells from a major innate immune cell population as an *in vitro* model, we have investigated signature cytokine responses as well as infectivity-replicative profiles of DENV clinical isolates from either a nonfatal case of classical dengue fever (strain DENV3/290; isolated in Brazil in 2002) or a fatal case of dengue fever with visceral complications isolated in Paraguay in 2007 (strain DENV3/5532). Strain DENV3/5532 was found to display significantly higher replicative ability than DENV3/290 in monocyte-derived dendritic cells (mdDCs). In addition, compared to DENV3/290 results, mdDCs exposed to DENV3/5532 showed increased production of proinflammatory cytokines associated with higher rates of programmed cell death, as shown by annexin V staining. The observed phenotype was due to viral replication, and tumor necrosis factor alpha (TNF-α) appears to exert a protective effect on virus-induced mdDC apoptosis. These results suggest that the DENV3/5532 strain isolated from the fatal case replicates within human dendritic cells, modulating cell survival and synthesis of inflammatory mediators.

Dengue virus (DENV) is currently one of the most serious public health problems worldwide. The World Health Organization (WHO) estimates that about 80 million people become infected annually in more than 100 countries. About 550,000 of the infected patients require hospitalization, and at least 25,000 die due to dengue fever (DF) disease (47). Dengue virus is a member of the *Flaviviridae* family and *Flavivirus* genus, with a single-stranded RNA genome of positive polarity (26). There are four serotypes (DENV-1, -2, -3, and -4), and the virus is transmitted to humans through the bite of mosquitoes of the genus *Aedes* (5).

DENV causes a wide spectrum of clinical manifestations, from DF to dengue hemorrhagic fever (DHF), which may progress to dengue shock syndrome (DSS) (20, 43). DF is characterized by nonspecific symptoms such as headache, fever, arthralgia, myalgia, nausea, vomiting and rashes. In addition to these symptoms, DHF is also characterized by bleeding, thrombocytopenia, and plasma leakage, which are attributed to increased vascular permeability (29, 42, 46). This condition may progress rapidly to hypovolemic shock and, in many cases, lead to death. Over the last 10 years, a growing number of unusual clinical manifestations of DENV infection, including cardiac, neurological, hepatic and pulmonary disorders, have been observed. During an outbreak in Paraguay (2007 to 2009), such unusual manifestations were implicated in about half of the deaths caused by dengue virus infection in the country (35).

The pathophysiological mechanisms involved in development of DHF or DSS or unusual dengue manifestations are still unclear, and a complex interplay between viral and host factors may determine disease severity. Antibody-dependent enhancement (ADE) phenomena (21), cytokine storms (hypercytokinemia) (36), viral strain virulence (38), host cellular response (31), and innate immune responses (32) are among the factors that may contribute to DHF- or DSS-associated pathology and/or unusual manifestations of dengue virus infection.

Once DENV is introduced into the skin by a mosquito bite, its primary target cells are probably the skin-resident dendritic cells (DCs), Langerhans cells (LCs), and dendritic cells of the dermis (DDCs) (32). DCs are thought to be the most efficient antigen-presenting cells and are essential for capture, processing, and transport of antigens to the lymph nodes, where they

* Corresponding author. Mailing address: Instituto Carlos Chagas, FIOCRUZ, Rua Prof. Algacyr Munhoz Mader 3775, CIC, 81350-010, Curitiba, PR, Brazil. Phone: 55 41 3316-3230. Fax: 55 41 3316-3267. E-mail for Claudia N. Duarte dos Santos: clsantos@tecpar.br. E-mail for Juliano Bordignon: jbordig@tecpar.br.

‡ Present address: Mississippi State University, Starkville, MS.

† Supplemental material for this article may be found at http://jvi.asm.org/.

▽ Published ahead of print on 30 March 2011.

present the peptides to T cells. Thus, DCs are a key link between the innate and adaptive immune responses and may play a role in severe dengue fever pathogenesis (9). DENV infection of DCs during severe disease could deregulate cell functions such as DC maturation and cytokine responses. Nevertheless, there remains a paucity of information on whether clinical strains of dengue virus from fatal cases affect DC function and survival.

In the present study, we demonstrated that a dengue virus isolate (DENV3/5532) from a fatal case replicated in human monocyte-derived DCs (mdDCs) and induced high rates of programmed cell death compared to the rates seen with the classical DENV3/290 case isolate. Such effects were found to be associated with increased production of cytokines such as tumor necrosis factor alpha (TNF-α). Together, these findings suggest that DENV strains associated with fatal dengue fever disease modulate DC function and may contribute to the clinical outcomes observed in such infections.

MATERIALS AND METHODS

Cell cultures. Monocyte-derived dendritic cells were generated using peripheral blood from healthy volunteers after informed consent and approval from the FIOCRUZ Research Ethics Committee (approval no. 514/09). Mononuclear cells were separated by density gradient centrifugation using lymphocyte separation medium (Lonza, Walkersville, MD), and $CD14^+$ cells were purified by magnetic immunosorting (Miltenyi Biotec, Auburn, CA) in accordance with the manufacturer's recommendations. $CD14^+$ cells were cultured for 6 to 7 days in RPMI 1640 medium containing 100 ng/ml interleukin-4 (IL-4) and 50 ng/ml granulocyte-monocyte colony-stimulating factor (GM-CSF; PeproTech, Rocky Hill, NJ), 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Gibco-BRL, Grand Island, NY). Cultures were assessed by flow cytometry, and only cultures with purity of more than 80% with respect to CD1a, CD11c, CD11b, CD209, and HLA-DR and less than 5% with respect to CD14 were used (see Fig. S2 in the supplemental material). C6/36 mosquito cells were grown in Leibovitz-15 medium supplemented with 5% FBS, 0.26% tryptose, and 25 μg/ml gentamicin (all reagents from Gibco-BRL, Grand Island, NY).

Virus isolation and nucleotide sequencing. The DENV3/5532 virus used in this study was isolated from a patient with a fatal case of acute primary dengue fever with visceral manifestations in Lambaré (Asunción metropolitan area; 25°35′S and 57°65′W), Paraguay, during the epidemic in 2007. The DENV3/290 virus was obtained from a patient with a case of primary classical dengue fever in 2002, in Rio de Janeiro, southeast Brazil (22°57′S and 43°12′W). Viruses were isolated from the C6/36 mosquito cell line directly from plasma and passaged five times for DENV3/290 and three times for DENV3/5532. Culture supernatants were titrated using a focus-forming assay (18) and used for infection of mdDCs.

The complete nucleotide sequence of the DENV3/5532 strain was determined directly using purified PCR fragments, a primer-walking strategy, a Thermo Sequenase kit (USB Inc., OH), an ABI 3100 device, and the BigDye Terminator method (Applied Biosystems Inc.). The Phred/Phrap/Consed system package (12, 13, 16, 17) was used to assemble the fragments.

The nucleotide sequences of DENV3/5532 (bankit accession no. 1388619) and DENV3/290 (Entrez accession no. EF629369) were compared by alignment of complete nucleotide sequences followed by manual editing with BioEdit version 7.0.9.0 (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). Amino acid sequences were translated from nucleotide sequences using BioEdit and MEGA version 4.0 (44). 3′ RNA secondary structures were predicted with the mfold program (49) available on the Mobyle@pasteur server (http://mobyle.pasteur.fr/cgi-bin/portal.py).

