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Dengue virus disrupts Daxx and NF-κB interaction to induce CD137-mediated apoptosis

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ABSTRACT

Dengue virus (DENV) is a positive-strand RNA virus of the *Flavivirus* family with 4 different serotypes. Clinical manifestations of DENV infection include dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. Following DENV infection, apoptosis of hepatic cells is observed both *in vitro* and *in vivo*. However, the molecular mechanisms revealing how viral components affect cellular apoptosis remain unclear. In the present study, the role of death domain-associated protein 6 (Daxx) in DENV-mediated apoptosis was characterized by RNA interference and overexpression studies, and the anti-apoptotic function of Daxx during DENV infection was identified. Furthermore, the viral component, DENV capsid protein (DENV C), interacted with Daxx to disrupt interaction between Daxx and NF-κB. The liberated NF-κB activated the promoter of CD137, which is a member of the TNF family, and is previously shown to induce apoptosis during DENV infection. In summary, DENV C disrupts Daxx and NF-κB interaction to induce CD137-mediated apoptosis during DENV infection.

1. Introduction

Dengue virus (DENV), a positive-strand RNA virus of the *Flavivirus* family with 4 different serotypes (DENV-1, -2, -3, and -4), is transmitted to man by the mosquito *Aedes aegypti* [1]. All four serotypes of DENV cause disease with a varied degree of severity. Most of the DENV-infected patients develop dengue fever (DF). However, some patients reach severe forms, which are classified into dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2]. Hepatic injury concerns one of the most clinical symptoms, which leads to severe diseases. Evidence of hepatic injury is also demonstrated by hepatomegaly and an increase in transaminase levels [3]. Hepatic biopsy specimens, obtained from fatal cases of DSS, show cellular apoptosis, which may be related to the pathogenesis of DHF/DSS [4].

Following DENV infection, apoptosis of hepatic cells was observed both *in vitro* and *in vivo* [5–12]. The transcription factor NF*k*B is activated concomitantly with synthesis of DENV proteins before the appearance of apoptotic cells in HepG2 cells [6]. Viral components, including membrane (DENV M) and capsid (DENV C), contribute to DENV-mediated apoptosis [7,8,13]. The molecular mechanisms confirming how viral components affect cellular apoptosis need further investigation. The interplay between DENV C and human death domain-associated protein Daxx in DENV-mediated apoptosis is reported [13]. The loss of nuclear localization of DENV C disrupts the interaction with Daxx in the nucleus, and decreases apoptosis [13]. However, how Daxx is involved directly in DENV-induced apoptosis is not known. Moreover, the mRNA expression of CD137, which is a member of the TNF receptor super family 9 (TNFRSF 9) and is involved in apoptosis [14]was up-regulated in HepG2 cells expressing DENV C [15]. However, how DENV C mediates CD137-induced apoptosis is not known.

This study characterized the role of Daxx during DENV infection by RNA interference and overexpression studies, and the anti-apoptotic function of Daxx during DENV infection is identified. To demonstrate the molecular mechanism how Daxx mediated an anti-apoptotic function when HepG2 cells were infected with DENV, co-immunoprecipitation studies were performed. The results show that DENV C disrupts interaction between anti-apoptotic Daxx and

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the p65 subunit of NF-κB. The liberated NF-κB then activates the CD137 promoter to induce apoptosis.

2. Materials and methods

2.1. Cell culture and preparation of DENV

Up to 6×10^5 HepG2 cells were seeded in a 6-well plate and cultured for 24 h prior to infection. HepG2 cells were grown in DMEM medium (Gibco-BRL), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml of penicillin and streptomycin at 37 °C in a humidified atmosphere, containing 5% CO_2. DENV serotype 2 strain 16681 was propagated as described previously [15,16].

2.2. Construction of plasmids

To construct a plasmid expressing Daxx, RNA was extracted from HepG2 cells and converted to cDNA. The nested-PCR was performed by using a set of outer primers including NTDaxx_F 5′CCATGCGAGGTTCTGAG3′ and NTDaxx_R 5′CACCAAAAGGGGA TTAG3′ and inner primers including DaxxBam_F 5′CTAGGA TCCGACATGGCCACCGCTAAC3′ and DaxxXba_R 5′CTA TAGACTAAT CAGAGTCTAAG3′; the cDNA was sub-cloned into pcDNA 3.1 His C (Invitrogen), and verified by DNA sequencing. The expression of Daxx was confirmed by Western blot analysis using both anti-Daxx (sc-7152; Santa Cruz Biotechnology) and anti-His antibodies (sc-8036; Santa Cruz Biotechnology).

