Antiviral and Immunomodulatory Effects of *Norantea brasiliensis* Choisy on Dengue Virus-2

Luciana Gomes Fialho^a Vagner Pereira da Silva^b Sônia Regina Nogueira Ignácio Reis^a
Elzinandes Leal Azeredo^a Maria Auxiliadora Coelho Kaplan^c
Maria Raquel Figueiredo^b Claire Fernandes Kubelka^a

^aLaboratório de Imunologia Viral, Instituto Oswaldo Cruz, FIOCRUZ, ^bLaboratório de Química de Produtos Naturais 3, Instituto Tecnológico de Fármacos, FIOCRUZ, and ^cInstituto de Pesquisas de Produtos Naturais, Centro de Ciências da Saúde, UFRJ, Rio de Janeiro, Brazil

Keywords

Dengue · *Norantea brasiliensis* Choisy · Antiviral effect · Immunomodulation

Abstract

Background/Aims: Severe dengue fever is a result of exacerbated immune responses and no specific treatments are available. We evaluated the antiviral and immunomodulatory effects of *Norantea brasiliensis* Choisy. **Methods:** Human adherent monocytes infected in vitro with dengue virus (DENV)-2 were incubated with the crude ethanol extract from leaves (NB1) or 3 derived fractions: dichloromethane (NB3), ethyl acetate (NB5), and butanolic (NB6) partitions. The antiviral and immunomodulatory activities were determined by intracellular detection of DENV antigen within monocytes and by secreted NS1 viral protein and cytokines. **Results:** The crude extract alone exhibited both antiviral activities (intracellular and secreted antigens) and all fractions derived from this extract modulated NS1 production. Regarding the immunomodulatory effect, among the secreted factors, TNF- α was inhibited by NB3 and NB6; IL-6 was inhibited by NB1, NB3, and NB6; IL-10 by NB1 and NB3; and IFN- α by NB6. The crude extract (NB1) presented the best antiviral

effect, whereas the dichloromethane fraction (NB3) presented an immunomodulatory effect in the inflammatory and anti-inflammatory cytokines. **Conclusion:** During in vitro DENV infection, *N. brasiliensis* Choisy exerts both antiviral and immunomodulatory effects that are likely associated, considering that less viral load may lead to less immunostimulation.

© 2017 S. Karger AG, Basel

Introduction

Among the viral diseases transmitted by mosquitoes, dengue is the one that spreads most quickly around the world. In the last 50 years, its incidence rate has increased 30% with geographic expansion into new countries [1]. In Brazil, it is placed among one of the most serious public health issues, with about 1.2 million cases reported until the nineteenth week of 2016 [2].

The disease is transmitted by mosquitoes, mainly by *Aedes aegypti*, which inoculates the virus while feeding [3]. Dengue virus (DENV) belongs to the *Flavivirus* genus, from the *Flaviviridae* family of 4 specific serotypes (DENV-1 to -4). Its genome encodes a polyprotein that is

translated into 3 structural proteins and 7 nonstructural proteins [4]. Among the nonstructural proteins, NS1 is expressed both in the resident form within the endoplasmic reticulum, associated with the membrane of infected cells or in soluble form in plasma of infected patients. It appears to be involved in viral replication [5, 6] and, moreover, it was recently described to be involved in the increase of endothelial permeability, which leads to vascular leakage during in vivo infections [7].

DENV has been reported to infect a wide range of cells including dendritic cells, monocytes, hepatocytes, and endothelial cells either in vivo [8, 9] or in vitro [10–15]. Infected cells become activated after infection, producing proinflammatory mediators including TNF, IL-6, and IL-8, among others that compromise the integrity of the endothelial cell layer, likely inducing vascular leakage [16–19]. On the other hand, monocytes may be involved in protective mechanisms by producing IFN- α in response to DENV [20] and becoming activated as CD14^{high} CD16⁺ monocytes that express Toll-like receptors and control exacerbated inflammatory responses, possibly by IL-10 production [21]. Previously, we reported immunomodulatory properties of *Uncaria tomentosa* extracts in a DENV-2 infection model of primary human monocytes, reducing TNF- α production and presenting antiviral activities [22]. Most studies involving natural products focus on their possible antiviral action against DENV, but do not pay attention to immunomodulatory effects.

Dysregulation of the immune system has long been treated in various diseases by herbal medicine and its therapeutic effects may be achieved by cytokine modulation [23, 24]. Natural products have become the main source of test material in the development of drugs based on traditional medical practices [25] and have been used to treat viral infections in animals and humans. Several plants have been reported to display antiviral activities [26–29]. More recently, these medicinal products are described as antiviral agents against DENV, inhibiting the viral load and/or their replication [30–32].

The Marcgraviaceae family is formed by 8 genera and approximately 110–130 species [33, 34], of which 4 genera and 35 species are present in the Brazilian flora. In this study, the Brazilian species *Norantea brasiliensis* Choisy, discovered by a Jacques Denis Choisy (1799–1859), was used [35]. This is a climbing shrub type that grows on the sandbanks of Rio de Janeiro, popularly known as “agarra-pé” (grab-foot) and is distributed in all Brazilian coastal sandbanks from Paraíba State to Santa Catarina, and along the coast of Rio de Janeiro State [36, 37]. The species is rich in flavonoids, considered chemo-

systematic markers for the Ericales order [38], and were detected in our samples by thin layer chromatography using specific reagents. Earlier, pharmacological tests were performed with this species in order to evaluate biodynamic potentials. The choice of *N. brasiliensis* species was based on prior knowledge of information on chemical features and biological activities of this plant's extracts, such as anti-inflammatory, analgesic, and trypanocidal properties [39]. The anti-inflammatory properties from the plant called our attention to new research on DENV infection since the disease has an inflammatory profile observed in patients known as a “cytokine storm,” which means there is a significant increase in the levels of soluble inflammatory mediators in the most severe forms of the disease [40–44]. Our aim was to investigate future potential for a new therapeutic agent for treating the disease.

