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Levels of CXCL10 Chemokine in Dengue Infected Hepatocyte Huh 7 it-1 Cell Line Co-cultured with Peripheral Blood Mononuclear Cells

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Dengue is a *mosquito borne virus* that spreads rapidly in the world. At present, it is estimated that more than 3.9 billion people are at risk of being infected with dengue virus (DENV) and there are 96 million clinical cases that have been reported annually in 128 countries worldwide. In DENV infected patients often associated with liver dysfunction which hepatocyte and kuppfer cells as the main target of viral infections. DENV infection induced the expression of several chemokines, which might play an important role during the inflammatory response and pathogenesis of a disease. CXCL10 is known as a chemokine that activated lymphocytes for innate and adaptive immunity, induces tissue damage, and modulates tumor formation. Therefore, we conducted an in vitro study using Huh 7it-1 cells co-cultured with Peripheral Blood Mononuclear Cells (PBMCs) to investigate CXCL10 chemokine induction during DENV infection. Huh 7it-1 cells were grown on 96 micro well plate until a monolayer was formed. The cells were infected with DENV-2 MOI 0.5 FFU/cell and 1 FFU/cell in the presence of PBMCs. Huh7 cell medium were used as negative control. After 2 hours of infection, cells were co-cultured with PBMCs and incubated at 37 ºC with 5% CO_2 for 48 hours. Cell supernatant was collected and CXCL10 chemokine levels were measured using CXCL10 Quantikine ELISA Kit. Statistical analysis was performed by SPSS 23. In the presence of PBMCs, CXCL10 levels from Huh 7it-1 infected by DENV-2 MOI 0,5 FFU/cell and 1 FFU/cell were 552,653 ± 22,779 pg mL^{-1} and 576,787 ± 16,901 pg mL^{-1}, those levels were higher than negative control. As conclusion, DENV-2 infected Huh 7it-1 cells were able to induce the secretion of CXCL10 from PBMC.

Key words: CXCL10 chemokine, Dengue, Huh 7it-1

Dengue merupakan *mosquitos borne virus* yang paling cepat menyebar di dunia. Diperkirakan saat ini lebih dari 3,9 miliar orang berisiko terinfeksi DENV, dengan 96 juta kasus klinis yang dilaporkan pertahun di 128 negara di seluruh dunia. Disfungsi hati sering dikaitkan pada pasien terinfeksi DENV dengan sel hepatosit dan kuppfer sebagai target utama infeksi virus. Infeksi DENV diketahui dapat menginduksi ekspresi beberapa kemokin, yang mungkin memainkan peran penting selama respon inflamasi dan patogenesis suatu penyakit. CXCL10 diketahui sebagai kemokin yang mengaktifkan limfosit untuk kekebalan bawaan dan adaptif, menginduksi kerusakan jaringan, dan memodulasi pembentukan tumor. Oleh karena itu, kami melakukan penelitian in vitro menggunakan sel Huh 7it-1 yang diko-kultur dengan PBMC untuk mengetahui profil kemokin CXCL10 selama infeksi DENV. Sel Huh 7it-1 ditumbuhkan pada 96 *micro well plate* hingga monolayer. Sel kemudian diinfeksikan DENV-2 dengan MOI 0,5 dan 1 FFU/sel dan diko-kutur bersama PBMC. Medium sel kultur Huh7 digunakan sebagai kontrol negatif. Setelah 2 jam infeksi, sel diko-kultur dengan PBMC dan diinkubasi pada suhu 37 ºC dengan CO2 5% selama 48 jam. Supernatan sel kemudian dipanen dan kadar kemokin CXCL10 diukur menggunakan Kit ELISA Quantikine CXCL10. Analisis statistik dilakukan dengan SPSS 23. Pada sel yang di ko-kultur dengan PBMC, kadar CXCL10 dari Huh 7it-1 yang diinfeksi dengan DENV-2 MOI 0,5 FFU/sel dan 1 FFU/sel adalah 552.653 ± 22.779 pg mL^{-1} dan 576.787 ± 16.901 pg mL^{-1}, kadar tersebut lebih tinggi daripada kontrol negatif. Sebagai kesimpulan, Sel Huh 7it-1 yang diinfeksi dengan DENV-2 mampu menginduksi sekresi CXCL10 dari PBMC.