To construct plasmids, containing the CD137 promoter and its three deleted constructs (Fig. 4A and B), genomic DNA was extracted from the monocyte cell line THP-1. The nested PCR was then performed by using outer primers, including CD137 Promoter_F 5′AGGTACCTGCCATGTTGGACGTC3′ and CD137 Promoter_R 5′TAAG CTTGATGAAATCTGGCACAG3′, and these sets of inner primers to construct CD137 full promoter and the three deleted constructs, including Full CD137_F 5′CTAGGTACCAATCCCTCCTAGCTCTCAG3′; Del1_F 5′CTAGGTACCCCATTATCAGGCAGG3′; Del2_F 5′CTAGGTAC CGAGACCCCGCCCCTG3′; Del3_F 5′TAGGTACCG ACCTGAAGTCCTC3′ and Full CD137_R 5′GTTAAGCTTAGATCTCAGGGCTGCCGG3′, respectively. PCR products were separately sub-cloned into the pGL3 basic vector (Promega), and verified by DNA sequencing.

2.3. RNA interference directed against Daxx during DENV infection, and in HepG2 cells either expressing DENV C or NLS-mutated DENV C (R85 DENV C)

HepG2 cells were transfected with either siRNA against Daxx (SiDaxx; 5′CAGCCAAGCUCUAUGUCUACAUCAA3′) (Invitrogen) or siRNA control (SiC; 5′CACGCCTCTTTGTCTTGTTTCGAAA3′) (Invitrogen). At 24 h post transfection, DENV serotype 2 was infected at MOI of 5. DENV-infected HepG2 cells were harvested 24 h after infection. Both life and dead cells were collected, subjected to an apoptosis assay by annexin V/PI staining (BD Biosciences), and analyzed by flow cytometry. The expression level of Daxx was verified by Western blot analysis using anti-Daxx antibody, and compared with β-actin antibody, which was used as a loading control.

HepG2 cells, stably expressing either DENV C or NLS-mutated DENV C (R85 DENV C) were also constructed according to methods, described previously [11]. The culture supernatant, containing infectious viral particles, was collected after 24 h post transfection and added to the HepG2 cell line, which was pre-incubated with 8 μg/ml of polybrene. At 24 h after incubation, the HepG2 cells stably expressing DENV C or R85 DENV C were selected with media containing 0.5 mg/ml G418 (Calbiochem). The G418-resistant cells were grown and maintained in DMEM medium containing 0.5 mg/ml G418, and the expression of DENV C was examined by flow cytometry and Western blot analysis using an antibody to DENV C (D2-C1) [17]. Up to 6×10^5 of stably HepG2 cells expressing DENV C or R85 DENV C were then placed into a 6-well plate for 24 h prior to transfection. The transfections of siRNA directed against Daxx or siRNA control were performed, and treated with 0.5 μg/ml anti-Fas mAb antibody (Sigma) and 1 μg/ml cycloheximide (Sigma) for 24 h in culture media. Both adherent and floating cells and culture supernatant were collected for an apoptosis assay by annexin V/PI staining, and analyzed by flow cytometry. The expression level of Daxx was verified by Western blot analysis using an anti-Daxx antibody compared with β-actin antibody.

2.4. Overexpression of Daxx during DENV infection, and in HepG2 cells expressing either DENV C or R85 DENV C

Up to 6×10^5 of HepG2 cells were plated into a 6-well plate and transfected with either plasmid, coding for Daxx (His/Daxx) or vector control (His). At 24 h post transfection, DENV serotype 2 was added at MOI of 5. The cells were harvested 24 h after infection. Apoptosis was then measured as described previously. The expression level of Daxx was verified by Western blot analysis using anti-Daxx antibody and compared with β-actin antibody.

Up to 6×10^5 of HepG2 cells, stably expressing DENV C or R85 DENV C, were also placed into a 6-well plate for 24 h prior to transfection. The transfection with either plasmid coding for Daxx (His/Daxx) or vector control (His) was performed, and the cells were treated with 0.5 μg/ml anti-Fas mAb antibody (Sigma) and 1 μg/ml cycloheximide (Sigma) for 24 h in culture medium. Apoptosis was measured as described previously. The expression level of Daxx was verified by Western blot analysis using anti-Daxx antibody compared with β-actin antibody.

