RESEARCH ARTICLE

Suppression of TGF-β/Smad2 signaling by GW788388 enhances DENV-2 clearance in macrophages

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Abstract

Dengue fever, caused by the dengue virus (DENV-1, −2, −3, and −4), affects millions of people in the tropical and subtropical regions worldwide. Severe dengue is correlated with high viraemia and cytokine storm, such as high levels of transforming growth factor-β1 (TGF-β1) in the patient's serum. Here, the TGF-β1 signaling was investigated in the context of in vitro viral clearance. Macrophages were infected with DENV-2 at MOI 5 and treated with the TGF-β receptor 1 and 2 inhibitor, GW788388. TGF-β1 expression, signal transduction and viral load were evaluated 48 h after DENV-2 infection by enzyme-linked immunoassay, immunofluorescence, and RT-qPCR assays. Total TGF-β1 level was reduced in 15% after DENV-2 infection, but the secretion of its biologically active form increased threefold during infection, which was followed by the phosphorylation of Smad2 protein. Phosphorylation of Smad2 was reduced by treatment with GW788388 and it was correlated with reduced cytokine production. Importantly, treatment led to a dose-dependent reduction in viral load, ranging from 6.6×10^5 RNA copies/ml in untreated cultures to 2.3×10^3 RNA copies/ml in cultures treated with 2 ng/ml of GW788388. The anti-TGF-β1 antibody treatment also induced a significant reduction in viral load to 1.6×10^3 RNA copies/ml. On the other hand, the addition of recombinant TGF-β1 in infected cultures promoted an increase in viral load to 7.0×10^6 RNA copies/ml. These results support that TGF-β1 plays a significant role in DENV-2 replication into macrophages and suggest that targeting TGF-β1 may represent an alternative therapeutic strategy to be explored in dengue infection.

1 | INTRODUCTION

Dengue is a viral infection transmitted to humans through the bite of female Aedes mosquitoes infected with any of the four dengue virus serotypes (DENV-1 to DENV-4).[1] DENV is an enveloped virus, and its genome contains 11 kb single-stranded positive-sense RNA encoding three structural proteins (C: capsid; prM: premembrane and E: envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).[2] Dengue is found in tropical and subtropical regions worldwide, and its global incidence has grown dramatically in recent decades, with an annual estimate around 400 million cases, and over 3 billion people at risk of contracting the disease.[3] In January and February 2022, there were 90 335 probable cases of dengue in Brazil, with an incidence rate of 42.3 cases per 100 000 inhabitants and an increase of 43.2% over the same period analyzed in 2021.[4,5] From 2021 to February 2022, the number of deaths was 224 in Brazil.[4,5] Despite several drugs has been explored in clinical trials, there is still no specific therapy for the treatment of dengue. However, in 2015 it was registered the first dengue vaccine termed Dengvaxia (CYD-TDV) produced by Sanofi Pasteur, but its efficiency is questioned due to possible instability, depending on DENV serotype, baseline serostatus and age of the patient.[6] Currently, other dengue vaccines are undergoing in ClinicalTrials.gov.[7,8]

Dengue infection can be asymptomatic or manifested with a self-limiting dengue fever (DF), both usually resulting in complete recovery; however, in some cases, the infection evolves to severe dengue (SD), including dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).[9] Although these nomenclatures are still used, in 2009 the World Health Organization replaced the previous classifications for dengue with or without warning signs and SD.[10] The factors involved in the transition from DF to DHF and DSS are still not well understood, but the high viraemia, NS1 protein, anti-DENV NS1 antibodies, cytokines and chemokines production (cytokines storm) have been considered as key factors in the pathogenesis of dengue.[11-15] Monocytes/macrophages are earliest targets for DENV infection and initiate the innate immune response being the major sources of cytokines and chemokines production during the initial phase of infection to control the disease.[16] However, a shift from Th1- to a Th2-type cytokine appears to be associated with the evolution from DF to DHF and DSS.[17] Literature data reported that a predominance of the Th1-type response occurred in 66% of cases of mild illness and the Th2-type response in 71% of severe DHF grade index of variation (IV) cases.[18]

Transforming growth factor-β1 (TGF-β1) is a cytokine that has multiple immunomodulatory effects in several infectious diseases.[19] In patients with dengue, previous studies have demonstrated a positive correlation between severity of disease and TGF-β1 levels.[20-23]

Children in French Polynesia with dengue showed no change in serum levels of TNF-alpha between the groups of patients with DF and DHF, but TGF-β1 levels were significantly higher in children with DHF than with DF.[20] A study including patients with different grades of dengue illness showed increased TGF-β1 levels in the group of patients with DHF grade IV as compared to those with DF.[21] Circulating levels of interleukin (IL)-18, TGF-β1 and sICAM-1 were significantly elevated in dengue patients as compared to healthy subjects.[22] Elevated levels of TGF-β, IL-6 and IL-18 were also demonstrated in the liver of patients with DHF who had hepatic injury and progression to more severe forms of the disease, which was correlated to apoptosis, increased vascular permeability and plasma leakage.[23] In addition, higher levels of TGF-β1 are also reported in other viral infections.[24,25] A preclinical study demonstrated that BALB/c mice infected with influenza virus had high levels of active TGF-β correlated to induction of apoptosis.[24] Hepatitis C virus (HCV) infection in human hepatoma cells also leads to elevated levels of TGF-β directly correlated to viral persistence and hepatic fibrogenesis, contributing to liver damage as seen in human pathology.[25] Increased levels of TGF-β have also been associated with apoptosis of liver cells infected with the yellow fever virus.[26]

