Human TLR3 recognizes dengue virus and modulates viral replication in vitro

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Summary

The elicitation of large amount inflammatory cytokine in serum has been developed as the cause of the plasma leakage in dengue fever (DF)/dengue haemorrhagic fever (DHF) infection. Virus recognition in innate immunity is the key. The Toll-like receptors (TLRs) play an important role in pathogen recognition towards cytokine induction among several viruses; however, the role of TLRs on innate immune recognition against DENV remains unclear. This study aims at the interaction between dengue virus (DENV) and human TLRs at the incipient stage of infection in vitro. Our experiment reveals that stably expression of TLR3, 7, 8 on HEK293 enables IL-8 secretion after DENV recognition. By the model of human monocytic cells U937, we demonstrated the trigger of IL-8 after viral recognition of human monocytic cell is primary through TLR3 following endosomal acidification. Silencing of TLR3 in U937 cells significantly blocks the DENV-induced IL-8 production. Besides, the interaction is further corroborated by colocalization of TLR3 and DENV RNA upon DENV internalization. Furthermore, in this study we found the expression of TLR3 can mediate strong IFN-α/β release and inhibit DENV viral replication significantly, thus limit the cytopathic effect.

Introduction

Dengue virus (DENV), genus flavivirus in the Flaviviridae family, is a single positive strand RNA packaged in an icosahedral nucleocapsid and wrapped with an envelope. DENV causes diseases such as dengue fever (DF) and dengue haemorrhagic fever (DHF)/dengue shock syndrome in human that are the most prevalent arthropod-borne viral diseases in subtropical and tropical regions of the world (Halstead, 1980). In natural human infections, peripheral monocytes/macrophages, myeloid dendritic cells (DC), dermal Langerhans cells and reactive splenic lymphoid cells are known as the major target cells of DENV replication (Jessie et al., 2004; Kwan et al., 2005). Clinical evidence manifests that, during DENV infection, the grades of DF/DHF closely correlate with persisting high viral load (Wang et al., 2003) and inflammatory cytokine overproduction in plasma (Vitarana et al., 1991; Raghupathy et al., 1998; Gagnon et al., 2002; Chakravarti and Kumaria, 2006) that mainly released from monocytes, CD4+ and CD8+ T lymphocytes (Rothman and Ennis, 1999; Dewi et al., 2004; Cardier et al., 2005). The direction of DENV-triggered inflammatory response should be highly emphasized.

Recently, the innate immune recognition at the incipient stage of virus infection has shown its predominant role in priming and regulating the inflammatory response through pattern recognition receptors (PRRs) (Medzhitov, 2001; Kabelitz and Medzhitov, 2007). Toll-like receptors (TLRs), one of the most important category of PRRs that especially abundant on monocytes/macrophages and DCs, have established to play a key role in regulating the inflammatory response against infectious viruses (Akira et al., 2006; Trinchieri and Sher, 2007). TLRs capture signals derived from viral particles and subsequently initiate signalling for inflammatory cytokine response such as IL-8, IFN-α/β, TNF-α production (Iwasaki and Medzhitov, 2004). By far, the TLRs including TLR2, 3, 4, 7, 8 and 9 have been well-described involved in priming inflammatory response against various viruses such as herpes simplex virus (HSV) (Sato et al., 2006), influenza A virus (Le Goffic et al., 2007), respiratory syncytial virus (Rudd et al., 2005) or coxsackie B virus (Triantafilo et al., 2005). Up to the present, in flavivirus, TLR2 and TLR3 are reported to participate in hepatitis C virus-induced cellular activation and cytokine production (Naka et al., 2006; Chang et al., 2007); multiple TLRs including TLR3, 7, 8 and 9 are highly suggested implicated in yellow fever virus YF-17D infection; besides, the TLR3 signalling has been highlighted during West Nile virus infection not only for...
eliciting cellular antiviral response but also for shaping a detrimental innate and adaptive immune response in vivo (Wang et al., 2004). The meaning of TLRs in counteracting viral infection and disease development is increasing, however, till now a comprehensive investigation on TLRs in DENV recognition is yet unavailable.

