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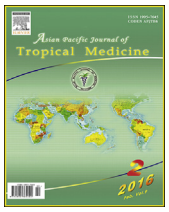

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Dengue virus non-structural 1 protein interacts with heterogeneous nuclear ribonucleoprotein H in human monocytic cells

Drishya Diwaker, K.P. Mishra*, L. Ganju, S.B. Singh

Immunomodulation Lab, Defence Institute of Physiology & Allied Sciences, Delhi, India

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ABSTRACT

Objective: To study protein–protein interaction between heterogeneous nuclear ribonucleoprotein H (hnRNP H) and Dengue virus (DENV) proteins.
Methods: DENV proteins were screened against the host hnRNP H protein, in order to identify the host-viral protein–protein interactions in DENV infected THP-1 cells by co-immunoprecipitation. The co-localization of the interacting proteins was further confirmed by immunofluorescence microscopy.
Results: The host protein hnRNP H was found to interact with DENV non-structural 1 protein and help the virus to multiply in the cell.
Conclusions: The non-structural 1 glycoprotein is a key modulator of host immune response and is also involved in viral replication. Therefore, disruption of this key interaction between hnRNP H and DENV non-structural 1 could be an important therapeutic strategy for management of DENV infection.

1. Introduction

The heterogeneous nuclear ribonucleoproteins (hnRNPs) are a group of cellular proteins that are RNA binding proteins in association with heterogeneous nuclear RNA. They are primarily associated with pre-mRNA and help in the maturation process of these mRNA molecules. They have also been known to regulate gene expression by acting in trans [1]. The hnRNP protein family comprises of around 20 members whose size ranges from 34 to 120 kDa and other less abundant proteins [1–3]. hnRNP proteins are abundant and share similar RNA binding motifs and sequence homology. They also have an additional domain, which is rather divergent, for protein–protein interactions and sub-cellular localization [2]. hnRNP H contains two glycine rich domains separated by a quasi-RNA recognition motif [4]. hnRNP H binds to intronic G-triplets or glycine rich repeats and mediates the cleavage and polyadenylation of the pre-mRNA [5].

Viruses depend on the host cell for their propagation and seize the host machinery, utilizing it to their benefit. The interplay between the host and virus could be at various levels. An understanding about the molecular interactions and regulation of cellular functions is necessary to determine the immunopathogenesis mechanism of the viral infection. Dengue virus (DENV) has been reported to interact with specific cellular proteins at different stages of its propagation within the host cell, such as the interaction with certain Golgi apparatus proteins during viral replication and with calreticulin during viral assembly [6]. hnRNP H has been shown to be involved in splicing regulation in viral infections such as human papillomavirus [5,7], Rous sarcoma virus [8], Simian vacuolating virus [9] and HIV [10,11].

Members of the Flaviviridae family of viruses are causative agents of several severe infections in human beings. DENV is a Flavivirus having a positive sense, RNA viral genome which codes for three structural (capsid, C; premembrane, prM; and envelope, Env) and seven-non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). The viral NS1 protein is a glycoprotein that has been reported to be involved in viral replication, maturation and signal transduction. Newly formed NS1 exists as a monomer, however, it dimerizes on subsequent

*Corresponding author: Dr K.P. Mishra, Scientist-D, Immunomodulation Laboratory, Defence Institute of Physiology & Allied Sciences, Lucknow Road, Timarpur, Delhi, 110054, India.
Tel: +91 11 23883163
Fax: +91 11 23932869
E-mail: kpmpgi@rediffmail.com

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glycosylation events. Recently, NS1 has been shown to remodel the lipid bilayer resulting in the formation of hexameric NS1 proteolipid particles and finally secreted [12]. On entering circulation NS1 interacts with the immune system. For instance, the viral NS1 protein has been reported to interact with a key component of the complement system called the human complement component 1 [13].

The proteomic profiles of cellular proteins up-regulated or down-regulated during DENV infection and extensive interaction networks have been reported by several groups. However, several discrepancies have been found particularly in the expression of the various hnRNPs in different cell types [14]. In this study, we have screened the DENV proteins against the host hnRNP H protein, in order to identify the host-viral protein–protein interactions in DENV infected THP-1 cells. From our previous study we know that hnRNP H helps the virus to multiply in the cell, so further to that, we wish to see how hnRNP H interacts with the viral proteins as it could throw some light upon the mechanism by which the host helps the virus to propagate itself [15].