Cytokines could be an alternative target for viral clearance and could contribute for preventing the DSS. Although TGF-β1 is not the only cytokine involved in dengue evolution and pathogenesis, in the present study we show that TGF-β1 plays a significant role in DENV-2 replication into macrophages. We observed a significant increase of biologically active TGF-β1 in infected macrophages, leading to activation of Smad2 classical signaling pathway. Importantly, the viral load was significantly decreased by suppression of the TGF-β1 signaling with the pharmacological inhibitor GW788388 and by TGF-β1 neutralizing antibody. In conclusion, targeting TGF-β1 may represent an alternative therapeutic strategy to be explored in dengue infection.

2 | MATERIALS AND METHODS

2.1 | Virus

The DENV-2 sample used to infect macrophages was originated from an isolate of a human case of DF (56344; Vitória, state of Espírito Santo).[27] It was propagated in mosquito cell line (C6/36 cell), in which the monolayers were inoculated with 100 μl DENV-2 (MOI 0.01) and led to adsorption for 1 h at 37°C under agitation every 15 min. Afterwards, the cultures were maintained at 28°C in Leibovitz L-15 supplemented medium (Cultilab) for approximately 7 days, when the cytopathic effect could be observed. The infected culture supernatants were collected and titrated

onto Vero cells by the plaque forming unit assay and stored at −70°C.

2.2 | Peritoneal macrophage primary cultures and DENV-2 infection

Peritoneal macrophages were obtained from male BALB/c mice by peritoneal lavage with RPMI 1640 medium (Cultilab). At the end, cells were centrifuged, reconstituted in RPMI medium supplemented with 2% fetal bovine serum (Cultilab), 2% L-glutamine (200 mM) (Gibco) and 0.2% solution of penicillin (10 000 U/ml) and streptomycin (10 mg/ml) (Sigma-Aldrich). After quantification, 2×10^6 cells were seeded on 35 mm plates containing glass coverslips and the cultures were maintained at 37°C in 5% CO_2 atmosphere. For the infection assays, peritoneal macrophages cultures were washed with phosphate-buffered saline (PBS), inoculated with 100 µl of DENV-2 (MOI 5) and maintained by 1 h at 37°C, under agitation every 15 min for virus adsorption. The infected cultures were then washed for removal non-adsorbed viral particles and the cells were treated as described below. All procedures were performed in accordance with the guidelines established by the FIOCRUZ Ethics Committee for the Use of Animals, resolution 242/99. License LW-4/15.

2.3 | Immunofluorescence labeling

Viral envelope protein E was detected by immunofluorescence in cultures of macrophages infected with DENV-2 at a MOI 5 for 48 h. A total of 4% paraformaldehyde fixed cultures were incubated with 3% bovine serum albumin (Sigma-Aldrich) for 30 min. at room temperature, washed 3 times and incubated with the monoclonal anti-DENV antibody (LATAM/Biomanguinhos) for 1 h at 37°C, washed 3 times and incubated with Alexa Fluor 488-conjugated secondary antibody for 1 h at 37°C (Thermo Fisher Scientific). In addition, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Thermo Fisher Scientific) was used to stain the nuclei of the host cell in blue, and then the samples were mounted with antifading 2.5% 1.4-diazabicyclo(2.2.2)-octane (DABCO) (Sigma-Aldrich) and examined using a Nikon microscope coupled with image acquisition systems (Nikon Eclipse Ci).

2.4 | Detection of sNS1 viral protein in the supernatant of macrophages

The secretion of soluble nonstructural protein NS1 (sNS1) was investigated in the supernatant of macrophage cultures mock-infected and infected with DENV-2 for 48 h, using the commercial Platelia Dengue NS1 AG kit (Bio-Rad) according to the manufacturer's instructions. The results were expressed as sample ratio (SR), calculated from the ratio between the sample optical density (OD) and the cut-off value (SR = OD sample/cut-off). The cut-off value

corresponds to the mean of negative control duplicate divided by two. OD was obtained at microplate reader at 620 nm (VersaMax Microplate Reader; Molecular Devices).

2.5 | TGF-β1 quantification in the supernatant of macrophages

Total and active TGF-β1 secreted by the macrophages were quantified in the supernatant of the cultures using a TGF-β1 specific commercial enzyme-linked immunosorbent assay kit (Quantikine TGF-β1 enzyme-linked immunoassay [ELISA]; R&D Systems) according to the manufacturer's instructions. The measurements of total (active + latent) TGF-β1 in these samples were performed after heat activation of latent TGF-β1 at 80°C for 10 min. The quantification of active TGF-β1 was performed without heating of samples. Absorbance was read at 450 nm (VersaMax Microplate Reader; Molecular Devices) and the results were expressed as an IV, considering always the value 1 for mock-infected culture in each test (Figure 3) or TGF-β1 levels in pg/ml (Figure 5). All samples were tested in triplicate.

