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Dengue virus envelope protein domain III induces pro-inflammatory signature and triggers activation of inflammasome

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ABSTRACT

Dengue virus poses a considerable clinical problem, with the four closely related serotypes of dengue virus (DENV) infecting around 50–100 million people per year world-wide. The drastic increase in the dengue infection could be partly attributed to geographic expansion of the vector due to increasing urbanization, unavailability of specific antiviral therapies, licensed dengue vaccine, and poor understanding of the host immune responses. It has been reported that the immune-dominant envelope protein (E protein) domain III region (EDIII) of DENV is one of the most potent vaccine candidates because of its ability to trigger host immunity by inducing production of protective neutralizing antibodies. However, its role in the modulation of innate inflammatory responses hitherto remains unexplored. Herein, we demonstrate that EDIII protein of DENV induces pro-inflammatory signature by inducing production of inflammatory cytokines such as IL-1β and TNF-α in THP-1 cells through NF-κB pathway. Also, we observed increase in the maturation of IL-1β, which was found to be associated with increased ROS production and potassium efflux. Further, our findings reveal that the IL-1β production by EDIII protein is mediated through caspase-1 and NLRP3 inflammasome activation. In conclusion this study unearths the role of DENV EDIII protein in modulating innate inflammatory responses, which might provide possible mechanism of pathogenesis and open-up new avenues for the development of therapeutics against DENV.

1. Introduction

Dengue is a common viral infection prevalent worldwide, which has shown an increase in the spread as well as epidemic incidences over the past few decades [1–3]. This is evident from the numbers reported in the WHO report, which clearly indicates that there has been a gradual upsurge in the Dengue cases globally from less than a thousand cases in 1950 to around three million cases annually in 2015 [4].

Dengue is a mosquito-borne viral disease, transmitted by the bites of female mosquitoes, mainly of the species *Aedes aegypti,* and *Aedes albopictus* in a few cases [5,6]. It is primarily caused by four different but quite related Dengue virus (DENV) serotypes, namely, DENV-1,DENV-2, DENV-3,and DENV-4 [5]. The presence of four different causative serotypes of the virus lead to cross-reactivity and hence, additional risk on subsequent dengue infections [2]. Clinically, dengue infection is characterised by a plethora of symptoms ranging from asymptomatic mild flu called as Dengue fever (DF)to the more severe condition, which includes coagulopathy, enhanced vascular fragility and thrombocytopenia known as Dengue Haemorrhagic fever (DHF), which may lead to hypovolemic shock called Dengue Shock Syndrome (DSS) [7].

Dengue virus (DENV) is an enveloped virus with positive-sense single stranded RNA genome that codes for seven non-structural and three structural proteins [8]. The non-structural proteins (NSPs) (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) play a role in replication of viral RNA and assembly as well as regulation of the host cellular and immunological responses [9,10]. DENV NS1 protein acts as immunogen during severe phase of infection and elicits a potent anti-NS1 response [11]. Emerging evidences highlight a vital role of NS1 in the activation of immune complement system during infection [12]. NSPs like NS2A, NS2B, NS4B and NS5 have been reported to modulate type I interferon (IFN) signaling [13,14] while simultaneously NS5 and NS4B proteins induce production of chemokines and proinflammatory mediators [13,15,16]. DENV structural proteins such as envelope glycoprotein (E) is a major component of the envelope of viral membrane, which helps the virus attachment to the host cell and subsequent fusion to the cellular membrane during the endosomal-mediated virus internalization [17]. The E protein is constituted of three structural domains, namely, EDI, EDII and EDIII [18], held by a helical stem region hitched to virus membrane by *trans*-membrane anchor [17,19]. EDIII is an Ig-like domain involved in binding of the virus, which makes it to be an

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imperative target for the humoral immune response during infection [11,20]. The EDIII is the region which has the highest variability amongst the four serotypes, congruently, the highly specific antibodies generated against this particular region are the ones with a high neutralising ability [21–23].

The innate immune responses mediated by host plays a primary role in limiting Dengue infection, however the immunological mechanisms exploited by the dengue virus to trick the host immune response still needs to be explored. Dengue virus has been known to escape from host immune responses through multiple routes and triggering of inflammation associated with host tissue damage is one such approach [24]. It is also evident from the studies which report that the DENV-infected cells produce a significantly enhanced levels of the proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and interleukin 12p70 (IL-12p70), as well as prostaglandin E2 (PGE2) [25]. Moreover, higher levels of pro-IL-1β, pro-IL-18, and NLRP3 in association with caspase-1 activation has also been observed in DENV-infected macrophages [26,27]. Hence, identifying the DENV component which triggers such response would enable in the design of antiviral therapies. The DENV EDIII region has been investigated as a potential candidate for DENV vaccine development owing to its ability to trigger potent antibody response [28,29], nevertheless its role in the modulation of host innate inflammatory responses is not yet completely investigated. Therefore, in the present study, we have investigated the role of domain III of Dengue envelope protein (DENV EDIII) in tailoring innate immune responses in the differentiated human monocyte cell line (THP-1).

2. Materials and methods

2.1. Chemicals and cell culture

The human monocyte leukaemia cell line THP1 (American Type Culture Collection, Rockville, Md. USA) was cultured in RPMI 1640 culture medium (Himedia, India and Gibco, Life Technologies, USA) augmented with 10% fetal bovine serum (FBS) (Invitrogen, UK) and 1% penicillin/streptomycin (P/S) (Invitrogen, UK) at 37 °C and 5% CO_2 in a humidified incubator. LPS (*E.coli* 0111:B4) (Sigma Aldrich), Phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich), Proteinase K, Isopropyl β-D-1-thiogalactopyranoside (IPTG) (G.Biosciences USA), Caspase-1 inhibitor Ac-YVAD-CHO (Calbiochem- Merck Germany) and Polymyxin B was purchased from Sigma Aldrich, USA.

2.2. Macrophage differentiation

The THP-1 monocytes were differentiated by treating 10^6 cells/ml of THP-1 cells for 24 h with 100 ng/ml phorbol 12- myristate 13-acetate (PMA) in a 12-well cell culture plate with 1 ml cell suspension in each well. It is well reported that the PMA-induced THP-1 cell differentiation results in the expression of certain macrophage associated surface markers, CD11b and CD36, and these differentiated cells demonstrate phagocytic activity as well [30]. Differentiated, adherent cells were washed with sterilized phosphate-buffered saline (PBS; pH 7.4) and subsequent addition of fresh RPMI 1640 medium without PMA prior to further treatment and stimulation.

