Dengue virus non-structural protein 1 disrupts the TGF-β/Smad signaling

Anismrita Lahon (anismrita.niv@gmail.com)
Laboratory of Virology, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi, India-110067
Ravi Arya
Kusuma School Of Biological Sciences, Indian Institute of Technology Delhi, New Delhi, India-110016
Vivek Kumar
Laboratory of Virology, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi, India-110067
Ritu Mishra
Laboratory of Virology, National Institute of Immunology
Akhil Banerjea (akhil@nii.res.in)
Laboratory of Virology, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi, India-110067

Article

Keywords: Dengue virus, NS1, TGF-β, Smad2, Smad3, Smad4, E3 ubiquitin ligase, Smurf2

Posted Date: October 26th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-37505/v2

Abstract

TGF-β signaling is tightly regulated to ensure cellular functions. Role of DENV on the TGF-β/Smad signaling has not been well established. Therefore, we aimed to study the association between DENV infection and TGF-β/Smad signaling. We observed significant impairment in the expression of Smad2, Smad3, Smad4, Smad6 and Smad7 during DENV replication, which are the key players in TGF-β signaling. Significant reduction in the expression of phosphorylated Smad3 was also documented. Overexpression of Smad2/3/4/6 provided the evidence of slight inhibition on DENV replication indicating these Smads may work against the establishment of DENV replication. DENV non-structural protein 1 (NS1) was noted as crucial viral factor that impaired the expression of Smad2, Smad3 and Smad4 and also physically interacts with these proteins as confirmed by co-immunoprecipitation assay. Additionally, we observed NS1 is also capable of blocking the nuclear translocation of Smad3 and thus further ensuring inhibition of Smad signaling. To figure out degradation mechanisms, we studied the role of two distinct E3 ligases, CHIP and Smurf2, which are essential for the degradation of Smad proteins. Co-expression of Smad2/3/4 and NS1 with Smurf2, $Smurf2^{mut}$, CHIP or use of $CHIP^{-/-}$ cells suggests that only Smurf2 has significant role in the degradation of Smad proteins during DENV infection. NS1 may acts as a co-factor with Smurf2 to escalate the proteasome and lysosome mediated degradation of Smad3 and Smad4 proteins respectively. Therefore, our results confirm that NS1 interacts with Smad proteins and reduces their expression by utilizing E3 ligase and disrupt the TGF-β/Smad signaling.

Introduction:

Transforming growth factor-β (TGF-β) is a multifunctional cytokine involved in immense number of biological activities. TGF-β is secreted by many cell types such as lymphocytes, macrophages and platelets. Presence of three TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3) have been reported in mammalian tissues. Among these, TGF-β1 is the most commonly expressed isoform [1, 2].

TGF-β signaling results in the activation of two different downstream pathways; Smad-dependent and Smad-independent pathways [3]. TGF-β binds to a TGF-β type II receptor, which phosphorylates and heterodimerizes with a type I receptor. Afterward, type I receptor recruits and phosphorylates cytoplasmic receptor called regulated Smads (R-smads; Smad2 and Smad3) which makes complex with Smad4 (co-Smad). This whole complex translocates into nucleus and binds to its promoter sites as transcription factors to regulate the expression of target genes [4,5]. TGF-β has been shown to act as a growth activator of several cell types, including mesenchymal stem cells, fibroblasts, smooth muscle cells, endothelial cells and other different cell types [6]. TGF-β works as double edged sword as it can induce cell proliferation (growth) or inhibition (apoptosis) on the same cells according to stimulus [7]. Degradation of proteins involved in the signaling pathways is a way to control the excess activation of the pathway. Ubiquitin mediated pathways are essential part of the degradation via using E3 ligases [8]. Smurf2 and CHIP are the two important E3 ligases which have been reported to degrade Smad proteins and regulate the TGF-β/Smad signaling pathway [9, 10].

Certain viruses have been reported to modulate TGF-β pathway by utilizing viral factors and induce or suppress associated receptors and intermediatory signaling molecules. Human Papilomavirus, a DNA virus dysregulates TGF-β pathway by interrupting TGF-β type II receptor expression and nuclear translocation of p-Smad2/Smad4 complex [11]. However, one of the RNA virus, influenza A virus utilizes a viral protein such as neuraminidase to induce TGF-β signaling and enhanced secondary bacterial infection by up-regulating different host factors [12].

Flaviviruses such as Hepatitis C virus (HCV) has been also reported to modulate TGF-β expression. NS5A, one of the non-structural proteins of HCV has been observed to down regulate TGF-β expression via blocking transcriptional activator for TGF-β gene expression, AP-1 [13]. NS3 of HCV inhibits the binding of Smad3 on its promoter site and suppresses its activation [14], while NS3-4A increases TGF-β signaling by inhibiting Smurf2, an E3 ligase that controls the activities of Smads [15].

Dengue virus (DENV) is one of the medically important flaviviruses of the *Flaviviridae* family responsible for growing global economic and disease burden in humans. DENV is mainly transmitted by mosquitoes of *Aedes* sp. Clinically apparent DENV infection leads to either mild Dengue Fever or severe Dengue Hemorrhagic Fever and dengue shock syndrome [16].

The viral particle encapsulates a positive strand RNA of ~11 kb that constitutes the viral genome, with 5’ and 3’ untranslated regions (UTR). Upon infection, the positive strand RNA is translated into a single polypeptide chain and processed into three structural (envelope, precursor-membrane/membrane and capsid) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) by viral and host cell proteases [17]. DENV utilizes these non-structural proteins for replication and to impair the host antiviral response and modulate the host-machinery in favor of virus propagation.

TGF-β signaling is involved in majority of cellular processes; hence it could be an attractive target to study during DENV infection. In this article, we aimed to uncover the role of DENV replication and involvement of viral and host factors on the TGF-β/Smad signaling.

Results

DENV infection disrupts TGF-β/Smad signaling by inhibiting phosphorylation and degrading Smad proteins

Smad2 and Smad3 are important members of receptor-activated Smads. Upon activation of TGF-β signaling, Smad2/3 get phosphorylated and translocated into nucleus and regulate TGF-β inducible transcription. To understand the association of DENV infection and TGF-β/Smad signaling, we utilized cell culture system of DENV replication with Huh-7 and HEK-293T cells. These cells are well documented with DENV replication.

Huh-7 cells were infected with DENV-2 strain NGC and viral replication was observed by specific qPCR at 24 and 48 hr post infection (hpi) (**Fig. 1a**). To check the phosphorylation status of Smad2 and Smad3,

Huh-7 cells were infected with DENV followed by TGF-β treatment or left un-treated. TGF-β treated control cells were observed with higher expression of phosphorylated Smad2 and Smad3. A significant reduction was noted in the expression of phospho-Smad3 and no effect was observed on phosphorylation of Smad2 in DENV infection ($p<0.05$, **Fig.1b and 1c**). However, un-treated cells were noted with low expression of phospho-Smad2/phospho-Smad3 in controls and only difference was observed with phospho-Smad3. Therefore, it appears that DENV-2 infection may reduce the phosphorylation of Smad3 which leads to possible disruption of TGF-β signaling.