2.6 | Treatment of macrophage cultures

After 1 h of infection, the cultures were treated with GW788388 (0.5–2 µM) (Glaxo Smith Kline), recombinant TGF-β1 (10 ng/ml) (R&D systems) or anti-TGF-β1 antibody (10 ng/ml) (R&D systems) and maintained for 48 h at 37°C in 5% CO_2 atmosphere. Afterwards, the culture supernatants were collected and stored at −20°C for ELISA or −70°C for RT-qPCR assays. GW788388 (4-{4-[3-(pyridin-2-yl) −1H-pyrazol-4-yl] pyridin-2-yl}-N-(tetrahydro-2H-pyran-4-yl) benzamide) (Figure 1) is a pharmacological selective inhibitor of TGF-β1 types I and II receptors (TβRI/II).[28]

2.7 | Phosphorylated Smad-2 protein labeling

The cultures were fixed in 4% paraformaldehyde at 4°C for 20 min and permeabilized with 0.1% Triton X-100 diluted in PBS for 5 min, washed and saturated for 30 min with 3% bovine serum albumin following an overnight incubation with rabbit immunoglobulin G
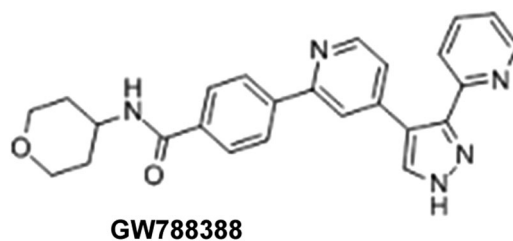

GW788388

FIGURE 1 Chemistry structure of GW788388, a pharmacological inhibitor of TGF-β1 type I and II receptors. TGF, transforming growth factor

Primer/direction	Sequence	Concentration
DENV-2 forward	5′-CAGGTTATGGCACTGTCACGAT-3′	0.5 pmols/µl
DENV-2 Reverse	5′-CCATCTGCAGCAACACCATCTC-3′	0.5 pmols/µl
DENV-2 probe	HEX-5′-CTCTCCGAGAACAGGCCTCGACTTCAA-3′–MGB-NFQ	0.18 pmols/µl

anti-phosphoSmad-2 (PS2) diluted in PBS (1: 200) (Cell Signaling Technology). After washings, the samples were incubated with the secondary antibody Alexa Fluor 594 (1:500) (Invitrogen) for 60 min. After washings, the cultures were incubated with 10 µg/ml DAPI (Sigma-Aldrich) for cell nucleus visualization. The coverslips were mounted in 2.5% DABCO (Sigma-Aldrich) to prevent loss of fluorescence and examined immediately using a Nikon microscope coupled with image acquisition systems (Nikon Eclipse Ci). Images were quantified using CellProfiler image analysis software (http://www.cellprofiler.org).

2.8 | RT-qPCR

Viral RNA was extracted from 200 µl of the culture supernatant through the High Pure Viral Nucleic Acid Kit (Roche) and 60 µl of Elution buffer was added to the RNA, following the manufacturer's instructions and stored at −70°C until use. The complementary DNA synthesis was obtained from 25 ng viral RNA through the SuperScript III kit (Life Technologies), according to manufacturer's instructions.

The RT-qPCR was performed according to the protocol of Johnson et al.[29] (Table 1). The reaction was carried out on a 7.500 Fast Real Time PCR System (Applied Biosystem), using the following cycling: 50°C/2 min, 40 cycles of 95°C/10 min, 95°C/15 s, 60°C/1 min. Absolute quantification was used to analyze the viral load obtained from culture supernatants. Forty-eight hour postinfection, the samples were processed simultaneously with a standard six-point curve of DENV-2 (10^7–10^2 virus genome equivalents/ml/ml).

2.9 | Statistical analysis

Statistical significance was evaluated by the unpaired t test, conducted with GraphPad Prism software version 8 for Windows. Differences were considered statistically significant when *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, and ****$p < 0.0001$.

3 | RESULTS

3.1 | Peritoneal macrophages infection

The macrophages were infected with MOI 5 of DENV-2 for 48 h. The infection was confirmed by the expression of the viral envelope protein (E) observed by green labeling by immunofluorescence

(Figure 2B,C). Note that mock-infected culture showed no green labeling (Figure 2A). At a higher magnification, it is possible to observe intense perinuclear protein labeling, as expected as flaviviruses presents a characteristic replication in the endoplasmic reticulum (Figure 2C, arrow). In addition to the evaluation of the expression of the envelope protein, the soluble nonstructural protein NS1 (sNS1) was also detected in the culture supernatants by Platelia Dengue NS1 AG kit (Figure 2D). The results of sNS1 measurement were expressed as SR, in which positive results are considered when SR ≥1 (cut-off). After 48 h of infection, the SR value in the supernatant of infected cultures was 2.97 ± 0.34, while the value for mock-infected cultures was 0.40 ± 0.08 (Figure 2D).

3.2 | DENV-2 increases the secretion of active TGF-β1 in peritoneal macrophages

To investigate the TGF-β1 involvement in DENV infection, the secretion of total and active cytokine was determined in the supernatant of mock-infected and DENV-2 infected cultures (Figure 3). The results were expressed as an index variation (IV), considering always the value 1 for mock-infected culture in each test, which media value of total TGF-β1 levels was 718.3 ± 0.5 pg/ml (1 = 718.3 ± 0.5 pg/ml). It was observed that the DENV-2 infection led to a 15% downward trend in total TGF-β1 levels (606.5 ± 48 pg/ml) when compared to mock-infected cultures (Figure 3A). On the other hand, a statistically significant difference was observed in the secretion of the biologically active form of TGF-β1, reaching an IV of 2.83 after DENV-2 infection (36.5 ± 1.3 pg/ml) when compared to mock-infected cultures (1 = 13 ± 1.4 pg/ml), an increase about threefold (Figure 3B).