To clarify the involvement of TLRs in the innate immune recognition of DENV, this study examined the process of viral internalization and TLR-silencing effect in human monocytic cells in vitro. The experimental results revealed that DENV-2 internalization for the immune recognition is endosomal acidification-dependent in human monocytic cell U937. By silencing and overexpressing of TLRs, we demonstrate that internalized DENV-2 particles can potently elicit signalling mainly through TLR3 molecules to trigger subsequent pro-inflammatory cytokine release. Furthermore, compared with TLR7 or TLR8, DENV-2 recognition-triggered TLR3 signalling in particular, initiates a stronger IFN-α/β response and can effectively restrain intracellular DENV-2 replication that eventually moderate the cytopathic effect (CPE) of DENV-2.

Results

Recognition of DENV-2 NGC in monocytic cells at the early stage of virus infection requires endosomal acidification

The process of lysosomal acidification is required for the early replication phase of several viruses (Seth et al., 2006), moreover, the acid environment of endosome is also crucial for virus uncoating to release their nucleic acid structures from capsid (Stiasny and Heinz, 2006). To investigate whether or not this process is required for DENV-2 recognition in human monocytic cells, chloroquine and bafilomycin A1, the two endosomal acidification inhibitors, were used in this experiment. In this experiment, U937 and THP-1, both abundantly expressing TLRs (Mueller et al., 2006; Indoh et al., 2007; Uehara et al., 2007), were infected with DENV-2 New Guinea C (NGC). The IL-8 cytokine release from the two cells both increased about 4–5 times from the basal line when infected with DENV-2 NGC at 5 multiplicities of infection (moi; P < 0.05, Fig. 1). However, the release was significantly blocked in U937 and THP-1 when treated with 10 μM chloroquine (reduction > 70% in both cells, P < 0.05) and 20 nM bafilomycin A1 (reduction > 90% in both cells, P < 0.01). Real-time PCR assay also showed similar results of reduction in mRNA level (data not shown). The treatment of the two inhibitors on virus or cell cultures at indicated concentration neither inactivates DENV-2 alone nor exhibits cytotoxic effect on the cell cultures (data not shown). These experimental results indicated that endosomal acidification is a necessary process for the innate recognition of DENV-2 and for the induction of cytokine production during the initial stage of virus infection in U937 and THP-1 cells.

The profiles of IL-8 and IL-6 cytokines response after DENV-2 recognition and different TLR agonist stimulation in the two human monocytic cells are also compared (Fig. 2). In both U937 and THP-1 cells, similar IL-8 responses in a dose-dependent manner is observed in polyinosinic-polycytidylic acid [poly(I:C)] and CL075 stimulation as well as in DENV-2 infection. In contrast with IL-8, IL-6 arose fecklessly in THP-1 in all groups with the excep-
tion of high-dose treatment with CL075 (5 and 10 μg ml⁻¹); on the other hand, U937 potentially sustained IL-6 secretion upon DENV-2 NGC infection at 15 moi and poly(I:C) stimulation at 25 μg ml⁻¹ (P < 0.01). In particular, neither THP-1 nor U937 revealed sensitivity to TLR9 agonist CpG-oligodinucleotide (CpG-ODN), even at a dose of 10 μM.

In all the experiments, no signal noise was observed in the C6/36 cell supernatant control experiments either the U937 or THP-1 cells.

**TLR3 mediates IL-8 production after DENV-2 recognition**

To comprehend the role TLRs play in DENV-2 recognition at early stage of infection, HEK293 cell clones that heterologously expressed human TLRs were used. After 24 h of DENV-2 infection, HEK293-TLR3, TLR7 and TLR8 elevated 7–20 times IL-8 release from basal line (P < 0.05, Fig. 3); however, the membrane TLR-bearing HEK293 cells (HEK293-TLR2 and TLR4) failed to induce IL-8 production after infection. The strongest IL-8 response was elicited by TLR3, which implies a cardinal role of TLR3 molecules implicated in the innate recognition of DENV-2 NGC. We further confirm that whether replication competence of DENV-2 NGC is required for the activation of TLR3, UV-inactive DENV-2 NGC was used to infect HEK293-TLR3 cells; however, HEK293-TLR3 cells that can respond in IL-8 release against DENV-2 NGC infection fails to effectively induce the production of IL-8 (data not shown). This result implies that replication-competent DENV-2 is required for TLR3 activation. The potency of TLR3 to induce IL-8 production was further confirmed by TLR3-knockdown assay on U937 monocytic cells. As