Kata kunci: Dengue, Huh 7it-1, Kemokin CXCL10

Dengue is a *mosquito borne virus* that spreads rapidly in the world. This virus transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes and causes of febrile illnes in the tropics and subtropics region. At present, it is estimated that more than 3.9 billion people are at risk of being infected with DENV and there are 96 million clinical cases that have been reported annually in 128 countries worldwide (Gyawali & Taylor-Robinson 2017). Furthermore, DENV infection is known to cause dengue hemorrhagic fever (DHF) with a mortality rate of 0.5% -3.5% in Asia. In Indonesia, DENV infections cause outbreak cases every year, with the largest cases occurring in 1998 and

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2004 with 79,480 sufferers and over 800 deaths. In the following years the number of cases by DENV infection was known to continued to increase and categorized as an epidemic disease with an increasing area (Candra 2010).

DENV is an RNA virus belongs to the *Flaviviridae* family and consist of four serotypes, ie DENV-1, DENV-2, DENV-3, and DENV-4 (Whitehorn 2012). All of serotypes cause a wide spectrum of clinical presentation, ranging from asymptomatic infections, Dengue Fever (DD), Dengue Hemorrhagic Fever (DHF) or Dengue Syock Syndrome (DSS). DHF and DSS are severe clinical forms due to DENV infection which can cause bleeding, vascular leakage, shock and organ damage, including the liver (Dhanoa *et al.* 2016, Shah and Rathi 2017).

DENV infection in human are often associated with liver dysfunction with hepatocyte and Kuppfer cells as the main target of viral infections (Samanta 2015). In addition, a number of in vitro studies have shown that DENV is able to infect human hepatoma cell lines such as HA22T, Hep3B, PLC, Chang liver cells, HLCs, Huh7, and HepG2 (Matsuda *et al.* 2005, Lin *et al*. 2000). *In vivo* studies, introducing DENV-2 intravenously into BALB/c mice producing virus particles in the liver and showing clinical signs that are almost similar to those seen in infected humans (Sakinah *et al.* 2017). Overall, these findings support the idea that hepatocytes are likely target cells for DENV.

The pathogenesis of liver cell damage due to DENV infection cannot yet be explained with certainty and many different mechanisms are involved, especially the direct cytopathic effect of the virus and immune response to virus infection (Trung *et al.* 2010). One reason is the excessive production of cytokines and chemokines in infections (Rathakrishnan *et al.* 2012). Chemokine is a small protein (8-10 kDa) that is commonly secreted during a viral infection. The main function of chemokine is for recruitment of leukocytes, which are important for leukocyte homeostasis and mediate immune and inflammatory responses. In addition, the different chemokine expressions in various inflammatory and infectious diseases indicate that they play an important role during the inflammatory response and pathogenesis of a disease. According to this idea, many viral infections are known to induce chemokine expression, which is most likely involved in regulating the recruitment of effector leukocytes to the site of infection. DENV infection is known to induce the expression of several chemokines, including CXCL1/IL-8, CCL3/MIP-1, CCL5/RANTES, and CCL4/MIP-1 in various human cell lines, but until now, not much is known what chemokines may play a role in DENV infection (Chen *et al.* 2006).

CXCL10 levels was increased in dengue patients with warning sign compared by dengue fever patients (P=0,046) (Masood *et al.* 2018). Other than that CXCL10 can be secreted from leukocytes, eosinophils, neutrophils, monocytes, endhotelial, epithelial, stroma cells and keratinocytes in response to INF-γ. CXCL10 is known to be able to induces chemotaxis, apoptosis, cell growth inhibition and angiostasis. Abnormal levels of CXCL10 have been observed in body fluids of individuals infected with viruses such as ebola, hepatitis B and C, dengue, HIV, and several other viruses. It was also found to be increasing in bacterial, fungal and parasitic infections, which showed that CXCL10 played an important role in the pathogenesis of these diseases(Liu *et al.* 2011).

Therefore, we conducted an in vitro study using hepatocyte cell line, Huh 7it-1 cells co-cultured with PBMCs to investigate CXCL10 chemokine induction during DENV infection, because CXCL10 is the most prominent chemokine and may be induced quickly after DENV infection (Chen *et al.* 2006). Hence, these study results can be used as a reference for further research to explore the association of CXCL10 in causing liver cell damage.