Microarray experiments, bioinformatic analysis, and quantitative PCR (qPCR). Monocyte-derived dendritic cells derived from 10 different healthy donors were subjected to mock infection or infected with DENV3/5532 at a multiplicity of infection (MOI) of 5 for 2 h in the absence of FBS. The viral inoculum was removed, and cells were washed and cultured in RPMI 1640 medium plus 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Gibco-BRL, Grand Island, NY). Cells were collected at 6, 12, 24, and 48 h postinfection (hpi), and total RNA was extracted using an RNeasy minikit according to the manufacturer's recommendations (Qiagen, Valencia, CA). The RNAs (75 μg each) of all 10 mdDC cultures were pooled for microarray experiments and compared to the RNAs from mock-infected cells by the use of Human Gene 1.0 st version 1 array GeneChip slides from Affymetrix (Santa Clara, CA). The RNA pool was processed according to the manufacturer's recommendations (Affymetrix, Santa Clara, CA) for the amplification and *in vitro* transcription, purification, tagging, hybridization, and scanning of the slides using GeneChip Scanner 3000.

Generated files were analyzed for quality using Expression Console software (Affymetrix, Santa Clara, CA). The same software was used to normalize the data by the robust multiarray average (RMA) method (23). Log ratios for DENV3/5532 infected mdDC and mock-infected mdDC intensity signal values were generated, and a list of differentially expressed genes was obtained, consisting of those with 2-fold or greater differences in values. Data clustering was performed using Cluster 3.0 software with a Euclidian distance determination. The tool for analysis of GO Enrichments (TANGO) in Expander software was used to obtain GO results with statistical enhancer values of 0.05. The NCBI Entrez Gene database (www.ncbi.nih.gov) and the Gene Ontology database (www.geneontology.org) were used to define the cell signaling and metabolic pathways that were most relevant during mdDC infection with DENV3/5532.

To confirm the microarray data, we selected three modulated genes (OAS2, EIF2KA2, and IFIT1) for quantitative PCR (qPCR) analysis. Monocyte-derived DCs from six different donors were exposed to DENV3/5532 or DENV3/290 (MOI of 5) for 24, 48, and 72 hpi. Total RNA was extracted with an RNeasy minikit (Qiagen, Valencia, CA) according to the manufacturer's recommendations, and cDNA was generated using random primers (Invitrogen, Carlsbad, CA) and reverse transcriptase (Promega, Madison, WI). PCR amplifications were performed with SYBR green master mix (Applied Biosystems, Inc.). The following cycles were used for DNA amplification: 50°C for 2 min and 96°C for 10 min, followed by 40 cycles of 96°C for 15 s, 59°C for 30 s, and 72°C for 1 min (primer sequences are given in Table S1 in the supplemental material). Melting curves were used to verify product specificity. The 18S housekeeping gene was used to normalize the amplification reaction. Gene modulation was calculated from the delta of the threshold cycle (ΔC_T) as described by Fonseca et al. (15).

***In vitro* infections and cytokine and apoptosis measurements.** Monocyte-derived dendritic cells (4.0×10^5) from 4 different healthy donors were infected with DENV3/290 and DENV3/5532 (MOI of 5) for 2 h at 37°C in a 5% CO_2 atmosphere. Cells were then washed with fresh medium and plated in 24-well plates. Cells and supernatants were recovered at 24, 48, and 72 hpi. Supernatants were used to determine viral titers by the use of a focus-forming assay and to test for the presence of inflammatory cytokines (human inflammatory cytokine kit [IL-8, IL-1β, IL-6, IL-10, TNF-α, and IL-12p70]) and chemokines (human chemokine kit [IL-8, RANTES, MIG, monocyte chemoattractant protein 1 [MCP-1], and IL-10]) by cytometric bead array (CBA) technology (Becton & Dickinson, San Jose, CA) according to the manufacturer's instructions.

Infected and apoptotic cells were assessed by flow cytometry. To determine the number of infected mdDCs, cells were washed with phosphate buffered-saline (PBS) and blocked with fluorescence-activated cell sorter (FACS) buffer (5% FBS and 1% human serum type AB) for 20 min at room temperature (RT) (PBS and human serum type AB from Lonza, Walkersville, MD). Cells were fixed and permeabilized using a Cytofix-Cytoperm kit (Becton & Dickinson, San Jose, CA), stained with (flavivirus-specific) monoclonal antibody 4G2 for 30 min at 37°C, washed with Perm/Wash buffer, and incubated for 30 min with secondary antibody (donkey anti-mouse polyclonal antibody conjugated with phycoerythrin [PE]) (eBioscience, San Diego, CA). Cells were washed twice using Perm/Wash buffer and analyzed using a FACSCalibur system (Becton & Dickinson, San Jose, CA) and FACSCanto II (LAMEB/UFSC). FACS data were analyzed using FlowJo 2.2.8 software.

Apoptotic mdDCs were analyzed using an annexin V apoptosis detection kit (Becton & Dickinson, San Jose, CA) according to the manufacturer's recommendations as follows. Briefly, mdDCs were recovered at 24, 48, and 72 hpi, washed once with PBS, and stained with annexin V and propidium iodide. A sample of uninfected cells was tested for baseline apoptosis on day 0 (data not shown). Cells that were positive for annexin V and negative for propidium iodide were considered to be apoptotic.

TNF blockade experiments. Monocyte-derived dendritic cells from 5 healthy donors were infected with DENV3 (strain 5532 or 290; MOI of 5) for 2 h and exposed to RPMI media (nontreated cells), TNF-α (10 ng/well) (eBioscience, San Diego, CA), anti-TNF-α neutralizing antibody (eBioscience, San Diego, CA) (1:250), or isotype control monoclonal antibody (anti-green fluorescent protein [GFP]) (20 ng/well). After 72 h of infection, percentages of infected cells and numbers of apoptotic cells (annexin V) were determined by flow cytometry and TNF-α concentrations were measured by CBA as described above for all treated infected mdDC cultures.

Experiments involving inactivated virus. Monocyte-derived dendritic cells (4.0×10^5 cells) from 5 healthy donors were infected with DENV3/5532, DENV3/290 (MOI 5) or inactivated viral strains (DENV3/5532i and DENV3/290i) or subjected to mock infection. The inactivated viral preparations were obtained by gamma irradiation at the Center for Radiation Technology, CTR IPEN-CNEN/SP, Brazil. Viral inactivation was confirmed by determining the virus titers in culture supernatants of C6/36 cells infected with inactivated virus and by reverse transcription-PCR (data not shown). After treatment, cells were washed with fresh medium and plated in 24-well plates. Cells and supernatants were recovered at 72 hpi. Supernatants were used for viral and inflammatory cytokine and chemokine titrations as described before. Cells were used to determine the numbers of infected and apoptotic cells by flow cytometry as described above.

Statistical analysis. All data are reported as means ± standard deviations (SD) and used for one-way analysis of variance (ANOVA) or two-way ANOVA followed by a Bonferroni test. The level of significance for these analyses was set at $P \leq 0.05$. The analyses were performed using GraphPad Prism 3.0 software (GraphPad Software, San Diego, CA).

Accession numbers. The entire set of supporting microarray data has been filed in the Gene Expression Omnibus (GEO) public database under registration number GSE23986. The nucleotide sequence was deposited in GenBank under accession number HQ235027.

TABLE 1. Amino acid substitutions between DENV3/5532 and DENV3/290

Protein	DENV3/290 vs DENV3/5532
C	K(35)R
pre-M	R(86)H
E	T(266)I
	N(302)K[a]
	H(345)Y[a]
	E(360)D[a]
	T(471)I
NS1	H(77)Y
	I(93)T
NS2b	I(105)V
NS5	L(27)R
	I(50)T
	V(181)I
	N(835)D

[a] Mutations located in domain III of protein E (28a, 28b).

RESULTS

Clinical presentation, viral isolation, and nucleotide sequencing. A dengue virus type 3 infection outbreak in 2007 in which unusual clinical manifestations were associated with approximately 50% of the deaths caused a major public health problem in Paraguay, South America (35). Viral strain DENV3/5532 was isolated from a 56-year-old female patient from Lambaré, Paraguay. She presented with an acute dengue virus infection, which was further confirmed by anti-NS1 IgM-positive and IgG-negative enzyme-linked immunosorbent assay (ELISA) results (data not shown). The patient presented with shock syndrome 5 days after the onset of symptoms, with a 35% hematocrit value and metabolic acidosis. She was diagnosed as having classical dengue virus infection, with myocarditis and hepatitis confirmed by pathological analysis (data not shown). The patient died due to cardiogenic shock (reference 35a and personal communication).