In the present study, we assessed the antiviral and immunomodulatory activities of *N. brasiliensis* ethanol crude extract from leaves and 3 of its partitioned fractions in an in vitro model using a DENV-2-infected monocyte model, and identified the constituents of the fractions with potential for developing a future herbal medicine for dengue treatment.

Materials and Methods

Plant Material and Extraction

Leaves of *N. brasiliensis* were collected in Rio de Janeiro, Rio de Janeiro State, Brazil. The material was identified by the botanist Dr. Geisa Lauro Ferreira and a voucher specimen was deposited at the Herbarium of Jardim Botânico in Rio de Janeiro, Brazil (No. 152017). Dried and ground leaves (975 g) were submitted to extraction at room temperature with ethanol. The solvent was removed under low pressure yielding 170 g of the dried extract.

After sample preparation, they were solubilized in 1% DMSO, with the help of an ultrasonic bath. Then, samples were diluted in PBS pH 7.4, and stored in a concentration of 1 mg/mL. For sample sterilization, a Gammacell 220 Irradiator, with a cobalt-60 source from the Nuclear Instrumentation Laboratory (LIN)-UFRJ, was used at 62,700 rad. Samples were aliquoted and stored at –20°C. To confirm the sterilization, a Limulus Amebocyte Lysate (LAL) test (LONZA cat. N184-06) was used for endotoxin detection. The result of this test was negative for all samples (data not shown).

Liquid-Liquid Partition of Crude Extract

To perform the liquid-liquid partition procedure, part of this extract was solubilized in methanol:water 1/9 v/v. The total volume obtained in this hydroalcoholic solution was exactly the same obtained in each of the organic solvents used in the partition: hexane, dichloromethane, ethyl acetate, and butanol. The fractions resulting from partition techniques and the aqueous residue were concentrated in a rotary evaporator and lyophilized.

The subtitle for each extract/fraction used in this work is defined below:

- NB1 – Crude ethanol extract Leaves
- NB3 – Dichloromethane fraction
- NB5 – Ethyl acetate fraction
- NB6 – Butanolic fraction

Thin Layer Chromatography Analyses

Thin layer chromatography (TLC) analyses were performed in pre-coated silica gel 60 F₂₅₄ (Merck) plates using mobile phase butanol/acetic acid/water 4:1:5 v/v and sprayed with NP-PEG reagent and sequential UV irradiation at 365 nm. The profiles from the 4 samples are represented in Figure 1, with hyperoside as a chemical marker for the ethanol extract.

High-Performance Liquid Chromatography Profile of the Extracts and Fractions

Crude extract and fractions were analyzed by a high-performance liquid chromatography (HPLC) apparatus, which consisted of a Shimadzu (Kyoto, Japan) Nexxera Series 20 liquid chromatograph, 2 Shimadzu LC-20DXR pumps equipped with a binary gradient valve, a Shimadzu SPD-M20A diode array detector, a Shimadzu SIL-30AC autosampler, a Shimadzu CTO-20AC oven, a Shimadzu CBM 20-A communications bus module, and a Shimadzu DGU-20A5R degasser. Fractionation was achieved using a Supelco (Bellefonte, PA, USA) Supelcosil LC-18 column (250 × 4.6 mm i.d.; 5-μm particle size). The mobile phase was a methanol: water gradient, the injection volume was of 20 μL at 10 mg/mL, the flow rate was 1.0 mL/min and the effluent was monitored at 254 nm. The profile from the 4 preparations is represented in Figure 2.

Cell Viability Assay by MTT

Sample cytotoxicity was evaluated by an MTT assay. Human peripheral blood was provided by the Hospital Universitário Clementino Fraga Filho, UFRJ. Buffy coat cells from healthy donors were submitted through density gradient centrifugation (350 g, 30 min in Ficoll-Paque Plus, GE Healthcare Life Sciences) to isolate the human peripheral blood mononuclear leukocytes (PBMLs). PBMLs were plated in 96-well flat microtiter plates (3 × 10⁵ cells/well) with complete RPMI 1640 medium (with 200 mM glutamine, 100 U/mL penicillin, and 10 μg/streptomycin), supplemented with 10% fetal bovine serum (FBS) (both from Gibco, Life Technologies), and incubated for 24 h at 37°C with 5% CO₂ for monocyte adherence. After this period, the medium was removed with nonadherent cells and adherent cells were incubated with different dilutions (10 and 1 μg/mL) from NB extract/fractions for 48 h. For performance of cytotoxicity assays, RPMI 1640 without phenol red (Gibco, Life Technologies) was used, and then 10 μL of the 12 mM MTT reagent (Vybrant® MTT Cell Proliferation Assay Kit, Life Technologies cat. No. M6494) was added per well. The cells were incubated for 4 h at 37°C with 5% CO₂, adding 50 μL DMSO to solubilize the formazan formed in the process. The concentration was determined by D.O. at 570 nm by the SpectraMax spectrophotometer, using Paradigm Software SoftMax® Pro 6. Tested concentrations used in this work were determined as noncytotoxic for all samples at 48 h (>90% viability as compared to untreated cells; data not shown).