2.3. Expression and purification of EDIII

The cloned construct DENV 2 pET28a-Domain III was transformed into *E. coli* Rosetta cells to check the expression. Single isolated colony from the transformed plates was used as an inoculum for the primary culture in kanamycin (50 μg/ml) and chloramphenicol (35 μg/ml) containing Luria Bertani (LB) media. The overnight primary culture was inoculated into 1L LB media (containing 50 μg/ml kanamycin and 35 μg/ml chloramphenicol) and was grown in the shaking incubator at 37 °C for about 2–3 h at 150 rpm or until the OD_{600} of the culture attained ~0.5–0.6. A minor aliquot of the uninduced culture was kept aside for subsequent SDS-PAGE analysis, while the remaining bulk was subjected to induction by adding IPTG to a final concentration 1 mM. Cells were harvested after 4 h of the induction period. The induced culture was centrifuged and the cell pellet obtained was resuspended in the buffer containing 8 M urea and 1 mM PMSF (Tris 50 mM, NaCl 300 mM, 20 mM imidazole, lysozyme and DNAase). The resuspended cell pellet was sonicated for 10 min and centrifuged at 12000 rpm for 30 min. The supernatant was collected, kept for binding with Ni-NTA beads for 1 h at 4 °C, and was passed through a Ni-NTA column (GE Healthcare). Flow-through and different fractions (eluted with 300 mM imidazole) were collected, and run on SDS-PAGE. The desired band size of 14.5 kDa DEN 2 EDIII was observed after Coomassie staining of SDS gels. For the purification DEN 3 EDIII, cloned construct of DENV 3 pET28a-Domain III was transformed into *E.coli* Rosetta cells for expression and purification. The purification of DEN 3 EDIII was carried out by the same methodology which was employed for DEN 2 EDIII; however size of DEN 3 EDIII was 15 kDa. The purified EDIII proteins were also confirmed through western blot using anti-His Tag antibody (Cell Signaling Technology, USA).

2.4. Immunoblotting

Immunoblotting was done to analyze the expression of different proteins. Cells were either left unstimulated or stimulated with the DEN 2 EDIII protein for 12 h, LPS was used as a positive control. Post incubation, adherent cells were washed gently with ice-cold 1XPBS. The cells were scraped and lysed in cell lysis buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton-X 100, 100 mM NaF, 1 mM EDTA, 17.5 mM β-glycerophosphate, 10% glycerol, 1 mM Phenyl methyl sulphonyl fluoride, 2 mg/ml pepstatin and 4 mg/ml aprotinin), supplemented with protease cocktail inhibitor (Sigma Aldrich, St. Louis, MO, USA) and incubated on ice for 30 min with intermittent vortexing as described earlier [31]. Lysates were later pelleted down at 12,000 rpm for 15 min at 4 °C. Equal amount of proteins were loaded and run on SDS-PAGE (12% Tricine gel). Separated proteins on SDS-PAGE were transferred onto the nitrocellulose membrane (Pall Corporation, New York, USA) following electroblotting. 5% skimmed milk powder (Hi-Media, India) was used for blocking the nitrocellulose membrane for 1 h, followed by overnight incubation with appropriate primary antibodies at 4 °C. The antibodies used for immunoblot analysis were rabbit anti-pro caspase-1 and goat anti-cleaved caspase-1 (Santa Cruz, USA), rabbit anti-IL-1β (Santa Cruz, California USA), mouse anti-β-actin (Cell Signaling Technology, USA), rabbit anti-P65 (NFκB) (Cell Signaling Technology, USA), rabbit anti-Phospho-IKBα, and Total-IKBα (Cell Signaling Technology, USA) . The membranes were washed thrice with 1X TBST to remove any non-specifically bound antibodies and were then incubated with Horse radish peroxidase (HRP) conjugated appropriate secondary antibodies for 1 h. After the incubation with secondary antibodies, the membrane was again washed thrice to remove non-specifically bound antibodies. Protein bands were finally visualized with femto-ECL-prime chemiluminescent substrate (Geno Biosciences, USA) and chemiluminescence was captured on a Chemidoc (BioRad, California USA).

2.5. Measurement of mature IL-1β and cleaved Caspase-1

PMA differentiated THP-1 cells were stimulated with different concentrations of DENV EDIII protein or LPS or were left unstimulated. After the requisite treatment, culture supernatants were harvested and methanol-chloroform precipitation was employed to precipitate the proteins [32]. Briefly, to 500 μl of the culture supernatant harvested, 500 μl of methanol was added. The mixture was vortexed and then 100 μl of chloroform was added and vortexed, followed by centrifugation at 14,000 rpm for 2 min. The top aqueous layer was pipetted off and again 500 μl methanol was added, vortexed, and centrifuged at

14,000 rpm for 2 min. Supernatant was again pipetted off as much as possible without disturbing the pellet. The pellet obtained was then dried by heating at 50 °C for 5 min. Later the pellet was dissolved in 50 µl of 2X sample buffer and then boiled at 95 °C for 5 min. Equal amount of protein was loaded and run on 12% SDS-PAGE (Tris-glycine gel). Separated proteins on SDS PAGE were transferred onto nitrocellulose membrane (Pall Corporation, USA) following electro blotting. Expression levels of mature IL-1β and cleaved caspase-1 protein were evaluated by immunoblotting as described earlier.

2.6. Subcellular fractionation for p65 translocation

Isolation of the nuclear and cytosolic proteins was carried out by differential centrifugation as described earlier [33,34]. Briefly 5×10^6 THP-1 treated cells were pelleted down and resuspended in 200 µl of cold buffer A (10 mM HEPES/KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.4% (vol/vol), NonidetP-40, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail) by pipetting gently. The homogenate was centrifuged for 3 min at 500g at 4 °C in a microfuge and the supernatant demonstrating the cytosolic fraction was collected. The pellet comprising the nuclei of cells was incubated at 4 °C for 10 min in buffer (20 mM HEPES/KOH (pH 7.9), 420 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, 25% (vol/vol) glycerol 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and protease inhibitor cocktail) and was further incubated for 15 min on ice with brief vortexing every 3 min. The sample was centrifuged at 13,800g for 10 min at 4 °C; supernatant was collected and examined by Western blotting.