Viral strain DENV3/5532 was confirmed to be DENV serotype 3, genotype III (Sri Lanka), by nucleotide sequencing. Comparison with the sequence of the DENV3/290 strain (reference sample of classical dengue virus type 3 in Brazil) revealed 14 amino acid substitutions, located in proteins prM, C, E, NS1, NS2B, and NS5 (Table 1); there were also two substitutions, T(151)G and C(332)T, in the 3′ untranslated region (UTR), changing the RNA secondary structures (see Fig. S1 in the supplemental material). No differences were observed in the NS2A, NS3, NS4A, and NS4B proteins or in the 5′ UTR.

Gene expression in mdDCs infected with the DENV3/5532 viral strain. Dendritic cells are thought to be important cell targets for dengue virus infection in humans (48). They are among the most important antigen-presenting cell types, establishing a link between innate and adaptive immunity (32). To evaluate the response of DCs to dengue virus infection, mdDC cultures from 10 healthy volunteer donors were infected with DENV3/5532 or subjected to mock infection for 6, 12, 24, and 48 h. Microarray analyses revealed an upregulation of 96 genes with a fold change (FC) ≥ 2 and a downregulation of 28 genes with a FC ≥ 2 (Fig. 1A). Gene ontology analyses classified the genes into the following categories: immune response ($n = 46$ [$P \leq 0.05$]); catalytic activity ($n = 7$; nonsignificant [NS]); cell adhesion ($n = 6$; NS); cell proliferation ($n = 5$; NS); other functions ($n = 29$; NS); and not defined ($n = 31$; NS). A more specific analysis of the functions of the genes affected by infection showed response to virus ($n = 26$ [$P \leq 0.05$]), ATP binding ($n = 7$ [$P \leq 0.05$]), inflammatory response ($n = 5$ [$P \leq 0.05$]), hydrolase activity ($n = 4$; NS), zinc ion binding ($n = 3$; NS), other functions ($n = 37$; NS), and not classified ($n = 42$; NS). The main biological processes related to the modulated genes were innate immunity response ($n = 8$ [$P \leq 0.05$]), oxidation-reduction ($n = 3$; NS), and chemotaxis ($n = 3$; NS) (Fig. 1B). Moreover, to biologically validate the microarray results, the transcription profiles of genes OAS2, EIF2KA2, and IFIT1 in mdDCs from 6 volunteer donors exposed to DENV/5532 or DENV3/290, a reference strain isolated from a case of classical DF, were analyzed by qPCR. The results confirmed that expression of all three selected genes in mdDCs was affected by DENV3/5532 infection. The DENV3/290 strain also affected the expression of OAS2 (Fig. 1C). These results suggest that DENV3/5532 may induce expression of a variety of genes during *in vitro* infection of mdDCs.

Human mdDCs display increased susceptibility to DENV3/5532 infection associated with an elevated rate of apoptosis. The ability of DENV to replicate in human cells differs between strains, which is consistent with the hypothesis of the dengue viral strain as a determinant of pathogenesis (20). mdDCs (from four different healthy donors) were found to be more susceptible to infection with DENV3/5532 than with DENV3/290 at 72 hpi ($P < 0.01$; Fig. 2A). To determine whether the higher infection rates resulted in production of increased numbers of infective viral particles, the viral titers in mdDCs culture supernatants were determined (Fig. 2B). Although, during the observed period, the difference in the viral titers (viral progeny produced) between the two groups (i.e., those infected with strain 5532 and those infected with strain 290) was approximately 0.5 to 1.0 $\log_{10}$, these results did not reach statistical significance. In addition, only $CD11c^+$ populations were found to be associated with viral infection (Fig. 2D and E).

The induction of apoptosis following viral infection contrib-

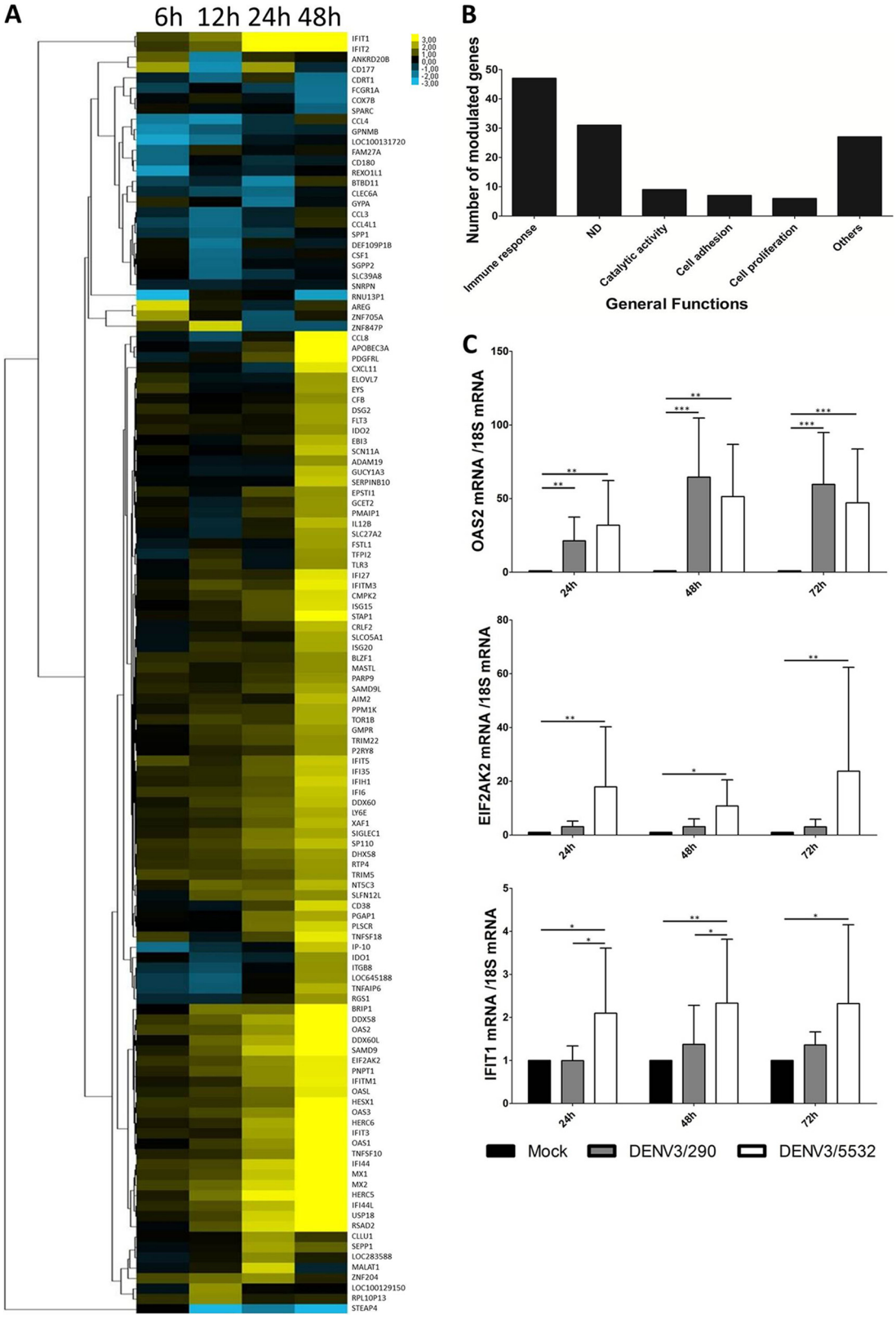

FIG. 1. (A) Hierarchical clustering (cluster 3.0) of the 124 modulated genes in mdDCs infected with DENV3/5532 (upregulated genes are indicated in yellow and downregulated genes in blue). (B) The functional annotation of 124 selected genes determined using Expander software and the NCBI Entrez Gene (www.ncbi.nih.gov) and Gene Ontology (www.geneontology.org) databases. ND, not defined. (C) Quantitative PCR analyzes of OAS2, IFIT1, and EIF2AK2 genes in mdDCs after infection with DENV3/5532 and DENV3/290 and mock infection. Data were analyzed using two-way ANOVA followed by a Bonferroni test; values represent means ± SDs of the results of six different experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