2.5. Co-immunoprecipitation

Up to 6×10^5 of HepG2 cells were plated for 24 h, followed by DENV infection at MOI of 1. Cells were harvested, and immunoprecipitation was carried out with either 5 μg of purified mouse antibody to DENV C (D2-C1) or 5 μg of rabbit antibody anti-Daxx antibody (sc-7152). The mixture was then incubated with a soft rotation at 4 °C overnight. The incubation was continued for 4 h after addition of protein G Sepharose beads. The bound proteins were eluted and subjected to Western blot analysis either with an antibody to DENV C (D2-C1), or a rabbit antibody anti-Daxx antibody (sc-7152), or a rabbit anti-p65 polyclonal antibody (sc-372; Santa Cruz Biotechnology), or a rabbit anti-AP1 polyclonal antibody (sc-1694; Santa Cruz Biotechnology). Cell lysates from HepG2 cells expressing DENV C or vector control were also immunoprecipitated either with a purified anti-DENV C antibody or an anti-Daxx antibody. The complexes were detected either with an anti-DENV C antibody, or an anti-Daxx antibody or an anti-NF*k*B antibody (p65) or an anti-AP1 antibody. The membranes were washed and incubated with HRP-conjugated secondary antibody. Immune complexes were detected by enhanced chemiluminescence (Pierce).

2.6. Dual-luciferase® reporter assay

Up to 1.2×10^5 of HepG2 cells were placed in a 24-well plate and transfected with either a plasmid, containing the full CD137 promoter or with a deleted CD137 promoter; Del1, Del2 or Del3 CD137. The luciferase empty vector was used as a negative control, and the vector containing the p65 subunit of NF-κB was used as a

positive control, respectively. All of these experiments were co-transfected with pRLSV40, which was a reporter plasmid. At 12 h post transfection, cells were infected with DENV at MOI of 5, and harvested at 36 h post infection. The luciferase activity was then measured by using the Dual Luciferase Assay kit (Promega), following the manufacturer's recommendations. The luciferase activity was reported as a relative light unit (RLU), which was calculated following this formula: RLU = {(firefly luciferase value of test/Renilla luciferase value of test)/(firefly luciferase value of control/Renilla luciferase value of control)}. Luciferase activity of HepG2 cell lysates, carrying the CD137 promoter or CD137 promoters with deletions (of stable HepG2 cells expressing DENV C or DENV C with R85 mutations) were also performed using protocol described previously.

2.7. Statistical analysis

Data were obtained from three independent experiments and reported as mean ± SEM. Statistical analysis between the groups were tested by unpaired *t*-test using StatView version 5.0 programs and *P* value less than 0.05 was considered as a significant difference.

3. Results and discussion

3.1. Daxx acts an anti-apoptotic protein during DENV infection

Daxx was firstly identified to be a pro-apoptotic protein [18,19]. However, the anti-apoptotic role of Daxx has been shown in several studies [20,21]. In the present study, HepG2 cells were transfected with either siRNA against Daxx or siRNA control. At 24 h post siRNA transfection, DENV was infected at MOI of 5. HepG2 cells were harvested 24 h after infection and apoptosis was measured by annexin V/PI staining (Fig. 1A). In the RNA interference study, apoptotic cells increased from 9.7% to 23.91% after DENV infection and increased to 33.96% in Daxx-depleted DENV-infected HepG2 cells (Fig. 1A), suggesting the anti-apoptotic function of Daxx. In the overexpression study, apoptotic cells decreased from 27.29% to 18.68% in DENV-infected HepG2 cells with overexpression of Daxx (Fig. 1B). The efficiency of knockdown and expression of Daxx was also shown by Western blot analysis (Fig. 1A and B). Hence, the anti-apoptotic role of Daxx during DENV infection was identified.