Virus and Cell Culture

The DENV-2 16681 original strain was obtained from Dr. S. Halstead, Military Institute of Research Walter Reed (Bethesda,

Fig. 1. Silica gel TLC chromatogram showing samples and standard derived from *N. brasiliensis*. Ethanol crude extract from leaves (NB1) and its fractions derived from liquid-liquid partition: dichloromethane fraction (NB3), hyperoside standard (H), ethyl acetate fraction (NB5), and butanol fraction (NB6). Mobile phase: butanol/acetic acid/water (4:1:5 v/v); spray reagent: NP-PEG.

MD, USA) [45] and used for virus stock preparation. Viruses were cultured and propagated in an *Aedes albopictus* cell line C6/36 at 28°C with Dulbecco's modified Eagle Medium (Gibco, Life Technologies) containing 5% FBS. The supernatant from the infected C6/36 cell was collected 8 days later and stored at -70°C for titer determination as well as subsequent experiments.

Virus Titration

The viral mass from DENV-2 was titrated by serial dilution cultures in 96-well microtiter plates and detected by immunofluorescence. Virus titer was calculated as 50% tissue culture infectious dose (TCID₅₀), according to the Reed and Munch formula [46]. Virus stock used showed titers of 1.37 × 10⁸ TCID₅₀/mL.

Human Peripheral Blood Mononuclear Phagocyte Enrichment

Human peripheral blood was provided by the Hospital Universitário Clementino Fraga Filho, UFRJ. Buffy coat cells from healthy donors were submitted to a density gradient centrifugation (350 g, 30 min in Ficoll-Paque Plus, GE Healthcare Life Sciences) to isolate the human PBMLs. After this procedure, cells were suspended in complete RPMI 1640 and incubated at 37°C under 5% CO₂ for 90 min to allow monocyte enrichment. Nonadherent cells were removed by washing while adherent cells were detached by mechanical cell harvesting with cell scrapers in a cold medium. Then, the recovered adherent cells – enriched monocytes – were suspended in complete RPMI supplemented with 10% FBS, seeded at 2 × 10⁶ cells in 1 mL on 48-well plates and incubated for 24 h.

Monocyte Infection and Treatment with *N. brasiliensis* Samples

After incubation, adherent monocytes were infected with a virus inoculum diluted (1/5) in cell culture medium (2.74 × 10⁷ TCID₅₀/mL in 300 μL) with a multiplicity of infection (MOI) of 4.1 per well. Cells were incubated for 2 h for virus adsorption and then

Fig. 2. High-performance liquid chromatograms from samples derived from *N. brasiliensis*. Ethanol crude extract from leaves (NB1) and its fractions derived from liquid-liquid partition: dichloromethane fraction (NB3), ethyl acetate fraction (NB5), and butanol fraction (NB6). Arrows: hyperoside standard.

the supernatant was replaced with a 2% FBS containing *N. brasiliensis* crude ethanol extract from leaves or either NB3, NB5, or NB6 fractions at different concentrations (10/1 µg/mL) and subsequently incubated at 37°C under 5% CO₂. After 48 h, supernatants were collected and stored at -20°C for cytokine measurement and cells were recovered for viral antigen determination. Wells containing no treatment or infection, fractions without infection, and infectious DENV were assayed.

Viral Antigen Detection by Flow Cytometry

Adherent monocytes previously treated or not with *N. brasiliensis* were recovered from plates by scraping with a plastic microtip in a cold cell culture medium. They were set at 1×10^6 cells/well in a 96-well plate, then centrifuged (350 g, 7 min) while supernatants were collected for subsequent measurement of cytokines/chemokines. The cells were then suspended in a wash buffer (phosphate-buffered saline pH 7.4 containing 1% bovine serum albumin and 0.1% NaN₃ (PBS/BSA). Afterwards, cells were fixed with a 2% paraformaldehyde PBS/BSA solution at 4°C for 20 min and permeabilized with a 0.15% saponin in PBS/BSA solution for 10 min. Permeabilized cells were blocked with 5% inactivated human plasma in PBS/BSA at 4°C for 30 min, washed, and incubated with mouse anti-dengue complex monoclonal antibody (1/100 dilution, Chemicon, Merck) at 4°C for 60 min. After washing, the cells were incubated with anti-mouse IgG labeled with Alexa Fluor™ 488 (1/400 dilution) for 30 min at 4°C. Cells were washed again

with PBS/BSA, recovered in 2% paraformaldehyde, and kept at 4°C until cell acquisition (10,000 events for gated monocytes) by a FACSCalibur flow cytometer (Beckon and Dickinson) and analyzed with FlowJo Software (TreeStar Inc.). An isotype-matched antibody was adopted as a negative control.

NS1 Semiquantitative Detection by ELISA

To evaluate the antiviral activity from *N. brasiliensis* samples, the DENV nonstructural protein, NS1, was evaluated. For this, the culture supernatants submitted to different treatment conditions and previously stored at -20°C were used for determining NS1 ratios with the Platelia Dengue NS1 Ag Kit (BioRad No. 72830), according to the manufacturer's instructions. Plates were read at 450 nm in the SpectraMax Paradigm Software SoftMax® Pro 6. To calculate the NS1 ratio, the optical density (OD) from tested samples was divided by the OD from the "cutoff" sample supplied by the kit (average of duplicates from kit calibrator, CO).