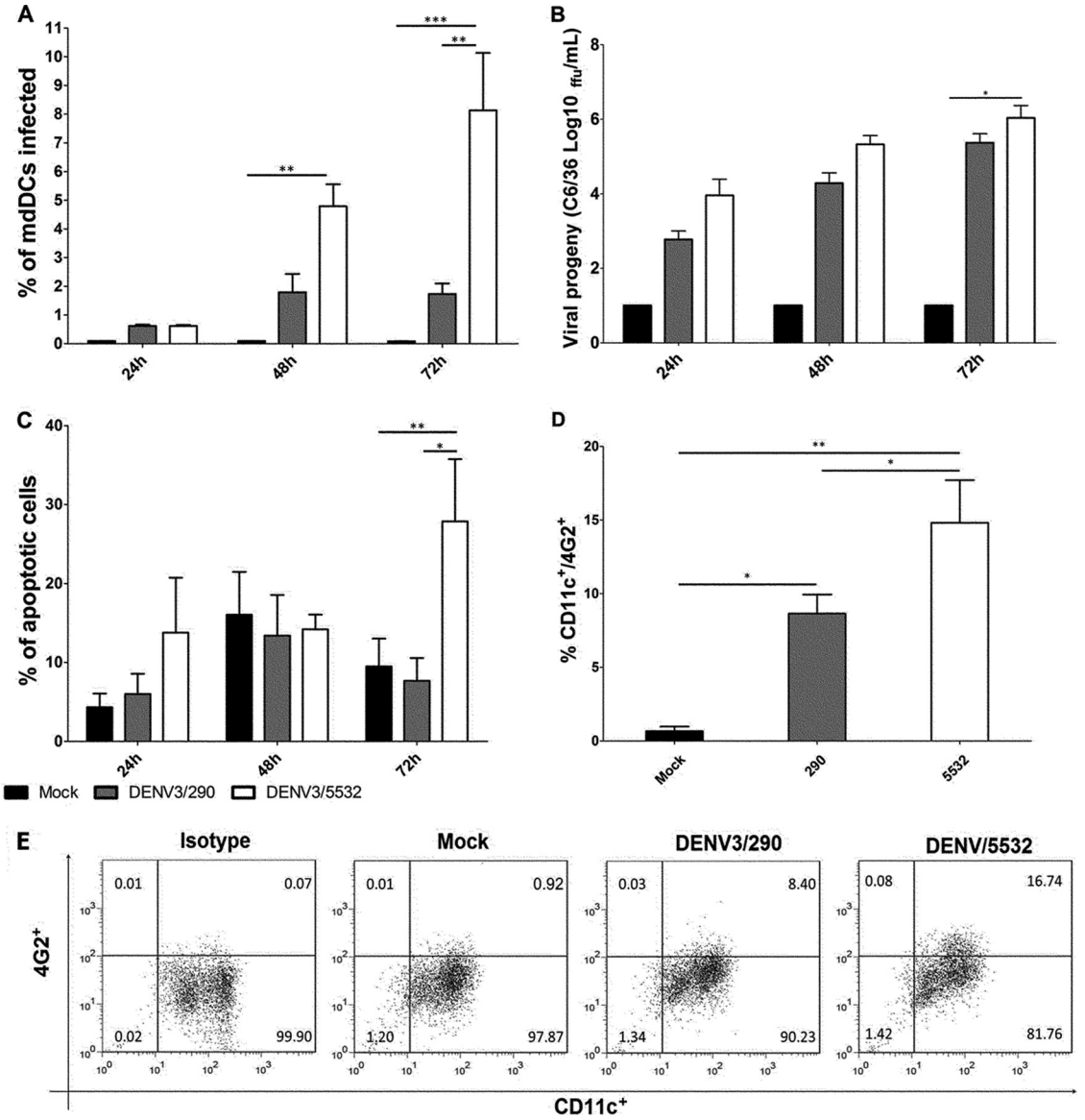

FIG. 2. DENV3/5532 displays higher infectivity and increased mdDC apoptosis compared to DENV3/290. (A) Percentages of infected mdDCs exposed to DENV3/290 or DENV3/5532 or mock infected. (B) Viral progeny in mdDC culture supernatants. For viral progeny, results are expressed in $\log_{10}$ focus-forming units (ffu) in C6/36 cells/ml. (C) Percentages of apoptotic cells. mdDCs were infected as described for panel A and assessed for annexin V-positive events by flow cytometry. Data were analyzed using two-way ANOVA followed by a Bonferroni test; values represent means ± SD of the results of four different experiments. (D) Percentages of mdDCs costained for $CD11c^+$ fluorescein isothiocyanate (FITC) and $4G2^+$ anti-flavivirus antibody plus anti-mouse PE conjugated by flow cytometry after 72 hpi. Data were analyzed using one-way ANOVA followed by a Bonferroni test; values represent means ± SD of the results of three different experiments. (E) Dot plot analyzes of one representative mdDC culture costained for $CD11c^+$ and $4G2^+$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

utes to the pathophysiological manifestations observed in flavivirus infections (8). To investigate whether the two strains differ in their levels of induction of programmed cell death, mdDCs (from four healthy donors) were infected with DENV3/5532 or DENV3/290 and apoptosis was analyzed by means of annexin V staining at 24, 48, and 72 hpi (Fig. 2C). At later time points (96 and 120 hpi), mdDC survival is compromised in the absence of IL-4 and GM-CSF (reference 22 and data not shown); thus, our analyses was restricted to 72 hpi. We observed that DENV3/5532 induces higher apoptosis rates than DENV3/290 at 72 hpi ($P < 0.05$). No differences in the induction of apoptosis at 24 and 48 hpi were observed for the two strains, suggesting that several rounds of viral replication are required to influence DC survival. These findings suggest that DENV3/5532 modulates DC survival, which appears to be dependent on viral replication.

DENV3/5532 infection induces increased production of inflammatory cytokines by mdDCs. Production of cytokines and chemokines by immune cells associated with dengue virus infection is thought to make a substantial contribution to the development of hemorrhagic manifestations, targeting vascular endothelial cells and causing fluid and protein leakage (36).

We evaluated the production by infected mdDCs (from four healthy donors) of inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-12p70, and IL-10) and chemokines (RANTES, interferon protein 10 [IP-10], MIG, and MCP-1), important DC function markers. TNF-α ($P < 0.05$) and IL-6 ($P < 0.05$) concentrations in culture supernatants at 72 hpi were enhanced in DENV3/5532-infected mdDCs (Fig. 3). Additionally, IP-10, MCP-1, and RANTES production was found to be elevated in mdDCs infected with both viral strains compared to mock-infected cell results (see Fig. S3 in the supplemental material). No differences in the synthesis of IL-10, IL-12p70, or IL-1β were found (data not shown).