Stable HepG2 cells, stably expressing DENV C, were transfected with either siRNA against Daxx or siRNA control. Apoptosis was

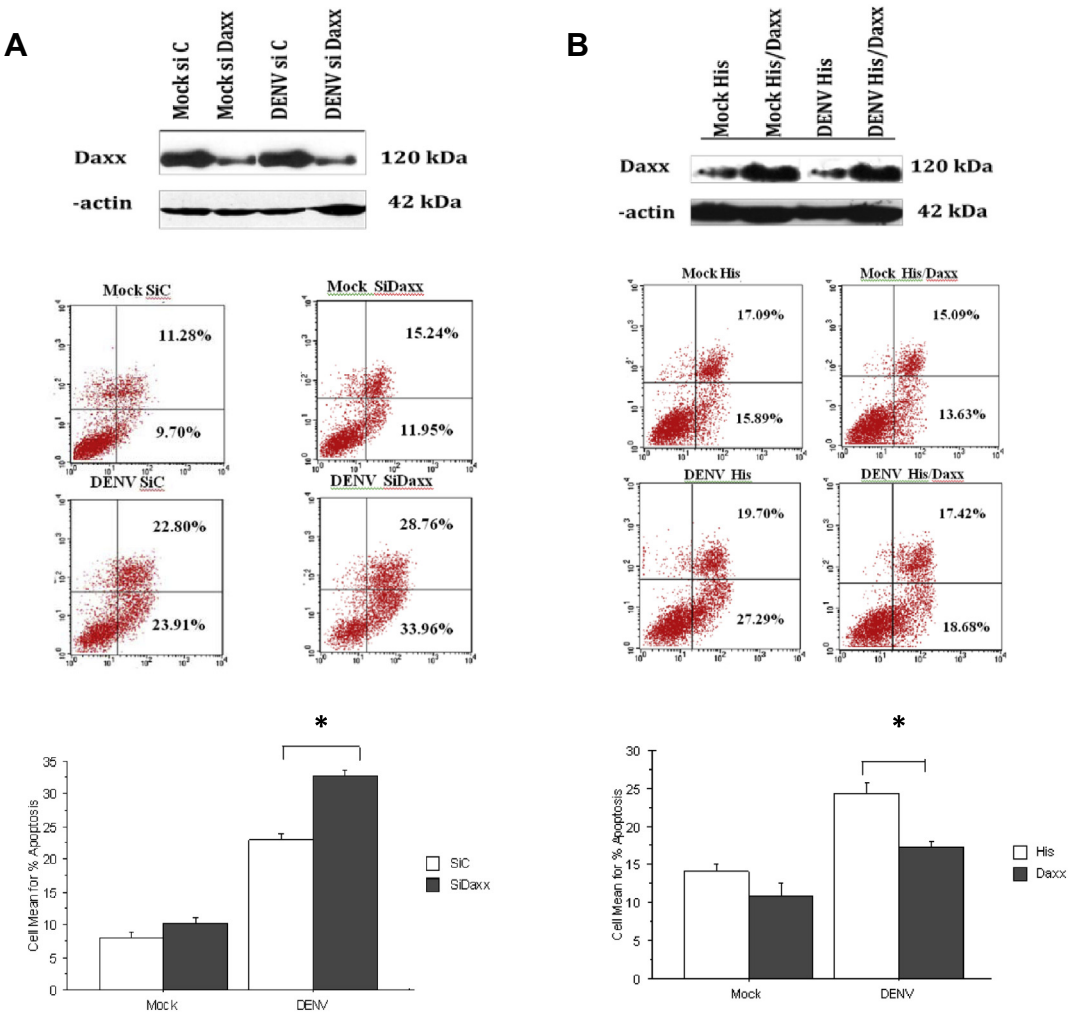

Fig. 1. The anti-apoptotic function of Daxx in DENV-infected HepG2 cells. (A) HepG2 cells were transfected with either siRNA against Daxx or siRNA control. At 24 h post siRNA transfection, DENV was infected at MOI of 5. HepG2 cells were harvested 24 h after infection and apoptosis and Daxx expression were measured. (B) HepG2 cells were transfected with either plasmid containing Daxx (His/Daxx) or vector control (His). At 24 h post transfection, DENV was added at MOI of 5 and harvested 24 h after DENV infection. Apoptosis and Daxx expression were measured. Data were obtained from three independent experiments and reported as mean ± SEM. The asterisks indicate statistically significant differences between groups ($p < 0.05$).

measured by annexin V/PI staining. RNA interference by siRNA against Daxx increased apoptosis in HepG2 cells, expressing DENV C, from 15.74% to 25.31% (Fig. 2A). The finding is similar to that in DENV infection, where Daxx acts an anti-apoptotic protein (Fig. 1A and B). Overexpression of Daxx decreased apoptosis in HepG2 cells, expressing DENV C, from 28.33% to 22.57% (Fig. 2B) and supports the anti-apoptotic role of Daxx in HepG2 cells, expressing DENV C. The expression of Daxx was verified by Western blot analysis (Fig. 2A and B).