2.7. qRT-PCR

After specific treatment with or without recombinant protein, media was aspirated off and the cells were washed gently with ice cold 1X PBS. Cells were then scraped in cold 1X PBS and centrifuged at 1200 rpm for 5 min at 4 °C. Total RNA was isolated from the cells using the Trizol-chloroform extraction protocol as described earlier [7]. Briefly, 1 ml Trizol reagent was added to the cell pellet, resuspended and kept at room temperature (RT) for 5 min. After the incubation, 200 µl of chloroform was added and the tube was shaken vigorously for 15 s by hand and was further incubated on ice for 15 min. The samples were centrifuged at 12,000 rpm for 15 min at 4 °C. After centrifugation, aqueous phase of the sample was carefully removed without disturbing the interphase and was transferred into a new tube. 500 µl of isopropanol was added to the aqueous phase, mixed gently and was incubated on ice for at least 15 min, followed by another centrifugation at 12,000 rpm for 30 min at 4 °C. The supernatant was removed to obtain the RNA pellet, which was resuspended in TE buffer. A total of 1 µg of RNA was reverse transcribed into cDNA using Verso cDNA synthesis kit (Thermoscientific, USA) according to the manufacturer's protocol. The cDNA were amplified using Syber-Green master mix (Kappa Biosystems, USA) with gene specific primers (See Supplementary Table) using the following thermal cycler parameters : initial cycle at 94 °C for 2 min, followed by 40 cycles of 30 s at 94 °C, 30 s annealing at 58 °C and 40 s extension at 72 °C, in Master cycler ep realplex (Eppendorf, Germany). The relative mRNA expression of each sample was calculated relative to the house-keeping gene GAPDH/βActin as described [35].

2.8. Elisa

Human monocytes differentiated with PMA were treated with or without DENV EDIII proteins, LPS was used as a positive control, and cytokines level were quantified in the culture supernatants by ELISA as described earlier [32], using human TNF-α, IL-1β, IL-6 ELISA kits (BD OptEIA, BD Biosciences, USA) as per the manufacturer's instructions. Briefly, 96-well poly-vinyl chloride microtiter plates (NUNC maxisorp, Thermoscientific, USA) were coated with the respective capture antibodies diluted in suitable coating buffer and were incubated overnight at 4 °C. The plates were washed and blocked with 10% FBS in PBS, followed by incubation with standard or test samples. After washing, plates were incubated with anti-cytokine detection antibodies along with horseradish peroxidase (HRP) conjugate. Next, 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (BD Biosciences, USA) was added and the plates were incubated at room temperature in dark, until the development of colour. 2 N Sulphuric acid (H_2SO_4) was used to terminate the reaction, and absorbance was read at 450 nm with background correction at 570 nm.

2.9. ROS estimation

For measurement of reactive oxygen species (ROS) levels, $1.5–2 \times 10^6$ THP-1 cells were seeded; PMA treated and allowed to differentiate for 24 h. Fresh media was added to the cells and treated with LPS or different concentrations of DEN 2 EDIII protein. Following the treatment, cells were washed in 1X PBS, harvested and incubated with 25 µM CM-H_2DCFDA (Life Technologies, USA) for 30 min at 37 °C in FACS buffer as described earlier [36]. Later, these cells were washed extensively with Hank's balanced salt solution (HBSS) and resuspended finally in FACS buffer before flow cytometry analysis.

2.10. Statistical analysis

All data are represented as mean ± S.E.M of three or four independent experiments. Two sample unpaired student's *t*-test was executed using Graphpad Prism software to evaluate the significance of difference between groups. A p value less than 0.05 was considered to be significant, and a p value less than 0.005 were considered to be highly significant.

3. Results

3.1. Expression and purification of DENV domain III proteins (DEN 2 EDIII and DEN 3 EDIII)

In order to check expression of EDIII of DENV-2 serotype (DEN 2 EDIII), the cloned construct DENV-pET28a-Domain III plasmid was transformed in *E.coli* Rosetta cells. Few colonies were picked from the transformed plate and were grown in LB media composed of kanamycin (50 µg/ml) and chloramphenicol (35 µg/ml) under Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. A typical experiment comparing the induced and uninduced culture reveals that the IPTG induction resulted in the expression of DEN 2 EDIII protein (Fig. 1A) We optimized the protein expression conditions by subjecting cultures to different concentrations of IPTG and observed better expression at 0.6 mM concentration of IPTG at 37°C (Fig. S1A). Next, we investigated the solubility of the expressed proteins. Culture was induced with IPTG and the pellet obtained was resuspended in lysis buffer, with and without detergent (TritonX-100 0.2%). The expressed protein was found in pellet and was going to inclusion bodies (Fig. S1B). To solubilize the proteins, 8 M urea was used in the lysis buffer. Protein was further purified using Ni-NTA kit. A single major protein band of expected size was observed upon purification (Fig. 1B). The purified protein was also subjected to western blotting using anti-His antibody, a band was observed corresponding to the Hexa-His tag peptide (6X-His) EDIII (Fig. 1C). Purified protein was concentrated using centricons (Amicon pro-affinity concentrator from Millipore) and refolded by dialysis in appropriate buffer without urea, using Dialyzer tubes. EDIII of DENV-3 (DEN 3 EDIII) was also purified with the same methodology and purified protein (Fig. S2A–C) was also subjected to western blotting using Anti-His for confirmation (Fig. S2D). The purified recombinant proteins were treated with Polymyxin B for the removal of any endotoxins [37] prior to *in-vitro* treatment or functional analysis.

