Infectivity and viability of dengue virus infected hepatocytes cocultured with peripheral blood mononuclear cells from a healthy subject

Sekar Asri Tresnaningtyas,¹ Fithriyah Sjatha,²,³ Beti Ernawati Dewi²,³

ABSTRACT

BACKGROUND Dengue virus (DENV) can infect and replicate in monocytes, resulting in antibody-dependent enhancement. The liver is the main target of DENV, and the infection mechanisms of DENV include direct cytopathic effects (CPEs) of the virus, mitochondrial dysfunction, and effect of cellular and humoral immune factors in the liver. This study was aimed to explore the infectivity of DENV and viability of human hepatocytes using Huh 7it-1 cells cocultured with peripheral blood mononuclear cells (PBMCs).

METHODS Huh 7it-1 cells were infected with dengue virus serotype-2 (DENV-2) New Guinea C strain at multiplicity of infection of 0.5 and 1 FFU/cell, and cocultured in vitro with and without adherent PBMCs. The infectivity of DENV was assessed by immunoperoxidase staining. The viability of Huh 7it-1 cells was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, a tetrazole) assay and trypan blue staining. Data were statistically analyzed by Shapiro–Wilk and analysis of variance for normality significances.

RESULTS The result showed that addition of PBMCs to DENV-2 infected Huh 7it-1 cells decreased the infectivity of DENV (15–37%). DENV-2 infection decreased the viability of Huh 7it-1 cells (15.5–20.8%). Despite the decrease in infectivity of DENV, the addition of PBMCs increased the Huh 7it-1 cells viability (4.5–10.2%).

CONCLUSIONS Addition of PBMCs to Huh 7it-1 cells that are infected with DENV-2 decreased the infectivity of DENV and increased Huh 7it-1 cells viability.

KEYWORDS dengue, hepatocytes, in vitro, liver, monocytes

Dengue virus (DENV) is a mosquito-borne virus transmitted by vectors Aedes aegypti and A. albopictus. DENV infection is widespread in all tropical and subtropical regions.¹ World Health Organization estimated that the incidence of DENV infection reaches 50–100 million cases in the world every year, and the incidence of DENV infection has increased 30-fold over the last 50 years.² Based on the prevalence of dengue studies, 3.9 billion people in 128 countries in the world have a risk of being infected with DENV.³

Based on an in vivo study, it was observed that DENV can infect monocytes,⁴ platelets,⁵ endothelial cells,⁶ hepatocytes,⁷ and Kupffer cells.⁸ Monocytes and macrophages are thought to be cells that can support DENV replication and lead to antibody-dependent enhancement hypothesis, which explains the risk of severity of DENV infection caused by a secondary infection with heterologous serotypes.⁹ In an in vitro study, it was seen that DENV can infect various hepatocyte strains, including Huh7 cells,
primary liver carcinoma (PLC), Hep3B, Chang liver cells, and HA22T. Some mechanisms involved in the damage of hepatocytes during DENV infection are direct and indirect cytopathic effects (CPEs). The direct CPE is a damage due to viral infection, while indirect CPE is due to hypoxia and the influence of cellular and humoral immune factors in the liver. The pathogenesis of DENV related to liver cell damage is still not clearly known. To determine the role of DENV and monocytes in liver cell damage, we studied DENV infection in Huh 7it-1 hepatocyte cell lines cultured in vitro with peripheral blood mononuclear cells (PBMCs) and assessed the infectivity and cell viability.

METHODS

This research was conducted from February to December 2018 in the laboratory of virology and molecular biology, Department of Microbiology, Faculty of Medicine, Universitas Indonesia. This study used PBMCs from a healthy subject, Huh 7it-1 liver cancer cells (obtained from Dr. Chie Aoki at Kobe University), and dengue virus serotype-2 New Guinea C (DENV-2 NGC) strain from the collections of the laboratory of virology and molecular biology, Department of Microbiology, Faculty of Medicine, Universitas Indonesia. This research has ethical permission from the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (No. 248/UN2.F1/ETIK/2018). In this study, we arranged 12 treatment groups as shown in Table 1.

This study started with infecting Huh 7it-1 cells using DENV-2 NGC strain at multiplicity of infection (MOI) of 0.5 and 1 focus-forming units (FFU)/cell (Table 1). After 2 hours of incubation, the treatment was continued with in vitro coculturing with and without adherent PBMCs for 72 hours. The infectivity of DENV was assessed through immunoperoxidase staining. The viability of Huh 7it-1 cells was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, a tetrazole) assay and trypan blue staining. The workflow diagram is shown in Figure 1.