DENV C localizes to both the cytoplasm and nucleus of DENV-infected HepG2 cells. DENV C contains three nuclear localization signals (NLS), (6)KKAR(9), (73)KKSK(76), and the bipartite signal (85)RKeigrmlnilnRRRR(100) [22]. HepG2 cells, expressing DENV C, exhibit more apoptosis compared to HepG2 cells, expressing DENV C with (85)RK mutations [13]. Apoptosis of HepG2 cells, expressing DENV C with (85)RK mutations in the presence of siRNA against Daxx (24.81%) or in the presence of siRNA control (22.81%), was relatively similar (Fig. 2A), suggesting that the nuclear localization of DENV C is required for the anti-apoptotic function of Daxx. This is further supported by an apoptosis assay in an over-expression study of HepG2 cells, expressing DENV C with (85)RK mutations in the presence of Daxx overexpression (21.92%) or vector control (23.99%); the result was relatively similar (Fig. 2B). The expression of Daxx was verified by Western blot analysis (Fig. 2A and B).

3.2. Disruption of Daxx and NF-kB interaction by DENV C

Nuclear localization of DENV C is required for Daxx interaction and apoptosis [13]. However, interplay of Daxx and DENV C involved in apoptosis is unknown. Daxx is an anti-apoptotic regulator [21], which interacts with NF-*k*B [23]. Although NF-*k*B supports not only cell death, but also supports cell survival in different settings [24], replication of DENV in HepG2 cells was shown to activate NF-*k*B, which further induces apoptotic cell death [6]. We hypothesized that DENV C may hijack Daxx from