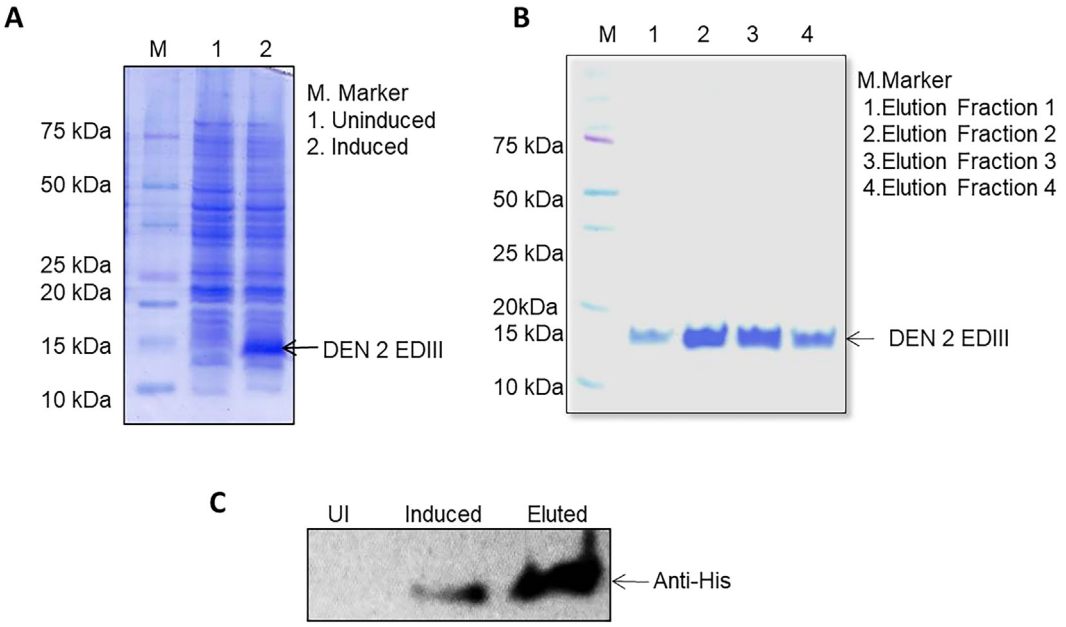

Fig. 1. Expression and purification of recombinant DEN 2 EDIII. (A) SDS-PAGE showing overexpression expression of EDIII in *E.coli* Rosetta cells. (B) SDS-PAGE analysis of purified serotype EDIII protein. (C) Western Blotting analysis of EDIII purified protein using anti-His antibody.

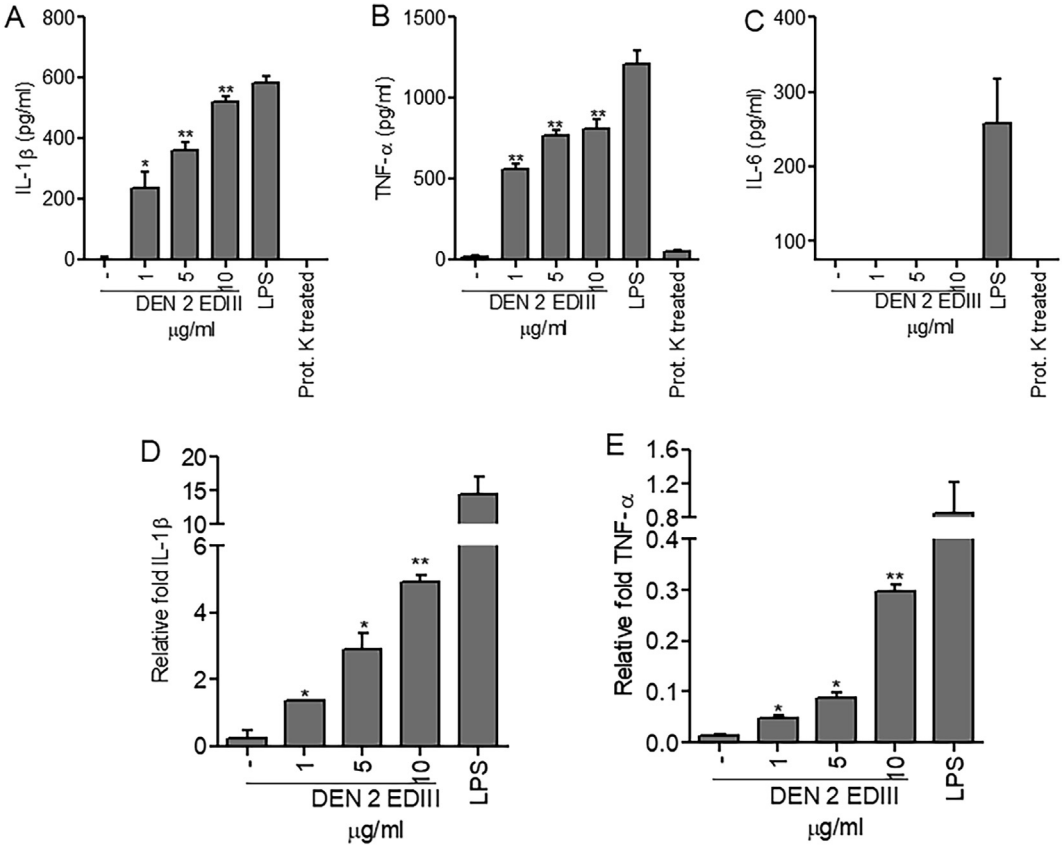

Fig. 2. Cytokine profile in differentiated THP-1 cells stimulated with DEN2 EDIII protein. ELISA for cytokine estimation from THP-1 cells untreated or treated with different concentrations of EDIII for 24 hrs. LPS (500 ng/ml) was used as positive control and Proteinase K treated protein was used as negative control. (A) IL-1β (B) TNF-α (C) IL-6 is the ELISA data. (D) IL-1β (E) TNF-α mRNA expression through qRT-PCR analysis in macrophages treated with EDIII and LPS. Statistical significance was calculated by student *t* test. $^{*}P \leq 0.05$, $^{**}P \leq 0.005$, were considered statistically significant. Error bars represent mean +/− SEM. Data are representative of one of three independent experiments.