Propagation of Huh 7it-1 cells

The frozen Huh 7it-1 cell stock in the cryotube was thawed and quickly resuspended with Dulbecco’s modified Eagle medium (DMEM) (Gibco, USA), and then centrifuged at 1,200 rpm for 4 min. The pellet was resuspended with 5 ml DMEM 10% FBS (fetal bovine serum) (Gibco) and replaced to a T25 flask followed by incubation at 37°C with 5% CO₂. The medium was replaced daily until a monolayer had formed on the flask surface (observed with an inverted microscope). Cell passaging was carried out by removing the medium in the flask and washing it with 2 ml phosphate buffered saline (PBS) 1×, followed by adding 500 µl trypsin 0.25 ethylenediaminetetraacetic acid (Gibco). The flask was then incubated at 37°C with 5% CO₂ for 3–5 min until the cells were detached from the surface of the flask (observed with an inverted microscope). Next, the flask was filled with 4 ml of DMEM without

![Figure 1. Workflow diagram. DENV-2=dengue virus serotype-2; PBMCs=peripheral blood mononuclear cells; MOI=multiplicity of infection; FFU=focus-forming units; UV=ultraviolet.](image-url)

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**Table 1.** The 12 treatment groups of this study

<table>
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<tr>
<th>Treatment group*</th>
<th>DENV-2</th>
<th>PBMCs</th>
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<tr>
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<tr>
<td>II</td>
<td>MOI 0.5 FFU/cell</td>
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<tr>
<td>III</td>
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<td>IV</td>
<td>MOI 1 FFU/cell</td>
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<td>√</td>
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<tr>
<td>VI</td>
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<tr>
<td>VIII</td>
<td>Heat-inactivated DENV-2</td>
<td>-</td>
</tr>
<tr>
<td>IX</td>
<td>Supernatant of Huh 7it-1 cell culture</td>
<td>√</td>
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DENV-2=dengue virus serotype-2; PBMCs=peripheral blood mononuclear cells; MOI=multiplicity of infection; FFU=focus-forming units; UV=ultraviolet

*All treatment groups were cultured in Huh 7it-1 cell
FBS, resuspended and transferred into a tube, and centrifuged at a speed of 1,200 rpm for 4 min. Pellets were resuspended in the DMEM 10% FBS and then kept in several flasks. These flasks were then incubated at 37°C with 5% CO₂ until confluence.

**Propagation of DENV-2 NGC strain**

DENV-2 NGC strains were propagated in Vero cells in a T75 flask. Medium of the monolayer Vero cells was infected with 500–1,000 µl of DENV-2 NGC strains. After 2 hours of incubation, 10 ml MEM 2% FBS was added to the flask and incubated at 37°C with 5% CO₂. The harvesting of the supernatant (which contains the DENV-2) was carried out on the 5–6th day when CPE appeared. Virus titers were measured by immunoperoxidase staining and stored in a 1 ml tube at −80°C until used.

**Isolation of PBMCs**

In this study, we used 20 ml of whole blood from a healthy subject who was vaccinated with Japanese encephalitis virus vaccine. Serum from whole blood was tested for IgG, IgM, and dengue NS1. The isolation of PBMCs from whole blood were done according to the previous study.¹¹ Five milliliters of whole blood was suspended with 2 ml PBS in a tube and transferred slowly through the tube wall into a falcon tube, which contained 5 ml of Ficoll-Paque (GE Healthcare, USA). Then, it was centrifuged at 2,500 rpm for 30 min with swing rotor and brake off. The PBMC rings were collected in a tube and washed with Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, USA) without FBS, and then centrifuged at 1,200 rpm for 5 min.

Adherent PBMCs were isolated as described in the previous study.¹² The pellets were resuspended in 5 ml RPMI 10% FBS with 1% Penicillin-Streptomycin (Gibco). The suspension was placed on a T25 flask and incubated at 37°C with 5% CO₂ for 2 hours. Non-adherent PBMCs that were not attached to the flask were removed, and the flask was washed twice with RPMI medium without FBS. The cells attached in the flask were pipetted to obtain adherent PBMCs. The suspension contained adherent PBMCs centrifuged at a speed of 1,200 rpm for 5 min. Pellets were resuspended in RPMI 10% FBS. The calculation of the number of adherent PBMCs was undertaken using hemocytometer, with the cell concentration of 1 × 10⁶ cells/ml.

