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Role of the myeloid differentiation primary response (MYD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) pathways in dengue

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ABSTRACT

Aims: Dengue disease courses with high viremia titers and high cytokine production suggesting viral replication and active immune response that could be related to viral evasion. One of the main targets of dengue virus (DENV) is monocyte/macrophage cells; however, little information regarding viral evasive mechanisms and pathway activation in monocytes infected by DENV is available. The aim of this study was to determine the role of myeloid differentiation primary response (MyD88), TIR-domain-containing adapter- inducing interferon-β (TRIF) and NF-kB pathways in viral replication and cytokine production in human monocyte cultures infected by DENV2.
Main methods: In this regard Pepinh- TRIF, Pepinh- MYD and pyrrolidine dithiocarbamate (PDTC) were used to inhibit TRIF, MYD88 and NF-kB pathways. Cytokine production was measured by ELISA.
Key findings: Increased DENV replication and IFNα/β, TNF-α, IL-12 and IL-18 in infected cultures at 24 h were found. All of these parameters were significantly decreased after TRIF, MYD88 or NF-kB inhibition. Association analysis between viral replication and cytokine production showed high significant positive correlation in TRIF and MYD88 treated cultures.
Significance: This study shows that DENV2 induces activation of innate-immune response and transcription factors to drive viral expression and replication in the face of pro-inflammatory antiviral responses in vitro.

1. Introduction

Dengue virus (DENV) is a single positive-stranded RNA virus of the *flavivirus* genus that encodes three structural and seven non-structural proteins [1]. DENV causes the most prevalent arthropod-borne viral disease of humans [2]. Identifying the targets of DENV infection is crucial for understanding virus spread and disease pathogenesis. Previously, it has been reported DENV replication in macrophages in lymph nodes, spleen, lung, and liver and monocytes in blood from patients with lethal dengue disease [3]. In earlier stages of dengue, most DENV-infected cells in the peripheral blood of acute dengue patients are activated monocytes [4], suggesting that monocyte/macrophages play an important role as targets for early DENV replication. In vitro studies have also shown that dendritic cells generated from monocytes support DENV infection [5]. In addition, in humans, DENV efficiently suppresses the interferon (IFN) response, replicates and causes disease [6]. These findings suggest immune evasion strategies where by DENV impairs the function of infected monocyte/macrophages. There is little information regarding viral evasive mechanisms and activation pathways in circulating monocytes after DENV infection. Activation of myeloid differentiation primary response (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) pathways, lead to the expression of numerous cytokines, such as TNF-α, IL- 1 β, IL-6, IP-10 and IFN-γ, through transcriptional factors (NF-kB, AP-1 and IRF-3) [7], that could lead to host defense or deleterious effects (viral replication). Information about the activation of those pathways in DENV evasion is also limited. Since, dengue disease is characterized by high viremia titers and high production of cytokines [8–10], suggesting viral replication and activation of immune system, the aim of this study was to determine the role of TRIF, MYD88 and NF-kB-dependent pathways on viral replication and production of IFN α/β, TNF-α, IL-12, and IL-18 during early in vitro infection of human monocyte/macrophage cultures by dengue virus type 2 (DENV2).

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2. Materials and methods

2.1. Reagents and medium

Pepinh- TRIF (TRIF inhibitor peptide) and Pepinh- MYD (MYD88 inhibitor peptide), were purchased from InvivoGen (San Diego, CA, USA). Pyrrolidine dithiocarbamate (PDTC: NF-kB inhibitor) was obtained from Sigma (St. Louis, MO, USA). RPMI 1640 culture medium, fetal bovine serum (FBS), penicillin and L-glutamine were purchased from (Invitrogen, Grand Island, USA).

2.2. Preparation of virus stock and virus titration

Dengue virus (DENV2, New Guinea C) was propagated in C6/36 mosquito cells that were cultured in Eagle's MEM medium containing 10% (*v*/v) fetal bovine serum (FBS). The virus culture medium was harvested after 5 days of incubation and after removal of cell debris by centrifugation; the virus supernatant was aliquoted and stored at −70 °C until used. Virus was titrated by plaque formation assays on Vero cells. Cells were placed at 1×10^6 cells/well in 24-well plates and subsequently, serial dilutions of virus were added and the mixtures were incubated at 37 °C for 7 days. Afterwards, the plaques were visualized by staining with a dye solution composed of 1% crystal violet. Virus concentrations are given as plaque forming units (PFU)/ml [11].

2.3. Monocyte cultures

Mononuclear leukocytes were obtained from heparinized venous blood from five DENV seronegative healthy adult male donors. All of them fulfilled the inclusion criteria: absence of dengue virus infection antecedents, not evidence of hepatitis or HIV infection (determined by the absence of antibodies against the viruses), absence of any other current infectious processes and not taken any medications known to influence the inflammatory pathway (antibiotics, anti-allergic, steroids, analgesics) at least in the last 4 weeks. Mononuclear cells were isolated through density gradient centrifugation in Hystopaque 1.077 (Sigma Chemical Co., St Louis, MO, USA). Cells were suspended in RPMI 1640 supplemented with 100 U/ml penicillin, 10 μg/ml streptomycin and 10% FBS and afterwards incubated at 37 °C in a humid atmosphere with 5% CO_2. Cells were incubated for 3 h and adherent cells were enriched by washing away unattached cells twice. After washing, a mean of 3×10^5 adherent cells/well was obtained. Thereafter, monocyte cultures were treated with the studied inhibitors and infected with DENV2. Cell viability was determined by trypan blue exclusion at 24 h of cultures. Cultures from each donor were performed in triplicate. All human studies have been approved by the Ethics Committee of Instituto de Investigaciones Clínicas Dr. Américo Negrette and FONACIT (Caracas, Venezuela) in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from all individuals prior to blood collection.