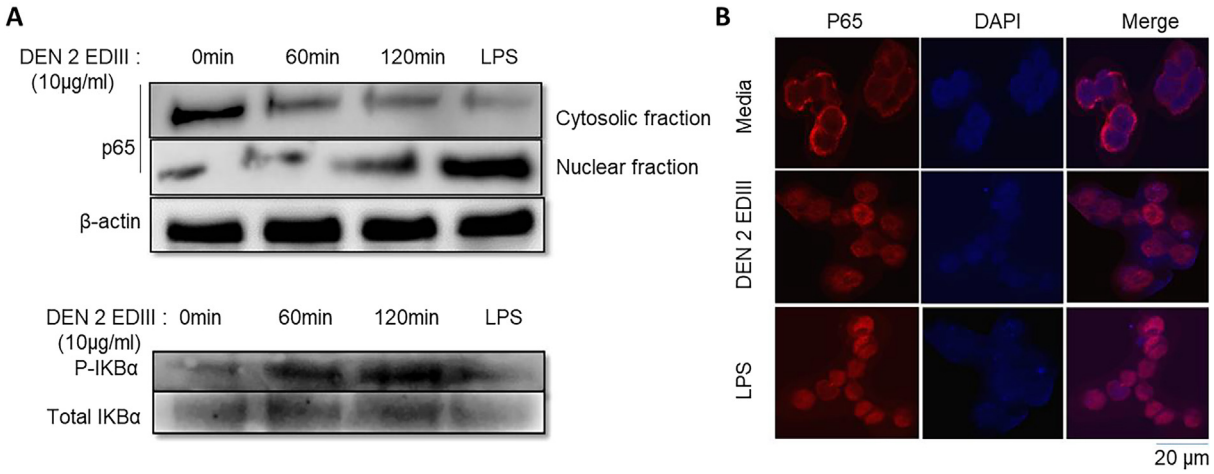

Fig. 3. Activation of NF-Kappa β following stimulation with DEN 2 EDIII protein in THP-1 cells. (A) Immunoblot showing nuclear translocation of p65 and phosphorylation of IKBα in THP-1 cells after stimulation with EDIII in a time-dependent manner, LPS was used as a positive control. (B) Confocal microscopy images showing p65 (red) translocated to nucleus in EDIII and LPS stimulated macrophages, nuclei were stained with DAPI (blue). Scale bars, 20 μm. Cells were stimulated for 1 h. Data are representative of one of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. DENV EDIII protein induces a robust pro-inflammatory cytokine signature in human macrophage cell line

The excessive production of inflammatory mediators including cytokines and chemokines from the deregulated immune cells has been mainly attributed towards the severity of Dengue. However the components and the mechanisms involved in triggering such response is not well understood [38]. Although several non-structural proteins of DENV have been shown to modulate inflammatory response, however very less is known about the innate immune responses elicited by structural proteins like E protein. The EDIII of DENV E protein encapsulated in nanospheres has been recently shown to induce innate antiviral responses by producing cytokines and chemokines in dendritic cells [39]. However, the mechanisms through which DENV EDIII protein induces innate inflammatory responses in immune cells like monocytes/macrophages have not been extensively investigated. Therefore, we have demonstrated the effect of EDIII protein on innate-inflammatory responses in the differentiated THP-1 cells. For this, we have incubated the purified recombinant EDIII of DENV 2 serotype (DEN 2 EDIII) with the differentiated THP-1 cells and the levels of pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 were detected using ELISA and qRT-PCR in the culture supernatants and cells respectively. The differentiated THP-1 cells were treated with different non-toxic concentrations of DEN 2 EDIII (Fig. S3), while LPS was used as a positive control. Following stimulation, the cells and culture supernatants were harvested for the examination of cytokines levels. Interestingly, the data (Fig. 2A and B) reveals that the levels of cytokine IL-1β and TNF-α showed a remarkable increase with increasing concentrations of DEN 2 EDIII protein. However, IL-6 did not show any dose dependent change with protein treatments (Fig. 2C). To rule out the possibility of endotoxin contamination, the purified heterologous protein was subjected to proteinase K treatment [40,41]. The EDIII protein treated with proteinase K was not able to induce pro-inflammatory cytokines in THP-1 cells, thereby suggesting that the observed phenomenon is the result of protein activity and not because of the LPS contamination. We also used human methionine aminopeptidase-1(MetAP-1) recombinant protein purified from *E.coli* as a control to address the concern of LPS contamination and further confirm that the response observed above is indeed triggered by the DEN 2 EDIII protein. Interestingly, the data showed that MetAP-1 could not produce significant IL-1β as compared to DEN 2 EDIII and LPS treated cells (Fig. S5). Concomitantly, we also examined the mRNA levels of these cytokines using qRT-PCR. For qRT-PCR, the cells were treated with different concentrations of EDIII protein and LPS for 6 h. The qRT-PCR results were in line with the ELISA data and showed a similar trend (Fig. 2D, E), thereby suggesting that the DEN 2 EDIII protein influences transcriptional machinery to program the proinflammatory environment of the cells.

Having observed that DEN 2 EDIII protein influences enhanced production of IL-1β and TNF, we were interested to examine whether this phenomenon was restricted to the EDIII of DENV 2 serotype or mediated by other serotype EDIII proteins. To test this hypothesis, we used EDIII protein of DENV3 serotype (DEN 3 EDIII). Interestingly, we observed similar results with DEN 3 EDIII proteins (Fig. S4), thereby suggesting that EDIII mediated tailoring of inflammatory cytokine production is not serotype restricted. Furthermore, the above finding suggests that EDIII protein induces proinflammatory signatures by increasing the production of proinflammatory cytokines IL-1β and TNFα in THP-1 cells.

3.3. Pro-inflammatory response induced by recombinant DEN 2 EDIII protein is mediated by activation of NF-κB

Having observed that EDIII proteins trigger IL-1β and TNF-α at mRNA and protein level, we were next interested to understand the mechanism through which these cytokines might be regulated. It is well documented that the transcription factor NF-κB regulates wide range of genes involved in regulating inflammatory responses [42,43]. NF-κB represents family of transcription factors composed of five structurally related members including p50, p52, p65, RelB and c-Rel [44]. Even though all of these members are well characterized but p65 has received the most attention. The phosphorylation of NF-κB results in the nuclear translocation of p65 subunit thereby activating the transcription of downstream genes by binding to target DNA elements [45,46]. Therefore, in order to determine whether IL-1β and TNF-α production is regulated by NF-κB activation in response to DEN 2 EDIII stimuli, we evaluated the NF-κB levels in nuclear and cytoplasmic fractions in EDIII protein stimulated THP-1 cells. A clear and enhanced nuclear translocation of p65 in DEN 2 EDIII treated cells was observed in a time dependent manner as indicated (Fig. 3A; upper panel). Time course measurements suggested an upsurge in nuclear p65 levels 2 h post treatment when compared to untreated control. This was in line with the declining p65 levels in the cytoplasm and its rising translocation to the nucleus. Under resting conditions NF-κB is bound by inhibitory IKB proteins including IKBα, thereby rendering it into inactive form [47]. In

response to external stimuli IKB proteins are phosphorylated which leads to their degradation by proteasome. Thus NF-κB is released from complex and is translocated to the nucleus [48]. Therefore we checked for IKBα phosphorylation under the same conditions as were employed for p65. We observed time dependent increase in the phosphorylation of IKBα (Fig. 3A; lower panel, Fig. S6A).