**Infecting Huh 7it-1 cells with DENV2**

Huh 7it-1 cells were grown on 96 microwell plates with a concentration of 5 × 10⁶ cells/ml. The microwell plates were incubated for 24 hours at 37°C with 5% CO₂ until a monolayer was formed, then the medium was discarded from the well. Cells on the microwell plates were infected with 50 µl DENV-2 at various titers according to the treatments including UV- and heat-inactivated DENV-2, supernatant cell, and medium as negative control. The microwell plates were incubated for 2 hours at 37°C with 5% CO₂ and agitated every 30 min.

**Coculturing of DENV-2-infected Huh 7it-1 cells with PBMCs**

After 2 hours of incubation, 50 µl of adherent PBMCs with a concentration of 1 × 10⁶ cells/ml was added into each well, meanwhile, RPMI 1640 medium (Sigma) was added in the control treatment. The microwell plates were then incubated at 37°C with 5% CO₂ for 72 hours.

**Observation of cell viability with trypan blue staining**

Cell viability was observed through trypan blue staining by adding 50 µl/well of trypan blue dye. Cells on the microwell plates were observed using an inverted microscope with 100× magnification. The colored cells were dead cells and transparent cells were living cells.

**Calculation of cell viability using MTT assay**

Cell viability was also observed using MTT assay by adding 150 µl of 10% MTT solution into each well in microwell plates. Microwell plates were then incubated at 37°C with 5% CO₂ for 3 hours. The solution in microwell plates was then discarded, and 100 µl of 100% dimethyl sulfoxide was added to every well. MTT result was read using an enzyme-linked immunosorbent assay (ELISA) reader at a wavelength of 490 nm. In this study, the viability of Huh 7it-1 cells infected with DENV-2 in various treatments after 72 hours of incubation was assessed using MTT assay and trypan blue staining. MTT assay result was obtained in terms of absorbance value/ optical density (OD). The percentage of cell viability was obtained by comparing the OD of each treatment with that of the negative control treatment (Huh 7it-1 cells which treated with the addition of Huh 7it-1 cell medium).
Determination of DENV-infected Huh 7it-1 cells

The number of DENV-infected cells was measured by immunostaining assay according to previous study with a slight modification by replacing the treatment using methyl cellulose with Huh 7it-1 cells for 72 hours. The solution in the microwell plate was discarded after 72 hours of treatment. Cells were fixed by adding 200 µl of formalin 3.7% in PBS 1× into each well, which were then incubated for 15 min at room temperature and washed three times using PBS 1× with each washing followed by 5 min of incubation. Next, 100 µl of Triton X 0.5% (Sigma) in PBS was added into each well in the microwell plate, incubated for 10 min at room temperature, and washed with 200 µl PBS 1× three times without incubation. Cells were added with 50 µl of primary antibodies (human IgG anti-DENV) in skim milk blocking buffer of 1/500 dilution, continued with incubation for 1 hour at room temperature. After 1 hour of incubation, microwell plates were washed with 200 µl PBS 1×. Washing was done three times, with each washing followed by 5 min of incubation. The process was continued by adding 50 µl of secondary antibody (Goat Anti-Human IgG-labeled HRP [Invitrogen, USA]) in skim milk blocking buffer (Sigma) 1/500 dilution into each well and washed as previously done. Then, 100 µl of 3,3'-diaminobenzidine substrate 1× was added, incubated for 15 min at room temperature, and washed as previously done. Furthermore, stained cells were observed with an inverted microscope and calculated to determine the number/percentage of the infected cells.

Statistical analysis

Data were analyzed using SPSS version 23 (IBM Corp., USA). Normality of the data was analyzed with Shapiro–Wilk test. The significance of differences of each treatment was analyzed by analysis of variance compared with control treatment. The differences were considered significant when the p-value <0.05.