Cytokine/Chemokines Detection by Multiplex Microbead Immunoassay

To evaluate the immunomodulatory activity from *N. brasiliensis* samples, culture supernatants from cells submitted to different treatment conditions and previously stored at -20°C were also used for cytokine determinations of TNF-α, IL-6, IL-10, and IFN-α. A high-sensitivity multiplex biometric immunoassay containing fluorescent dyed microspheres conjugated with a mono-

clonal antibody specific for a target protein was employed according to the manufacturer's instructions (Upstate) and following an earlier description [22]. The assay's sensibility range was 1.95–8,000 pg/mL. Cytokine levels were determined with a multiplex array reader from Luminex Instrumentation System (Bio-Plex workstation, Bio-Rad Laboratories). The analyte concentration was calculated with a software provided by the manufacturer (Bio-Plex Manager Software). It provided a regression analysis to derive the equation for cytokine concentration prediction in samples.

Statistical Analysis

Data were initially tested for normality, with Prism GraphPad Software version 6, using the Kolmogorov-Smirnov test. Once data groups mostly did not pass the normality test, we performed a 2-group comparison between virus infected cultures submitted to plant treatment with untreated ones that were evaluated for significance with the paired and nonparametric statistical Wilcoxon test. Altered parameters were considered significant when $p < 0.05$.

Results

In vitro Antiviral Activity of N. brasiliensis Choisy in DENV-Infected Monocyte-Enriched Cells Detected by Intracellular Antigen Labeling and NS1 Secretion

DENV-2 strain 16681 has been efficient in inducing monocyte infection and the intracellular antigen detection peak occurred on the second day after infection [47]. This model has already been used for testing *U. tomentosa* antiviral and immunomodulating activities [22]. Here, the strain 16681 was used for in vitro infection of human adherent PBMLs – monocyte-enriched cells – to test the effects of *N. brasiliensis* Choisy on dengue infection and cytokine production.

In vitro antiviral activities of *N. brasiliensis* on DENV-2 were assessed by 2 different approaches from the same culture: DENV-infected adherent PBMLs, enriched for monocytes, treated with 1 or 10 µg/mL of the crude extract or its 3 partition fractions derived from the *N. brasiliensis*. After 48 h of infection, the culture supernatant was removed. Cells were labelled for intracellular detection of DENV-Ag⁺ cell rates and analyzed by flow cytometry in the regions containing cells with monocyte morphology accordingly to FSC and SSC parameters (Fig. 3a; 80–90% gated cells are CD14⁺). The crude NB1 extract significantly decreased monocyte DENV-Ag detection at 1- and 10-µg/mL doses (Fig. 3a, b). Treatments with NB3, NB5, and NB6 fractions demonstrated no effect on DENV infection rates in any of the tested concentrations. The 10-µg/mL dose of NB3 and NB5 presented unspecific Ag labeling in uninfected cells (data not shown).

The decrease in NS1 secretion detected by ELISA was statistically significant when the infected monocytes were

treated either with the crude NB1 extract or its 3 fractions, NB3, NB5, or NB6, with a 1-µg/mL dose. None of the tested samples demonstrated antiviral activities by reducing NS1 rates with a 10-µg/mL dose (Fig. 4).

In general, NB1 presented more efficient effects in DENV antiviral activity, reducing both intracellular detection of DENV Ag and NS1 viral protein reduction at a 1-µg/mL concentration.

In vitro Immunomodulatory Activity of N. brasiliensis Detected by Immunofluorescent Assays in Cytokine Production by DENV-Infected Monocyte-Enriched Cells

The in vitro *N. brasiliensis* immunomodulatory activity was detected on the same DENV-2 infected adherent PBMLs; monocyte-enriched culture supernatants assayed for DENV antigens at 48 h postinfection and TNF-α, IL-6, IL-10, and IFN-α determinations were assessed by the LuminexTM technique as described in materials and methods. Considering that the 10-µg/mL dose did not present effect in the NS1 antiviral assay, only the 1-µg/mL dose was used for the immunomodulation assay.

In TNF-α determination, a significant modulation of this cytokine was observed when infected monocytes were treated with NB3 and NB6 fractions (Fig. 5a). No significant changes were detected with the crude NB1 extract or with NB5 fraction treatments. NB1, NB3, and NB6 extracts significantly inhibited IL-6 (Fig. 5b), and IL-10 levels were downregulated significantly by NB1 and NB3, with a tendency for IL-10 reduction by NB6 (Fig. 5c) in monocyte supernatants. The NB6 fraction also significantly reduced IFN-α production in the infected monocyte culture supernatants (Fig. 5d). The NB5 fraction was ineffective in inducing immunomodulatory activities that were assayed.

Discussion

Physiopathology of DENV in patients with severe dengue characteristically involves a “cytokine storm,” presenting high levels of circulating cytokines and chemokines detected in circulating blood and associated with severe outcomes, including shock and hemorrhages. Proinflammatory cytokines such as TNF-α, IL-6, and IFN-γ, as well as anti-inflammatory factors such as IL-10, and chemokines such as IL-8, MIP-1β, and MCP-1, among others, are believed to be acting on endothelium integrity, disturbing hemostasis and the coagulation cascade [41–44, 48]. There-

(For legend see next page.)

Fig. 4. Nonstructural protein NS1 production by enriched monocytes in DENV-2-infected cultures treated with NB compounds derived from *N. brasiliensis*. Enriched human monocytes were infected with DENV-2 (2.74×10^7 TCID₅₀/mL; MOI = 4.1) and treated for 48 h with 1 µg/mL from the ethanol crude extract (NB1) and its dichloromethane (NB3), ethyl acetate (NB5), and butanolic (NB6) fractions (gray), or untreated (black). Medium, crude ex-

tract, or fraction was used in uninfected cultures as controls (white). The NS1 ratio was calculated using the sample optical density (OD) and NS1 cutoff OD. Values from 8 independent experiments (mean $\pm$ SEM). Treated samples were compared to untreated ones by Wilcoxon test. * $p < 0.05$.