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**Fig. 2.** Responding profiles of THP-1 and U937 monocytic cells after DENV-2 NGC infection and endosomal TLR agonists stimulation. THP-1 and U937 monocytic cells were infected with DENV-2 at the indicated moi or treated with TLR3 agonist poly(I:C), TLR8/7 agonist CL075 or TLR9 agonist CpG-ODN (ODN2006) at different concentrations then incubated at 37°C for 24 h. Cell culture supernatants were collected for IL-8 and IL-6 cytokine measurement. Medium: RPMI medium control; C6/36 sup. mock infected C6/36 cell culture supernatant; 5, 10 and 15 moi: cells infected with DENV-2 NGC only at 5, 10 or 15 moi. One of two reproducible experiments is shown. (A) and (C): IL-8 secretion profiles in the culture supernatants of activated THP-1 and U937 respectively. (B) and (D): IL-6 secretion profiles of activated THP-1 and U937. The IL-8 and IL-6 cytokine values are expressed as pg/ml and represented as mean ± SD.
Fig. 4A indicates, specific shRNA TRCN0000056849 against TLR3 was used to downregulate TLR3 expression in U937 human monocytic cells. The IL-8 release against DENV-2 infection significantly decreased 69.9% in TLR3-knockdowned U937 than in normal U937 cells ($P < 0.05$) when the specific shRNA targeting luciferase did no effect on IL-8 release. The stimulation on TRCN0000056849-treated cells with poly(I:C) at 12.5 $\mu$g ml$^{-1}$ (84.6% lower, $P < 0.01$) and CL075 at 5 $\mu$g ml$^{-1}$ (no significant effect) served as TLR3-specific knockdown control. We further examine this phenomenon in TLR3-knockdowned HEK293-TLR3 cells, similar reducing effect after DENV-2 infection was also observed (Fig. 4C). The reduction effects of downregulated TLR3 on U937 or HEK293-TLR3 cells in transcripts and proteins expression were also demonstrated in Fig. 4B and D respectively.

**Dengue viral RNA is colocalized with TLR3, 7 and 8 intracellularly**

TLR3 molecules revealed the potential in recognizing DENV-2 NGC. To further demonstrate intracellular ligation

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**Fig. 4.** Downregulation of TLR3 significantly reduces IL-8 responding intensity in human monocytic cell U937 and fibroblast HEK293-TLR3. The indicated cells were infected with DENV-2 NGC (moi = 5). Following incubation at 37°C for 24 h, the culture supernatants were then analysed by IL-8 ELISA. The IL-8 response against DENV-2 infection after TLR3-knockdown in U937 (A) and HEK293-TLR3 (C) were shown respectively. Poly(I:C) and CL075 serve as specific knockdown control. U937 and HEK293-TLR3: TLR3 positive control; -shTLR3: TLR3-knockdowned group; -shLuc: negative knockdown control. The IL-8 cytokine values are represented as mean ± SD ($n = 2$, independent experiments).

For TLR3-knockdowned validation, TLR3 transcript expression in U937 was examined by real-time PCR (B); the TLR3-YFP protein expression in HEK293-TLR3 was examined by flow cytometry (D), the grey shadow area represents the fluorescence signal of original HEK293-TLR3 cells and the black line represents the signal of HEK293-TLR3 cells following shRNA-knockdown.
of TLRs with viral RNA, the colocalization of TLR and dengue RNA structure was visualized by confocal microscope. The BrU-DENV was used to infect HEK293 cells bearing individual TLRs and stained by anti-BrdU antibody conjugated with fluorophore Alexa-568 (Red). An additive anti-fluorescent protein (YFP) fluorescein-labelled antibody (Green) was used to enhance the fluorescence of YFP post paraformaldehyde fixing. The 488 nm laser line was used for excitation of FITC (detected at 504 nm < λFITC < 530 nm), and the 543 nm laser line was used for Alexa-568 fluorescence (detected at 600 < λAlexa-568 < 670 nm). As Fig. 5 shows, the colocalization sites of TLR3, 7 and 8 and dengue viral RNA in the cytoplasm of HEK293 cells are evidenced by the significant signal overlapping spots (yellow colour) at 30 min post virus infection.