We also examined the NF-κB nuclear translocation with the help of confocal microscopy. THP-1 cells were seeded on coverslips and were treated with recombinant DEN 2 EDIII protein and LPS. The microscopic images clearly show NF-κB translocation in nucleus of the treated cells as compared to media control (Fig. 3B). It is reported that toll-like receptors (TLRs) signalling is involved in phosphorylation of IKB proteins and their subsequent degradation allows NF-kB to move into the nucleus thereby triggering induction of inflammatory cytokines [49]. Since TLRs are involved in the activation of NF-kB, we wanted to check which TLR is involved the production of IL-1β mediated by DEN 2 EDIII. We incubated cells with TLR2 and TLR 4 neutralizing antibodies (20 μg/ml) to block respective receptors followed by treatments of DEN 2 EDIII. An isotype control IgG2a was also used. We observed significant inhibition in IL-1 β production in the cells where TLR4 antibodies were preincubated compared to TLR2 and isotype control Fig. S6B. This shows that DEN 2 EDIII mediates signalling via TLR 4.

3.4. IL-1β maturation induced by DEN 2 EDIII requires caspase-1 activation and involves the NLRP3 inflammasome

IL-1β is one of the most pro-inflammatory cytokines which is produced by activated monocytes and macrophages in response to proinflammatory signals like LPS [50]. IL-1β is produced as biologically inactive 31 kDa polypeptide called as pro- IL-1β and is cleaved by caspase-1 to produce 17 kDa mature IL-1β (biologically active form) [51]. Caspase-1 is itself activated to active enzyme by autocatalytic reaction which is tightly controlled by molecular scaffold termed as inflammasome [52]. Since IL-1β maturation and secretion is controlled by caspase-1 upon inflammasome activation, we tested whether DEN 2 EDIII protein has any role in the maturation of pro- IL-1β and inflammasome activation. We assessed the release of both, the active caspase 1 and IL-1β, from the differentiated human monocytes by immunoblotting. Cells were seeded in Opti-MEM media containing reduced serum followed by treatment with different concentrations of protein as well as LPS. Immunoblotting results clearly show increase in pro- and mature IL-1β, as well as cleaved caspase 1, however, procaspase 1 levels were comparable (Fig. 4A;Fig. S7A–C). This data suggests that the IL-1β maturation induced by DEN 2 EDIII protein is dependent on caspase-1 activation. In order to check whether caspase-1 is required for DEN 2 EDIII induced maturation of IL-1β we used caspase-1 inhibitor (Ac-YVAD-CHO) and examined its effect on mature IL-1β [53]. We incubated cells with DEN 2 EDIII in presence or absence of Ac-YVAD-CHO and observed that mature IL-1β was drastically reduced in presence of Ac-YVAD-CHO compared to only DEN 2 EDIII treated cells (Fig. 4B). Further, we also investigated whether NLRP3 inflammasome activation has a role to play in maturation of IL-1β; we stimulated the cells with recombinant protein and probed for the expression levels of NLRP3. Immunoblotting results show that DEN 2 EDIII treatment induces NLRP3 activation (Fig. 4D). NLRP3 expression was also evaluated using qRT-PCR, which also indicates an increased expression of NLRP3 in LPS and DEN 2 EDIII protein treated cells as compared to the untreated (Fig. 4C). This data, therefore, demonstrates that the NLRP3 inflammasome activation is involved in DEN 2 EDIII protein induced IL-1β maturation and is mediated via caspase-1.

3.5. Recombinant DEN 2 EDIII induced IL-1β maturation is dependent on ROS and potassium efflux

Since NLRP3 expression was observed in the DEN 2 EDIII treated cells, we tried to look into the mechanism of inflammasomes activation. Several mechanisms have been proposed for its activation. It is evident from previous reports that production of ROS plays a crucial role in the activation of NLRP3 inflammasomes. As described above IL-1β maturation is dependent on caspase 1 which in turn is regulated by Inflammasomes. Therefore we were interested to find out whether DEN 2 EDIII triggers ROS production. To estimate intracellular ROS production, cells were stimulated with different concentrations of DEN 2 EDIII protein and LPS for 12 h, followed by incubation with CM-H_2DCFDA for intracellular ROS estimation. A significant increase in ROS production was observed in cells stimulated with EDIII protein as compared to the untreated cells (Fig. 5A). Next we examined the levels of IL-1β and TNF-α in DEN 2 EDIII stimulated cells in presence and absence of ROS inhibitor, N-acetyl-L-cysteine (NAC), to find any correlation between ROS production and IL-1β maturation. Interestingly, IL-1β levels were reduced in the cells treated with (NAC) plus DEN 2 EDIII as compared to the cells stimulated with only protein (Fig. 5B). However, there was no change in the levels of TNF-α (Fig. 5C). This data suggests that the maturation of IL-1β induced by DEN 2 EDIII protein is dependent on ROS production which is in agreement with previous studies showing correlation of ROS with IL-1β maturation [54,55].

It is well established that potassium efflux is an important signal for the activation of NLRP3 inflammasome and IL-1β maturation [56], we next examined the role of these signals in the DEN 2 EDIII mediated IL-1β processing and secretion. Since it is well established that IL-1β maturation is dependent on potassium efflux [57], therefore, in order to check the role of potassium efflux on maturation of IL-1β, we treated the cells with NaCl and KCl [58] prior to the recombinant protein stimulation. We observed reduction in the IL-1β levels in the DEN 2 EDIII protein treated cells with higher extracellular K^+ levels as compared to the cells treated with only protein, however not much decrease was observed in the cells with higher extracellular Na+ levels (Fig. 5D). However TNF-α levels remained unchanged (Fig. 5E), which is in line with the previous findings [59], thereby suggesting that DEN 2 EDIII induced IL-1β maturation is indeed dependent on ROS and Potassium efflux which supports the previous findings about the IL-1β regulations [60].

4. Discussion

Dengue infection is considered as one of the most pervasive viral disease in humans transmitted by a mosquito. A spectrum of ailments from symptomless flu-like conditions to fatal Dengue Shock Syndrome/Dengue Haemorrhagic Fever (DSS/DHF) is caused by the four closely related serotypes of DENV (DENV-1 to DENV-4) [7,61]. Dengue infection can be mild illness lasting for about one week, whereas severe dengue (DSS/DHF), is characterized by plasma leakage and considered to be life threatening [62]. The upsurge in the incidence of dengue epidemics globally has led to an increase in the investigations on dengue virus biology and its pathology, so as to design therapeutics for the prevention and containment of the infection. However, designing of adequate therapies and vaccines needs in-depth knowledge about the disease pathogenesis. Even though many efforts have been made to understand DENV pathogenesis, the underlying mechanisms of this disease still remains elusive, and need to be explored further.