3.3 | DENV-2 infection activates TGF-β1 signaling pathway in peritoneal macrophages

Since TGF-β1 was detected in its active form in the supernatant of DENV-2 infected cultures, it was investigated whether TGF-β1 signaling pathway was activated during infection (Figure 4). In the classical pathway, active TGF-β1 binds to the receptor-type II, which phosphorylates and forms a complex with receptor-type I and lead to the phosphorylation of intracellular proteins, such as Smad-2, one of the major effector proteins in this pathway. We observed a significant increase in the expression of phosphorylated Smad-2 protein (PS2) in the cell nucleus of DENV-2 infected cultures
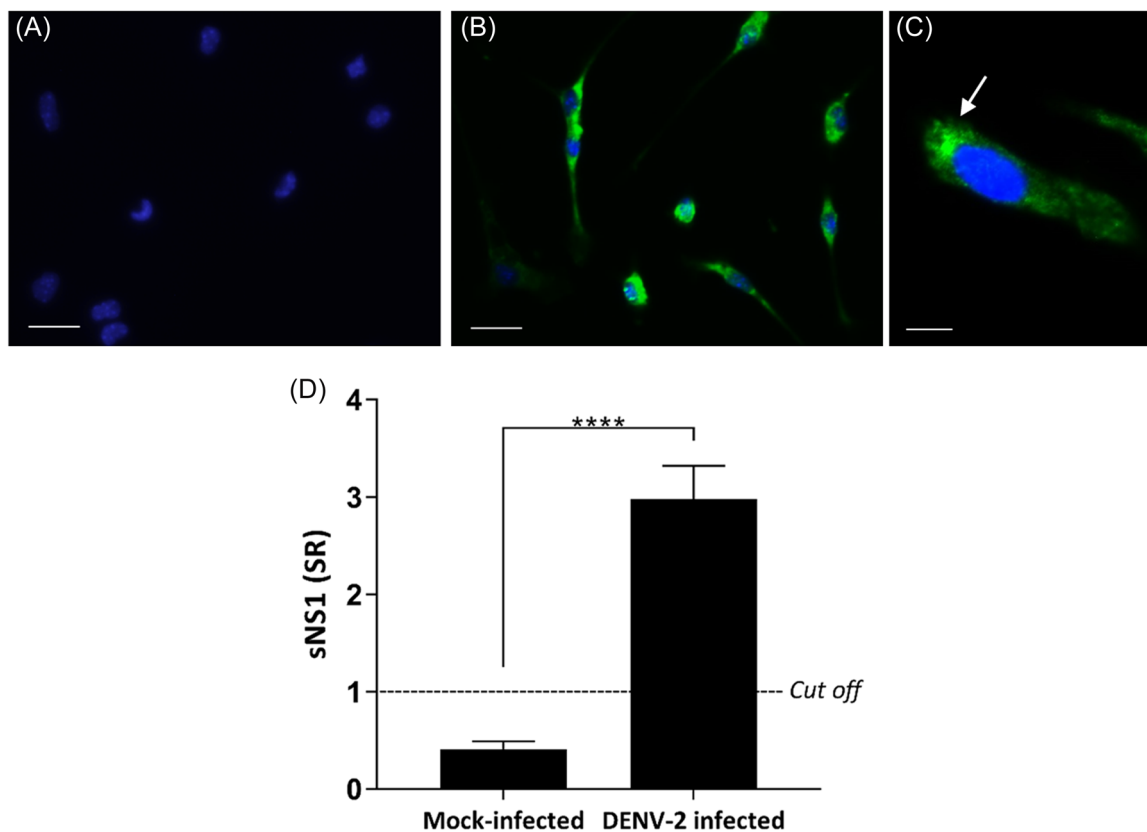
FIGURE 2 Detection of DENV-2 infection in macrophages at MOI 5 for 48 h. The host cells nucleus in blue (A–C) and viral envelope protein in green (B and C) were detected by immunofluorescence. The nonstructural viral protein sNS1 was measured in the supernatant of mock-infected (A and D) and DENV-2 infected (B–D) macrophages. The results of sNS1 measurement were expressed as sample ratio (SR) and represent the mean ± standard deviation of five independent experiments (D). Statistical significance was determined by a one-way ANOVA, followed by Dunnett's multiple-comparisons test (****$p < 0.0001$). Bars 20 μm (A and B) and 5 μm (C). ANOVA, analysis of variance
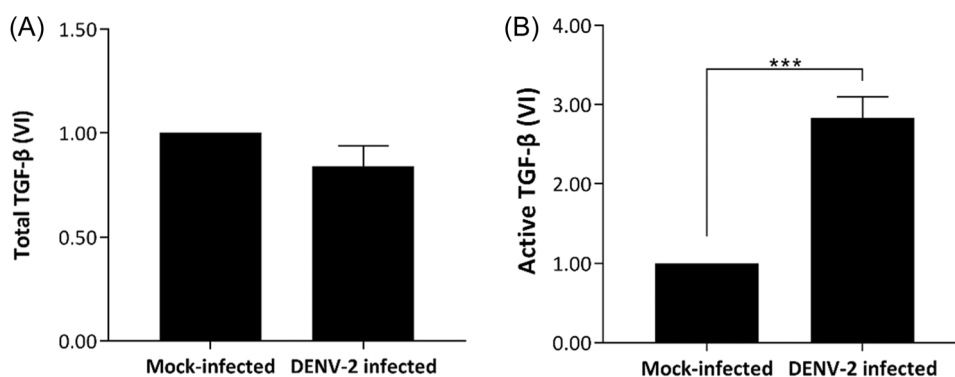