**Fig. 5.** TLR3, 7 and 8 colocalizes with DENV-2 viral RNA at early infection stage. HEK293-TLR3, 7 and 8 cells were infected with DENV-2 NGC labelled with BrUTP (BrU-DENV) at room temperature for 30 min. Double immunofluorescent staining was performed to detect viral RNA and TLR-YFP fusion protein. HEK293-TLR3 cell infected with non-labelled virus was served as control. TLR-YFP: stained with anti-GFP, FITC-labelled (green); Viral RNA: stained with anti-BrdU, Alexa-568-labelled (red); Nuclei: stained with DAPI (blue); Merge: merged image of double immunofluorescent staining of the same field. The arrows indicate the densest colocalization sites of viral RNA and TLR-YFP. The white bar indicates a length of 4 μm.

**TLR3 expression significantly reduces the cytopathic effect of DENV-2**

DENV-2 NGC–induced cytocidal effect in HEK293 and HEK293-TLRs cells was initially observed at day 3 post infection and continued for 3 days. However, the DENV-2 infected HEK293-TLR3 cells exhibited notably the least CPE when compared with other infected HEK293 TLR-bearing cells (Fig. 6A). According to 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the cytocidal ratio in all TLR-transfected or original HEK293 cells significantly increased (up to 45% to 75%) at day 5 post infection except in HEK293-TLR3, which reveals only 6.1% of the cytocidal ratio (Fig. 6B). To really determine whether the antiviral effect is indeed mediated by TLR3, we have used the shRNA-expressing...
lentivirus to knockdown TLR3 level and then monitor the
effect on virus replication (Fig. 6C). The result indicated
that the TLR3-knockdowned HEK293-TLR3 cells can
revert to the highly intense CPE caused by DENV-2.

We found the reduction of CPE in HEK293-TLR3
noticeably correlates with restrained viral replication;
intracellular immunofluorescent staining with anti-DENV-2
envelope monoclonal antibody HB-46 clearly evidence
the reduction of viral envelope protein synthesis in
HEK293-TLR3 cells in comparison to HEK293 cells by
lower fluorescent intensity and percentage of positive
stained cells (Fig. 7A). Furthermore, expression of TLR3
on HEK293 cells also significantly reduced the viral yield
over 40 times at 48 h post infection (Fig. 7B).

To determine how TLR3-mediated signalling inhibits
virus replication and promotes the survival ratio of
infected cells, we examined the cytokine responses of
IFN-α and IFN-β, the vital TLR-inducing interferons,
among all HEK293-TLR clones at 6 or 24 h after DENV-2
challenge. As indicated in Fig. 8, late but strong
responses of IFN-α and IFN-β were induced in transla-
tional and also transcriptional (data not shown) level in
HEK293-TLR3 cells at 24 h post virus infection while
weaker responses were elicited in HEK293-TLR7 and
HEK293-TLR8 cells. No IFN-α or IFN-β response was
observed in HEK293 cells or TLR2, 4 or 9 – bearing
HEK293 cells, and that, the noise effect of overexpressing
TLR molecules can be excluded.

Discussion

Circulating cytokines abnormally increasing in DF and
DHF patients (Raghupathy et al., 1998; Gagnon et al.,
2002; Avila-Aguero et al., 2004) has been proposed to be
the central role of pathogenesis (Rothman and Ennis,
1999; Pang et al., 2007). The priming and regulating of
innate and adaptive immune response are now heavily
emphasized towards this issue. This study performed a
series of in vitro experiments to investigate the role of TLR
molecules, one important category of PRRs, in immune
recognition of DENV at early infection stages.

Initially, chloroquine or bafilomycin A1, the two inhibitors
of endosomal acidification were applied to examine
whether or not the immune recognition of DENV is endo-
somal acidification-dependent in the TLR-abundant
monocytic cells U937 and THP-1. When the inhibitors
were used, the secreted IL-8 levels of U937 and THP-1
after DENV-2 recognition were significantly suppressed.
This experimental result indicates that, viral internalization

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and the process of uncoating, are necessary for DENV-2 recognition and the trigger of cytokine secretion in monocytic cells U937 and THP-1 cells at early infection stage; furthermore, an intracellular sensor rather than a surface one should be responsible for recognizing DENV in these cells. Comparatively, this conclusion also implies a marginal role of the plasma membrane-bound TLRs in the recognition of DENV-2 – at least for the structural proteins (e.g. Env, M protein).