2. Material and methods

2.1. Cells

Human monocytic cell line THP-1 and C6/36 mosquito cell line were obtained from National Centre for Cell Sciences, Pune, India and maintained in RPMI (Sigma, USA) and Eagle's MEM (Sigma, USA), respectively, supplemented with 10% (v/v) FBS, 100 U/mL and 100 μg/mL streptomycin at 37 °C in 5% CO_2 atmosphere in an incubator (Sanyo, Japan).

2.2. Virus

DENV type-2 New Guinea C strain was obtained from International Centre for Genetic Engineering and Biotechnology, New Delhi, India. Virus was expanded in C6/36 mosquito cell line for 14 d. Culture supernatant was collected and centrifuged at 2 000 r/min for 15 min. Again supernatant was collected and stored at −80 °C which served as virus stock.

2.3. Infection of cells

THP-1 cells (2×10^6 cells/mL) were infected with the DENV serotype 2 at a multiplicity of infection (MOI) 3 and 5 in serum free medium at 37 °C for 1 h to permit viral adsorption. The culture plates were gently agitated for optimal virus cell contact. Thereafter, the unadsorbed virus was removed by washing the cells with plain medium. The DENV infected and mock infected cells were replenished with fresh medium supplemented with 2% (v/v) FBS and further incubated for 24, 48 and 72 h at 37 °C. At the end of the incubation, cells were harvested and the cell free supernatant was stored in aliquots at −70 °C until assayed for further analysis.

2.4. Apoptosis by propidium iodide staining

THP-1 cells (2×10^6 cells/mL) were infected with DENV at MOI-3 and 5, and cultured for 24, 48 and 72 h. Cells were harvested and washed twice with PBS in FACS tubes (BD Biosciences, USA). Supernatant was discarded and 500 μL of 70% (v/v) ethanol was added to the cells and incubated for 1 h on ice. After incubation, cells were centrifuged at 1 200 r/min for 10 min and washed with PBS twice. Supernatant was discarded and 500 μL PBS with 2 μL RNaseA (1 mg/mL) were added to the cells and incubated for 30 min. Cells were washed with PBS by centrifuging at 1 200 r/min for 10 min. Supernatant was discarded and cells were suspended in 500 μL PBS with 10 μL propidium iodide (1 mg/mL) prior to analysis. The sub-G_1 peak was detected and considered as apoptotic death. Cells (10 000 events) were acquired using flow cytometer (BD, FACScalibur).

2.5. Immunoblotting

Whole cell extract was prepared from C6/36 and THP-1 cells either mock or DENV infected as per standard protocol. Samples containing 40 μg of proteins were separated on 12% sodium dodecyl sulphate-polyacrylamide gels following the method described by Laemmli [16] and transferred to polyvinylidene difluoride membranes, as described by Towbin *et al.* [17]. The membranes were incubated for 1 h with 3% (v/v) BSA in TBS buffer (0.1 mol/L Tris–HCl, pH 7.4, 0.9% NaCl) to block non-specific binding followed by washing with $TBST_{20}$ (0.1% Tween-20 in TBS) and incubation with primary antibody. Immunoblotting for DENV NS1 was done in mock or DENV infected C6/36 lysates and β-actin was used as loading control. Antibody against DENV protein(s) (Env, prM, C, NS1, NS3, NS4B and NS5) and hnRNP H was used to probe the whole cell extract proteins of mock or DENV infected THP-1 cells, separated and transferred onto polyvinylidene difluoride membrane. Subsequently, the membranes were washed thrice, for 10 min with $TBST_{20}$ and incubated with secondary antibody horseradish peroxidase-conjugated IgG (AbD Serotec, UK) against the DENV and host primary antibody, respectively. The proteins were detected by chemiluminescence (Sigma, USA).