MATERIALS AND METHODS

This research was conducted in Laboratory of Virology and Molecular Biology, Department of Microbiology, Faculty of Medicine, Universitas Indonesia from January to July 2019. In this study we used hepatocyte Huh 7it-1 cell line, Vero cell, and Peripheral Blood Mononuclear Cells (PBMCs) from healthy control with ethical permission from the FKUI RSCM Health Research Ethics Committee, with ethical number: KET-472/UN2.F1/ETIK/PPM.00.02/2019.

Treatment Group. There are 6 treatment groups in this study, which are summarized in Table 1.

Propagation of Huh 7it-1 Cells. In this study, we used hepatocyte cells of Huh 7it-1 cells from cryopreservation Huh 7it-1 cell from Virology and Molecular Biology, Department of Microbiology, Faculty of Medicine, Universitas Indonesia's collection, then resuspended with DMEM medium (Gibco) and centrifuged at 1200 rpm for 4 minutes at 4 ºC. The

supernatan was discarded and pellets resuspended with 5 ml DMEM 10% FBS medium, transferred into the T25 flask and incubated at 37 ºC with 5% CO_2 until the monolayer cell was formed. To propagate Huh7 cells, passages are performed according to previous study and incubated in an incubator 37 ºC with 5% CO_2 (Tresnaningtyas *et al.* 2018).

Propagation of DENV-2 Strains New Guinea C. DENV-2 strain New Guinea C was provided from Virology and Molecular Biology, Department of Microbiology, Faculty of Medicine, Universitas Indonesia's collection and propagated in Vero cells on T75 flask based on previous study (Tresnaningtyas *et al*. 2018). For DENV-2 NGC propagation, MEM 2% FBS medium was used, and incubation was carried out at 37 ºC with 5% CO_2. Cells observed every day to see the cytopathic effects (CPE). DENV-2 was harvested on 5-7th days of incubation or after CPE were seen. DENV-2 NGC titers were measured by focus assay method, then stored at -80 ºC until used for experiment.

Isolation of PBMCs. PBMCs was obtained from the blood of healthy control who have been vaccinated with the Japanese Encephalitis Virus (JEV) vaccine (Dewi *et al.* 2008). Ten mL of healthy control's blood was tested with Dengue Ag+Ab Duo (SD Biosensor) rapid test to confirm that the subjects were negative anti-DENV IgG, anti-DENV IgM, and NS1 DENV antigen. PBMCs isolation process was carried out by the ficoll gradient centrifugation method according to previous study (Tresnaningtyas *et al.* 2018, Dewi *et al.* 2008). The number of adherent PBMC cells was counted by haemocytometer, then resuspended in RPMI 1640 (Sigma) with 10% FBS and 1% penstrep (Gibco) to prepare the cells with a concentration of 1 x 10^6 cells mL^{-1}.

Infection of DENV-2 NGC to Huh 7it-1 Cells. Huh 7it-1 cells with a concentration of 5 x 10^5 cells mL^{-1} were grown on 96 microwell plates by incubating at 37 °C with 5% CO_2 for 24 hours until the monolayer cells formed. The medium in 96 microwell plates was removed when the cells are monolayer and the cells were infected with 50 µl DENV-2 NGC at various titers according to the treatment. Infected cells were incubated at 37 ºC and 5% CO_2 for 2 hours with agitation every 30 minutes. In this study, we used culture cell medium as negative controls.

Co-cultured PBMCs on Huh 7it-1 Cells Infected with DENV-2. Fifty µL of adherent PBMCs with a concentration of 1 x 10^6 cells mL^{-1} and 50 µL RPMI 1640 medium was added to each well according to the treatment group. Plates were then incubated for 48 h at 37 ºC with 5% CO_2. After incubation, the supernatant in each well was collected and stored at -80 ºC until used for the measurement of chemokine.