Tumor necrosis factor alpha (TNF-α) is a proinflammatory cytokine that has been demonstrated to induce apoptosis of endothelial cells, contributing to the pathogenesis of DHF (4). To evaluate the role of TNF-α in mdDC survival, cells were stimulated with TNF-α or the cytokine activity was blocked with anti-TNF-α neutralizing antibody. Apoptosis was less frequent in DENV-infected mdDCs (mainly in those infected with DENV3/5532) treated with TNF-α than in anti-TNF-α-treated control cells ($P < 0.001$ [five different cultures]) (Fig. 4A), suggesting a protective role for TNF-α in DENV3-induced mdDC death. Additionally, a higher number of DENV3/5532-infected mdDCs was observed in the TNF-α-treated group, possibly due to increased cell survival induced by TNF-α. The isotype control was found to behave similarly to the nontreated group (filled bars in Fig. 4) in all experiments performed. The difference between the isotype-treated and anti-TNF-α groups in apoptosis observed with DENV3/5532-infected mdDCs was due to an outlier (data not shown). Together, these results suggest that TNF-α may play a protective role in DENV3-infected DC survival *in vitro*.

Viral replication is involved in mdDC apoptosis and cytokine production. Viral replication is an important factor in the development of dengue hemorrhagic fever. To determine whether viral replication affects apoptosis and cytokine production, mdDCs (from five healthy donors) were exposed to infective or radiation-inactivated DENV3 strains (Fig. 5). Apoptosis ($P < 0.01$) as well as production of proinflammatory cytokines (TNF-α, IL-6, and IL-8) and chemokines (MIG) ($P < 0.001$; data not shown) observed in DENV3/5532- or DENV3/290-infected mdDCs was found to be due to viral replication. These results suggest that mdDCs are infected by DENV3, which replicates within the cell, modulating DC function.

DISCUSSION

The nature of the pathophysiological mechanisms triggering severe forms of dengue virus infection (DHF and DSS) or unusual dengue fever manifestations is still unclear. However, the characteristics of the infecting viral strain certainly contribute to determining dengue pathogenesis (38). Comparison of the amino acid sequences of DENV3/5532 and DENV3/290 revealed 14 differences (Table 1) mapping both to structural proteins (one in protein prM, one in protein C, and five in protein E) and nonstructural proteins (one in NS2B, two in NS1, and four in NS5). Three of the mutations in protein E are located in its III domain, an Ig-like domain that has been implicated in receptor recognition (40). Differences in the

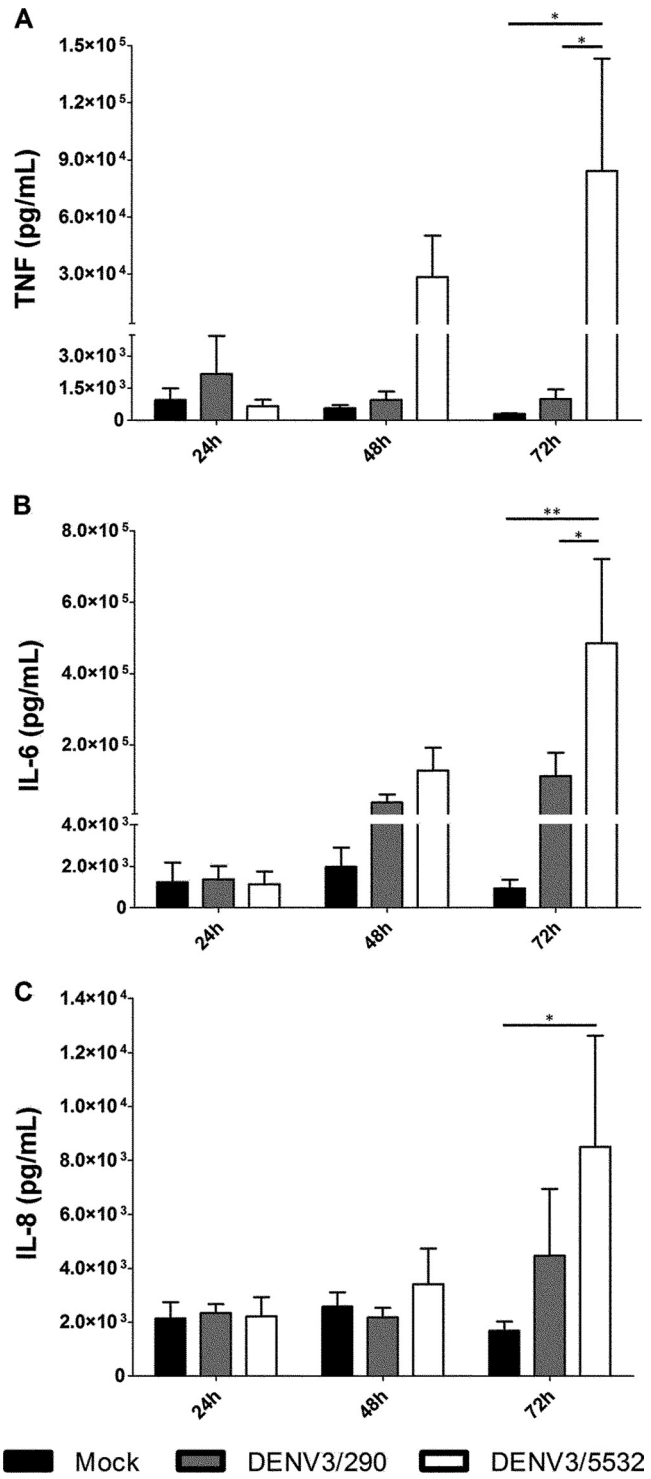

FIG. 3. DENV3/5532 enhances TNF-α, IL-6, and IL-8 production by mdDCs. mdDCs from four healthy donors were infected with DENV3/5532 or DENV3/290 or mock infected for several time points. Inflammatory cytokines were measured in cell culture supernatants by CBA as described in Materials and Methods. Data were analyzed using two-way ANOVA followed by a Bonferroni test; values represent means ± SD of the results of four different experiments. *, $P < 0.05$; **, $P < 0.01$.

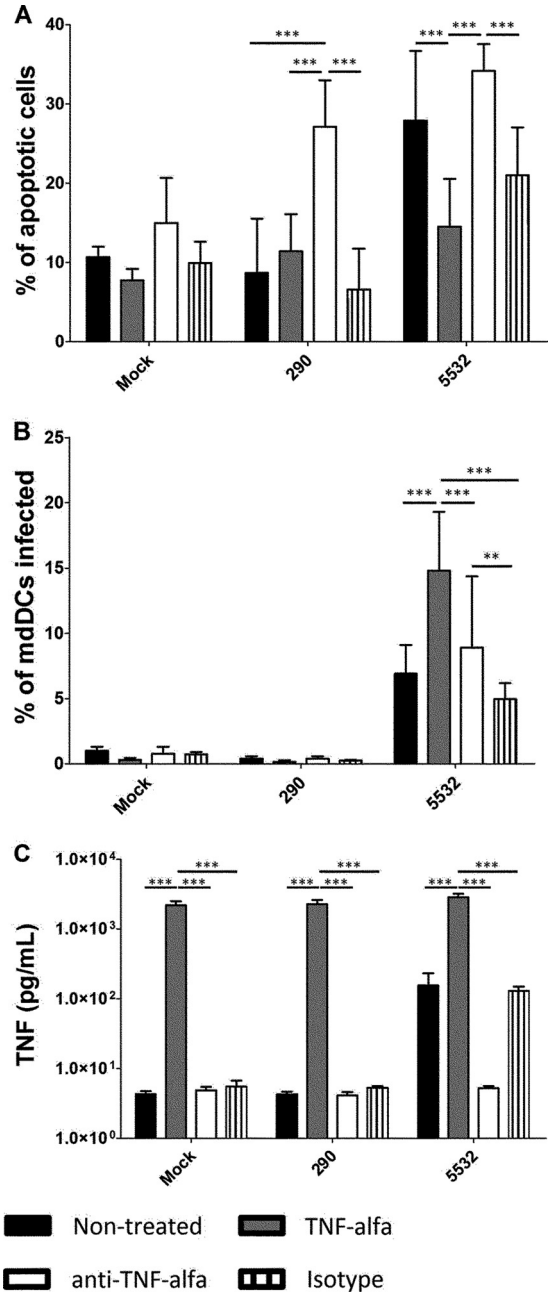

FIG. 4. Role of TNF-α in virus fitness and mdDC apoptosis. Cells undergoing apoptosis (A), percentages of infected cells (B), and levels of TNF-α (C) after 72 hpi with DENV3/290 or DENV3/5532 or mock infection and treatment with RPMI media (nontreated group), TNF-α (10 ng/well), anti-TNF-α neutralizing antibody, and an isotype control are indicated. Data were analyzed using two-way ANOVA followed by a Bonferroni test; values represent means ± SD of the results of five independent experiments. **, $P < 0.01$; ***, $P < 0.001$.