2.6. Co-immunoprecipitation

Co-immunoprecipitation was carried out using Pierce Co-immunoprecipitation Kit (Thermo Scientific) in accordance to manufacture's protocol. Briefly, 15 μg of hnRNP H specific antibody (Thermo Scientific, USA) was coupled to 50 μL of AminoLink plus amine-reactive resin (aldehyde-activated beaded agarose) prior to the addition of 400 μg of crude cellular lysate to the resin beads. The suspension was rotated at 4 °C for 16 h, followed by washing with 200 μL of immunoprecipitation lysis buffer and eluted with 60 μL of elution buffer. The supernatant (or flow through) from washes was discarded or saved for later testing to measure free (unbound) protein. Immunoblotting was performed as described above to probe hnRNP H protein (bait) and DENV protein(s) (prey) such as DENV Env, prM, NS1 and NS4B (Abcam, UK) Capsid, NS3 and NS5 (GeneTex, USA).

2.7. Immunofluorescence staining

THP-1 cells were either mock or DENV infected as described above, at MOI-3 for 24 h. Cells were fixed and resuspended in blocking buffer (10% human serum in wash buffer) and incubated for 30 min on ice. Subsequently, cells were washed twice with wash buffer and incubated with anti-hnRNP H primary antibody for 60 min on ice. Cells were then washed thrice in

wash buffer and incubated with anti-mouse IgG-PE secondary antibody and anti-DENV NS1-FITC conjugated antibody for 60 min on ice. Cells were then washed thrice in wash buffer and prepared for immunofluorescence imaging.

2.8. Statistical analysis

The results are expressed as mean ± SEM. All statistical analyses were performed by SPSS 15.0. All comparisons were carried out using Student's *t*-test. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Propagation of DENV in C6/36 cell line

The DENV serotype 2 NGC strain was propagated in C6/36 mosquito cell line for 14 d. Infected cells, 7 d post infection (*pi.*) began to show cytopathic effect. Dengue virus infected cells appeared crenated and granulated as compared to healthy uninfected cells (Figure 1A,B). Viral infection was confirmed by immunoblotting for DENV NS1 in the whole cell lysate of uninfected and infected C6/36 cells (Figure 1C).

3.2. Determination of optimum MOI of DENV infected THP-1 cells

Human monocytic cell line THP-1 was infected with DENV type 2, NGC strain at MOI-3 and 5, for 24, 48 and 72 h. The mean fluorescence intensity of DENV infected cells at MOI-3 and 5, was found to be 441 and 438 at 24 h *pi.*; 314 and 333 at 48 h *pi.*; and 102 and 114 at 72 h *pi.*, respectively (Figure 2A,B). Apoptotic death percentage of DENV infected cells was analysed by propidium iodide staining. There was 5.0% and 5.6% increase in cell death as compared to control in MOI-3 and MOI-5, respectively at 24 h *pi.* At 48 h *pi.*, there was 14.6% and 18.0% increase in cell death as compared to control in MOI-3 and MOI-5, respectively. Whereas, at 72 h *pi.*, there was 26.0% and 28.0% increase in cell death as compared to control in MOI-3 and MOI-5, respectively. We found that cell death increased with DENV MOI and time, *ie.*, in a dose and time dependent manner. Therefore, the optimum dose for productive viral replication and expression of the viral proteins in THP-1 was standardized at MOI-3, 48 h *pi.* as this dose showed optimum infectivity and viability for further necessary experiments (Figure 2C). DENV infection in THP-1 cells was confirmed by image flow cytometry (Figure 3). DENV infected cells showed cytopathic effects as compared to the control cells (Panels A &

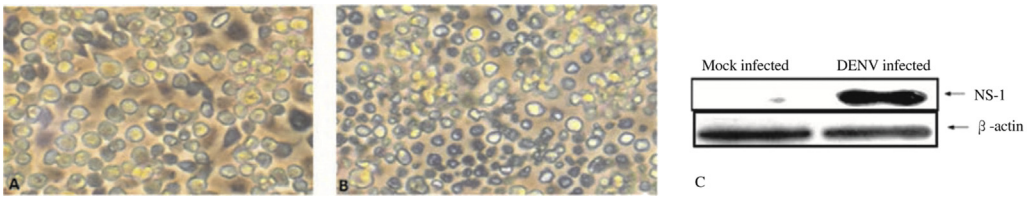

Figure 1. Propagation of DENV and NS1 expression in C6/36 insect cells.
Propagation of DENV in C6/36 insect cells: Bright field image of A) mock cells; B) DENV infected cells 7 d *pi.*; C) DENV NS1 expression: Immunoblotting for viral NS1 protein was done in the whole cell extract of mock and DENV infected C6/36 cells 48 h *pi.* DENV NS1 expression was very high in the infected cell lysate.