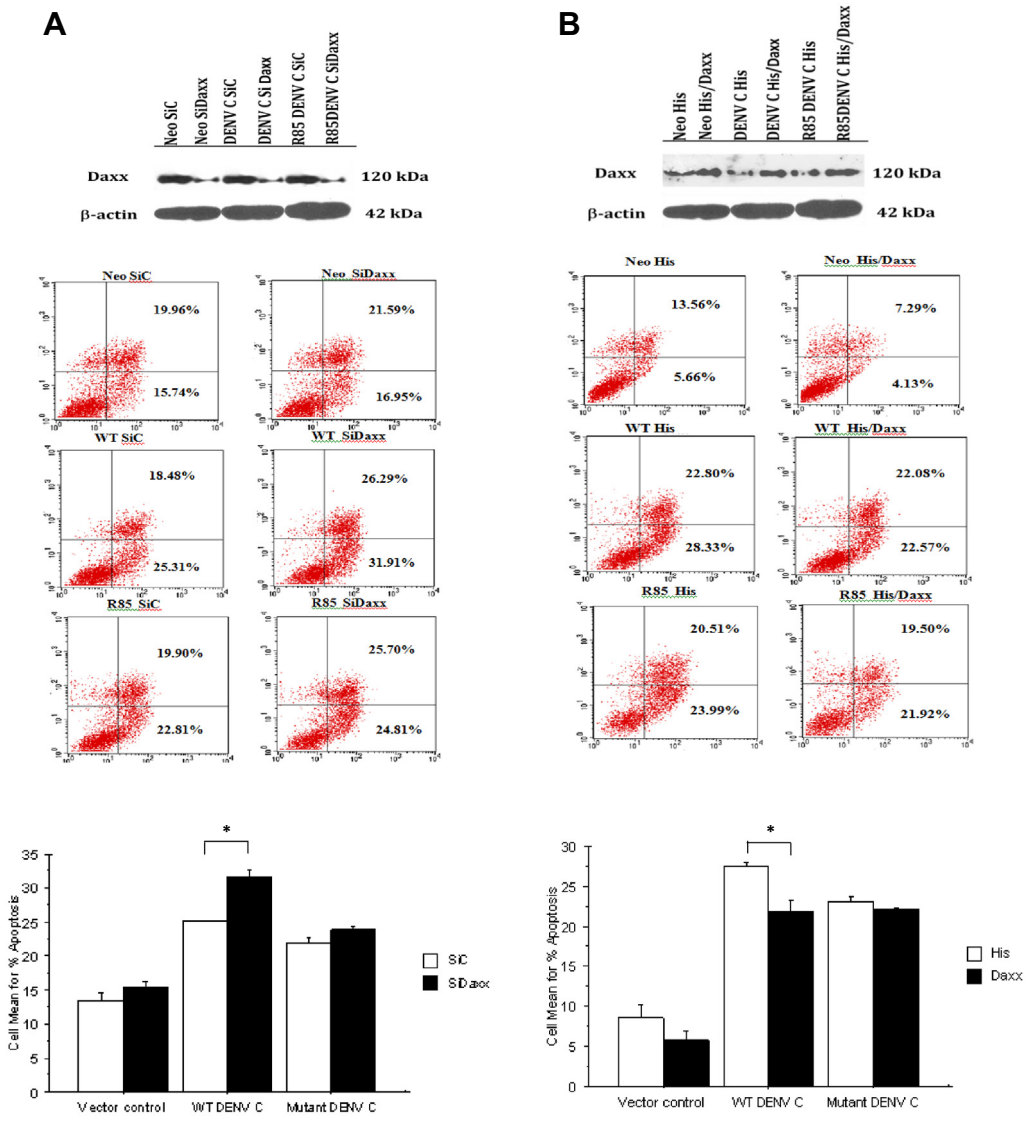

Fig. 2. The anti-apoptotic function of Daxx in HepG2 cells expressing DENV C. (A) HepG2 cells, stably expressing DENV C or DENV C with R85 mutations, were transfected with either siRNA against Daxx or siRNA control, and treated with 0.5 μg/ml anti-Fas mAb antibody (Sigma) and 1 μg/ml cycloheximide (Sigma) for 24 h in culture medium. Apoptosis and expression of Daxx were then measured. (B) HepG2 cells, stably expressing DENV C or NLS-mutated DENV C, were placed for 24 h prior to transfection. The transfection of either plasmid containing Daxx (His/Daxx) or vector control (His) was performed, and the cells were treated with 0.5 μg/ml anti-Fas mAb (Sigma) and 1 μg/ml cycloheximide (Sigma) for 24 h in culture medium. Apoptosis and expression of Daxx were then measured. Data were obtained from three independent experiments and reported as mean ± SEM. The asterisks indicate statistically significant differences between groups ($p < 0.05$).

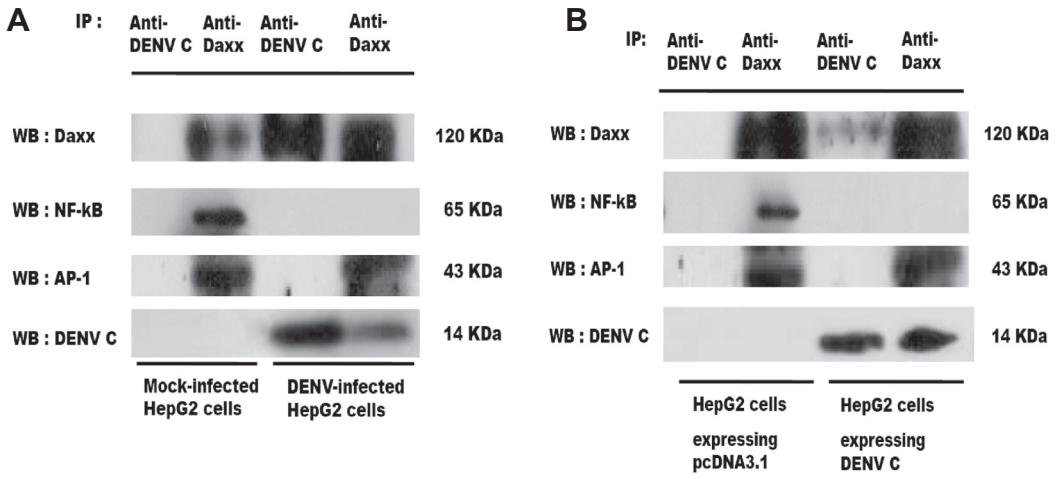

Fig. 3. DENV C disrupts Daxx and NF-κB interaction. (A) Cell lysates from mock-infected (Lane1, 2) and DENV-infected HepG2 cells (Lane3, 4) were immunoprecipitated either with purified anti-DENV C antibody or anti-Daxx antibody. The complexes were detected either with anti-DENV C antibody, or anti-Daxx antibody or anti-NF*k*B antibody (p65) or anti-AP1 antibody. (B) Cell lysates from HepG2 cells expressing DENV C (Lane3, 4) or vector control (Lane1, 2) were immunoprecipitated either with a purified anti-DENV C antibody or an anti-Daxx antibody. The complexes were detected either with anti-DENV C antibody, or an anti-Daxx antibody or an anti-NF*k*B antibody (p65) or an anti-AP1 antibody.