Further we investigated whether DENV infection also disrupts the non-phosphorylation status of Smad protein. To achieve this, we used HA or flag tagged constructs of Smad2 or Smad3 in HEK-293T cells. HEK-293T cells were transfected with constructs of Smad2 and Smad3 and 24h after transfection, these cells were infected with DENV and maintained till 48 hours post infection (hpi). Western blot analysis revealed expression of Smad2 and Smad3 was significantly disrupted by DENV infection at 48 hpi when compared with mock infected cells ($p<0.05$ and $p<0.01$; **Fig. 1d** and **1e**). Smad signaling and regulation requires other assessor proteins such as Smad4 (co-smad), Smad6 and Smad7 (inhibitory Smads). We also utilized constructs of Smad4, Smad6 and Smad7 to see their expression pattern in HEK-293T cells with DENV infection. The western blot analysis of control and infected HEK-293T cells revealed significant reduction in the expression of Smad4, Smad6 and Smad7 at 48h post DENV infection ($p<0.05$; **Fig. 1d** and **1e**). However, the gene expression analysis could not reflect any change in the Smad transcription level with DENV replication (**Supplementary Fig.1**). Overall, these results showed that DENV was clearly impairing the TGF-β signaling pathway.

Effect of Smads on DENV replication

To establish the effect of different Smads on DENV replication, we checked for viral load in the supernatants of DENV infected Smad overexpressing HEK-293T cells. qPCR data showed slight reduction in viral replication with Smad2, Smad3, Smad4 and Smad6 over expressing cells ($p<0.05$; **Fig. 2a**). Therefore we believe that these Smads may potentially reduce the viral replication.

Dengue virus NS1 Protein Inhibits TGF-β signalling by reducing the expression of Smads

DENV replication derailed the TGF-β/Smad signaling pathway by impairing the expression of Smad proteins. Several viral proteins have been known for their interfering role in the TGF-β/Smad signaling pathway [13,14,18]. We used DENV non-structural protein 1 (NS1), as it has been reported to interact with different host proteins [19] to find out whether NS1 has any role in the TGF-β/Smad pathway. To achieve the same, NS1 protein was co-expressed with other Smad proteins i.e. Smad2, Smad3, Smad4, Smad6 or Smad7, in HEK-293T cells. Except Smad7, we observed significant reduction in the expression of all Smads when co-expressed with NS1 ($p<0.05$ and $p<0.01$, **Fig. 2b** and **2c**). Thus confirms the involvement of viral factor in TGF-β/Smad signaling.

Dengue virus NS1 blocks the nuclear translocation of Smad3

Nuclear translocation of Smad proteins is essential step of TGF-β/Smad signaling. As we confirmed earlier that NS1 reduced the expression of Smad2 and Smad3, so our next logical step was to examine whether or not NS1 alter nuclear translocation of these Smad proteins. Cells expressing Smad2 and Smad3 in the presence or absence of NS1 were fractionated into distinct cytoplasmic and nuclear fractions and analyzed by western blot. We have observed significant decrease in the expression of Smad3 in nucleus suggesting the possible inhibition of Smad3 nuclear translocation by NS1 ($p<0.05$; **Fig. 3a and b**).

E3 Ubiquitin ligase Smurf2 is required for regulation of Smads during DENV infection

Smad ubiquitination regulatory factor 2 (Smurf2) is an E3 ligase of HECT family. Smurf2 plays a key role in the editing of TGFβ/Smad signaling via ubiquitination of Smad proteins [9, 20]. Stub-1 or CHIP, another E3 ligase has been reported to promote ubiquitination of Smad proteins [10,21]. Here, we investigated the role of these E3 ligases, Smurf2 or CHIP during DENV infection.

NS1 and Smad protein constructs were co-transfected with Smurf2 or CHIP in HEK-293T cells. Western blot analysis revealed that Smurf2 has significant role in degradation of Smad3, Smad4, Smad6, Smad7 and had less effect on Smad2 ($p<0.05$; **Fig. 4a and b**). However, no difference was observed with CHIP co-transfection (**Fig. 4c**).

Smurf2 mutation reduce the effect of NS1 on Smad expression but not in CHIP$^{-/-}$ cells

We utilized construct of Smurf2 (C176A), which has mutation in the catalytic domain that make Smurf2 E3 liagse inactive [20,22-23] and also CHIP knockdown HEK-293T cells to see the effect on the expression of Smads with or without NS1. Smad4 forms a complex with Smad2 and Smad3 and translocates into the nucleus. So, we continued further experimentation only with these Smad proteins.

CHIP knockdown cells were prepared by using CRISPER-Cas9 method [24]. Smad2, Smad3, Smad4 and NS1 were co-expressed in the CHIP$^{-/-}$ HEK-293T cells. We observed higher expression of Smad3 and Smad4 in CHIP$^{-/-}$ cells and NS1 had less effect on their expression in these cells **(Fig. 4d)**. Catalytically inactive Smurf2 had no effect with or without NS1 on the expression of Smad3 and Smad4, which confirmed the active involvement of Smurf2 during DENV infection (**Fig. 4e**).

Proteasome and lysosome mediated degradation of Smad3 and Smad4 by dengue virus NS1

E3 ligases marked their target proteins and promote their degradation via proteasome or lysosome mediated pathways. Host/cellular protein degradation is mediated by these two mechanisms [8]. Therefore, to understand the mechanism behind the escalated degradation of Smad3 and Smad4 by NS1, we utilized inhibitors of proteasome or lysosome mediated pathways, i.e., MG132 and chloroquine. Smad3, Smad4 were co-transfected with NS1 and transfected cells were treated with MG132 and chloroquine for 8 hrs prior harvesting and investigated levels of Smads by immunoblotting.

Following analysis of transiently transfected HEK-293T cell lysates by immunoblotting, we observed treatment with the proteasome inhibitor MG132 restores the expression of Smad3 compared to control or chloroquine-treated cells (**Fig. 5a**). However, we observed restoration in the expression of Smad4 with the addition of chloroquine but not with MG132, which clarified the mechanism of degradation of Smad4 was lysosomal mediated (**Fig. 5a**). Overall, this in vitro study suggesting NS1 exploits both of the proteasomal and lysosomal pathway to lower the expression of these Smad proteins.

NS1 interacts with Smad2, Smad3 and Smad4 complex

Smad2 and Smad3 form complex with Smad4 and translocate in to nucleus. To find out the physical interaction of Smad proteins and DENV NS1, we performed co-immunoprecipitation (co-IP) assay by using Smad proteins or NS1 as bait. Our co-IP data suggested that NS1 physically interacts with Smad proteins. We observed increase in degradation of these Smad proteins with NS1 when co-expressed with Smurf2. Therefore, we wanted to know whether Smurf2 is also interacting with NS1. Co-IP of NS1 and Smurf2 verified their interactions. Interaction of NS1 with Smad proteins and Smurf2 complex may inhibit the TGF-β/Smad signaling pathway (**Fig. 5b**).

Discussion:

TGF-β/Smad signaling in viral infection has been widely explored earlier. This is a crucial host-defense mechanism against viruses. Different viruses and viral proteins were found to abrupt the signaling by interacting with downstream signaling molecules such as TGF-β receptors and Smad proteins. In DENV infection, the role of Smads and their interaction with dengue viral proteins has not been studied.