Fig. 3. DENV Ag detection in monocyte population infected with DENV-2 after treatment with ethanol crude extract (NB1) from *N. brasiliensis*. Enriched human monocyte coats were infected with DENV-2 (2.74×10^7 TCID₅₀/mL; MOI = 4.1) and treated with ethanol crude extract (NB1) or untreated. DENV Ag was intracellularly detected by flow cytometry analysis (Alexa-647). **a** Gated monocyte population accordingly to size (FSC) and granularity (SSC) parameters and representative dot plots within the mono-

cyte gate from uninfected, DENV-infected, or DENV-infected and NB1 (1 or 10 µg/mL) treated cultures (SSC vs. FL8-Alexa-647). **b** Histograms obtained from data of 8 PBML donors. Enriched monocytes were cultured with medium (white), treated with 1 or 10 µg/mL NB1 (gray), DENV infected (black), or DENV infected in presence of 1 or 10 µg/mL NB1 (light gray). Values from 8 independent experiments (mean $\pm$ SEM). Treated samples were compared to untreated ones by Wilcoxon paired test. * $p < 0.05$.

Fig. 5. Cytokine production by enriched monocytes in DENV-2-infected cultures treated with NB compounds derived from *N. brasiliensis*. Enriched human monocytes were infected with DENV-2 (2.74×10^7 TCID₅₀/mL; MOI = 4.1) and treated for 48 h with 1 µg/mL of the ethanol crude extract (NB1) and its dichloromethane (NB3), ethyl acetate (NB5), and butanolic (NB6) frac-

tions (gray), or untreated (black). Medium, crude extract, or fraction was used in uninfected cultures as controls (white). TNF-α, IL-6, IL-10, and IFN-α were assayed using Luminex™ technology. Values from 8 independent experiments (mean ± SEM). Treated samples were compared to untreated ones by Wilcoxon test. * $p < 0.05$, ** $p < 0.01$, • $p = 0.0781$ (IL-10) and • $p = 0.0625$ (IFN-α).

fore, serum levels of these molecules may serve as a laboratory tool for predicting severe disease [49].

Here we adopted our earlier model of human primary monocytes [22] to evaluate the immunomodulatory potential of extracts from *N. brasiliensis* Choisy and observed downregulation of TNF-α, IFN-α, IL-6, and IL-10, associated with antiviral effects detected by reduction in cellular antigenic viral load and secreted NS1 protein.

This study stands out for its use of primary monocytes, the main target cells for DENV in patients [14], and its aim to identify medicinal plants with effects against DENV. This cell type is relevant for the in vitro performed assays, regarding its susceptibility to DENV-2 infection and capability to induce cytokines and other inflammatory mediators related to dengue severity during infection [22, 47, 50].

There are currently no specific treatments for dengue fever [51], only standard treatment for management of fever offered, i.e., nursing care, fluid balance, electrolytes, and blood clotting parameters [1]. Therefore, the need for an effective treatment for dengue is urgent and, considering the cytokine role in dengue pathogeny, it is still important to search for medicinal plants that act as immunomodulatory agents.

We showed that the hydroalcoholic extract from *N. brasiliensis* leaves and 3 derivate fractions presented antiviral activity, significantly reducing the percentage of DENV Ag⁺ cells or the NS1 protein, a viral load indicator. Also an immunomodulatory effect is reported by reducing the production of some important cytokines such as TNF- α , IL-6, and IL-10. Considering that severe clinical manifestations are associated with an exacerbated cytokine production, we believe that this immunomodulation is an important step for reducing or avoiding undesired outcomes of the disease. Furthermore, it is important to note that possibly the actual reduction in viral load may lead to a reduction in the immune response and contribute to a good prognosis.

In this study, NS1 was used for the first time to evaluate the antiviral activity of natural products in an in vitro DENV infection model using human primary monocytes as targets. This protein have already been used for this activity in mice models, in which NS1 levels were related with the circulating virus detected by plaque-forming assay and RNA quantification by PCR [52] and more recently in studies involving Vero cell infection by the 4 DENV serotypes [31]. The role of IL-10 in association with hepatic dysfunctions and effusions in children with dengue was reported earlier [42]. Moreover, both DENV NS1 antigen and IL-10 serum levels were associated with severity in acute dengue infection, and IL-10 has suppressed dengue-specific T-cell responses [53]. Malavige and colleagues [54] demonstrated that NS1 is positively correlated with IL-10 production in monocytes, and induces its production, leading to severity. Therefore, our results showing an IL-10 reduction could be a consequence of *N. brasiliensis* effects in reducing NS1 and DENV production, acting simultaneously in an antiviral and immunomodulatory manner, and also improving the prognosis of infection.

The data presented here strongly suggest that the assay for NS1 determination is more indicated for screening tests to evaluate the antiviral activities of medicinal plants in the monocyte study model since it presented a higher sensitivity in detecting viral load changes than in labelling of intracellular antigen. The stable NS1 protein accumu-

lates in culture, unlike the flow cytometry analysis that may not detect cells destroyed by the virus.

A predominant role for IL-8 and IL-6 as markers of acute disease severity in dengue Indian children was reported, and reduction in IL-8 and IL-10 levels identified markers of recovery from severe disease [55]. TNF- α has been classically associated with more severe cases of dengue fever [43, 56] and is also known to be an endothelial cell activator, inducing the expression of adhesion molecules and disrupting bridges with adherent molecules such as catenin, thereby favoring endothelial permeability and fluid leakage, resulting in severe outcomes such as hypotension and hemoconcentration [57, 58].