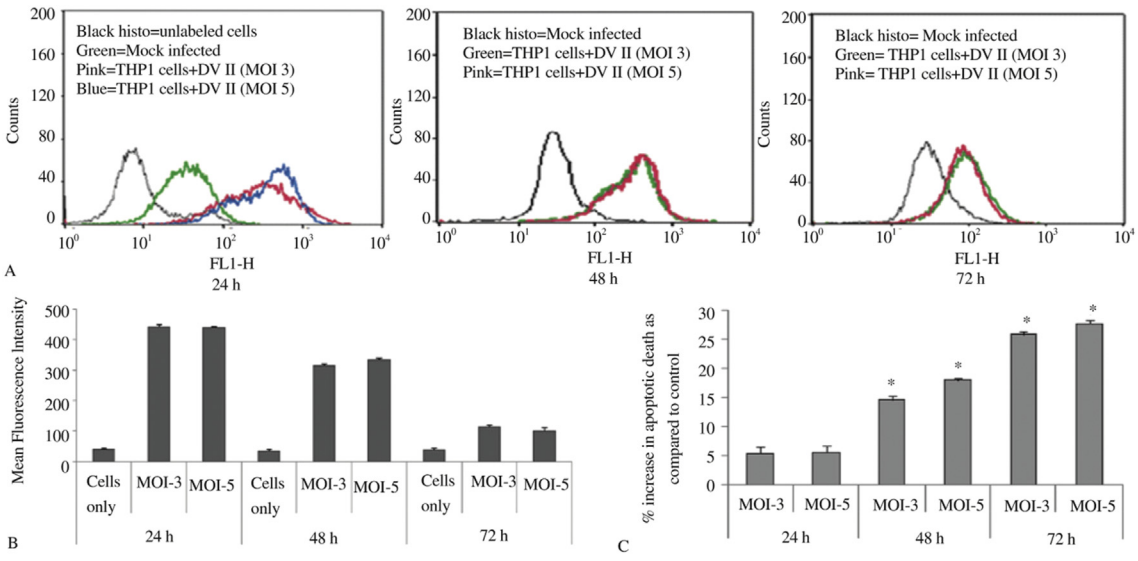

Figure 2. Increase of apoptosis in DENV infected THP-1 cells.
A) MOI dose standardization by flow cytometry: THP-1 cells infected with DENV were harvested and immunostained for the viral Env protein as described in Methods. B) Mean fluorescence intensity of cells only (mock cells), MOI-3 (cells infected with MOI-3 of DENV) and MOI-5 (cells infected with MOI-5 of DENV) obtained 24, 48 and 72 h *pi.* has been shown. C) Percentage of apoptosis in DENV infected THP-1 cells: MOI-3 (THP-1 cells infected with DENV at MOI-3) & MOI-5 (THP-1 cells infected with DENV at MOI-5), 48 & 72 h *pi.* were stained with propidium iodide and analysis using flow cytometry was done as described in Methods. *$P < 0.05$ *vs.* respective control.