To uncover the mechanism of TGF-β signaling during DENV infection, we utilized in vitro cell culture system with virus infection; constructs of different Smads, Smurf2 and CHIP, Smurf2 mutant with no catalytic activity and CHIP knockdown cells. Smad2 and Smad3 are called R-smads, which get phosphorylated upon induction of TGF-β and translocate in to nucleus. Viruses affect the expression of Smad proteins to maintain suitable environment for their own replication. With DENV infection, we found that DENV disrupts the expression of Smad2 / Smad3 and may inhibit the TGF-β signaling, more specifically by reducing the phosphorylation of Smad3 during replication. Downregulation of R-Smads was used as a strategy by some viruses to improve their survival. Respiratory syncytial virus inhibits the Smad2/3 signaling in macrophages to block interferon secretion [25]. Hepatitis C virus (HCV) protein NS3, NS5 or core interact with Smad2/3 and block the phosphorylation and function of these Smad proteins [14, 26]. Herpes Simplex virus, human cytomegalovirus and HIV inhibit the Smad3 activity to maintain their survival in different cells [27-29]. Not all viruses require suppression of Smad2/3 activities; Rift Valley fever virus requires the phosphorylation of these proteins for its own replication [30]. In other report influenza virus activates the TGF-β signaling to promote secondary bacterial infection [12].

R-Smads form the complex with Smad4, also called co-Smad and translocate in to nucleus for the transcription of specific genes. Therefore, we were curious to know what will be the fate of the co-Smad Smad4 involved in the Smad signaling during DENV replication. Over expression of Smad4 in HEK-293T

cells followed by DENV infection resulted in its degradation. Similar results had been observed with Human T-cell leukemia virus type 1 that blocks Smad3 and Smad4 binding with respective promoters to activate the transcription of associate genes [31]. Likewise, BARF1 protein of Epstein-Barr virus downregulates Smad4 to promote cell proliferation and virus induced stomach cancer progression [18]. In TGF-β signaling the activity of Smad2/3/4 is regulated by inhibitory Smads, i.e. Smad6 and Smad7. Lower expression of Smad6 and Smad7 were observed with viral infection. Additionally, all the Smad proteins had slight impeding effect on virus replication. This may be the key for developing antiviral therapy against DENV infection. However, Smad6 and Smad7 promote HCV replication by inducing the expression of SDC1 and LDLR [32].

The viral non-structural proteins have been reported to evade the host response by interacting with host proteins. Different RNA and DNA viruses use their viral proteins to evade TGF-β signaling by blocking the Smad complex [14, 18, 26]. DENV NS1 has been known to its multifunctional nature [19]. Over expression of these Smads with NS1 results their degradation at different levels. Since NS1 does not possess ubiquitin function, it must do so by taking help from ubiquitins involved in TGF-β signaling. A handful of ubiquitins such as Smurf2 and CHIP/Stub1 are known to interact with Smad family proteins [15, 33-35]. Our results confirmed that these E3 ligases have different preferences during DENV replication and NS1 expression. CHIP and Smurf2 has been reported for their specific regulation of Smad proteins in different environments [34]. $CHIP^{-/-}$ cells showed higher expression of Smad3 and Smad4 suggesting constant requirement of CHIP in maintaining their basal level in normal condition. However, a catalytic mutant of Smurf2 [23] confirmed that DENV utilizes Smurf2 to reduce the expression of Smads.

We found that E3 ligases utilize the proteasomal and lysosomal pathway for the degradation of different Smad proteins. To uncover the mechanism behind the degradation of Smad proteins during DENV infection, we used inhibitors of proteasomal and lysosomal pathway. Utilization of both proteasomal and lysosomal mediated pathway by E3 ligases in enveloped/RNA virus infection is well reported [24, 35]. Smurf2 has been reported to degrade inhibitory Smad via proteasomal and lysosomal pathways [23]. It appears that DENV NS1 utilized this unique feature of Smurf2 to degrade Smad3 and Smad4. Utilization of such selective pathways during infection may be the one of the biological signatures of DENV pathogenesis.

Reduction in expression of Smad proteins in the presence of NS1 allowed us to look for the physical interaction between NS1, Smads and E3 liagses. Our co-IP experimentation established the physical interaction between E3 ligases and Smads which may be facilitated by NS1 as a connecting bridge. Smad2, Smad3 and Smad4 form a complex and accumulated in nucleus. Further experimentation showed lower level of accumulation of Smads into nucleus due to NS1 suggesting abrogation of Smad signalling during DENV infection. Likewise NS5 of HCV has been earlier reported to block the translocation of Smad proteins into nucleus and reduce its accumulation [26]. Such kind of mechanisms may provide strategic benefit to the viral pathogenesis.

DENV uses its non-structural proteins to evade the host defense. This study has demonstrated the novel regulatory role of NS1 in TGF-β/Smad signaling, and confirmed that DENV NS1 utilizes E3 ligase Smurf2 to regulate the expression of regulatory, co and inhibitory Smads during DENV infection. We have observed another evidence of multifunctional viral protein NS1 playing as an antagonist of host-defense mechanism as depicted in the graphical presentation. Thus NS1 would be a perfect target for developing novel therapeutics against DENV infection.

Materials And Methods:

Cell culture and virus propagation

Dengue virus serotype 2 (DENV-2) strain New Guinea C (NGC) was used in our experiments, prepared by propagating in C6/36 cells. These cells were maintained in L-15 medium (Gibco Life Technologies), supplemented with fetal bovine serum and antibiotic antimycotic solution (Himedia Laboratories). Virus containing cell supernatants were harvested upon observation of cytopathic effect or physical changes in the cells, filtered and stored at -70^{o}C till further use. Virus stocks were titered by plaque assays using BHK21 cells.

The STUB1/CHIP gene in HEK293T cells was knocked out with CRISPR-Cas9 plasmid containing CHIP sgRNA as described previously [24, 36]. DNA oligonucleotides were synthesized by using the sgRNA designer tool available in the web interface of Broad Institute, USA from cDNA sequence of CHIP. Source of control sgRNA was firefly (*Photinus pyralis*) luciferase gene (**Supplementary Table S1**). Oligonucleotides were mixed with phosphonucleotide kinase enzyme reagent mix and kept for 40 min at 37^{o}C. Reaction was stopped by adding 0.1M NaCl and incubated at 65^{o}C for 20 min. Oligonucleotides were annealed by boiling the mix for 5 min at 100°C and allowed the mix to bring to room temperature. BbsI digested pSpCas9 (BB)-2A-GFP (pX458) plasmid was mixed with reaction mix for ligation with annealed oligos and transformed in to *E. coli* to propagate the plasmid. Clone was confirmed by Sanger sequencing and used to transfect HEK-293T cells. The said plasmid has GFP protein to sort the positive cells by sorter (BD FACS Aria-II). Sorted cells were maintained in DMEM containing 10% FBS and antibiotics and gene knockdown was checked by western blot.