Type 1 IFN (α/β) plays a significant role in establishing protection against virus infections [59]. However, DENV viral proteins, such as nonstructural NS4b can inhibit IFN signaling, downregulating the JAK/STAT pathway and impairing this antiviral effect of IFN-regulated gene expression [60–62]. IFN- α in excess may induce autoimmune diseases, such during the treatment for hepatitis [63]. Therefore, IFN- α downregulation, detected by tested NB6 fraction, may represent a decrease in immunological stimulation associated with reduced virus replication. Moreover, the virus elimination could be achieved by other antiviral molecules than IFNs, such as nitric oxide, which is known to inhibit DENV [14, 64].

Acknowledgements

We would like to thank Amanda Torrentes-Carvalho and Mariana Gandini for technical assistance, and Cláudia Kamel for the linguistic revision of the manuscript. The work was supported by Technological Platforms RPT03C-Luminex – RJ and RPT11D-/Bioensaios IV (Dengue) from VPPLR, PROEP/IOC, Farmanguinhos, all at FIOCRUZ; CNPq; FAPERJ and CAPES in Brazil. The NMR spectra were performed at the Analytical Platform, Farmanguinhos/FIOCRUZ.

Disclosure Statement

No conflicts of interest are declared.

References

- WHO/TDR: Dengue: guidelines for diagnosis, treatment, prevention and control – New edition. World Health Organization (WHO) and the Special Programme for Research and Training in Tropical Diseases (TDR). http://whqlibdoc.who.int/publications/2009/9789241547871_eng.pdf (accessed December 5, 2015).
- Brazilian Federal Government: Monitoramento dos casos de dengue, febre de chikungunya e febre pelo vírus Zika até a Semana Epidemiológica 32, 2016. <http://portalsaude.saude.gov.br/images/pdf/2016/setembro/16/2016-028-Dengue-SE32.pdf> (accessed October 26, 2016).
- Gubler DJ: Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends Microbiol* 2002;10:100–103.
- Mukhopadhyay S, Kuhn RJ, Rossmann MG: A structural perspective of the flavivirus life cycle. *Nat Rev Microbiol* 2005;3:13–22.
- Flamand M, Megret F, Mathieu M, Lepault J, Rey FA, Deubel V: Dengue virus type 1 non-structural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. *J Virol* 1999;73:6104–6110.
- Chambers TJ, Hahn CS, Galler R, Rice CM: Flavivirus genome organization, expression, and replication. *Ann Rev Microbiol* 1990;44:649–688.
- Beatty PR, Puerta-Guardo H, Killingbeck SS, Glasner DR, Hopkins K, Harris E: Dengue virus NS1 triggers endothelial permeability and vascular leak that is prevented by NS1 vaccination. *Sci Transl Med* 2015;7:304ra141.
- Jessie K, Fong MY, Devi S, Lam SK, Wong KT: Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. *J Infect Dis* 2004;189:1411–1418.
- Wu SJ, Grouard-Vogel G, Sun W, Mascola JR, Brachtel E, Putvatana R, Louder MK, Filgueira L, Marovich MA, Wong HK, Blauvelt A, Murphy GS, Robb ML, Innes BL, Bix DL, Hayes CG, Frankel SS: Human skin Langerhans cells are targets of dengue virus infection. *Nat Med* 2000;6:816–820.
- Dalrymple NA, Mackow ER: Roles for endothelial cells in dengue virus infection. *Adv Virol* 2012;2012:840654.
- Ho LJ, Wang JJ, Shaio MF, Kao CL, Chang DM, Han SW, Lai JH: Infection of human dendritic cells by dengue virus causes cell maturation and cytokine production. *J Immunol* 2001;166:1499–1506.
- Suksanpaisan L, Cabrera-Hernandez A, Smith DR: Infection of human primary hepatocytes with dengue virus serotype 2. *J Med Virol* 2007;79:300–307.
- Tassaneeritthep B, Burgess TH, Granelli-Piperno A, Trumpfheller C, Finke J, Sun W, Eller MA, Pattanapanyasat K, Sarasombath S, Bix DL, Steinman RM, Schlesinger S, Marovich MA: DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J Exp Med* 2003;197:823–829.
- Neves-Souza PC, Azeredo EL, Zagne SM, Valls-de-Souza R, Reis SR, Cerqueira DI, Nogueira RM, Kubelka CF: Inducible nitric oxide synthase (iNOS) expression in monocytes during acute dengue fever in patients and during in vitro infection. *BMC Infect Dis* 2005;5:64.
- Castañeda-Sánchez JJ, Domínguez-Martínez DA, Olivar-Espinosa N, García-Pérez BE, Loroño-Pino MA, Luna-Herrera J, Salazar MI: Expression of antimicrobial peptides in human monocytic cells and neutrophils in response to dengue virus type 2. *Intervirology* 2016;59:8–19.