In this study, we found the characters of the two monocytic cells – U937 and THP-1 cells are only competent in responding to poly(I:C) and CL075 but not CpG-ODN2006 stimulation. The feckless response to CpG-ODN2006 might due to the use of B type CpG-ODN that is recently proposed less efficient than type A CpG-ODN (Sanjuan et al., 2006; Nordone et al., 2007); or perhaps might indicate an insufficient level of TLR9 molecules expressing on U937 and THP-1 cells. On the other hand, IL-6 was only incited by DENV-2 in U937 but not in THP-1. This result is in conflicting with the previous report that described the potency of DENV-216681 strain to elicit IL-6 expression in THP-1 cells (Chareonsirisuthigul et al., 2007). The existent evidence might indicate that, variation among virus strains/serotypes or cell types can result in distinct cellular responses. Recent study of HSV has also come up with a similar conclusion as the diverse capabilities among different strains in stimulating TLR2-mediated NF-κB activation (Sato et al., 2006). Besides, there is a huge difference in IL-6 production on U937 cells between stimulation with an moi of 10 and an moi of 15 (Fig. 2D). The reason for this difference remains unclear. However, it may be due to the threshold-dose-dependent manner of the cytokine. Similar phenomenon was reported in the report of RSV (Samransamruajkit et al., 2000).

Next to clarifying the necessity of endosomal acidification for DENV-2 at early stage of infection in vitro, the HEK293-TLR clones were further applied to demonstrate the involvement of individual TLRs in DENV-2 recognition. As Fig. 3 shows, DENV-2 infection potently activated

![Image](https://example.com/image.png)

**Fig. 7.** Expression of TLR3 restrains the replication of DENV-2 NGC in HEK293 cells. A. Immunofluorescence staining with anti-DENV-2 envelope protein monoclonal antibody (HB46) at 48 h post infection. Left column: the arrows indicate the presence of DENV-2 envelope protein synthesis in the cytoplasm of infected cells. Right column: the fields corresponding to the same field as the left column were observed by phase microscope. The white bar indicates a length of 20 μm. B. HEK293 and HEK293-TLR3 cells were infected with DENV-2 NGC. The virus yield in the supernatants collected at indicated time points post infection was determined by plaque assay. Ordinate: plaque-forming unit titre expressed in log scale; Abscissa: the time points post infection. (n = 3, independent experiments)

**Fig. 8.** TLR3 elicits IFN-α and IFN-β response in DENV-2 NGC-infected HEK293 cells. HEK293 cells and HEK293-TLR cells were infected with DENV-2 NGC. The release of IFN-α and IFN-β cytokines in the culture supernatants of infected cells were determined by ELISA, as presented in (A) and (B) respectively. Ordinate: concentration of IFN-α (pg ml⁻¹) or IFN-β (IU ml⁻¹) in the supernatants; Abscissa: the time points post infection. (n = 2, independent experiments)
HEK293-TLR3, TLR7 and TLR8 cells and subsequently triggered IL-8 cytokine release in the supernatants. After entry of DENV through endocytosis, the virus will be followed by uncoating, RNA transcription, translation, assembling and release of new viral particles. Regarding whether replication competent DENV-2 is required for TLR3 activation, we set the UV-inactive DENV-2 control and demonstrate the replication competent DENV-2 is required for TLR3 activation. Our results strongly evidence the involvement of TLR molecules especially TLR3 in DENV-2 recognition at the early infection stage and meantime reinforce the involvement of TLR7 obtained from an antagonist assay (Wang et al., 2006). Among these TLRs, TLR3 exhibits the greatest potential in response to DENV-2 infection. The capacity to drive IL-8 response in U937 cells demonstrated in TLR-silencing experiment also implies a fundamental role that TLR3 can play during DENV infection. In studies of flavivirus, TLR3-signalling pathway has been already reported to activate during West Nile virus (Wang et al., 2004; Daffis et al., 2008) and hepatitis C virus (Naka et al., 2006) infection. However, recent studies reveal that the another class of PRRs, the RNA helicase family is also implicated in driving antiviral immune response during Japanese encephalitis virus (Chang et al., 2006), WNV (Frederiksen et al., 2008) and DENV (Loo et al., 2008) infection. Taken the above findings with our data, DENV-2 may stir intracellular antiviral response through both of the TLR and the RNA helicase pathway. Further clarification on the contribution between the two pathways in natural infections in vivo is required.