Transfections and plasmids

Transfections were performed using linear Polyethyleneimine (MW 25,000; Polysciences Inc., USA) reagents in accordance with the manufacturer's instructions. Addgene plasmids pCMV5B-HA-Smad2 (# 11734), pCMV5B-Flag-Smad3 (# 11742), pCMV5-Smad7-HA (# 11733), pCMV5B-Flag-Smurf2 wt (# 11746) and pCMV5B-Flag-Smurf2 C716A (# 11747) were gifts from Dr. Jeffrey Wrana. pcDNA Flag-Smad4M (# 14959) and CS2 HA-Smad6 (# 14962) was a gift from Joan Massague via Addgene. DENV NS1 encoding plasmid (pCDNA 3.1-His-NS1) was kindly provided by Dr. Ronaldo Mohana-Borges, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

Western blotting and antibodies

Supernatant of lysed HEK-293T cells were resuspended in SDS sample loading buffer, resolved on 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Cat # SCNJ8101XXXX101, MDI, Advanced Microdevices Ltd., India). Membrane was blocked with nonfat milk or BSA and probed with different specific primary antibodies, followed by probing with respective HRP-conjugated secondary Abs (Jackson ImmunoResearch, USA). Membrane was washed and developed using chemiluminescence solutions.

Anti-Smad2 (cat#5339), phospho-SMAD2 (Ser465/Ser467; cat#18338), anti phospho-Smad3 antibody (Ser423/425; cat#9520), anti-Lamin A/C antibody (cat#2032) antibodies were obtained from Cell Signaling Technology; anti-GAPDH antibody (cat#Sc-32233) from Santa Cruz Biotechnology; anti-HA tag antibody (cat #M1001010), anti-His tag antibody (cat#M1001020) from Immunotag/G-Biosciences and anti-FLAG® M2 antibody (cat #F1804), anti-DENV NS1 antibody (cat# SAB2702308) from Sigma-Aldrich.

Activators and inhibitors

Human Transforming Growth Factor-beta 1 (#TC298; Himedia) was used to stimulate Smad signaling. Lysosomal function inhibitor chloroquine (#C6628, Sigma-Aldrich) and MG132 (#C2211, Sigma-Aldrich), a membrane permeable proteasome inhibitor were used to treat HEK-293T cells for 8 hours prior harvesting for immunoblotting.

Preparation of nuclear and cytoplasmic extracts

Nuclear and cytoplasmic fractions were prepared from cells transfected with different plasmids of interest using Nuclear and Cytoplasmic Extraction Kit (G-Biosciences, USA) as per manufacturer's instructions and subjected to immunoblotting. In our experiments, Lamin A/C and GAPDH were used as a nuclear and cytosolic loading control respectively.

Gene expression analysis

Total cellular RNA was extracted from DENV-2 infected or control HEK-293T cells using RNeasy Mini Kit (Qiagen) as per the manufacturer’s instructions. The quantity and quality of RNA were determined on a Nanodrop instrument (ThermoFisher Scientific). 500 ng of RNA was subjected to cDNA synthesis by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative Real time PCR was performed on cDNA samples using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) on a ABI 7500 Fast Real Time PCR system (Applied Biosystems) as described in manufacturer’s protocol. Set of primers used in this study were described in **Supplementary Table 2**. Gene expression analysis was performed by ddCt method and fold changes were calculated.

Quantitative real-time PCR for viral replication

Viral RNA was extracted from cell supernatants harvested from DENV infected HEK-293T cells using QIAamp Viral RNA Mini Kit as per manufacturer instructions (Qiagen). Eluted RNA samples were

subjected to qRT-PCR with very specific primers and probe of DENV-2 capsid region and TaqMan fast virus one step master mix (Applied Biosystems) as described previously [37]. Estimation of viral RNA (copies/mL) was carried out by using the standard curve generated from DENV-2 transcripts.

Statistical analysis

Western blot images were analyzed by using imageJ software. All results represented here as mean± standard error obtained from three independent experiments. For the calculation of fold changes GAPDH was used as endogenous control. Statistical significance was calculated by using student t test and data was considered significant if $p<0.05$.

Abbreviations

TGF- β : Transforming growth factor-β

Smad: Small mothers against decapentaplegic

Smurf2: Smad ubiquitination regulatory factor 2

CHIP: C terminus of HSC70-Interacting Protein

DENV: Dengue virus

NS1: Non-structural protein 1

Declarations

Acknowledgements

This study was supported by funds from DST-INSPIRE Faculty award (Department of Science and Technology DST, Government of India, New Delhi, India) and Institutional core funding (National Institute of Immunology, New Delhi, India) received by A.L. and A.C.B. respectively.

Author contributions

A.L., R.P.A and A.C.B. conceived and designed the experiments, analyzed data and wrote the manuscript. A.L., R.P.A., V.K. and R. M. performed the experiments.

Competing interests

The authors declare no competing interests.

References

1. Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, Roberts AB, Sporn MB, Goeddel DV. Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. Nature. 1985;316,701–705.
2. Kubiczkova L, Sedlarikova L, Hajek R, Sevcikova S. TGF-β - an excellent servant but a bad master. J. Transl. Med. 2012;10,183.
3. Ikushima H, Miyazono K. TGFβ signalling: a complex web in cancer progression. Nat Rev Cancer. 2010;10(6),415-
4. Derynck R & Zhang YE. Smad-dependent and Smad independent pathways in TGF-β family signalling. Nature. 2003;425,577–584.
5. Shi Y & Massague´ J. (2003). Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell. 113,685–700.
6. Morikawa M, Derynck R & Miyazono K. TGF-β and the TGF-β Family: Context-Dependent Roles in Cell and Tissue Physiology. Cold Spring Harb Perspect Biol. 2016;8(5),a021873.
7. Spender LC, O'Brien DI, Simpson D, et al. TGF-beta induces apoptosis in human B cells by transcriptional regulation of BIK and BCL-XL. Cell Death Differ. 2009;16(4),593-602.
8. de Bie P, Ciechanover A. Ubiquitination of E3 ligases: self-regulation of the ubiquitin system via proteolytic and non-proteolytic mechanisms. Cell Death Differ. 2011;18(9):1393-1402.
9. Xu, Z., Greenblatt, M., Yan, G. et al. SMURF2 regulates bone homeostasis by disrupting SMAD3 interaction with vitamin D receptor in osteoblasts. Nat Commun. 2017;8,14570.
10. Lönn, P., Morén, A., Raja, E. et al. (2009). Regulating the stability of TGFβ receptors and Smads. Cell Res.19, 21–35.
11. French D, Belleudi F, Mauro MV, et al. Expression of HPV16 E5 downmodulates the TGFbeta signaling pathway. Mol Cancer. 2013;12(1), 38.
12. Li N, Ren A, Wang X, et al. Influenza viral neuraminidase primes bacterial coinfection through TGF-β–mediated expression of host cell receptors. Proc Natl Acad Sci. 2015;112(1),238-
13. Jee MH, Hong KY, Park JH, et al. New mechanism of hepatic fibrogenesis: hepatitis C virus infection induces transforming growth factor β1 production through glucose-regulated protein 94. J Virol. 2016;90(6),3044-
14. Cheng PL, Chang MH, Chao CH, et al. Hepatitis C viral proteins interact with Smad3 and differentially regulate TGF-β/Smad3-mediated transcriptional activation. Oncogene. 2004;23(47),7821-
15. Verga-Gérard A, Porcherot M, Meyniel-Schicklin L, et al. Virus/human interactome identifies SMURF2 and the viral protease as critical elements for the control of TGF-β signaling. FASEB J. 2013;27(10),4027-
16. Guzman MG, Gubler DJ, Izquierdo A, et al. Dengue infection. Nat Rev Dis Primers. 2016;2,16055.
17. Whitehead SS, Blaney JE, Durbin AP & Murphy BR. Prospects for a dengue virus vaccine. Nat Rev Microbiol. 2007;5(7),518-528.