RESULTS

Effect of DENV-2 infection and coculturing with PBMCs on infectivity

The results showed that Huh 7it-1 cells which were treated with DENV-2 at MOI of 1 FFU/cell (95%) was greater than the number of cells infected with DENV-2 at an MOI of 0.5 FFU/cell (57%) (Figure 2, a and b). Whereas there was no infection had occurred in control treatments, such as Huh 7it-1 cells, which were treated with supernatant of Huh 7it-1 cells, which were treated with supernatant of Huh 7it-1 cell culture (Figure 2c), UV-inactivated DENV-2 (Figure 2d), heat-inactivated DENV-2 (Figure 2e), and Huh 7it-1 cell medium (Figure 2f). It was observed that no cells were stained (brown color). However, the infectivity of Huh 7it-1 cells in the treatment group that was treated with cocultured PBMCs showed different results compared to that of the group without PBMCs. The number of infected cells from DENV-2 infection at an MOI of 1 FFU/cell without PBMCs showed different results compared to those treated without PBMCs. The number of infected cells from DENV-2 infection at an MOI of 0.5 FFU/cell; number of infected cells were 57% (without PBMCs) and 20% (cocultured with PBMCs) (Figure 2, a and h).

Effect of DENV-2 infection and coculturing with PBMCs on cell viability

The percentage of viability of Huh 7it-1 cells treated with UV-inactivated DENV-2, heat-inactivated DENV-2, and Huh 7it-1 cell medium were significantly higher (p = 0.001) compared to that of Huh 7it-1 cells that were infected with DENV-2 at MOI of 0.5 and 1 FFU/cell and supernatant of Huh 7it-1 cell culture as shown in Table 2. The result also showed that the viability percentage of Huh 7it-1 cells that were infected with DENV-2 at an MOI of 0.5 FFU/cell was significantly higher (p = 0.014) than those infected with DENV-2 at an MOI of 1 FFU/cell. The cell viability in treatment groups of Huh 7it-1 cells cocultured with PBMCs had the same results. The viability percentage of Huh 7it-1 cells treated with UV-inactivated DENV-2, heat-inactivated DENV-2, and Huh 7it-1 cell medium were significantly higher (p<0.001) compared to that of Huh 7it-1 cell infected with DENV-2 at MOI of 0.5 and 1 FFU/cell and supernatant of Huh 7it-1 cell culture.

The viability percentage of Huh 7it-1 cells infected with DENV-2 at an MOI of 0.5 FFU/cell cocultured with PBMCs was not significantly higher (p = 0.68) compared to those treated without PBMCs. A different result was found in the treatment of Huh 7it-1 cells infected with DENV-2 at an MOI of 1 FFU/cell. In this case, the viability percentage of Huh 7it-1 cells cocultured with PBMCs was significantly different (p<0.001) compared to those treated without PBMCs. In the treatment with and without cocultured PBMCs, the viability percentage of Huh 7it-1 cells which were...
not infected with DENV-2 was significantly higher (p < 0.001–0.03) than the treatments which involved cells infected with DENV-2.

The cell viability result from trypan blue staining showed that the number of dead cells after the treatment of Huh 7it-1 cells were infected with DENV-2 at an MOI of 0.5 (Figure 3a) and 1 FFU/cell (Figure 3b) was higher compared to when Huh 7it-1 cells with supernatant of Huh 7it-1 cell culture (Figure 3c), UV-inactivated DENV-2 (Figure 3d), heat-inactivated DENV-2 (Figure 3e), and Huh 7it-1 cell medium (Figure 3f). The difference between the staining results of Huh 7it-1 cells when cocultured with PBMCs and without PBMCs was not clearly visible.
The severity of certain DENV infections can cause interference with the liver, such as hepatomegaly, and even lead to liver dysfunction. In severe DENV infection cases, liver injury due to hepatocyte apoptosis can be observed.¹⁴ In this study, we have used Huh 7it-1 line cells, which are human hepatocyte line cells, to resemble the actual infection state. The results showed there were differences between morphology of Huh 7it-1 cells infected with DENV-2 and those not infected with DENV-2. DENV is known to infect and replicate in Huh 7it-1 cells; therefore these cells are often used as an in vitro model for the DENV research.⁹ The effect on the infectivity of DENV-2-infected cells cocultured with PBMCs were assessed using immunostaining assay after 72 hours of incubation. The infected cells appeared brown as shown in Figure 2. In this study, we also found that DENV-2 infections with higher MOI values had increased infectivity compared to DENV-2 infections with lower MOI values. Hence, it was proved that high titers could infect more Huh 7it-1 cells than low DENV-2 titers. DENV can infect hepatocytes including Huh 7it-1 cells, by attaching the protein E virion to receptors on host cells, such as heparan sulfate,¹⁵ glucose-regulated protein 78,¹⁶ and laminin, which are commonly found in the majority of liver cell lines.¹⁷ The pre-membrane structural protein in DENV-2 can bind to the claudin-1 receptor in Huh 7it-1 cells. Thus, DENV-2 can attach to the claudin-1 receptor and facilitate the entry of the virus into the cell. DENV-2 can also infect other cell lines, such as PLC, Hep3B, Chang liver cells, and HA22T. Experiments by Lin et al⁹ showed that Huh7 cells, PLC, Hep3B, and Chang liver cells have higher replication rates, virion production levels, and aspartate aminotransferase levels.