The inflammatory responses against DENV have been reported to play a crucial role in DENV pathogenesis [27]. The diverse clinical manifestations between mild and severe dengue patients suggest that inflammatory responses might differ significantly between the disease conditions [27]. It has been reported that DENV-infected cells produce substantially elevated levels of the proinflammatory cytokines TNF-α, IL-6, IL-12p70, as well as PGE2 [25]. The role of NSPs has been studied in pathogenesis and host immunomodulation of dengue, while structural proteins like Envelope (E) protein has been studied mostly from the perspective of vaccine development [63,64]. E protein which is a multi-domain protein (EDI, EDII, and EDIII domains) [17,18] is the

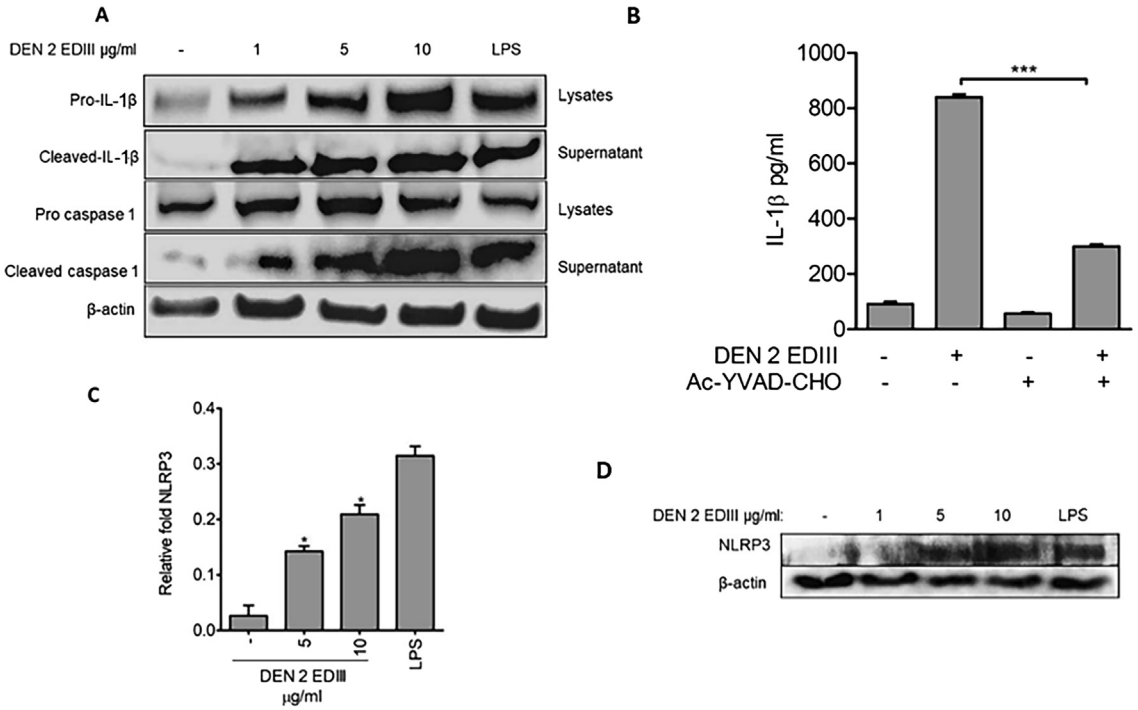

Fig. 4. Maturation of IL1-β requires Caspase-1 and NLRP3 inflammasome activation. (A) Immunoblot analysis for expression of IL-1β and caspase1 (pro and active) from differentiated THP-1 cells untreated or treated with different concentration of EDIII for 12 h and LPS (500 ng/ml) (Lys. cell lysates; Sup., culture supernatant;) (B) Estimation of IL-1β in supernatants of THP-1 cells untreated or treated with DEN 2 EDIII (10 µg/ml) in presence or absence of caspase-1 inhibitor Ac-YVAD-CHO (50 µM). (C) qRT-PCR to check expression of NLRP3. (D) Production of NLRP3 from DEN 2 EDIII stimulated THP-1 cells by immunoblotting. Statistical significance was calculated by student *t* test. $^*P \leq 0.05$ was considered statistically significant. Error bars represent mean +/− SEM. Data are representative of one of three independent experiments.

indispensable target of the antibody based immune response during dengue infection [65].The upper lateral surface of E from all the three domains has been defined as the one with maximum neutralizing ability; concomitantly, this region also reflects the premier variability amongst the four serotypes, accounting for the specificity of these antibodies [21,22]. As for the innate immune responses of EDIII of DENV E protein are concerned, it has been recently reported that EDIII when encapsulated in nanospheres triggers enhanced innate immune responses in dendritic cells [39]. Herein, through this study, we demonstrate that the EDIII protein induces a robust proinflammatory cytokine signature in human macrophages both at RNA as well as protein level. The levels of proinflammatory cytokines like IL-1β and TNF-α showed a dose-dependent increase in the EDIII treated cells, which is consistent with the earlier reports that these cytokines play a crucial role in DENV pathogenesis. However, IL-6 did not show an increase upon EDIII stimulation. In addition, the findings of this study reveal that EDIII induced increase in the levels of IL-1β and TNF-α is not serotype specific.

Furthermore, it is well-established that the NF-κβ family of transcription factors plays a vital role in inflammation and innate immunity [66,67]. It controls the expression of cytokines, which is in agreement with our study, as the increase in cytokine levels following stimulation with EDIII protein was found to be mediated via NF-κβ activation. Activation of the NF-κβ transcription factor is firmly controlled by group of proteins called inhibitory complex [48,68,69].These proteins block the nuclear localization signal (NLS) of NF-κβ, hence prevents its nuclear translocation. Phosphorylation of IKB proteins like IKB-α, responsible for masking p65 component of NF-κβ resulting in ubiquitination and proteosomal degradation and subsequent p65 translocation to the nucleus [47,68,70]. In our study, we observed a decrease in the levels of NF-κβ p65 component in the cytosolic fraction while a simultaneous increase in nuclear fraction was observed following the DEN 2 EDIII treatment in a time-dependent fashion, that correlated with the phosphorylation of IKBα. This suggests that DEN 2 EDIII induces cytokine expression via NF-κB activation and nuclear translocation of its NF-κβ p65 component, thereby suggesting that NF-κβ activation might be responsible for regulating these cytokines transcriptionally.