18. Kim DH, Chang MS, Yoon CJ, Middeldorp JM, Martinez OM, et al. Epstein-Barr virus BARF1-induced NFκB/miR-146a/SMAD4 alterations in stomach cancer cells. Oncotarget. 2016;7, 82213-82227.
19. Uno N, Ross TM. Dengue virus and the host innate immune response. Emerg Microbes Infect. 2018;7(1),167.
20. Bai Y, Ying Y. The Post-translational Modifications of Smurf2 in TGF-β Signaling. Front Mol Biosci. 2020;7:128.
21. Wang L, Liu YT, Hao R, Chen L, Chang Z, Wang HR, Wang ZX, Wu JW. Molecular mechanism of the negative regulation of Smad1/5 protein by carboxyl terminus of Hsc70-interacting protein (CHIP). J Biol Chem. 2011;286(18):15883-94.
22. Ogunjimi AA, Briant DJ, Pece-Barbara N, Le Roy C, Di Guglielmo GM, Kavsak P, Rasmussen RK, Seet BT, Sicheri F, Wrana JL. Regulation of Smurf2 ubiquitin ligase activity by anchoring the E2 to the HECT domain. Mol Cell. 2005;19(3):297-308.
23. Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, Wrana JL. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. Mol Cell. 2000;6(6):1365-1375.
24. Ali A, Farooqui SR & Banerjea AC. The host cell ubiquitin ligase protein CHIP is a potent suppressor of HIV-1 replication. J Biol Chem. 2019;294(18),7283-7295.
25. Pokharel SM, Shil NK & Bose S. Autophagy, TGF-β, and SMAD-2/3 Signaling Regulates Interferon-β Response in Respiratory Syncytial Virus Infected Macrophages. Front Cell Infect Microbiol. 2016;6,174.
26. Choi S-H & Hwang SB. Modulation of the transforming growth factor-β signal transduction pathway by hepatitis C virus nonstructural 5A protein. Biol Chem. 2006;281(11),7468-7478.
27. Nie Y, Cui D, Pan Z, Deng J, Huang Q, Wu K. HSV-1 infection suppresses TGF-beta1 and SMAD3 expression in human corneal epithelial cells. Mol Vis. 2008;14,1631-1638.
28. Hancock MH, Crawford LB, Pham AH, et al. Human Cytomegalovirus miRNAs Regulate TGF-β to Mediate Myelosuppression while Maintaining Viral Latency in CD34+ Hematopoietic Progenitor Cells. Cell Host Microbe. 2020;27(1),104-
29. Kang JH, Jung MY, Yin X, Andrianifahanana M, Hernandez DM, Leof EB. Cell-penetrating peptides selectively targeting SMAD3 inhibit profibrotic TGF-β signaling. J Clin Invest. 2017;127(7), 2541-2554.
30. de la Fuente C, Pinkham C, Dabbagh D, et al. Phosphoproteomic analysis reveals Smad protein family activation following Rift Valley fever virus infection. PLoS One. 2018;13(3), e0194633.
31. Lee DK, Kim BC, Brady JN, Jeang KT, Kim SJ. Human T-cell lymphotropic virus type 1 tax inhibits transforming growth factor-beta signaling by blocking the association of Smad proteins with Smad-binding element. J Biol Chem. 2002;277 (37), 33766-33775.
32. Zhang F, Sodroski C, Cha H, Li Q, Liang TJ. Infection of Hepatocytes With HCV Increases Cell Surface Levels of Heparan Sulfate Proteoglycans, Uptake of Cholesterol and Lipoprotein, and Virus Entry by Up-regulating SMAD6 and SMAD7. Gastroenterology. 2017 Jan;152(1):257-270.e7.

33. Li L, Xin H, Xu X, Huang M, Zhang X, Chen Y, Zhang S, Fu XY, Chang Z. CHIP mediates degradation of Smad proteins and potentially regulates Smad-induced transcription. Mol Cell Biol. 2004;24(2), 856-864.
34. Inoue Y, Imamura T. Regulation of TGF-beta family signaling by E3 ubiquitin ligases. Cancer Sci. 2008;99(11),2107-
35. Roy N, Pacini G, Berlioz-Torrent C, Janvier K. Characterization of E3 ligases involved in lysosomal sorting of the HIV-1 restriction factor BST2. J Cell Sci. 2017;130(9),1596-
36. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA & Zhang F. Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 2013;8:2281–2308.
37. Lahon A, Arya RP, Kneubehl AR, Vogt MB, Dailey Garnes NJ, Rico-Hesse R. Characterization of a Zika Virus Isolate from Colombia. PLoS Negl Trop Dis. 2016;10(9),e0005019.