pathogenicities of the American and Asian genotypes of DENV2 have been related to mutations in the E protein (N390D) and in the 3′ and 5′ UTRs. These mutations contribute to a lower ability of the American genotype strains to replicate in human monocyte-derived macrophages and dendritic cells (7, 38). Conversely, the five mutations we report in nonstructural proteins may interfere with viral replication (10) and inhibit type I interferon (IFN) signaling (1, 31, 41).

The clinical outcome of a viral infection is essentially dependent on the balance between the host response and viral replication. The ability of the virus to evade or modulate host immune responses is critical to the development of disease (34). DCs are key cells in generating effective immune responses; for this reason, they represent a useful *ex vivo* model for studying dengue pathogenesis (9). In the present study, we demonstrated that strain DENV3/5532, isolated from a patient with a fatal case of dengue fever, replicates in mdDCs and differentially modulates several genes involved in innate immunity. Additionally, *in vitro* experiments showed that this viral strain induces higher rates of apoptosis and enhances secretion of proinflammatory cytokines and chemokines. We speculate that this process could have contributed to the development of the myocarditis, hepatitis, and death by shock of the patient. Unbalanced DC survival and function could impair immune responses and enhance dengue-associated disease (34).

Furthermore, dengue pathogenesis correlates positively with viral load; indeed, viremia peaks are 100 to 1,000 times higher in patients who develop DSS than in those with DF (25, 43). We observed that strain DENV3/5532 replicates more efficiently than strain DENV3/290 (isolated from a patient with a case of classical DF) in mdDCs (Fig. 2A and B), which is consistent with the enhanced virulence reported *in vivo*. The small (0.5 to 1.0 log) differences in viral progeny observed in mdDCs infected with the two viral strains can be explained by the high level of apoptosis observed in DENV3/5532-infected mdDCs, impairing production of viral particles. Alternatively, DENV3/5532 could show deficient viral assembly compared with DENV3/290, or it may have reached an infection plateau, although that is more unlikely.

Several studies in recent years have evaluated the host response to dengue virus infections by the use of high-throughput technologies such as microarrays (3, 14, 33, 45). These studies generally demonstrated substantial activation of the innate immune response, mainly mediated through the presence of type I IFN. Our microarray analyses of mdDCs infected with DENV3/5532 revealed the activation of interferon-stimulated genes (ISGs) with antiviral activity, including genes corresponding to the oligoadenylate synthetase (OAS) family and encoding protein providing resistance to myxovirus (Mx) infection, protein with tetratricopeptide repeats (IFIT), ISG15, and ISG20. Despite the induction of expression of several innate immune genes with antiviral activity in DENV3/5532-infected mdDCs, it appears that such a response is not sufficient to control virus infection in these cells. Additionally, microarray data revealed the upregulation of some inflammatory cytokines and chemokines, notably IP-10 (interferon protein 10 or CXCL10), IL-8 (interleukin-8 or CCL8), IL-12p40 (interleukin-12, subunit p40), CCL3, and CXCL11, which are important for the recruitment of other immune cells to the site of infection. Some of these genes, such as IP-10 and IL-8, also had their expression confirmed at the protein level (Fig. 3; see also Fig. S3 in the supplemental material). It has been increasingly recognized that the inflammatory response and deregulated cytokine production play key roles in the development of severe clinical manifestations of dengue virus infection (6).

TNF-α and IL-6 production by mdDCs was enhanced fol-

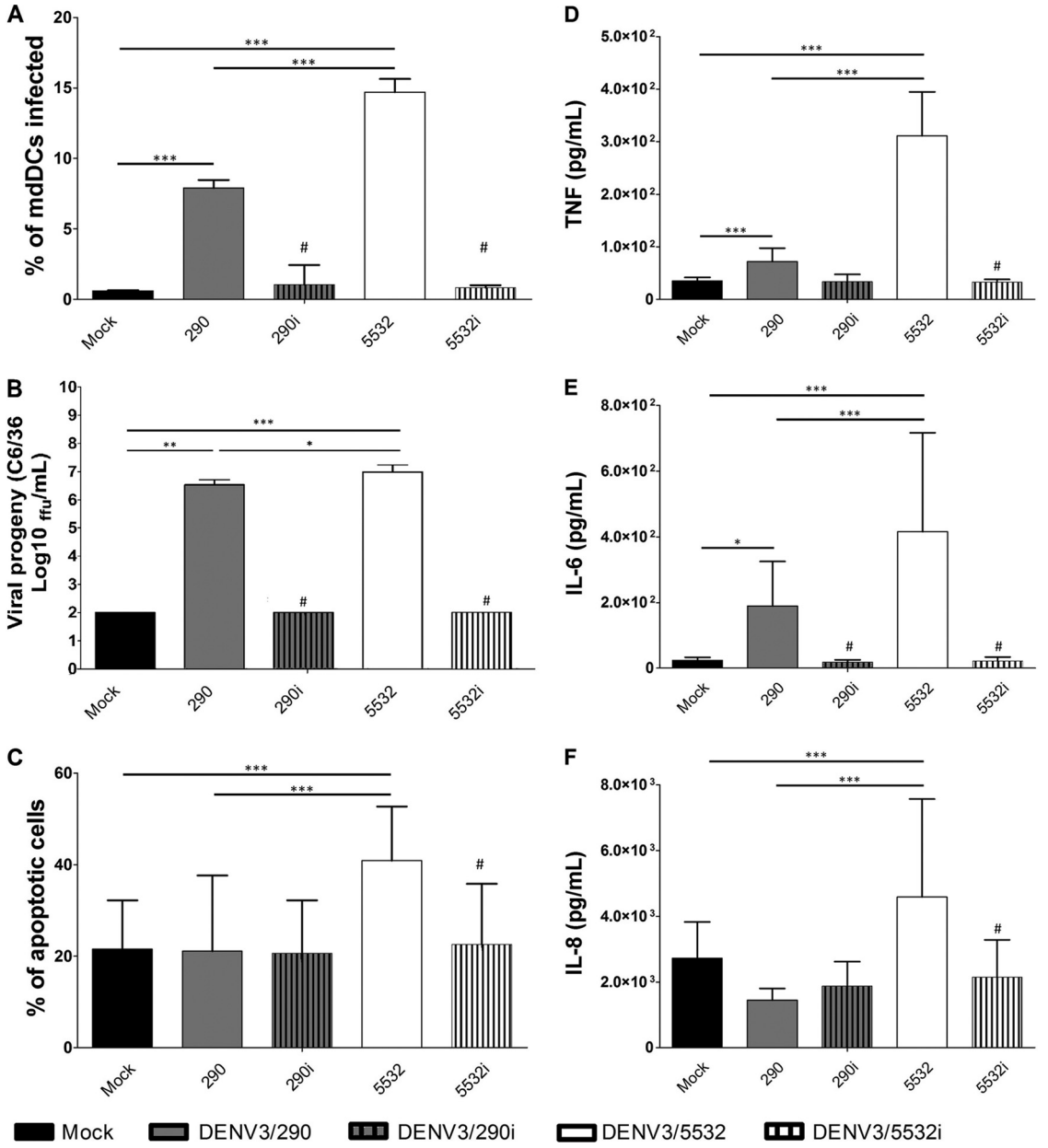

FIG. 5. Role of virus replication in fitness, apoptosis, and secretion of proinflammatory cytokines. Percentages of infected cells (A), viral progeny (B), and cells undergoing apoptosis (C) and levels of TNF-α (D), IL-6 (E), and IL-8 (F) after 72 hpi with DENV3/290, inactivated DENV3/290, DENV3/5532, or inactivated DENV3/5532 or mock infection are indicated. Data were analyzed using one-way ANOVA followed by a Bonferroni test; values represent means ± SD of the results of five different experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P < 0.05$ (compared to the native virus strain).