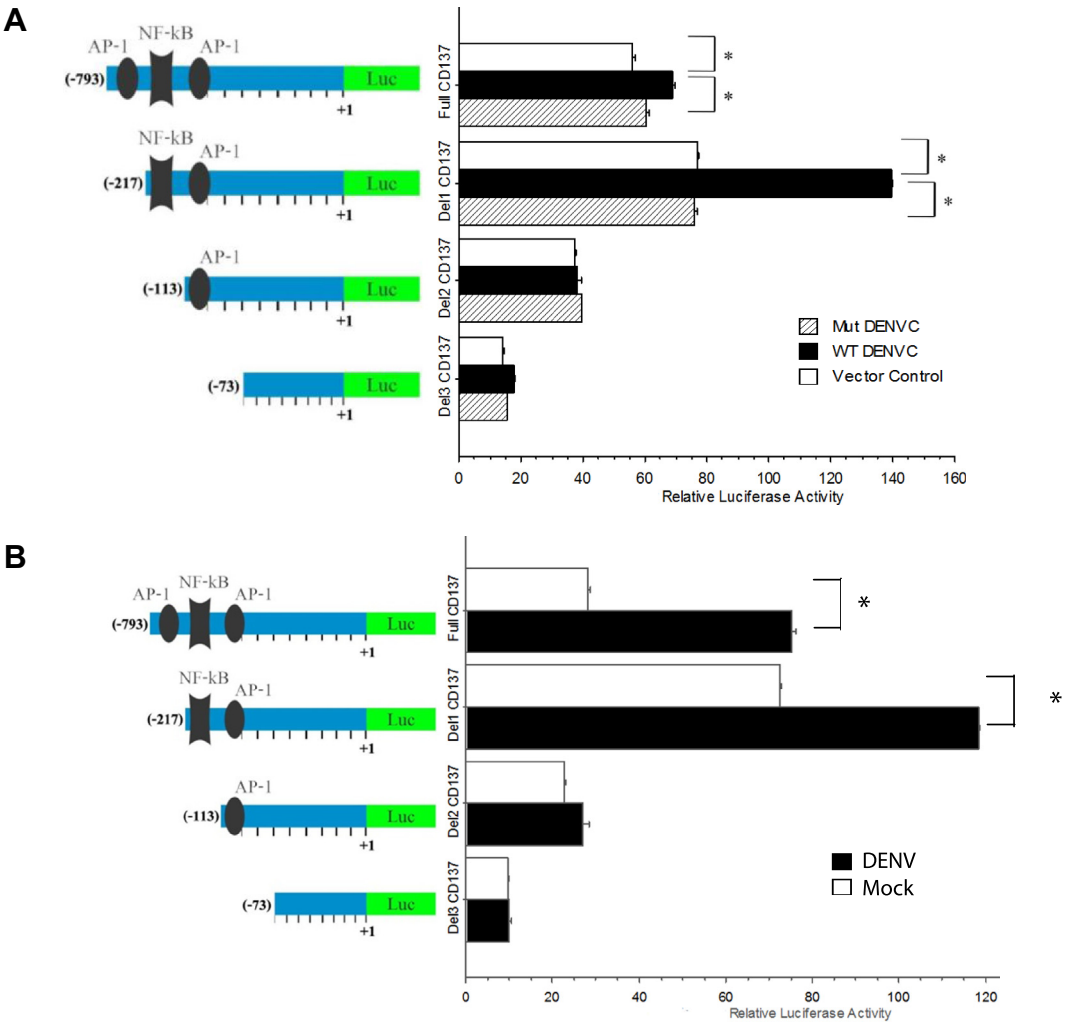

Fig. 4. NF-κB is required for CD137 activation during DENV infection (A) Luciferase activity of HepG2 cell lysates, carrying the CD137 promoter or CD137 promoters with deletions (of stable HepG2 cells expressing DENV C or DENV C with R85 mutations). (B) Luciferase activity of mock-infected and of DENV-infected HepG2 cells carrying the CD137 promoter or CD137 promoters with deletions. The results are expressed as the average of three independent experiments ± SEM. The asterisks indicate statistically significant differences between groups ($p < 0.05$).