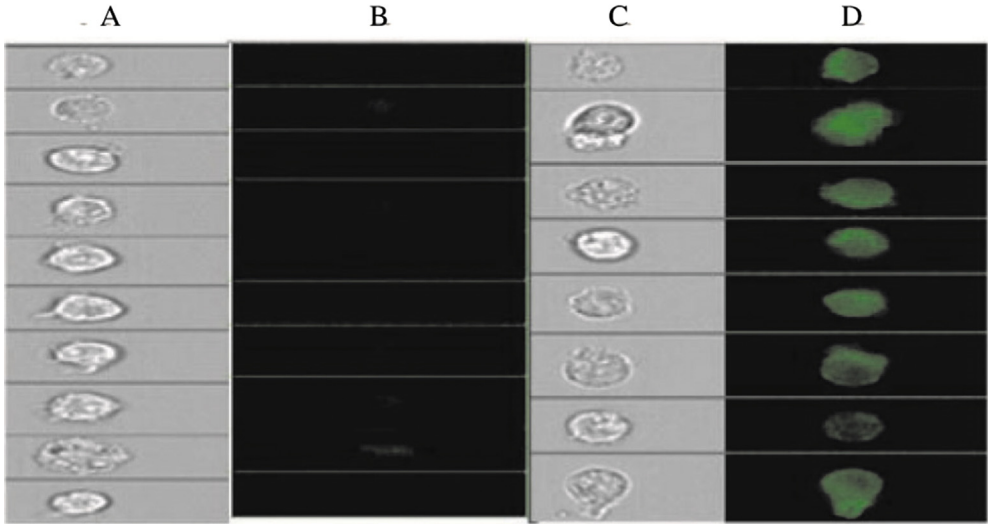

Figure 3. Confirmation of DENV infection by immunofluorescence imaging for DENV envelope protein by image flow cytometry.
Mock THP-1 cells, Mock THP-1 cells: A) Bright field, B) FL-1 (Fluorescence channel); DENV infected cells, C) Bright field, D) FL-1 (Fluorescence channel) showing cells immunostained for viral Env protein as described in Methods.

C, Figure 3). Cells infected with DENV showed green fluorescence for the viral Env protein (Panels B & D, Figure 3), whereas mock cells did not show immunofluorescence. The results confirmed infection of THP-1 cells with DENV at MOI-3, 48 h *pi.*

3.3. Interaction of DENV NS1 protein with host hnRNP H protein

The identification of protein–protein interaction between host protein hnRNP H and DENV proteins was performed by screening seven DENV proteins with hnRNP H. Co-immunoprecipitation of mock and DENV infected cell lysates was performed using specific antibodies against human hnRNP H. Presence of hnRNP H and viral protein(s) in the whole cell lysates (Input) of uninfected and infected cells was confirmed by immunoblotting in order to rule out any false positive signals, prior to performing co-immunoprecipitation with the lysates. Human anti-hnRNP H antibody was covalently coupled to the column and incubated with cell lysates as described in Methods. The bait–prey complex captured in the co-immunoprecipitation column was then eluted and subjected to simultaneous immunoblotting analysis for hnRNP H and DENV proteins (Env, prM, capsid, NS1, NS3, NS4B and NS5). This was repeated at least thrice for every interaction pair. Immunoprecipitation of hnRNP H mock cell lysate did not show any co-precipitation of DENV proteins, whereas, in DENV infected cell lysate, the viral NS1 protein was found to co-precipitate with hnRNP H, as shown by immunoblot analysis (Figure 4). However, the other DENV proteins (Env, prM, C, NS3, NS4B and NS5) did not show interaction with hnRNP H by co-immunoprecipitation.