- Bosch I, Khaja K, Estevez L, Raines G, Melichar H, Warke RV, Fournier MV, Ennis FA, Rothman AL: Increased production of interleukin-8 in primary human monocytes and in human epithelial and endothelial cell lines after dengue virus challenge. *J Virol* 2002;76:5588–5597.
- Carr JM, Hocking H, Bunting K, Wright PJ, Davidson A, Gamble J, Burrell CJ, Li P: Supernatants from dengue virus type-2 infected macrophages induce permeability changes in endothelial cell monolayers. *J Med Virol* 2003;69:521–528.
- Chen YC, Wang SY: Activation of terminally differentiated human monocytes/macrophages by dengue virus: productive infection, hierarchical production of innate cytokines and chemokines, and the synergistic effect of lipopolysaccharide. *J Virol* 2002;76:9877–9887.
- Spain-Santana TA, Marglin S, Ennis FA, Rothman AL: MIP-1 alpha and MIP-1 beta induction by dengue virus. *J Med Virol* 2001;65:324–330.
- Kurane I, Ennis FA: Production of interferon alpha by dengue virus-infected human monocytes. *J Gen Virol* 1988;69:445–449.
- Azeredo EL, Neves-Souza PC, Alvarenga AR, Reis SR, Torrentes-Carvalho A, Zagne SM, Nogueira RM, Oliveira-Pinto LM, Kubelka CF: Differential regulation of Toll-like receptor-2, Toll-like receptor-4, CD16 and human leucocyte antigen-DR on peripheral blood monocytes during mild and severe dengue fever. *Immunology* 2010;130:202–216.
- Reis SR, Valente LM, Sampaio AL, Siani AC, Gandini M, Azeredo EL, D'Ávila LA, Mazzei JL, Henriques M, Kubelka CF: Immunomodulating and antiviral activities of *Uncaria tomentosa* on human monocytes infected with dengue virus-2. *Int Immunopharmacol* 2008;8:468–476.
- Wang Q, Kuang H, Su Y, Sun Y, Feng J, Guo R, Chan K: Naturally derived anti-inflammatory compounds from Chinese medicinal plants. *J Ethnopharmacol* 2013;146:9–39.
- Williams JE: Review of antiviral and immunomodulating properties of plants of the Peruvian rainforest with a particular emphasis on Una de Gato and Sangre de Grado. *Altern Med Rev* 2001;6:567–579.
- Meneses R, Ocazionez RE, Martínez JR, Stashenko EE: Inhibitory effect of essential oils obtained from plants grown in Colombia on yellow fever virus replication in vitro. *Ann Clin Microbiol Antimicrob* 2009;8:8.
- WHO: Traditional Medicine. Fact Sheet. World Health Organization. 2003. <http://www.who.int/mediacentre/factsheets/fs134/en/>.
- Betancur-Galvis L, Saez J, Granados H, Salazar A, Ossa J: Antitumor and antiviral activity of Colombian medicinal plant extracts. *Mem Inst Oswaldo Cruz* 1999;94:531–535.
- Kudi AC, Myint SH: Antiviral activity of some Nigerian medicinal plant extracts. *J Ethnopharmacol* 1999;68:289–294.
- Cardile AP, Mbuy GKN: Anti-herpes virus activity of silibinin, the primary active component of *Silybum marianum*. *J Herb Med* 2013;3:132–136.
- Chu JJ, Yang PL: c-Src protein kinase inhibitors block assembly and maturation of dengue virus. *Proc Natl Acad Sci USA* 2007;104:3520–3525.
- Sood R, Raut R, Tyagi P, Pareek PK, Barman TK, Singhal S, Shirumalla RK, Kanoje V, Subbarayan R, Rajerethinam R, Sharma N, Kanaujia A, Shukla G, Gupta YK, Katiyar CK, Bhatnagar PK, Upadhyay DJ, Swaminathan S, Khanna N: *Cissampelos pareira* Linn: Natural source of potent antiviral activity against all four dengue virus serotypes. *PLoS Negl Trop Dis* 2015;9:e0004255.
- Lima-Junior RS, Mello Cda S, Siani AC, Valente LM, Kubelka CF: *Uncaria tomentosa* alkaloidal fraction reduces paracellular permeability, IL-8 and NS1 production on human microvascular endothelial cells infected with dengue virus. *Nat Prod Commun* 2013;8:1547–1550.
- Barroso GM: Marcgraviaceae; in: *Sistemática de Angiospermas do Brasil*. Vicoça, Imp. Univ. UFV, 1978, vol 1, pp 137–138.
- Hammel BE: Three new species of Marcgraviaceae from Costa Rica, with references to related species and notes on the generic placement of *Schwartzia jimenesii*. *Lankesteriana* 2006;6:73–81.
- Gledhill D: *The Names of Plants*, ed 3. Cambridge, Cambridge University Press, 2002.
- Soares C: *O Livro de Ouro das Flores – As Cem Variedades mais Belas, suas Características e Histórias*. Rio de Janeiro, Ediouro Publicações S/A 2002.