It is well documented that production of cytokines such as IL-1β is tightly controlled not only at transcriptional levels but also at the posttranscriptional level [32,71,72]. IL-1β is mainly produced as an inactive pro-protein and a cysteine protease caspase-1 mediates its activation and release, cleaves it to biologically active form [54]. Caspase-1 is itself cleaved to its active form by inflammasome activation. Our results indicate an increase in mature IL-1β and cleaved caspase-1 in THP-1 cells following DEN 2 EDIII treatment, which confirms that IL-1β activation is dependent on caspase-1. DEN 2 EDIII also induces NLRP3 inflammasome activation, which is important in DENV pathogenesis and host immune modulation during dengue infection. Reactive oxygen species (ROS) generation and potassium (K^+) efflux have been suggested for NLRP3 activation to trigger caspase-1 activation and IL-1β maturation [73,74]. The ROS production acts as both a signalling molecule as well as a mediator of inflammasome activation and inflammation [32]. DEN 2 EDIII treatments also induced the production of ROS and the increase in IL-1β levels, was found to be associated with the increase in ROS production. Mature IL-1β levels decreased in the presence of both ROS inhibitor N-acetyl cysteine (NAC) [60,75] as well as K^+ ions, which shows that release of mature IL-1β is dependent on both ROS production as well as K^+ efflux.

5. Conclusion

In summary, this study highlights the role of EDIII protein of Dengue Virus in the modulation of host innate inflammatory response, which possibly might be one of the major factors associated with exaggerated inflammation during severe Dengue infection cases. However, in depth mechanistic insight through which EDIII proteins regulate

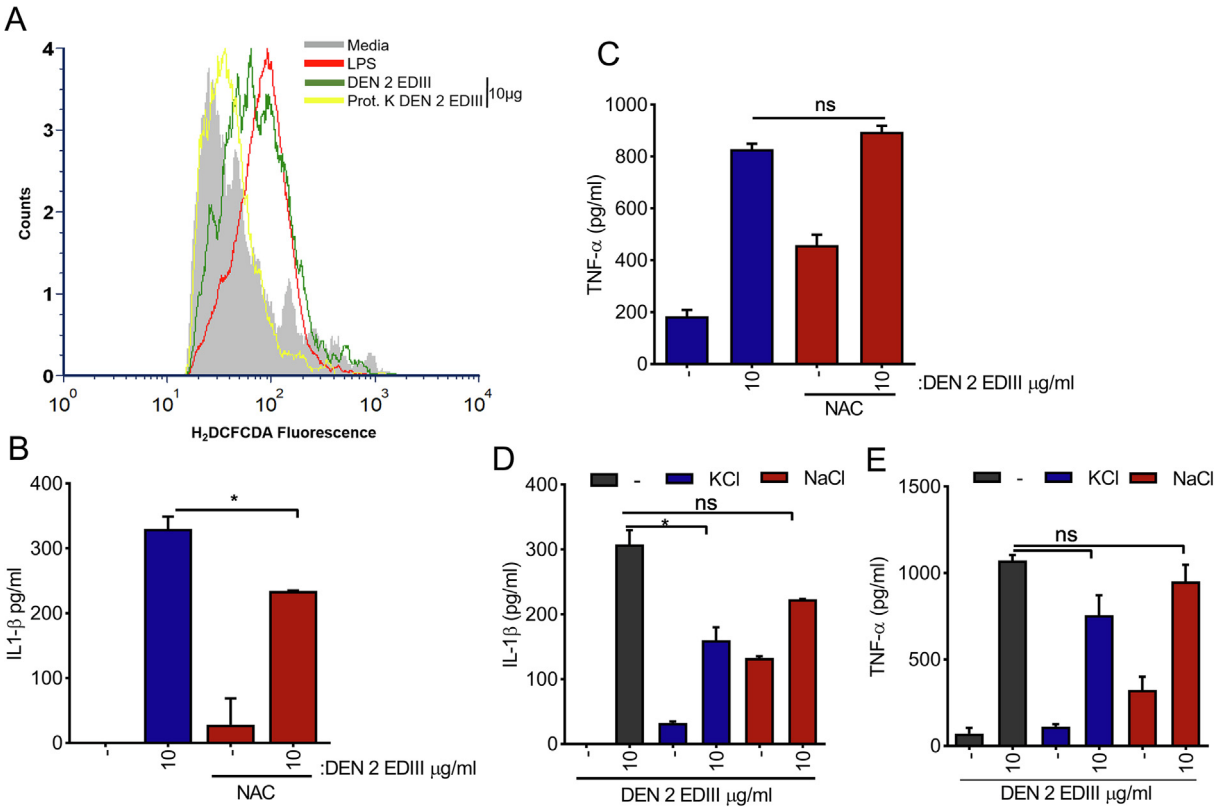

Fig. 5. ROS production and Potassium efflux regulates IL1-β maturation. (A) ROS estimation in differentiated THP-1 cells stimulated with EDIII and LPS (500 ng/ml). ELISA to check (B) IL1-β and, (C) TNF-α levels in THP-1 cells stimulated with EDIII in presence and absence of NAC (ROS inhibitor). Estimation of (D) IL1-β and, (E) TNF-α levels in EDIII stimulated THP-1 cells supplemented with NaCl and KCl to mimic intracellular Na + and K +. Statistical significance was calculated by student *t* test. $^*P \leq 0.05$ was considered statistically significant, ns = not significant. Error bars represent mean +/− SEM. Data are representative of one of three independent experiments.

inflammatory responses would help in the better understanding of DENV pathogenesis, which in future might aide in the design and development of the therapeutic interventions against Dengue Virus.

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Declaration of Competing Interest

The authors declare no competing financial interests.

Author contributions

N.K and R.A.K planned and designed the research. RAK performed experiments. S.A performed ROS estimation through FACS and assisted in few experiments. RAK, S.A and S.B and GM analysed and interpreted the data. Manuscript was written by R.A.K and N.K. All the authors read and approved the manuscript before submission.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.154780.

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