Figures

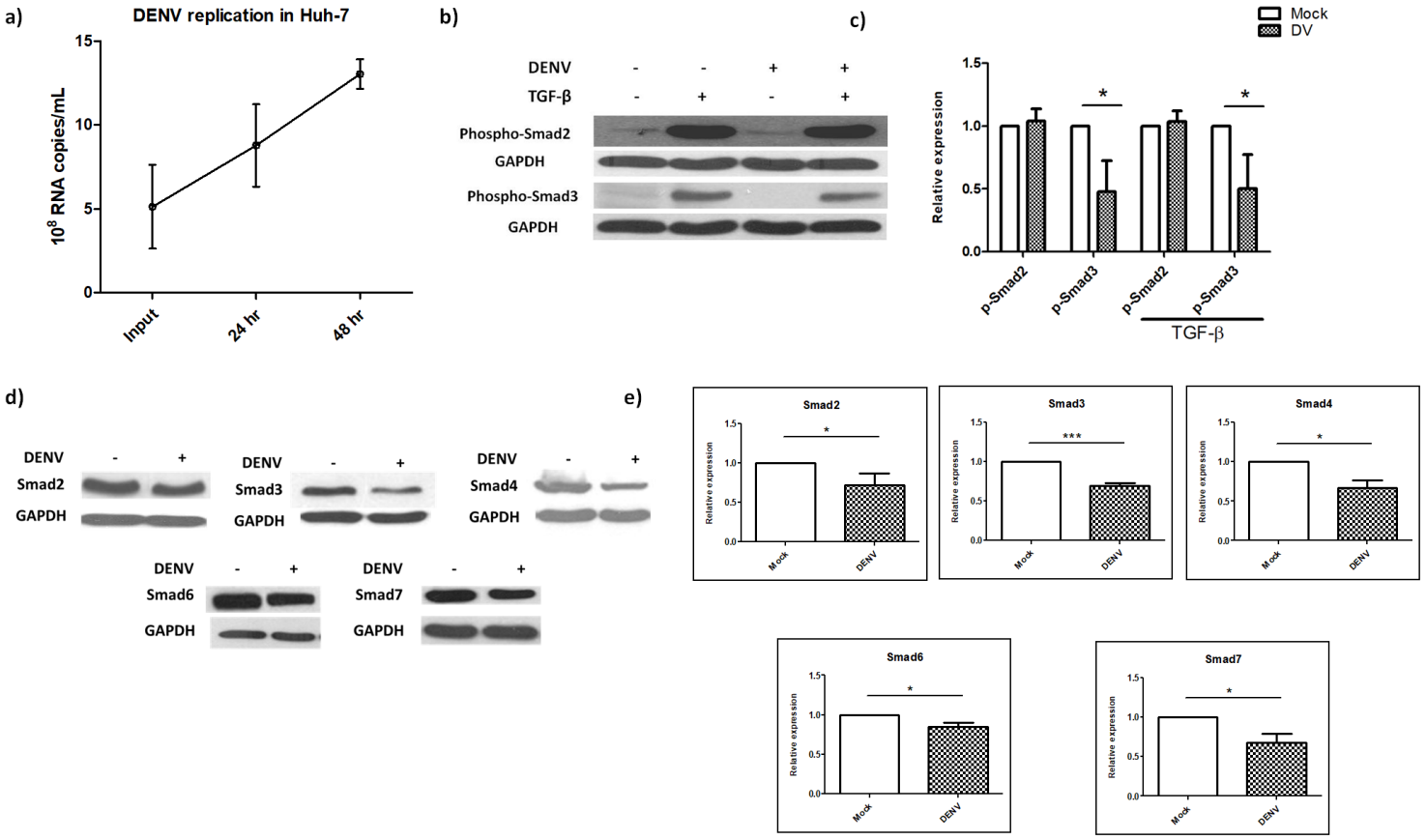

Figure 1

DENV interacts with Smad proteins and impairs their expression. (a) Huh-7 cells were infected with DENV (1 MOI) and virus replication was assessed at 24 and 48 hr post infection by quantitative RT-PCR. (b) DENV infected Huh-7 cells were treated with TGF-β or left untreated. Expression of phosphorylated Smad2/3 in DENV infected cells was observed by western blot. (c) Expression of p-Smad2/3 showed as

column graphs (mean±SE). (d) Smad2, Smad3, Smad4, Smad6 and Smad7 transfected HEK-293T cells were infected with DENV (1 MOI) and utilized to observed their expression by Western blot. Reduced expression of all Smads was noted with DENV infection. (e) Expression of Smad proteins with DENV infection is presented as column graphs (mean±SE).

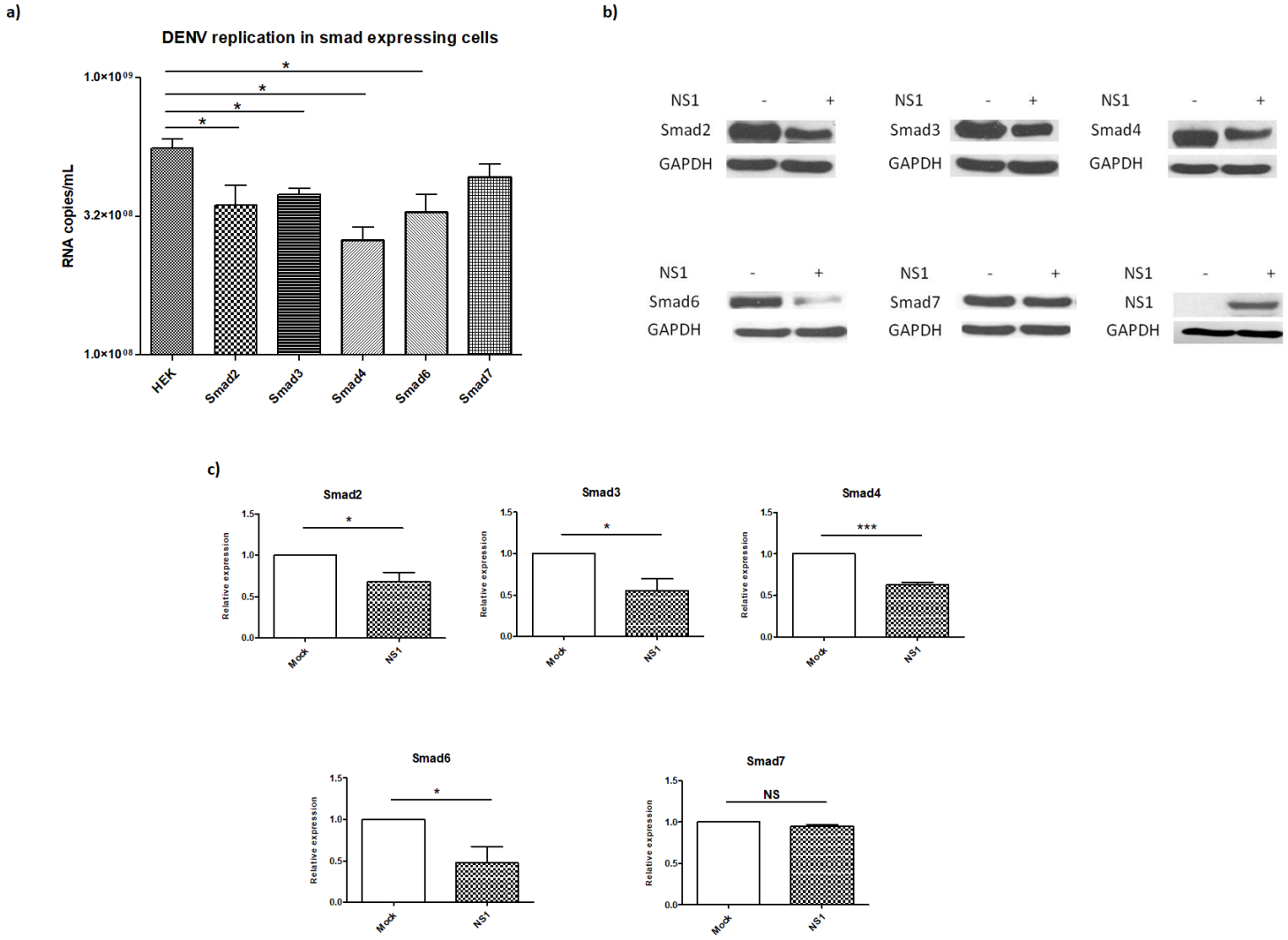

Figure 2

Effect of Smad over-expression on DENV replication and effect of NS1 on the expression of Smads. a) Identification of Smads that inhibit DENV replication. HEK-293T cells (1×105) were seeded on 6-well plates and the next day transfected with Smad constructs (Smads 2, 3, 4, 6 and 7) or left untransfected. At 24h post-transfection, cells were infected with 1 MOI of DENV. Viral RNA copy number was determined from isolated supernatants collected at 48hr post-infection by qRT-PCR. (b) DENV-NS1 is responsible for degradation of Smad proteins. HEK-293T cells were transfected with DENV NS1 and any one of the Smad constructs (Smad2/3/4/6/7). Western blot analysis reveals that the degradation is NS1 mediated. All the blots are representative of three independent experiments. (c) Expression of Smad with NS1 is presented as column graphs (mean±SE).