Measurement of CXCL10 Chemokine Levels. CXCL10 chemokine levels in supernatan DENV infected Huh 7it-1 co-cultured with PBMCs were determined using Human CXCL10 Quantikine ELISA Kit (R&D System, United States). CXCL10 measurement were carried out according to the assay procedure from Human CXCL10 Quantikine ELISA Kit (R&D System, United States). Absorbance values of each well were analyzed by ELISA reader with a wavelength of 450 nm (ELISA 2018).

Statistical Analysis. Data were analyzed using the Statistical Product and Service Solutions (SPSS) 23. Normality of the chemokine levels data were analyzed by Shapiro-wilk normality analysis and for the significance were analyzed by ANOVA.

RESULTS

Levels of CXCL10 Chemokine. Determination of CXCL10 chemokine levels were measured by the CXCL10 ELISA kit (R&D System, United States). The absorbance results of each treatment were then converted into chemokine levels by converting OD values into standard curves that have been made previously. DENV-2 infected Huh 7it-1 cells without PBMCs secreted CXCL10 in lowest levels and known to have no significant differences according to statistical analysis ($P>0.05$). These results are shown in the blue graph in Figure 1. On the contrary, PBMCs co-cultured with DENV-2 NGC infected Huh 7it-1 cells was secreted CXCL10 chemokine in higher levels. The CXCL10 levels produced by Huh 7it-1 cells infected by DENV-2 NGC MOI 0.5 and 1 FFU/cell were 552,653 ± 22,779 pg/ml and 576,787 ± 16,901 pg/ml respectively. These results indicated that CXCL10 levels produced by Huh 7it-1 cells infected by DENV-2 MOI 0.5 FFU/cell and 1 FFU/cell was higher than negative control ($P<0.05$).

DISCUSSION

DENV is known to infect cells in several important organs such as bone marrow, spleen, lymph nodes, central nervous system and liver (Martina *et al.* 2009). Liver is the most common organ involved in severe DENV infection. The ability of DENV to infect liver cells is due to the attachment of viruses to heparan sulfate receptors (HS) on the cell surface. HS has been

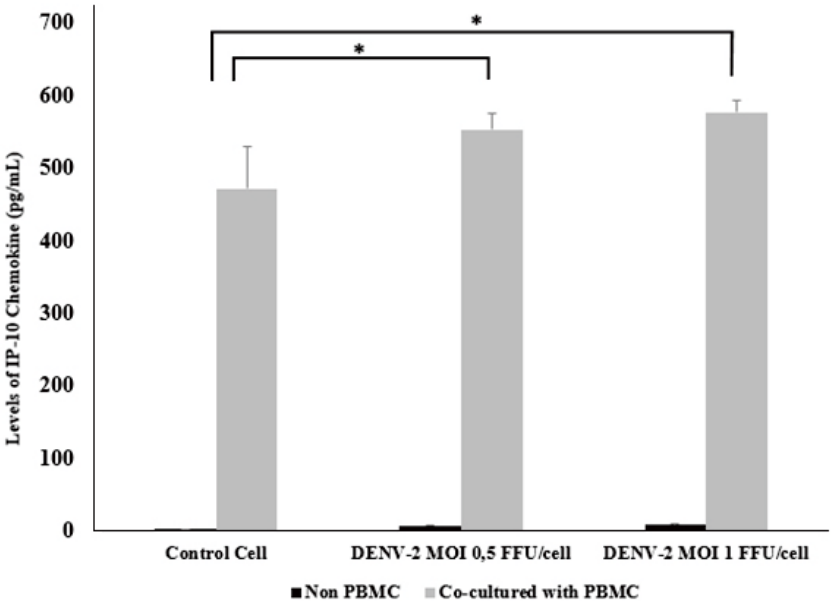

Fig 1 Levels of CXCL10 chemokine.*P value <0.05.

known to be a receptor for entry of all DENV strains (Seneviratne *et al.* 2006). In addition, there are also other receptors such as GRP78, DC-SIGN, and laminin receptors (Pando-Robles *et al.* 2014). The ability of DENV to infect liver cells in humans in this study was demonstrated through in vitro experiments using Huh 7it-1 cells. These cells are hepatocyte cell lines originating from human liver tumors, which are expected to be able to resemble the real situation.