2.4. Inhibitor treatments and viral infection of monocyte cultures

Six hours previously viral infection, monocyte cultures were treated with Pepinh- TRIF (40 μm/ml), Pepinh- MYD (40 μm/ml), PDTC (100 μm/ml) or combinations of these inhibitors. Doses used in this study were capable of inhibiting the studied pathways as previously demonstrated [12–15]. These values represent final concentrations in monocyte cultures. Cells were incubated in RPMI 1640 supplemented with 100 U/ml penicillin, 10 μg/ml streptomycin and 10% FBS at 37 °C in a humid atmosphere with 5% CO_2 for 6 h. Thereafter, monocyte cultures were infected (multiplicity of infection: MOI = 1) by DENV2 and cultured for additional 24 h. During this time, infected cultures and respective controls were under inhibitor's treatments. Thereafter, culture media were collected to determine cytokine concentration and monocytes were homogenized to determine cellular protein concentration. Media and homogenates were stored at −70 °C until use. Monocytes cultured in supplemented medium without virus or in medium with or without inhibitors were used as negative controls. Viral replication was determined at 6, 12 and 24 h by PFU assay, since DENV replication has been reported to take place as early as 3 h after infection [16]. In addition, DENV2/monocyte interaction induces apoptosis as early as one hour of post-infection [17]. Monocytes were fixed (5 min) with cold acetone or with 2% (w/v) paraformaldehyde to determine viral antigen or monocytes antigen (CD14), respectively. Intracellular viral antigens (viral infectivity) were detected by a direct immunofluorescence assay using a fluorescein-conjugated anti-DENV2 specific monoclonal antibody (Chemicon International Inc. MA, USA). Monocytes were also identified using a fluorescein-conjugated monoclonal antibody anti-CD14 (eBioscience, Inc. San Diego, CA. USA). Positive cells were determined using a fluorescent inverted microscopy (Model 872E, Zeiss, Gottingen, Germany). High percentage of CD14 positive cells (96 ± 3) was observed. In all analyses at least 200 cells per sample were counted.

2.5. Cytokine determinations

Cytokine concentrations were determined in culture media from controls and DENV2 infected cultures at 24 h of post-infection by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's indications. TNF-α ELISA kit was obtained from Thermo Scientific (Rockford, IL, USA). Interferon α and β ELISA kits were obtained from PBL Interferon Source (Piscataway, NJ, USA). IL-12 ELISA kit was obtained from R&D Systems (Minneapolis, MN, USA) and IL-18 from Abcam (Cambrigde, UK). Results were expressed as pg/mg of cellular protein. In this regard, protein content in the monocyte homogenates was measured by Bradford's method using a commercially available kit (Bio—Rad Protein Assay; Hercules, CA, USA).

2.6. Statistical analysis

The GraphPad InStat software package was used for statistical analyses. Data were expressed as mean ± standard deviation and were analyzed by analysis of variance (ANOVA) and Bonferroni Multiple Comparisons Test. Pearson's correlation was used to analysis associations between viral replication and cytokine production. Significance was assumed to be at two tailed $p < 0.05$.

3. Results

Effects of MYD88, TIRF and NF-kB blockers on viral replication and cytokine production.

This study was aimed to determine the role of MYD88, TIRF and NF-kB pathways on the DENV2 replication and cytokine production in human infected monocyte cultures. Treatment of monocyte/macrophage (Mn/MΦ) cultures with studied drugs did not induce cellular toxicity (Mn/MΦ: 98 ± 1; Mn/MΦ + Pepinh- TRIF: 97 ± 0.58; Mn/MΦ + Pepinh- MYD: 97 ± 1.53; Mn/MΦ + PDTC: 96 ± 0.58; Mn/MΦ + Pepinh- TRIF + Pepinh- MYD: 97 ± 1; Mn/MΦ + Pepinh- TRIF + PDTC: 97 ± 1; Mn/MΦ + Pepinh- MYD + PDTC: 98 ± 1; Mn/MΦ + Pepinh- TRIF + Pepinh- MYD + PDTC: 95 ± 0.58 percentage of trypan blue negative cells at 24 h of culture). DENV2 was capable of replication at 24 h of culture. Viral replication was reduced by treatments with Pepinh- TRIF, Pepinh- MYD, PDTC or combinations of these inhibitors (Fig. 1A). PDTC had a lower inhibitory effect on viral replication than those observed in Pepinh- TRIF or Pepinh- MYD; however, viral infectivity (presence of virus into the cells) was decreased by PDTC and PDTC combinations (Fig. 1B). These data suggest activation of MyD88, TIRF and NF-kB pathways during DENV2 replication in monocyte/macrophages and the capacity of NF-kB pathway to modulate cellular viral entry during DENV2 infection.