NF-*k*B to induce apoptosis of HepG2 cells. Co-immunoprecipitation for Daxx and NF-*k*B was performed in mock-infected and DENV-infected HepG2 cells. The results show that Daxx interacted with NF-*k*B in mock-infected HepG2 cells, but did not interact with NF-*k*B in DENV-infected HepG2 cells (Fig. 3A). Therefore, we concluded that DENV disrupts interaction between Daxx and NF-*k*B. As a control, interaction between Daxx and AP-1 was still intact in mock-infected and DENV-infected HepG2 cells suggesting interaction between Daxx and AP-1 was not disrupted during DENV infection.

To determine how DENV C is involved in this process, co-immunoprecipitation of Daxx and NF-*k*B was performed in HepG2 cells, expressing DENV C or expressing His. The results show that Daxx interacted with NF-*k*B in HepG2 cells expressing His (Fig. 3B), but did not interact with NF-*k*B in HepG2 cells expressing DENV C (Fig. 3B). As a control, interaction between Daxx and AP-1 was still intact in HepG2 cells expressing DENV C and expressing His, suggesting that DENV C disrupted Daxx and NF-*k*B interaction, but did not disrupt Daxx and AP-1 in HepG2 cells expressing DENV C. Interestingly, disruption of the Daxx complex with viral proteins is supported by several studies in other viruses, including disruption of the Daxx and NF-*k*B by matrix 1 protein (M1) in influenza A virus[25] and disruption of the Daxx-ATRX chromatin remodeling complex by major tegument protein (BNRF1) in Epstein–Barr Virus (EBV) [26].

3.3. NF-kB activates CD137 signaling during DENV infection

CD137 is a member of the TNF family and induces apoptosis during DENV infection [11,14,15]. CD137-mediated apoptotic activity was shown in both DENV infection of HepG2 cells and in HepG2 cells expressing DENV C [11,15]. In addition, the relationship between CD137 signaling and NF-*k*B activation has been reported [27,28]. Therefore, we next asked whether NF-*k*B activates the promoter of CD137 both in transfection and infection models [14].

The plasmid, expressing DENV C or DENV C with (85)RK mutations, was co-transfected into HepG2 cells with either the pGL3-basic plasmid, or the CD137 promoter reporter plasmid or the CD137 promoter reporter plasmid with deleted constructs, and a pRL-SV40 internal control plasmid. After 48 h post transfection, HepG2 cells were subjected to measurement of luciferase activity. The activity was presented in terms of RLU normalized to empty pcDNA3.1/Hygro transfection control. In the presence of the NF-*k*B binding site on the CD137 promoter, DENV C activated the CD137 promoter compared with that of the empty pcDNA3.1/Hygro transfection control (Fig. 4A). However, the ability of DENV C to activate the CD137 promoter was significantly decreased in the absence of the NF-*k*B binding site, suggesting the importance of NF-*k*B in activation of CD137 promoter in HepG2 cells expressing DENV C. As expected, in HepG2 cells expressing DENV C with (85)RK mutations, which lost its ability to enter into the nucleus, it could not activate the CD137 promoter (Fig. 4A). All together, this suggests the importance of NF-*k*B in activation of CD137 promoter in the nucleus of HepG2 cells expressing DENV C.

Furthermore, mock-infected and DENV-infected HepG2 cells were co-transfected with either the pGL3-basic plasmid, or the CD137 promoter reporter plasmid, or the CD137 promoter reporter plasmid with deletion (Fig. 4B), and a pRL-SV40 internal control plasmid. After 48 h post transfection, HepG2 cells were subjected to measurement of luciferase activity. Similar to the transfection model, in the presence of NF-*k*B binding site on the CD137 promoter, DENV activated CD137 promoter more than that of control (Fig. 4B). However, the CD137 promoter was notably less activated in the absence of the NF-*k*B binding site on the CD137 promoter, indicating the importance of NF-*k*B in activation of the CD137 promoter during DENV infection (Fig. 4B).

In summary, we propose that when DENV C enters into the nucleus, it interacts with Daxx and disrupts the interaction between Daxx and NF-*k*B, thereby releasing NF-*k*B to activate the promoter of the CD137 gene for DENV-induced apoptosis. However, how CD137 regulates its downstream signaling proteins to induce apoptosis during DENV infection and how DENV generates a new flux of NF-*k*B by other mechanisms need further investigation.

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