On the other hand, based on our findings, it can be inferred that the most predominant TLR3-bearing cell – CD11c+ myeloid DCs, the primary DENV target in human – might play a critical role during DENV infection; the strong interaction between TLR3 and DENV may heavily drive IRF and NF-κB activation for massive TNF-α, IFN-α and IL-12 p70 release when encounter persistent high viral load in circulation, those cytokines have been demonstrated significantly elevated from DENV-2-infected myeloid DCs (Libraty et al., 2001). Whether the existence of TLR3 during a long-term DENV infection is beneficial or detrimental requires further investigation within an in vivo model.

TLR3 acts crucially in triggering antiviral innate immunity. Despite the capacity of inducing pro-inflammatory cytokines, TLR3-mediated signalling is considered as one of the most essential response pathways to type I IFN production following exposure to viral dsRNA structure (Alexopoulou et al., 2001). Current evidence reveals that, moderate IFN response initiated by TLRs can help host eliminating hepatitis C virus (Lee et al., 2006; Kanda et al., 2007), hepatitis B virus (Isogawa et al., 2005), Coxsackie B3 (Padalko et al., 2004) and HSV-2 (Svensson et al., 2007) infection. In our experiments, DENV-2 recognition-triggered IFN-α and β through TLR3 also fundamentally participate in suppressing viral replication and mitigating the CPE. Interestingly, even if most of the TLRs share similar signalling pathway; however, it is noticeable that TLR7 and TLR8 exhibited feeble effectiveness in IFN-stimulation and the inhibition of viral replication. Different from TLR7 and TLR8, TLR3 pathway is well-known exclusively mediated by TRIF, which can recruit both TBK1/IKKi and RIP1 for IRF3/7 and NF-κB signalling activation in the absence of MyD88 or IRAKs — the essential adaptors for the other TLRs such as TLR7 and TLR8 in triggering IRF7 and NF-κB activation (Akira and Takeda, 2004; Moynagh, 2005; Trinchieri and Sher, 2007). This marked difference is likely to be the cause of diverse IFN induction in DENV-2 infection. A follow-up and further investigation is required for better understanding on this issue and for clarifying the other involvement of suspected host or viral factors.

In conclusion, this study provides the foremost evidence that TLR3 actively participates in the recognition of innate pro-inflammatory response at the early infection DENV stage and leads to the consequent antiviral cytokine/interferons induction in vitro. These findings may offer better understanding on host cell–DENV interaction and the trigger of inflammatory response against of DENV infection.

Experimental procedures

Cells

The human monocytic cells U937 and THP-1 were grown in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 4.2 g l⁻¹ glucose (Sigma) and 1% penicillin and streptomycin (P/S, Gibco). Human embryonic kidney cell line HEK293 and 293T cells were grown in DMEM supplemented with 10% FBS and 1% P/S.

To clarify the involvement of individual TLRs in DENV recognition, HEK293 cells naturally expressing minute levels of TLR1 and TLR6 were employed to clarify the interaction by modelling the heterologous expression of different TLR molecules. The HEK293 clones stably expressing TLR2, TLR4-MD2 or TLR9 (Latz et al., 2002) were kindly provided by Dr Golenbock Douglas (University of Massachusetts Medical School, MA). The TLR3, TLR7 and TLR8-encoded pcDNA3 plasmids constructs also obtained from Dr Douglas were used to construct HEK293 expressing these TLRs. Briefly, plasmids expressing TLR3, 7 and 8 were transduced into HEK293 cells by TransIT LT1 (Mirus Bio) and the trigger of inflammatory response against of DENV infection.

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DENV-2 preparation

DENV-2 NGC strain was propagated in the C6/36 mosquito cell line. Harvested viruses were aliquoted and stored at –80°C until use. The virus titre was determined by BHK cell-based plaque-forming assay as described previously (Kao et al., 2001).