lowing infection with DENV3/5532 (Fig. 3A and B). The late (72 hpi) production of TNF-α and IL-6, and the low gene modulation observed at early time points (6 and 12 hpi) by microarray analyses, could have been due to the need for several rounds of DENV replication to trigger their production. Consistent with this, Ho et al. (22) demonstrated that the peak of TNF-α production by DENV-infected mdDCs occurs at 72 hpi. TNF-α has been implicated in endothelial cell damage by activating human dermal microvascular endothelial cells (HMEC-1) through the induction of cellular adhesion molecules such as ICAM-1 (4). Furthermore, TNF-α has been shown to induce apoptosis of HMEC-1 cells and may contribute to the plasma leakage observed in cases of DHF and DSS (4). Higher levels of IL-6 were also observed in Asiatic children exhibiting severe forms of dengue (DSS) (24) and are related to the development of autoantibodies against platelets and endothelial cells, contributing to the thrombocytopenia and plasma leakage (39). Likewise, IL-6 seems to be an important mediator of sepsis through the induction of proinflammatory cytokines, C-reactive protein (CRP), and NO synthesis (19). No statistically significant differences between the two viral strains were observed with respect to the levels of secretion of

RANTES, CXCL9 or MIG, MCP-1 (monocyte chemoattractant protein 1), IP-10, and IL-8 (Fig. 3), although the levels were modulated compared to mock-infected cell results.

Our results suggest that TNF-α appears to display a protective effect in mdDC apoptosis (Fig. 4A). In agreement with our data, a protective role for TNF-α in follicular dendritic cells (HK cell line) has previously been demonstrated, where it was shown that these cells proliferate in response to TNF-α treatment (37). TNF-α or microbial stimuli such as lipopolysaccharide (LPS) have been demonstrated to induce DC maturation, enhancing cell survival due to upregulation of antiapoptotic molecules like Bcl-X_L and Bcl-2 (27). In our model (see Fig. S5 in the supplemental material), we propose that, compared to the DENV3/290 strain, DENV3/5532 displays higher rates of replication and apoptosis in mdDCs as well as enhanced production of TNF-α. In contrast, blockade of TNF-α produced by infected mdDCs reduces cell maturation and enhances mdDC apoptosis.

Despite the fact that TNF-α has been implicated in the pathogenesis of DHF (4), it is difficult to exclude the possibility of a direct effect of dengue virus replication on the induction of apoptosis. It has been reported that the level of dengue virus replication in monocytes (11), the mouse central nervous system (10), and a human hepatoma cell line (28) is associated with induction of apoptosis. Similarly, we have observed that DENV3/5532 displays enhanced infection of mdDCs and that the enhancement was associated with apoptosis at 72 hpi (compared to both inactivated virus and DENV3/290 results). At the same time point, higher levels of TNF-α, IL-6, and IL-8 were also observed. Using inactivated virus, we found that the apoptosis rates and proinflammatory cytokine secretion observed with DENV3-infected mdDCs are dependent on viral replication (Fig. 5). Our data suggest that active viral replication and an unknown bystander effect are responsible for the cell death of mdDCs (see Fig. S4 in the supplemental material). Further studies are necessary to reveal the characteristics of DENV3-derived molecules influencing DC function and survival.

The data presented here could be of help to enhance understanding of the severe or fatal outcomes observed in several dengue patients, as previously suggested (2). Finally, in view of our data and taking into account previous results by Palmer et al. (34) in studies of the deficient activation of T cells by DCs infected with DENV, the high infection and apoptosis levels observed in DENV3/5532-infected mdDCs could impair DENV3 antigen presentation to T lymphocytes, affecting adaptive immune responses. Whether DENV3/5532 DC infection decreases T cell priming remains to be determined.

ACKNOWLEDGMENTS

We are indebted to Maria Eugenia Ribeiro de Camargo and Jörg Kobarg (Laboratório de Micro-arranjos) of the Laboratório Nacional de Luz Sincrotron (LNLS/Campinas/SP/Brasil) for technical support with microarray experiments. We thank Vanessa Stella, Paula R. Zanello, and Felipe Leprevost of the Instituto Carlos Chagas, FIOCRUZ, and Lucas L. Nogueira (LIDI/UFSC) and Verônica Horewics (LIDI/UFSC) for technical assistance. We thank Monica Mathor of the Instituto de Pesquisa em Energia Nuclear, IPEN, São Paulo, Brazil, for gamma irradiation of viral supernatants. We also acknowledge Samuel Goldenberg (ICC/Fiocruz) for continuous support.

Financial support was received from CNPq, Fundação Araucária, FIOCRUZ, PROSUL, and Red Iberoamericana en Virosis Emergentes (RIVE-CYTED). C.N.D.D.S., A.D., A.L.P.M., A.B., and F.M. are CNPq fellows.