FIGURE 3 TGF-β measure by ELISA in peritoneal macrophage cultures. Total TGF-β (A) and active TGF-β (B) in the supernatant of mock-infected and DENV-2 infected cultures (MOI 5) after 48 h of infection. The results were expressed as an index of variation (IV = OD DENV-2/OD mock), considering always the value 1 for mock-infected culture in each test (1 = 718 ± 0.5 pg/ml of total TGF-β and 1 = 13 ± 1.4 pg/ml of active TGF-β levels). The graphics represent the mean ± standard deviation of five independent experiments. Asterisks indicate statistically significant differences (***$p < 0.001$). ELISA, enzyme-linked immunoassay; TGF, transforming growth factor

(Figure 4D), when compared to mock-infected cultures (Figure 4B, arrow). The percentage of PS2 positive cells was 15.1 ± 3.6% in mock-infected cultures (Figure 4B and 4I) and 53.5 ± 7.1% in DENV-2 infected cultures (Figure 4D and 4I). As expected, treatment with the TGF-β1 receptor inhibitor in DENV-2 infected cultures reduced the

percentage of PS2 + cells in 49% (Figure 4F and 4I) and 66% (Figure 4H,I) with 0.5 and 1 μM of GW788388, respectively. We also observed that the pharmacological inhibition of TGF-β1 signaling altered its secretion (Figure 5). The treatment of DENV-2 infected cultures with 0.5 and 1 μM of GW788388 reduced total TGF-β1 levels
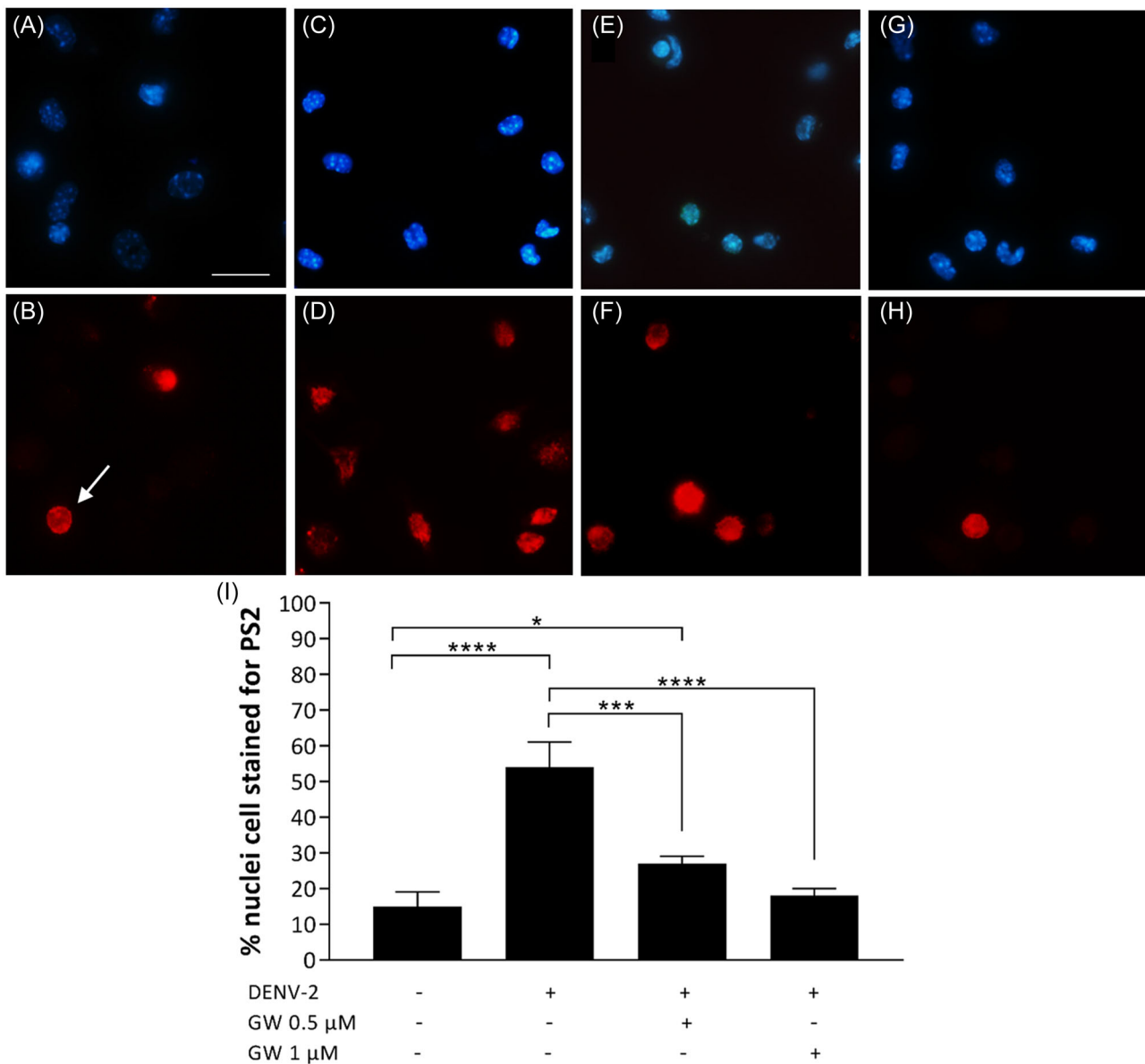
FIGURE 4 Phosphorylated Smad2 protein (PS2) immunolabeling. Fluorescence of host cells nucleus in blue by DAPI (A, C, E, and G) and red staining of PS2 by primary monoclonal antibody followed Alexa Fluor 594 secondary antibody (B, D, F, and H). Mock-infected (A and B) and untreated (C and D) and treated with 0.5 μM (E and F) or 1 μM (G and H) of GW788388 DENV-2 infected cultures (MOI 5) after 48 h of infection. (I) The graphic represents mean ± standard deviation of PS2 positive cells (%). Arrow indicates PS2 labeling in the cell nucleus. Bars 20 μm. Asterisks indicate statistically significant differences (*$p < 0.05$; ***$p < 0.001$, ****$p < 0.0001$). DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride

in 43% and 46%, respectively (Figure 5A), and the active TGF-β1 levels was reduced in 21% with 1 μM of GW788388 (Figure 5B).

3.4 | TGF-β1 signaling inhibition reduces viral load in peritoneal macrophages

To confirm the involvement of TGF-β1 in DENV replication, the viral load was quantified by RT-qPCR in the supernatants of treated and untreated cultures with 0.5, 1, and 2 μM of GW788388 (Figure 6). Interestingly, pharmacological inhibition of TβRI/II receptors reduced

the viral load for $4.8 \pm 0.8 \times 10^5$, $3.5 \pm 0.9 \times 10^4$, and $2.3 \pm 1.4 \times 10^3$ genome equivalents/ml, respectively, as compared to untreated cultures ($6.6 \pm 1.4 \times 10^5$ genome equivalents/ml) (Figure 6A). To corroborate with the effect of TGF-β1 signaling inhibition on viral replication, the neutralizing of TGF-β1 was carried out by the treatment of the cultures with 10 ng/ml of anti-TGF-β1 antibody for 1 h postinfection. It was observed a reduction of 2 logs in viral load, exhibiting values of $1.6 \pm 0.3 \times 10^3$ genome equivalents/ml, as compared to untreated cultures ($6.6 \pm 1.4 \times 10^5$ genome equivalents/ml) (Figure 6B). On the other hand, when DENV-2 cultures were treated with 10 ng/ml recombinant TGF-β, it was observed an
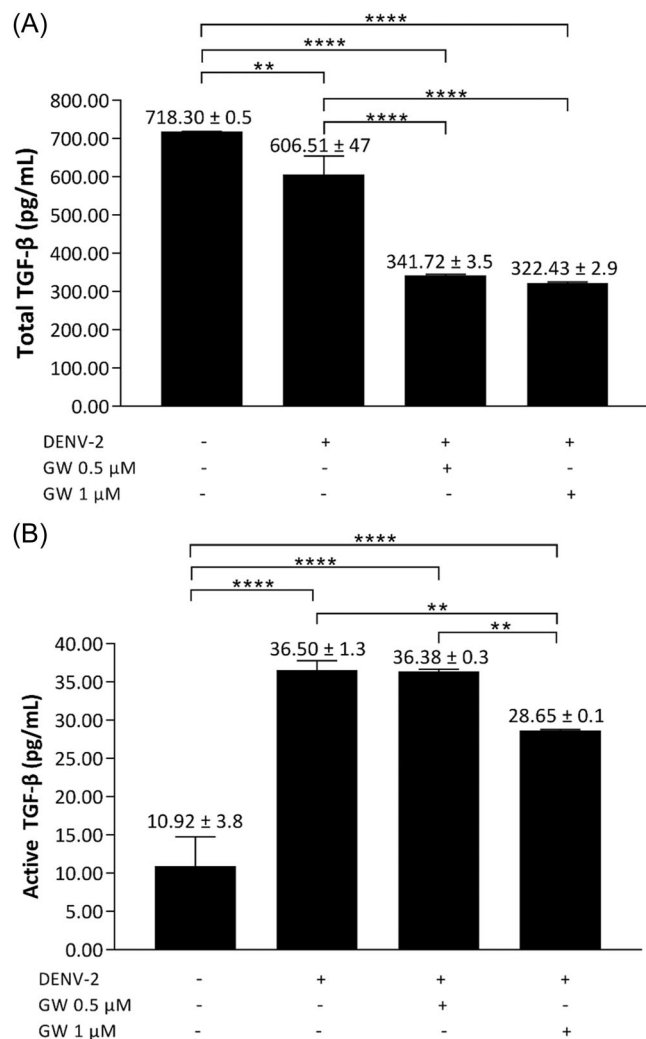
FIGURE 5 TGF-β secretion after GW788388 administration. Mock-infected and DENV-2 infected cultures were treated or not for 48 h with 0.5 and 1 μM of GW788388. Total TGF-β (A) and active TGF-β (B) were measure by ELISA in the supernatants of macrophage cultures. The results were expressed as pg/ml and represents mean ± standard deviation of five independent experiments. Asterisks indicate significant differences (**$p < 0.01$; ****$p < 0.0001$). ELISA, enzyme-linked immunoassay; TGF, transforming growth factor

increase of 1 log on viral load ($7 \pm 1 \times 10^6$ genome equivalents/ml) in the culture supernatants as compared to untreated cultures (Figure 6B). These results suggest that active TGF-β1 secretion by infected cultures may be contributing to viral replication.