Infection of cells with DENV-2

U937 or THP-1 cell lines were seeded in 48-well plates at 1 x 10^5 cells per well whereas HEK293 cells were seeded 1 day before infection in 24-well plates at 2 x 10^5 cells per well. Cells were infected by DENV-2 NGC with indicated moi and incubated at 37°C for 1.5 h before adding the complete medium. Virus infection was confirmed by indirect immunofluorescence assay using DENV-2-specific mouse monoclonal antibody HB46 (ATCC) as the primary antibody and anti-mouse IgG antibody conjugated with fluorescein (KPL) as the secondary antibody. An inverted fluorescence microscope (Zeiss Axiosvert 200 M) was used to observe the fluorescence.

Chloroquine and bafilomycin A1 pretreatment before infecting THP-1 with DENV-2

To investigate whether or not the process of endosomal acidification is necessary for the recognition of DENV-2 NGC, U937 and THP-1 cells were pretreated with chloroquine (Invivogen) or bafilomycin A1 (Sigma) for 2 h before virus infection. During virus infection and post-infection incubation, either chloroquine or bafilomycin A1 was also adjusted to the indicated concentrations. After 24 h incubation, the culture supernatants from infected cells were collected to measure IL-8 by enzyme-linked immunosorbent assay (ELISA).

Cell viability assay

The cell viability after experimental treatment throughout this study was determined by MTT method. Briefly, MTT reagent (Sigma) was added to the cell culture at the indicated time point post infection to a final concentration at 650 µg ml^-1 in a 96-well plate. The microplate was then incubated at 37°C for 30 min to allow formazan to form. After the incubation, the microplate was read at 570 nm using a microplate reader. The cell viability after experimental treatment throughout this study was determined by MTT method. The absorbance at 570 nm was then measured using a microplate reader. The percentage viability of cells was calculated by the formula: (OD of mock infected cells–OD of infected cells) / OD of mock infected cells) x 100%.

In vitro TLR agonist stimulation of human monocytic cell U937 and THP-1

TLR3 agonist poly(I:C) (Sigma), TLR8/7 agonist CL075 and TLR9 agonist CpG-ODN2006 (Invivogen) were used to compare IL-8 and IL-6 cytokine response profiles of DENV-2 infection and TLR agonist stimulation. After seeding of U937 and THP-1 cells in 24-well plates (1 x 10^5 cells per well), DENV-2 or TLR agonists were added individually into each well. After 24 h incubation, IL-8 and IL-6 release in the culture supernatant was measured by ELISA.

ELISA

The IL-8, IL-6, IFN-α and IFN-β cytokine levels in cell-free culture supernatants were determined by their respective ELISA development kit (Biosource) according to the manufacturer’s instructions. Samples were assayed in duplicate and diluted as necessary to ensure the analyte concentration fell within the reliable range of the standard curve. Reading OD at 450 nm was performed by SpectraMax M5 (Molecular Devices).

TLR3-knockdowned assay

Specific TLR shRNA constructs cloned into plasmid pLKO.1-puro were obtained from The RNAi Consortium (TRC) via National RNAi Core Facility (Academia Sinica, Taiwan ROC). The shRNA-encoding plasmid delivery was conducted by a 3-plasmid lentiviral vector system. Briefly, shRNA plasmid targeting tlr3 mRNA sequence: 5′-CCAGTTGAAAGAACC-GATA-3′ (TRCN0000055685) was mixed with plasmid pCMVΔR8.91 and pMD.G. This mixture was then transduced to the 293T cell lines using TransIT LT1 reagent (Mirus Bio) according to the manufacturer’s instructions. After 16 h incubation at 37°C, the culture medium was refreshed. The supernatant containing lentiviral pseudovirus was collected at 40 and 64 h post transduction. The supernatant was then centrifuged at 150 g for 10 min at 4°C to remove cell debris then immediately filtered through a 0.45 µm filter. The elution was further concentrated by ultra-centrifugation (Beckman L80) using an SW41 rotor at 70 000 g at 4°C for 90 min to harvest the pseudovirus. The virus titres were determined and expressed as transduction units on HEK293 cells (TU ml⁻¹).