REFERENCES

1. **Ashour, J., M. Laurent-Rolle, P. Y. Shi, and A. Garcia-Sastre.** 2009. NS5 of dengue virus mediates STAT2 binding and degradation. J. Virol. **83:**5408–5418.
2. **Balas, C., et al.** 2011. Different innate signatures induced in human monocyte-derived dendritic cells by wild-type dengue 3 virus, attenuated but reatogenic dengue 3 vaccine virus, or attenuated nonreatogenic dengue 1–4 vaccine virus strains. J. Infect. Dis. **203:**103–108.
3. **Becerra, A., et al.** 2009. Gene expression profiling of dengue infected human primary cells identifies secreted mediators in vivo. J. Med. Virol. **81:**1403–1411.
4. **Cardier, J. E., et al.** 2005. Proinflammatory factors present in sera from patients with acute dengue infection induce activation and apoptosis of human microvascular endothelial cells: possible role of TNF-α in endothelial cell damage in dengue. Cytokine **6:**359–365.
5. **Clark, G. G., C. L. Crabbs, C. L. Bailey, C. H. Calisher, and G. B. Craig, Jr.** 1986. Identification of Aedes campestris from New Mexico: with notes on the isolation of western equine encephalitis and other arboviruses. J. Am. Mosq. Control. Assoc. **2:**529–534.
6. **Clyde, K., J. L. Kyle, and E. Harris.** 2006. Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. J. Virol. **80:**11418–31141.
7. **Cologna, R., and R. Rico-Hesse.** 2003. American genotype structures decrease dengue virus output from human monocytes and dendritic cells. J. Virol. **77:**3929–3938.
8. **Courageot, M. P., A. Catteau, and P. Desprès.** 2003. Mechanisms of dengue virus-induced cell death. Adv. Virus Res. **60:**157–186.
9. **Dejnirattisai, W., et al.** 2008. A complex interplay among virus, dendritic cells, T cells, and cytokines in dengue virus infections. J. Immunol. **181:**5865–5874.
10. **Desprès, P., M. P. Frenkiel, P. E. Ceccaldi, C. N. D. Santos, and V. Deubel.** 1998. Apoptosis in the mouse central nervous system in response to infection with mouse-neurovirulent dengue viruses. J. Virol. **72:**823–829.
11. **Espina, L. M., N. J. Valero, J. M. Hernández, and J. A. Mosquera.** 2003. Increased apoptosis and expression of tumor necrosis factor-alpha caused by infection of cultured human monocytes with dengue virus. Am. J. Trop. Med. Hyg. **68:**48–53.
12. **Ewing, B., and P. Green.** 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res. **8:**186–194.
13. **Ewing, B., L. Hillier, M. Wendl, and P. Green.** 1998. Base calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res. **8:**175–185.
14. **Fink, J., et al.** 2007. Host gene expression profiling of dengue virus infection in cell lines and patients. PLoS Negl. Trop. Dis. **1:**e86.
15. **Fonseca, S. G., et al.** 2007. Locally produced survival cytokines IL-15 and IL-7 may be associated to the predominance of $CD8^+$ T cells at heart lesions of human chronic Chagas disease cardiomyopathy. Scand. J. Immunol. **66:**362–371.
16. **Gordon, D., C. Abajian, and P. Green.** 1998. Consed: a graphical tool for sequence finishing. Genome Res. **8:**195–202.
17. **Gordon, D., C. Desmarais, and P. Green.** 2001. Automated finishing with Autofinish. Genome Res. **11:**614–625.
18. **Gould, E. A., and J. C. S. Clegg.** 1985. Growth, titration and purification of togaviruses. *In* B. W. J. Mahy (ed.), Virology: a practical approach. IRL Press, Washington, DC.
19. **Hack, C. E., L. A. Aarden, and L. G. Thijs.** 1997. Role of cytokines in sepsis. Adv. Immunol. **66:**101–195.
20. **Halstead, S. B.** 1988. Pathogenesis of dengue: challenges to molecular biology. Science **239:**476–481.
21. **Halstead, S. B., and E. J. O'Rourke.** 1977. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. J. Exp. Med. **146:**201–217.
22. **Ho, L. J., et al.** 2001. Infection of human dendritic cells by dengue virus causes cell maturation and cytokine production. J. Immunol. **166:**1499–1506.
23. **Irizarry, R. A., et al.** 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4:**249–264.
24. **Levi, M., and T. van der Poll.** 2010. Inflammation and coagulation. Crit. Care Med. **38:**S26–S34.
25. **Libraty, D. H., et al.** 2002. High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. J. Infect. Dis. **186:**1165–1168.
26. **Lindenbach, B. D., and M. R. Rice.** 2001. Flaviviridae: the viruses and their replication, p. 1101–1152. *In* M. D. Knipe and M. P. Howley (ed.), Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
27. **Lundqvist, A., T. Nagata, R. Kiessling, and P. Pisa.** 2002. Mature dendritic cells are protected from Fas/CD95-mediated apoptosis by up-regulation of Bcl-X_L. Cancer Immunol. Immunother. **51:**139–144.
28. **Marianneau, P., A. Cardona, L. Edelman, V. Deubel, and P. Desprès.** 1997.

Dengue virus replication in human hepatoma cells activates NF-κB which in turn induces apoptotic cell death. J. Virol. **71:**3244–3249.

28a. **Modis, Y., S. Ogata, D. Clement, and S. C. Harrison.** 2003. A ligand-binding pocket in the dengue virus envelope glycoprotein. Proc. Natl. Acad. Sci. U. S. A. **100:**6986–6991.

28b. **Modis, Y., S. Ogata, D. Clement, and S. C. Harrison.** 2005. Variable surface epitopes in the crystal structure of dengue virus type 3 envelope glycoprotein. J. Virol. **79:**1223–1231.

29. **Mongkolsapaya, J., et al.** 2003. Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat. Med. **9:**921–927.

30. Reference deleted.

31. **Muñoz-Jordan, J., G. G. Sánchez-Burgos, M. Laurant-Rolle, and A. Garcia-Sastre.** 2003. Inhibition of interferon signaling by dengue virus. Proc. Natl. Acad. Sci. U. S. A. **100:**14333–14338.

32. **Navarro-Sánchez, E., P. Desprès, and L. Cedillo-Barrón.** 2005. Innate immune response to dengue virus. Arch. Med. Res. **36:**425–435.

33. **Nogueira, M. B., et al.** 2008. Evidence for the co-circulation of dengue virus type 3 genotypes III and V in the northern region of Brazil during the 2002-2004 epidemics. Mem. Inst. Oswaldo Cruz **103:**483–488.

34. **Palmer, D. R., et al.** 2005. Differential Effects of Dengue Virus on Infected and Bystander Dendritic Cells. J. Virol. **79:**2432–2439.

35. **Pan-American Health Organization.** 1995. Dengue type 3 infection. Nicaragua and Panama, October-November. Wkly. Epidemiol. Rec. **70:**41–43.

35a. **Pan-American Health Organization.** 2007. 15 November 2009, accession date. Emerging and reemerging infectious disease, region of the Americas—dengue outbreak in Paraguay: follow-up. Pan-American Health Organization, Washington, DC. http://www.paho.org/English/ad/dpc/cd/eid-eer-2007-03-15.htm.

36. **Pang, T., M. J. Cardosa, and M. G. Guzman.** 2007. Of cascades and perfect storms: the immunopathogenesis of dengue haemorrhagic fever-dengue shock syndrome (DHF/DSS). Immunol. Cell Biol. **85:**43–45.

37. **Park, S. M., H. Y. Park, and T. H. Lee.** 2003. Functional effects of TNF-a on a human follicular dendritic cell line: persistent NFκ-B activation and sensitization for Fas-mediated apoptosis. J. Immunol. **171:**3955–3962.

38. **Pryor, M. J., et al.** 2001. Replication of dengue virus type 2 in human monocyte-derived macrophages: comparisons of isolated and recombinant viruses with substitutions at amino acid 390 in the envelope glycoprotein. Am. J. Trop. Med. Hyg. **65:**427–434.

39. **Rachman, A., and I. Rinaldi.** 2006. Coagulopathy in dengue infection and the role of interleukin-6. Acta Med. Indones. **38:**105–108.

40. **Rey, F. A., F. X. Heinz, C. Mandl, C. Kunz, and S. C. Harrison.** 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2Å resolution. Nature **375:**291–298.

41. **Rodriguez-Madoz, J. R., et al.** 2010. Inhibition of the type I interferon response in human dendritic cells by dengue virus infection requires a catalytically active NS2B3 complex. J. Virol. **84:**9760–9774.

42. **Rothman, A. L.** 1997. Viral pathogenesis of dengue infections, p. 245–272. *In* D. J. Gubler and G. Kuno (ed.), Dengue and dengue hemorrhagic fever. CAB International, New York, NY.

43. **Rothman, A. L., and F. A. Ennis.** 1999. Immunopathogenesis of dengue hemorrhagic fever. Virology **257:**1–6.

44. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599.

45. **Warke, R. V., et al.** 2003. Dengue virus induces novel changes in gene expression of human umbilical vein endothelial cells. J. Virol. **77:**11822–11832.

46. **WHO (World Health Organization).** 1997. Dengue haemorrhagic fever: diagnosis, treatment, prevention and control, 2nd ed. World Health Organization, Geneva, Switzerland.

47. **WHO (World Health Organization).** 18 February 2010, accession date. Impact of dengue. http://www.who.int/csr/disease/dengue/impact/en/index.html.

48. **Wu, S. J., et al.** 2000. Human skin Langerhans cells are targets of dengue virus infection. Nat. Med. **6:**816–820.

49. **Zuker, M., D. H. Mathews, and D. H. Turner.** 1999. Algorithms and thermodynamics for RNA secondary structure Prediction: a practical guide. *In* J. Barciszewski and B. F. C. Clark (ed.), RNA biochemistry and biotechnology. Kluwer Academic Publishers, Boston, MA.