- 37 Pinheiro MCB, Ormond WT, Lima HA, Correia MCR: Biologia da Reprodução de *Norantea brasiliensis* Choisy (Marcgraviaceae). Rev Brasil Biol 1995;55:79–88.
- 38 Rocha MEdN, Figueiredo MR, Kaplan MAC, Durst T, Arnason JT: Chemotaxonomy of the Ericales. Biocheml Syst Ecol 2015;61:441–449.
- 39 Rocha MEN: Potencialidades biodinâmicas de *Norantea brasiliensis* Choisy (Marcgraviaceae). Dissertação (Mestrado em Biologia Celular e Molecular). Rio de Janeiro, Fundação Oswaldo Cruz, 2002.
- 40 Srikiatkachorn A, Green S: Markers of dengue disease severity. Curr Top Microbiol Immunol 2010;338:67–82.
- 41 de Azeredo EL, Monteiro RQ, de-Oliveira Pinto LM: Thrombocytopenia in dengue: interrelationship between virus and the imbalance between coagulation and fibrinolysis and inflammatory mediators. Mediators Inflamm 2015;2015:313842.
- 42 Ferreira RA, de Oliveira SA, Gandini M, Ferreira Lda C, Correa G, Abiraude FM, Reid MM, Cruz OG, Kubelka CF: Circulating cytokines and chemokines associated with plasma leakage and hepatic dysfunction in Brazilian children with dengue fever. Acta Trop 2015; 149:138–147.
- 43 Green S, Rothman A: Immunopathological mechanisms in dengue and dengue hemorrhagic fever. Curr Opin Infect Dis 2006;19: 429–436.
- 44 Bozza FA, Cruz OG, Zagne SM, Azeredo EL, Nogueira RM, Assis EF, Bozza PT, Kubelka CF: Multiplex cytokine profile from dengue patients: MIP-1beta and IFN-gamma as predictive factors for severity. BMC Infect Dis 2008;8:86.
- 45 Halstead SB, Marchette NJ: Biologic properties of dengue viruses following serial passage in primary dog kidney cells: studies at the University of Hawaii. Am J Trop Med Hyg 2003;69:5–11.
- 46 Reis SR, Sampaio AL, Henriques M, Gandini M, Azeredo EL, Kubelka CF: An in vitro model for dengue virus infection that exhibits human monocyte infection, multiple cytokine production and dexamethasone immunomodulation. Mem Inst Oswaldo Cruz 2007; 102:983–990.
- 47 Torrentes-Carvalho A, Azeredo EL, Reis SR, Miranda AS, Gandini M, Barbosa LS, Kubelka CF: Dengue-2 infection and the induction of apoptosis in human primary monocytes. Mem Inst Oswaldo Cruz 2009;104:1091–1099.
- 48 Srikiatkachorn A: Plasma leakage in dengue haemorrhagic fever. Thromb Haemost 2009; 102:1042–1049.
- 49 John DV, Lin YS, Perng GC: Biomarkers of severe dengue disease – a review. J Biomed Sci 2015;22:83.
- 50 Correa G, de ALC, Fernandes-Santos C, Gandini M, Petitinga Paiva F, Coutinho-Silva R, F Kubelka C: The purinergic receptor P2X7 role in control of Dengue virus-2 infection and cytokine/chemokine production in infected human monocytes. Immunobiology 2016;221: 794–802.
- 51 Beauté J, Vong S: Cost and disease burden of dengue in Cambodia. 2010 BMC Pub Health 2010;10:521.
- 52 Schul W, Liu W, Xu HY, Flamand M, Vasudevan SG: A dengue fever viremia model in mice shows reduction in viral replication and suppression of the inflammatory response after treatment with antiviral drugs. J Infect Dis 2007;195:665–674.
- 53 Malavige GN, Jeewandara C, Alles KM, Salimi M, Gomes L, Kamaladasa A, Jayaratne SD, Ogg GS: Suppression of virus specific immune responses by IL-10 in acute dengue infection. PLoS Negl Trop Dis 2013;7:e2409.
- 54 Adikari TN, Gomes L, Wickramasinghe N, Salimi M, Wijesiriwardana N, Kamaladasa A, Shyamali NL, Ogg GS, Malavige GN: Dengue NS1 antigen contributes to disease severity by inducing interleukin (IL)-10 by monocytes. Clin Exp Immunol 2016;184:90–100.
- 55 Singla M, Kar M, Sethi T, Kabra SK, Lodha R, Chandele A, Medigeshi GR: Immune response to dengue virus infection in pediatric patients in New Delhi, India – association of viremia, inflammatory mediators and monocytes with disease severity. PLoS Negl Trop Dis 2016;10:e0004497.
- 56 Braga EL, Moura P, Pinto LM, Ignacio SR, Oliveira MJ, Cordeiro MT, Kubelka CF: Detection of circulant tumor necrosis factor-alpha, soluble tumor necrosis factor p75 and interferon-gamma in Brazilian patients with dengue fever and dengue hemorrhagic fever. Mem Inst Oswaldo Cruz 2001;96:229–232.
- 57 Cardier JE, Rivas B, Romano E, Rothman AL, Perez-Perez C, Ochoa M, Caceres AM, Cardier M, Guevara N, Giovannetti R: Evidence of vascular damage in dengue disease: demonstration of high levels of soluble cell adhesion molecules and circulating endothelial cells. Endothelium 2006;13:335–340.
- 58 Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM, Stern DM: Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 1998;91:3527–3561.
- 59 Biron CA: Role of early cytokines, including alpha and beta interferons (IFN-alpha/beta), in innate and adaptive immune responses to viral infections. Semin Immunol 1998;10: 383–390.
- 60 Munoz-Jordan JL, Sanchez-Burgos GG, Laurent-Rolle M, Garcia-Sastre A: Inhibition of interferon signaling by dengue virus. Proc Natl Acad Sci USA 2003;100:14333–14338.
- 61 Morrison J, Aguirre S, Fernandez-Sesma A: Innate immunity evasion by dengue virus. Viruses 2012;4:397–413.
- 62 Munoz-Jordan JL, Fredericksen BL: How flaviviruses activate and suppress the interferon response. Viruses 2010;2:676–691.
- 63 Omür O, Daglyöz G, Akarca U, Ozcan Z: Subacute thyroiditis during interferon therapy for chronic hepatitis B infection. Clin Nucl Med 2003;28:864–865.
- 64 Takhampunya R, Padmanabhan R, Ubol S: Antiviral action of nitric oxide on dengue virus type 2 replication. J Gen Virol 2006;87: 3003–3011.