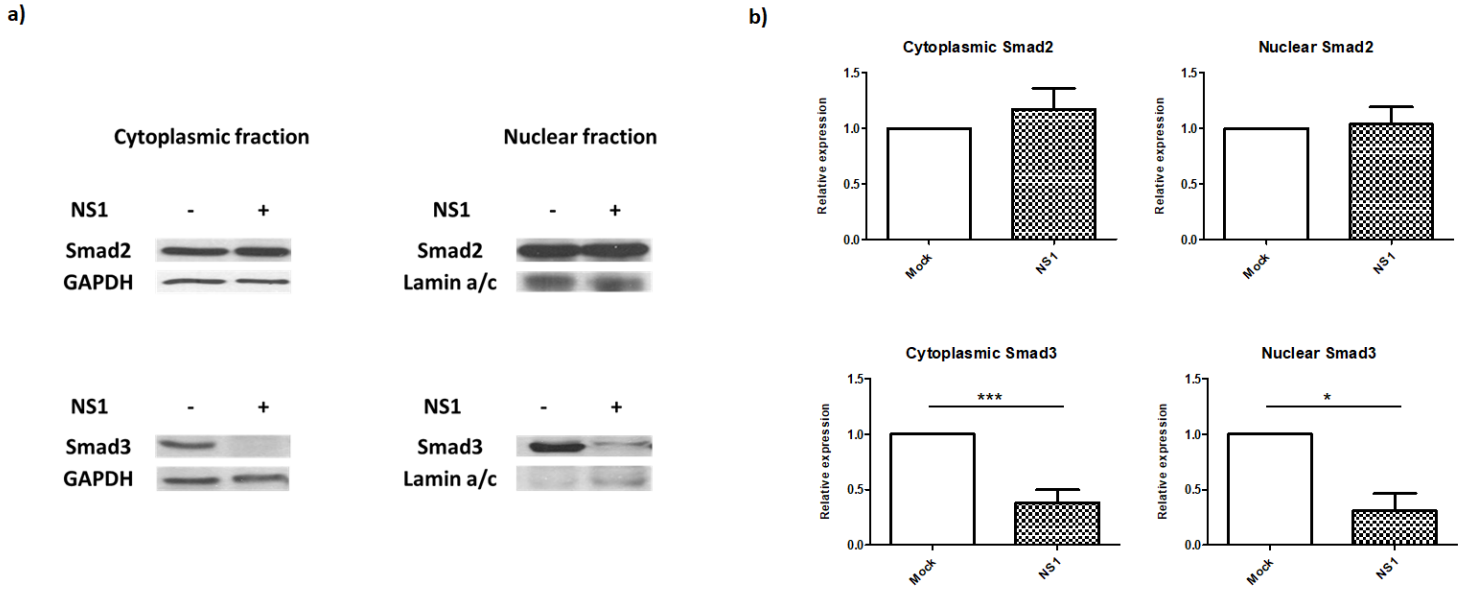

Figure 3

DENV NS1 blocks the nuclear translocation of Smad3 (a) Western blotting of nuclear and cytoplasmic extracts of HEK-293T cells transfected with DENV NS1 and respective Smads. Lamin A/C and GAPDH were used as markers for nuclear and cytoplasmic extract respectively. DENV NS1 blocks the nuclear translocation of Smad3. (b) Expression of Smad with NS1 in cytoplasm (GAPDH as loading control) and nucleus (lamin a/c as loading control) presented as column graphs (mean±SE).

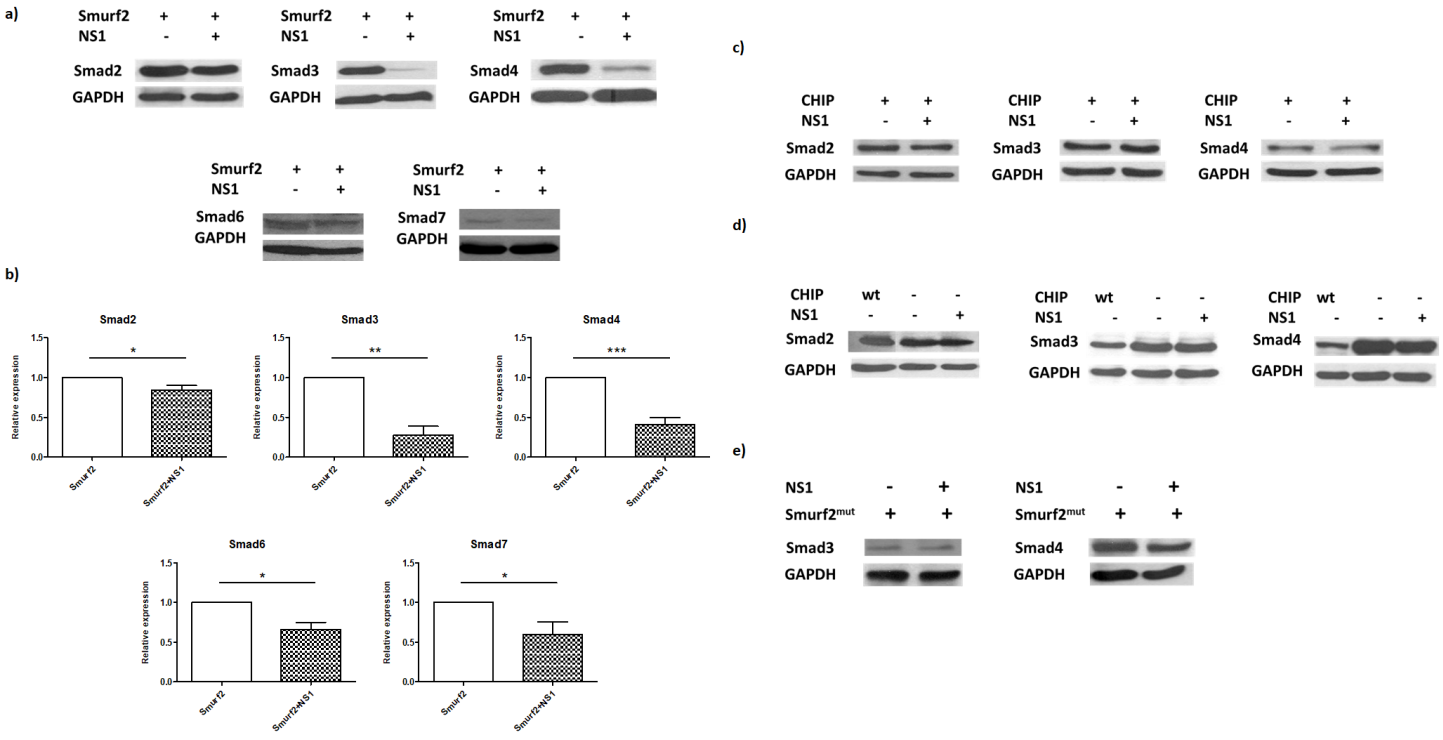

Figure 4

The ubiquitin ligase Smurf2 assists in DENV-NS1 mediated degradation of Smads (a) HEK-293T cells were co-transfected with 3 constructs, i.e., DENV-NS1, Smurf2 and Smad2/3/4/6/7 and harvested at 48h followed by western blot analysis. (b) Densitometry measurements indicate that Smurf2 has connection in degradation of Smad3, 4, 6, 7 and less affected on Smad2. (c) Effect of CHIP in the expression of Smad2, Smad3 and Smad4. Smad2/3 or Smad4 was transfected with CHIP construct in HEK-293T cells. Co-expression of CHIP did not change the normal level of expression of Smad2, Smad3 and Smad4. (d) Expression of Smad2, Smad3 and Smad4 in CHIP knockdown HEK-293T cells. CHIP knockdown cells showed higher expression of Smad3 and Smad4 and had no effect of NS1. (e) Smurf2 mutation reduced the degradation of Smad3 and Smad4 by NS1. HEK-293T cells were transfected with Smad3/4, Smurf2mut with or without NS1.