4 | DISCUSSION

The pathogenesis of SD is still under investigation, but it is already established that a high viral load can lead to extensive cellular activation at the beginning of the infection and subsequent release of high amount of cytokines and chemokines leading to endothelial damage and plasma extravasation.[12,30] Among these proteins,

TGF-β1 has been associated with disease severity and imbalance of the immune response.[31] TGF-β1 is a pleiotropic cytokine that is produced as a biologically inactive molecule termed latent TGF-β1 (LTGF-β) by a broad spectrum of cell types, including dendritic cells, monocytes and macrophages, which are the primary targets of DENV virus.[32–35] To exert its activities, TGF-β1 requires to be activated into its mature form of 25 kD, then being able to regulate many processes such as proliferation; differentiation; migration; fibrotic; apoptosis, among others.[32]

Despite the limitations of experimental models, several aspects of human dengue pathogenesis have been observed in immuno-competent mice infected with neuroadapted and non-neuroadapted DENV strains.[36–40] In vitro studies can contribute to investigate cellular factors related to host-pathogens interactions such as synthesis of immunomodulatory proteins that influence the resistance/susceptibility to infections. Here, the susceptibility of mouse macrophages to DENV-2 was demonstrated by measurement of NS1 viral protein, which is positively correlated with viral titers in patients' samples.[41,42] Also similar to those reported for humans, during the first week after mice infection the cellular targets of DENV-2 in the spleen were F4/80 + CD11b + macrophages and CD11c+ dendritic cells.[35] Corroborating our study, macrophages derived from the BALB/c mice strain have greater permissiveness to DENV-2 infection than macrophages from other rodent species.[43] Notably, data obtained from in vitro and in vivo mouse models, and in vitro human PBMCs suggest that NS1 protein activates the immunological cascade in monocytes and macrophages through Toll-like receptors contributing to the pathology observed in SD.[44,45] Similarly, in vitro differentiated macrophages from human monocytes can sustain DENV infection and produce cytokines and chemokines indicating the functional competence of these mature cells.[33]

We observed a reduction in the levels of total TGF-β1 in the supernatant of infected macrophages, probably due to the activation of its latent form since a significant increase of active TGF-β1 was observed in infected cells. Such increase suggests an induction of the TGF-β1 gene after DENV-2 infection, in fact, in hepatitis C (HCV) viral infection the structural core protein of HCV upregulates the promoter region of the TGF-β1 gene in HepsG2 cells stimulating the expression of cytokine.[46] Furthermore, alveolar macrophages obtained from horses infected with Equine Herpes virus 1 produced latent and active TGF-β, suggesting that these cells are an important source of circulating active TGF-β in these animals.[47] TGF-β1 has a variety of inhibitory effects including the ability to downregulate the activation and the effector function in macrophage. In addition, the supernatant of DENV-infected macrophages contains factors that increase the permeability of endothelial cells[48] and TGF-β1 induces the expression of endothelial adhesion molecules under inflammatory conditions to regulate leukocyte adherence and extravasation.[22,49] So, despite macrophages are essential components of the innate immune system and crucial for the elimination of pathogens, in dengue infection may represent an important TGF-β1 source and may contribute to elevated viral load and virus dissemination.
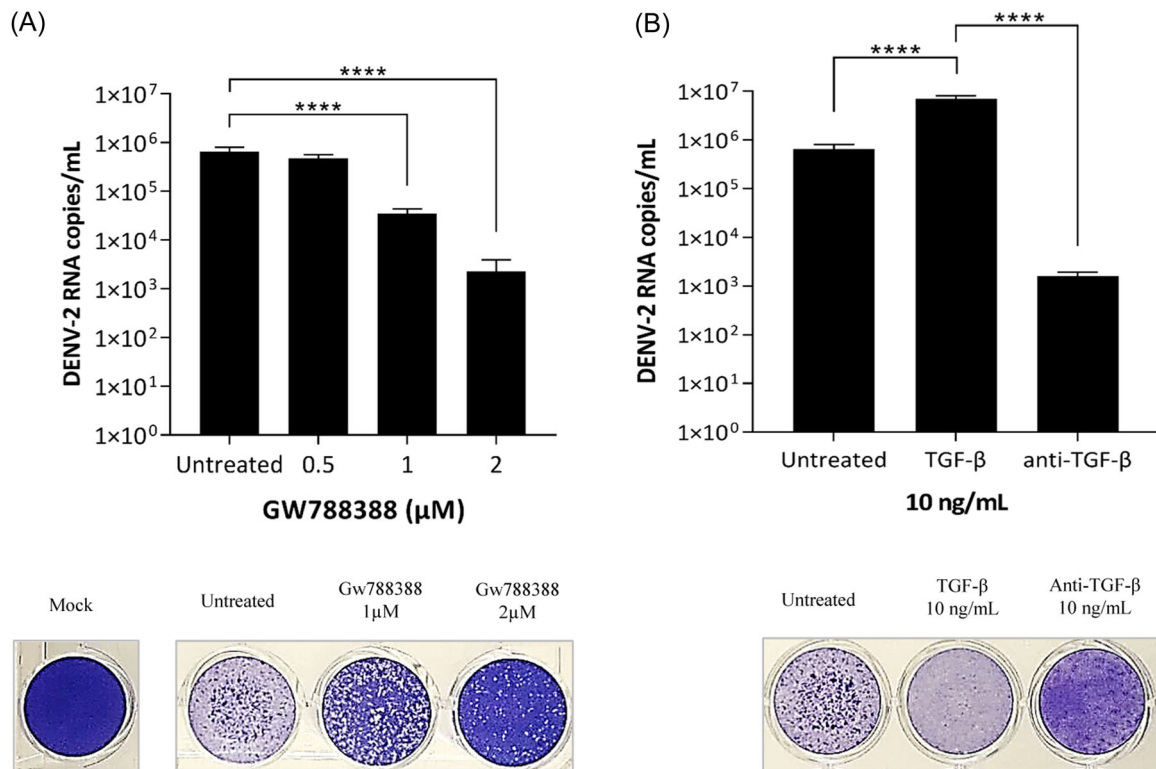
(A)