For knockdown assay, cells were infected with TLR3-targeting pseudovirus in the presence of 8 µg ml⁻¹ polybrene and centrifuged at 37°C for 30 min. The shRNAs targeting luciferase and lamin A/C served as knockdown-negative control and transduction-positive control respectively. Transduced cells were selected by puromycin at 24 h after transduction. Knockdown efficiency of the target cells was validated by relative quantitative real-time RT-PCR or flow cytometry. The efficiency of TLR3 downregulation was over 90% in our experiments (analysed by real-time PCR).

RNA isolation and RT real-time PCR for TLR3 mRNA

Cells were harvested at the indicated time post infection and lysed with TRIzol reagent (Invitrogen). Total RNA was isolated according to the manufacturer’s instructions. Treatment with DNase I (Invitrogen) was then performed to prevent genomic DNA contamination. The reverse transcription was conducted by Superscript II Reverse Transcription kit, and oligo-dT (Invitrogen) was used as the primer to generate cDNA. Real-time PCR method was performed using Syber Green mix (ABI, Foster City, CA) with Smart Cycler (Cepheid) to quantify gene expression level. The TLR3 mRNA expressing level was determined and normalized with house-keeping gene cyclophilin A. The PCR products were validated by melting curve and agarose gel electrophoresis. The following primers were used: TLR3 forward: 5'ACAACACCTAAGCAGCGTCTGGGA-3'; TLR3 reverse: 5′-ACCTCAAAGGGATCCTCGTCA-3'; Cyclophilin A forward: 5′-TGCACTCAACCACCTG-3'; Cyclophilin A reverse: 5′-CAATGCTGGACCAAACA-3′.

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Flow cytometry

Cells were harvested and washed twice with ice-cold FACS buffer. The cell suspension was adjusted to 1 × 10⁶ cells ml⁻¹ for flow cytometry. The experiment was performed on Beckman Coulter EPICS XL-MCL. The data were analysed by FACS Express software.

Preparation of 5-bromouridine 5'-triphosphate (BrUTP)-labelled dengue virus (BrU-DENV)

Subconfluent C6/36 mosquito cells were infected with DENV-2 NGC strain for 6 days (moi = 0.01). After removing the cell culture supernatant, BrUTP (Sigma) was introduced by using TransIT LT1 transfection reagent. Briefly, TransIT LT1 was diluted to 10% with OPTI-MEM (Invitrogen) for 10 min at room temperature. The BrUTP was added to the diluted TransIT LT1 solution at a final concentration of 10 mM then incubated at room temperature for 20 min. Next, 60 μl BrUTP/TransIT LT1 mixture was added to the C6/36 cell culture flask containing 1.5 ml complete medium. After 1 h of incubation at 28°C, the culture medium was refreshed and incubated for an additional 12 h. The culture supernatant was collected as the BrU-DENV and aliquoted for long-term storage at −80°C.

Confocal microscope

HEK293-TLR cells were seeded on sterile 10 mm coverslips for growing after approximately 50% confluent. The BrUTP-labelled DENV was then added and incubated at 37°C for 30 min. The infected cells were treated with 2 N HCl to expose the epitopes. After fixation with 4% paraformaldehyde for 1 h, the cells were permealized with 0.2% Triton X-100 containing 1% BSA for additional 30 min. Rat anti-BrdU antibody (also reactive to BrU, Novus Bio) and anti-rat IgG antibody conjugated Alexa-568 (Molecular Probes, Invitrogen) were used to detect BrUTP as described previously (El-Hage and Luo, 2003). Mouse anti-GFP (also reactive to YFP) antibody (LTK Biolaboratories, Taiwan) together with rat anti-BrdU antibody (also reactive to BrU, Novus Bio) and anti-rat IgG antibody conjugated Alexa-568 (Molecular Probes, Invitrogen) were used to detect BrUTP as described previously (El-Hage and Luo, 2003). Mouse anti-GFP (also reactive to YFP) antibody (LTK Biolaboratories, Taiwan) together with fluorescein-conjugated secondary antibody (Zymed, San Francisco, CA) were used to detect BrUTP as described previously (El-Hage and Luo, 2003). Mouse anti-GFP (also reactive to YFP) antibody (LTK Biolaboratories, Taiwan) together with fluorescein-conjugated secondary antibody (Zymed, San Francisco, CA) were used to detect BrUTP as described previously (El-Hage and Luo, 2003).

Statistical analysis

An ANOVA test with a one-way classification was used to calculate significant differences between test and control samples in ELISA analyses.

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