PBMCs are part of blood cells consisting of lymphocytes (T cells, B cells, and natural killer cells), monocytes, and dendritic cells. PBMCs can secrete cytokines. We have used adherent PBMCs, which attached to the surface of the flask when incubated for approximately 2 hours. PBMCs that attached to the
plate are known as monocytes. In a previous study, it was also stated that adherent PBMCs were able to reduce the transendothelial electrical resistance in endothelial cells, where it was suspected that adherent PBMCs were able to secrete more cytokines compared to non-adherent PBMCs. In addition, adherent PBMCs are also known as cells that support the replication of DENV in vivo. Cellular receptors reported to be used by DENV to enter cells are dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin, which is owned by monocytes derived from dendritic cells, and Fc receptors in cases of secondary infection.

In this study, the result of trypan blue staining showed that dead cells are colored by trypan blue or appear to be blue (shown by the red arrow in Figure 3), while living cells are not stained or appear to be transparent. It was also observed that the addition of PBMCs to Huh 7it-1 cells infected with DENV-2 resulted in decreased virus infectivity. This decrease in infectivity was caused by the mechanism of protection by interferons (IFNs), which are produced by monocytes in PBMCs. DENV can infect and replicate in monocytes. One of the host cell defense mechanisms against viral infections is IFN production. The results of research conducted by Kurane et al showed that IFN activity was detected in monocytes 12 hours after DENV infection and it reached the highest levels in 72 hours. Monocytes are thought to regulate T- and B-cell responses specific to DENV through antigen presentation and interleukin (IL)-1 production. In addition, monocytes also produce and induce IFN.

This study showed that DENV-2 infection caused a decrease in Huh 7it-1 cell viability, which may be due to the occurrence of apoptosis in Huh 7it-1 cells. In a study conducted by Nasirudeen et al, it was stated that DENV infection in baby hamster kidney, Huh 7it-1, and Vero cells can induce apoptosis. DENV infection in Huh 7it-1 cells showed cell death activity through the mitochondrial pathway, which resulted in low mitochondrial transmembrane potential in Huh 7it-1 cells. Increased p53 expression was also found in Huh 7it-1 cells infected with DENV.

Our results showed that DENV-2 infection increased the viability of Huh 7it-1 cells; however, the addition of PBMCs although decreased infectivity, still increased the cell damage. This increase in cell damage is due to the condition that PBMCs infected with DENV can produce cytokines. DENV can also infect monocyte-derived macrophage cells and induce cytokines. In a study conducted by Chen et al regarding pathogen recognition receptors toll-like receptor (TLR) 2 and TLR6, the results showed that DENV-infected PBMCs upregulates TLR2 and TLR6. The results of ELISA measurements of IL-6 and tumor necrosis factor (TNF)-α in PBMCs infected with DENV showed an increase in cytokines, and these cytokines were produced through TLR 2 and TLR6 signaling pathways.

The addition of PBMCs to DENV-infected Huh 7it-1 cells decreased infectivity of DENV. It may be due to protection by IFNs, which are produced by monocytes. The addition of PBMCs increased Huh 7it-1 cells viability. It may be due to the increase in IL-6 and TNF-α production by DENV-infected PBMCs as in the previous study. This study has some potential limitations, such as we could only include one variety of viruses (DENV) and only two varieties of MOI. The result of this study can contribute in knowledge of dengue pathogenesis related to the role of monocytes in liver damage. In conclusion, the addition of PBMCs to Huh 7it-1 cells infected with DENV-2 decreased the infectivity of DENV and increased Huh 7it-1 cells viability.

Conflict of Interest
The authors affirm no conflict of interest in this study.

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