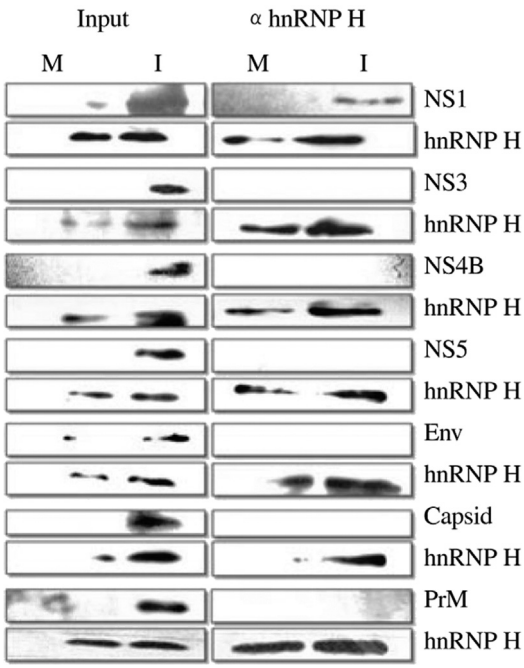

Figure 4. Co-immunoprecipitation and immunoblotting.
THP-1 cells, mock or DENV infected, were used to prepare the whole cell extract, using which, viral protein(s) interacting with hnRNP H was captured by coupling anti-hnRNP H antibody in the co-immunoprecipitation column as described in Methods. The figure depicts the results for screening of hnRNP H against seven DENV proteins (NS1, NS3, NS4B, NS5, Env, Capsid and prM). Immunoblotting was done for both hnRNP H and DENV protein simultaneously (M: Co-immunoprecipitation eluted proteins from mock cell lysate, I: Co-immunoprecipitation eluted proteins from DENV infected cell lysate). INPUT: whole cell lysate of M-mock cells, I-DENV infected cells, 48 h *pi.*

3.4. Co-localization of DENV NS1 protein with host hnRNP H protein

Furthermore, fluorescence image of mock and DENV infected cells immunostained for intra-cellular hnRNP H and NS1 using hnRNP H-PE labelled antibody and NS1-FITC labelled antibody were obtained (Figure 5). The FITC and PE channels were merged and we found that hnRNP H and NS1 co-localized in DENV infected cells (Merge, Figure 5A,B). The mock cells only showed hnRNP H expression. Thus, our results suggest that DENV NS1 protein interacted with the host protein hnRNP H and also co-localized with each other.