(B)

FIGURE 6 Effect of TGF-β signaling inhibition on viral load. DENV-2 infected cultures were treated or untreated for 48 h with 0.5, 1, and 2 μM of GW788388 (A) or 10 ng/ml of recombinant TGF-β and neutralizing antibody (B). The viral load was quantified by RT-qPCR in the culture supernatants and the results were expressed as DENV-2 virus genome equivalents/ml. The graphic represents mean ± standard deviation of five independent experiments. Asterisks indicate significant differences (****$p < 0.0001$). The images show equivalent PFU from DENV-2 infected cultures treated or untreated for 48 h with 1 and 2 μM of GW788388 (A) or 10 ng/ml of recombinant TGF-β and neutralizing antibody (B). TGF, transforming growth factor

The involvement of the TGF-β1 signaling has been observed in several infectious and noninfectious illnesses.[19,50] In the classic signaling pathway, active TGF-β1 exerts its biological role by binding to its specific transmembrane receptor type II (TβRII) that forms a heteromeric complex with serine/threonine kinases receptor type I (TβRI/ALK5); initiates intracellular signaling by phosphorylation of the Smad-2 protein and its association with Smad-4 and nuclear translocation of this complex to regulate a variety TGF-β1 responsive genes.[51] In the present work, as expected, we observed that the inhibition of TβRI/II by the treatment with the pharmacological inhibitor GW788388 reduced the phosphorylation of Smad-2 and the secretion of total TGF-β1.[28] Importantly, suppression of TGF-β/Smad-2 signaling led to the reduction in viral load into macrophages. We also inhibited TGF-β1 activity by anti-TGF-β1 antibody treatment and observed a large decrease in viral replication, which was enhanced by the addition of exogenous recombinant TGF-β. Reports on the involvement of TGF-β1 signaling during dengue infection are scarce, recently it was showed the upregulation of hsa-miR-30a microRNA in DENV-infected human monocytic cell line with significant association to TGF-β signaling, phosphatidylinositol mediated signaling, lipid metabolism process and blood coagulation.[52] In fact, it has been shown that in some pathologies the inhibition of TGF-β signaling can attenuate the development of

the pathogenesis observed on parasitological and viral infections. Our group demonstrated that inhibiting TGF-β pathway at early stages of the acute phase of Chagas disease, caused by *Trypanosoma cruzi* parasite, reduced parasitemia followed by reduced cardiac damage and fibrosis in mice infected during the acute phase of infection.[53,54] Regarding viral infections, during influenza A virus infection, it is observed an increase in the expression of cellular adhesion molecules mediated by TGF-β signaling which favors bacterial coinfections.[55] Another study showed that pharmacological inhibition of TGF-β/Smad and early endosomal antigen-1 (EEA1) pathways attenuated the deposition of beta-amyloid molecules in HIV- infected human brain endothelial cells.[56] Although the regulation of TGF-β1 signaling may attenuate dengue infection, the molecular factors responsible for this process should be further explored.

Here we show that TGF-β1 neutralization reduces DENV-2 replication into macrophages. Although reports on the involvement of TGF-β1 signaling during dengue infection are scarce, literature data of correlation between disease severity and serum TGF-β1 levels in dengue patients, as well as the regulation of infections by TGF-β1 signaling, support the evidence that targeting TGF-β1 may represent an alternative therapeutic strategy to be explored in dengue infection.

AUTHOR CONTRIBUTIONS

Gabrielly Sbano Teixeira: Investigation, writing—original draft preparation, methodology. **Audrien Alves Andrade, Lauana Ribas Torres, Dinair Couto-Lima, and Rayane Abreu**: methodology. **Otacilio C. Moreira**: formal analysis. **Mariana Caldas Waghabi**: formal analysis, writing—review and editing. **Elen Mello de Souza**: concept and design of the study, formal analysis, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data are available at ARCA dados FIOCRUZ. doi:10.35078/NOXRD2.

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