In addition, in this study there were also cell groups that were treated with PBMCs co-culture. PBMCs is a white blood cell consisting of B lymphocyte cells, T lymphocytes, natural killer cells (NK), and monocyte cells. These cells play an important role in humoral and cellular immunity in the human immune system (Warnasih *et al.* 2016). The presence of monocytes and macrophages in DENV infection is known to excrete dissolved mediators such as chemokines and cytokines that can influence DENV infection towards disease severity (Peiris *et al.* 1981). The existence of PBMCs in this research is expected to be able to provide an overview of the CXCL10 chemokine profile of Huh 7it-1 cells which infected with DENV and co-cultured with PBMCs.

CXCL10, also called interferon γ-induced protein or IP-10, is a 10 kDa-sized protein that belongs to the chemokine family. CXCL10 specifically activates CXCR3 receptors which are predominantly expressed by T lymphocytes, B lymphocytes, NK cells, dendritic cells, and activated macrophage cells. CXCL10 is a pleiotropic molecule that has strong biological functions, including supporting the chemotactic activity of CXCR3 cells, inducing apoptosis, regulating cell growth and ploriferation, and angiogenesis in infectious diseases, as well as inflammation and cancer. The strong CXCL10 chemotactic activity of activated lymphocytes modulates innate and adaptive immunity, induces tissue damage, and modulates tumor formation (Liu *et al.* 2011).

In this study, DENV infection in Huh 7it-1 cells co-cultured with PBMCs showed an increase in CXCL10 chemokine production compared with control cells after 48 hours of infection. This can be seen in Figure 1. which shows that infections of DENV-2 MOI of 0.5 and MOI of 1 FFU/cells have higher CXCL10 levels when compared to control cells that are only treated with Huh 7it-1 cell culture medium. This is similar to the results of in vivo studies conducted by Masood *et al*., which showed an increase of CXCL10 levels in DENV infected patients when compared to healthy controls (Masood *et al.* 2018). Improvement of CXCL10 levels also occurred in studies conducted by Sung *et al*., in infected mice with DENV-2 and showed elevated levels of CXCL10 in the serum and liver of mice. In addition, the infection also causes liver damage in mice that is associated with an increase of CXCL10 levels which is able to recruit NK cells into the liver and cause early cell death after DENV infection (Sung *et al.* 2012).

The increase of CXCL10 levels in this study is thought to occur as an antiviral response to viral

infection in target cells, this is because CXCL10 is a proinflammatory chemokine induced by IFN-γ, which is highly specialized in primary cells infected with DENV. CXCL10 plays a role in the antiviral response by competing to bind to heparin sulfate which is a receptor for the entry of DENV on target cells, to blocking entry and replication (Conroy *et al.* 2015). In addition, CXCL10 also has chemotactic abilities that play a role in innate immune responses, CXCL10 binds to the CXCR3 receptor, which dominantly expressed by T lymphocytes, B lymphocytes, NK cells, dendritic cells, and activated macrophage cells. CXCL10 is known to recruit T cells and NK cells that are activated to the location of DENV infection to carry out the process of virus elimination (Masood *et al.* 2018). In Chen *et al*. study it is known that an increase of CXCL10 levels in DENV infection is followed by the expression of other molecules such as perforin, granzim A and B and IFN, which is known to be produced by NK cells and causes apoptosis and target cell destruction to prevent the spread of the DENV virus in infected cells. On the other hand, high CXCL10 levels have been found in the serum of patients with chronic inflammatory conditions (Chen *et al.* 2006). Thus, CXCL10 is known to have a protective role against DENV, but could also be associated with a potentially destructive inflammatory response if it is produced in high quantities and uncontrolled(Becerra *et al.* 2009).

Whitout PBMCs, DENV infected Huh 7it-1 did not increase the CXCL10 level. Huh7it-1 cells are known to only produce certain cytokines and chemokines based on in vitro studies conducted by Rowell *et al.* (1997). Based on these studies it is not known whether CXCL10 chemokines can be secreted by hepatocyte cells or not, but in this study it was seen that CXCL10 levels did not increase significantly in Huh 7 cells infected with DENV-2 without PBMC when compared to control cells without PBMC (Rowell *et al.* 1997).

In conclusion, DENV-2 infection in Huh 7it-1 cells co-cultured with PBMCs is able to induce CXCL10 secretion, which may be induced as an antiviral response to DENV infection.

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