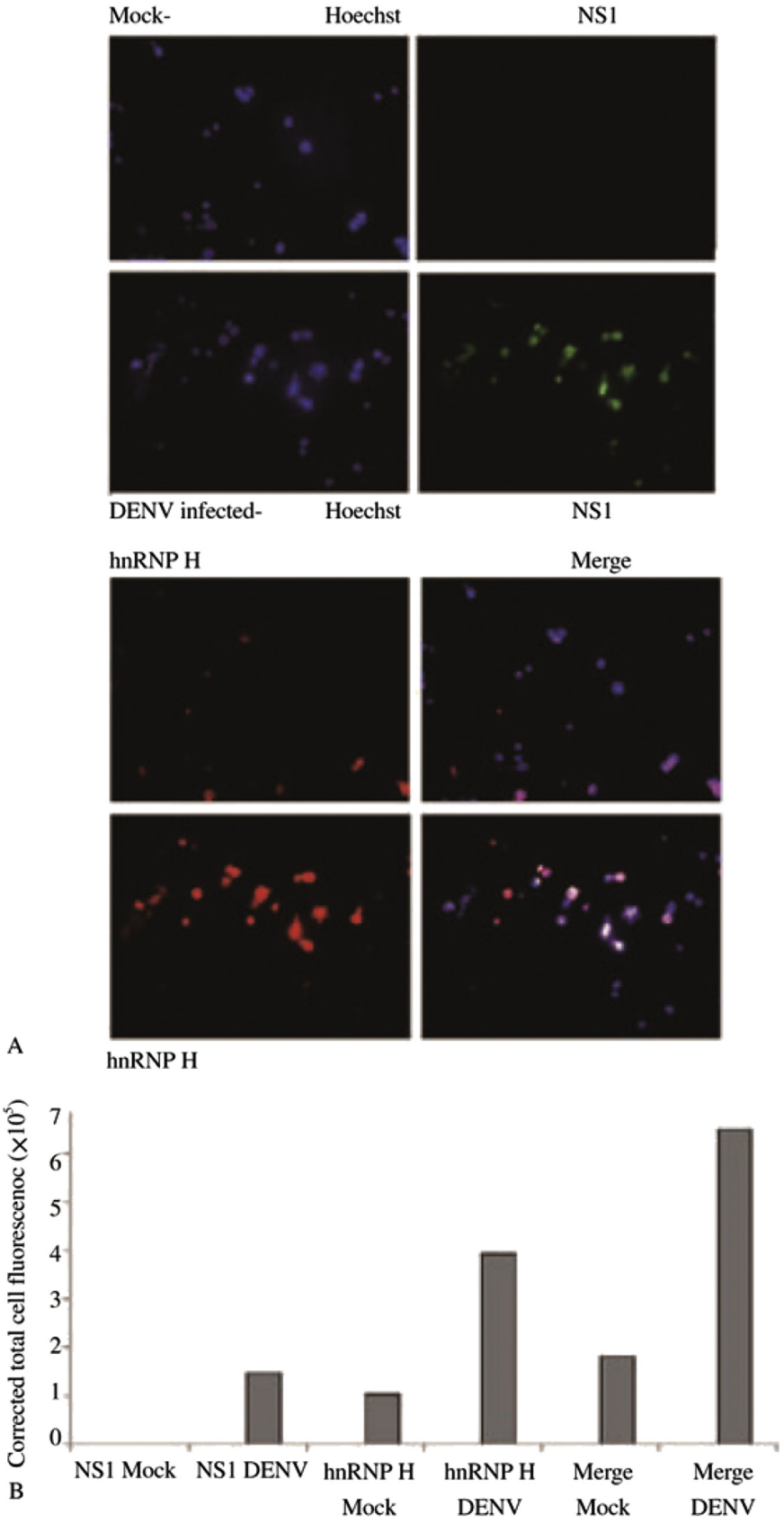

Figure 5. Co-localization of hnRNP H and NS1.
A) THP-1 cells were either mock or DENV infected. Cells were double immunostained for hnRNP H and NS1, 24 h *pi.* as described in Methods. B) Corrected total cell fluorescence.

4. Discussion

Cellular proteins, especially the ones involved in replication and translation, are exploited by viruses to propagate within the infected cell. Viruses adopt several strategies to survive and disseminate within the host. For instance, various strategic interactions of viral proteins with key cellular proteins occur with the intent to direct the cellular functioning in a manner that benefits the virus, particularly in replicating and producing mature viral particles [18,19]. Several viral proteins have the ability to mimic host proteins and regulate cytokine and chemokine production in order to divert the host response to infection [20]. hnRNPs are a family of multifunctional host proteins which can regulate the expression of several genes by directing alternative splicing of the mRNA transcript and can also modulate immunoglobulin gene recombination events [21], thereby having the power to influence the host immune response to infections and other stress or disease conditions.

During DENV infection in monocytic cells, the expression of the host protein hnRNP H gets up-regulated [15]. In this study, we reported that DENV NS1 protein interacts with hnRNP H. Further investigation may be required to understand the cellular repercussions of this interaction. Similar findings have been reported wherein hnRNP K interacts with the DENV core protein [22] and also hnRNP C1/C2 has been found to interact with DENV NS1 protein [23]. These reports suggest that the hnRNP family of proteins actively participate in the replication and maturation process of this virus and that the viral proteins either directly interact with the host hnRNP proteins or influence the expression of other vital cellular proteins indirectly by causing the induction of hnRNPs.

The viral NS1 protein can be found associated with intracellular membranes and the cell surface [24]. The glycoprotein is subsequently secreted and circulates within the bloodstream. DENV NS1 protein is expressed in a glycosyl-phosphatidylinositol-linked form that is capable of signal transduction. NS1 has also been suggested to be involved in viral replication [12]. This viral protein is a manipulative protein which not only participates actively in viral replication and signal transduction but also interacts with hnRNP H. The interaction between hnRNP H and NS1 perhaps causes an alteration in certain cellular responses to infection such as mRNA stability/processing, translation regulation, molecular chaperoning, oxidative stress response/regulation, cytoskeletal assembly, protein degradation, and cellular metabolisms that eventually benefits DENV in some way [25]. Moreover, it would be rather interesting to study which other genes and proteins are specifically expressed or repressed though alternative splicing mediated by hnRNP H that makes the host cell conducive for the maintenance of DENV in the cell.

In conclusion, this study suggests that DENV NS1 could serve as an effective target for inhibitors or drugs such that host-viral interaction may be inhibited and/or virus multiplication can be controlled. However, development of an effective treatment and prevention regime against DENV infection has not been possible owing to inadequate protection against all the DENV serotypes, resistance to anti-viral drugs and mutations in viral proteins. Targeting hnRNPs by inhibiting them for a transient period and/or the host-viral specific interactions may help counteract persistence of the virus. Thus, disruption of this key interaction between hnRNP H and DENV NS1 could be an important therapeutic strategy to help decrease the viral load during severe DENV infection and help in management of the disease.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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