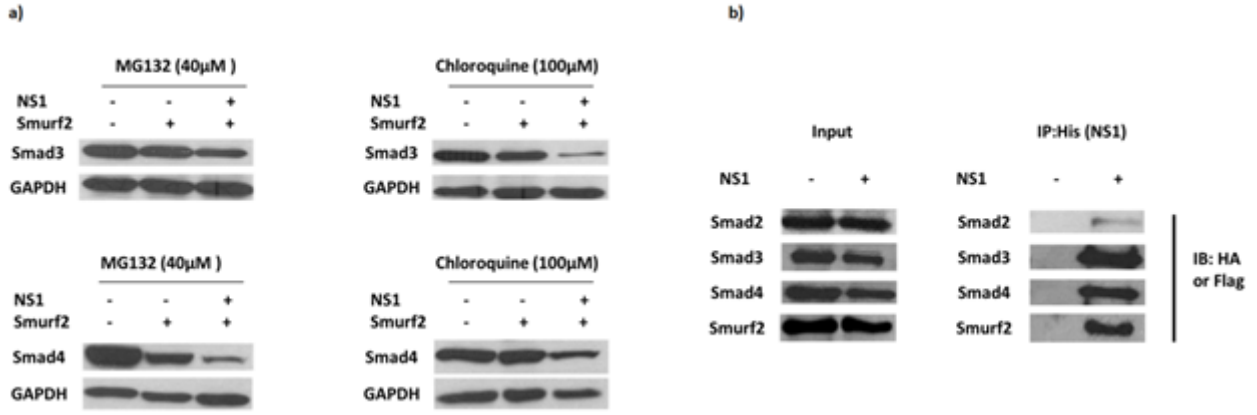

Figure 5

Co-immunoprecipitation of DENV-NS1 with Smad2/3/4 and Smurf2. (a) HEK-293T cells were transfected with Smad2/3/4 alone or with DENV NS1; at 36h post-transfection, cells were treated with proteasomal inhibitor, MG132 (40 uM) and lysosomal inhibitor Chloroquine (100uM) for 8h followed by western blotting. MG132 and chloroquine treatment reduced the effect of NS1 in Smad3 and Smad4 expression respectively. (b) His-NS1 and Smad2/3/4 or Smurf2 co-expressed in HEK-293T cells for 48 hr. Cells were lysed and supernatant was subjected with Ni-NTA resin. His-tagged NS1 bounded proteins were released from the cell lysate during elution. The eluted samples were subjected for western blot and probed for Smad or smurf2 proteins. Western blot analysis showed binding of Smad2/3/4 or smurf2 with NS1.

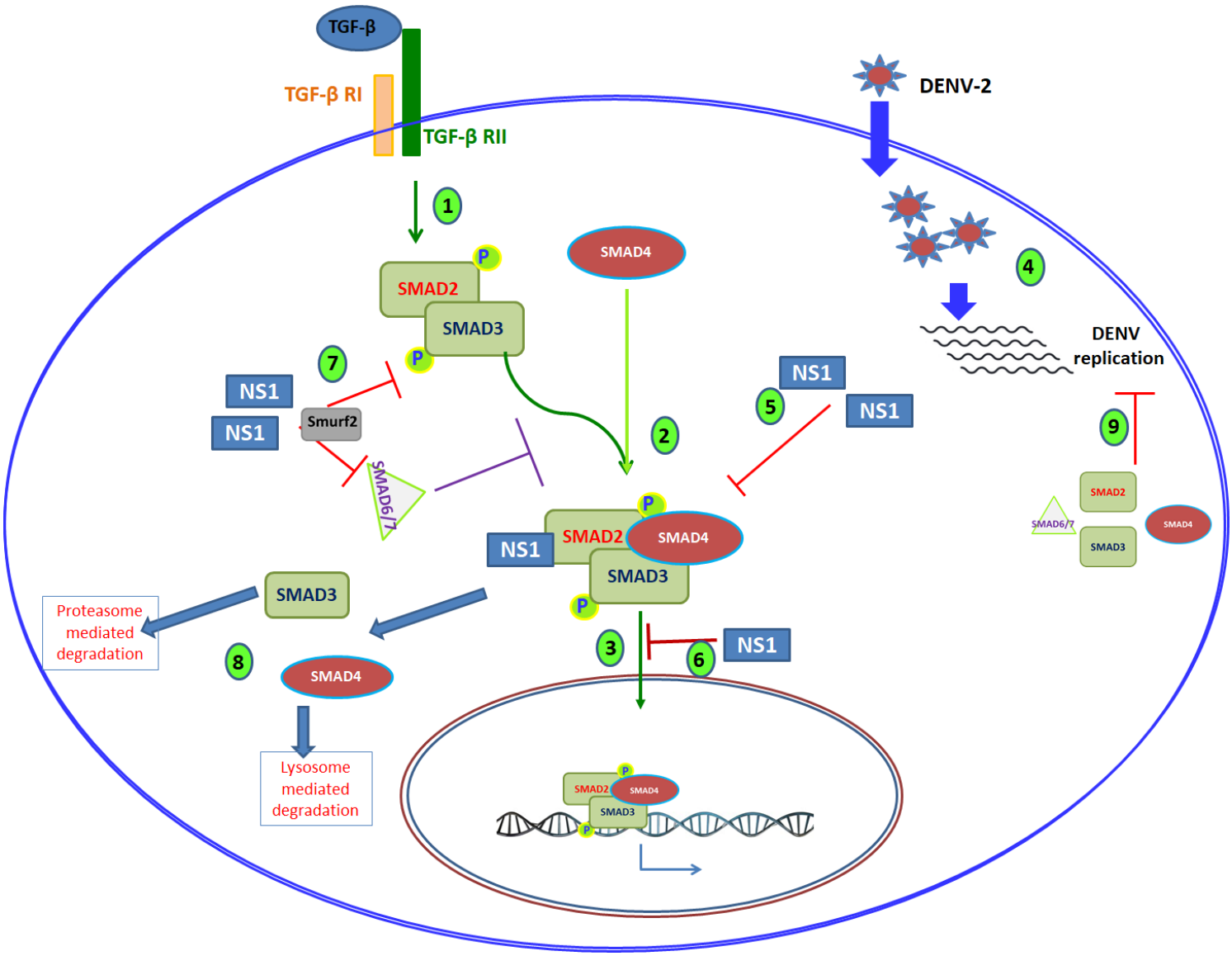

Figure 6

Graphical Presentation: The graphic represents the events during dengue virus infection in TGF-β signaling. (1) TGF-β binds to its receptors and the receptors activate the downstream signaling cascades. TGF-βRI and TGF-βRII phosphorylate Smad2/3. (2) p-Smad2/p-Smad3 binds with Smad4. (3) This complex goes into nucleus and binds to specific site of DNA for transcription. (4) DENV enters into the cells and releases its RNA genome for translation of viral structural and non-structural proteins and their replication. (5) NS1 binds with Smad2/3/4 and block translocation of Smads. (6) NS1 blocks the phosphorylated Smad2/3 and translocation of Smad2/3/4 complex into nucleus. (7) NS1 utilizes Smurf2 to degrade Smad2/3/4/6/7. (8) proteasomal and lysosomal degradation. (9) Smad2, 3, 4 and 6 has slight effect on the DENV replication.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTables.docx
- Suppfigure1.jpg