

Levels of TNF- α in PBMC (Peripheral Blood Mononuclear Cells) Induced by Recombinant Non Structural 1 Protein of Dengue Virus Serotype-2 *in Vitro*

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Dengue infection is a global health problem with an increasing incidence every year and now endemic in more than 100 WHO countries. Dengue infection is caused by dengue virus (DENV) which is an RNA virus with positive single strand, with ± 11 kb genome size encoding 3 structural proteins, 7 non-structural proteins, and two Untranslated Region (UTR). NS1 protein is known to have a very important role in the development of severe DENV infection, by the direct effect causing host cells damage and indirect effect by activating immune response to induce the secretion of excess cytokines. This study aims to evaluate whether recombinant pcNS1 plasmids which have been proven to be able to express recombinant NS1 proteins in previous studies is able to induce cytokine secretion from Peripheral Blood Mononuclear Cells (PBMC). Transfected Chinese Hamster Ovary-K1 (CHO-K1) cells with recombinant pcNS1 plasmid was co-cultured with PBMC from healthy donor. After 48 hours post co-cultured, cell supernatant was collected and TNF- α levels and NS1 recombinant were measured by ELISA. The results showed that recombinant NS1 protein was expressed in CHO-K1 mammalian cell line and able to induce TNF- α with higher levels compared to control.

Key words: CHO-K1 cell, dengue, NS1, TNF- α

Infeksi Dengue adalah masalah kesehatan global dengan kejadian yang terus meningkat setiap tahun dan sekarang endemik di lebih dari 100 negara WHO. Infeksi Dengue disebabkan oleh virus dengue (DENV) yang merupakan virus RNA rantai tunggal positif, dengan ukuran genom ± 11 kb yang mengkode 3 protein struktural, 7 protein non-struktural, dan 2 daerah yang tidak ditranslasikan (UTR). Protein NS1 diketahui memiliki peran yang sangat penting dalam pengembangan infeksi DENV yang parah, melalui efek langsung yang menyebabkan kerusakan sel inang dan efek tidak langsung yang mengaktifkan respon imun untuk menginduksi sekresi sitokin berlebih. Penelitian ini bertujuan untuk menguji apakah plasmid rekombinan pcNS1 yang telah terbukti mampu mengekspresikan protein NS1 rekombinan pada penelitian sebelumnya mampu menginduksi sekresi sitokin dari *Peripheral Blood Mononuclear Cells* (PBMC). Sel *Chinese Hamster Ovary-K1* (CHO-K1) pasca transfeksi dengan plasmid rekombinan pcNS1 di ko-kultur dengan PBMC dari orang sehat. Setelah 48 jam pasca ko-kultur, supernatan sel dipanen dan kemudian kadar TNF- α dan NS1 rekombinan diukur menggunakan ELISA. Hasil percobaan menunjukkan protein NS1 rekombinan terekspresi pada sel mamalia CHO-K1 dan dapat menginduksi TNF- α dengan kadar yang lebih tinggi dibandingkan dengan kontrol.

Kata kunci: dengue, NS1, sel CHO-K1, TNF- α

Dengue virus infection (DENV) is a major health problem in world's tropics and subtropics region, especially in Southeast Asia and the Western Pacific region. Based on WHO (World Health Organization) every year around 50 million cases of DHF (Dengue Fever) occur worldwide, including more than 500,000 cases of severe dengue. In Indonesia, DENV infection was firstly reported in Surabaya and Jakarta in 1968, with 58 total cases where 24 of them died. Nowadays, DENV infection has spread to all provinces in Indonesia with an increase tendency the number of

cases of DENV infection each year (WHO 2016; Kementerian Kesehatan RI 2017).

DENV is a member of Flavivirus family with ± 11 kb genome size consists of a single strand positive RNA which encoding 3 structural proteins (Capsid, Membrane and Envelope), 7 non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5), and two Untranslated Region (UTR). DENV divided into four distinct serotypes which antigenically different: DENV1-4, each serotype is divided into several genotypes based on sequential variability in the E (Envelope) gene (Simmonds *et al.* 2017; LiYun *et al.* 2013; Herman *et al.* 2017). DENV is an arthropod borne virus that spread through the bite of infected *Aedes aegypti* and *Aedes albopictus* mosquitoes (Aryu

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Candra 2010). Clinical manifestations of DENV infection can be asymptomatic and symptomatic. The symptomatic clinical manifestations are divided into non-typical fever, Dengue Fever (DF), Dengue Hemorrhagic Fever (DHF), and Dengue Shock Syndrome (DSS) (WHO 2011).

Several studies have shown an association between the severities of DENV infection with the presence of high level of circulating NS1 protein (Adikari *et al.* 2016). NS1 protein is a glycoprotein consists of 353-354 amino acids encoded by 1056bp nucleotides (Clyde, Kyle, and Harris 2006). NS1 is a key factor during DENV infection which along with other viral proteins can fulfill a structural role, helping to anchor the replication complex to the membrane and induce the formation of membrane components that facilitate viral RNA replication, assembly and release of viruses, immune avoidance, and various aspects of pathogenesis (Glasner *et al.* 2018). NS1 is found on cell surface as membrane bound NS1 protein (mNS1), and also secreted into the extracellular environment as soluble NS1 (sNS1) hexamer and circulates in blood during acute phase which is useful as a diagnostic biomarker. Membrane bound NS1 protein presented by the Major Histocompatibility Complex (MHC) molecule is known to induce immune response, whereas sNS1 can directly bind to various components of the complement that trigger or inhibit complement activity (Henrique *et al.* 2014; Watterson *et al.* 2016). NS1 DENV also directly capable to activating endothelial cells which causes decreased integrity of endothelial cell barrier via the dependent Toll-Like Receptors 4 (TLR4) pathway which results in endothelial cell damage and plasma leakage, activating many innate immune cells to produce any inflammatory cytokines via TLR4, and also inducing immunosuppressive cytokines from monocytes, thus potentially contributing to the pathogenesis of the disease. Dosage-dependent NS1 is known to induce an increase in the production of a number of pro-inflammatory cytokines such as IL-6, IL-1 β , TNF- α from PBMC cells via TLR4, which tends to contribute to cytokine storms in DENV infection (Modhiran *et al.* 2015; Avirutnan *et al.* 2006; Young *et al.* 2015; Adikari *et al.* 2016). Other studies also found that individual NS1 protein can induce secretion of IL-6 and TNF- α based on time-dependent manner (Chen *et al.* 2015).

NS1 DENV has crucial roles in cases of severe DENV infection and associated with excess cytokine secretion that causes severity of DENV infection. Hence, research is needed to determine whether the

recombinant NS1 protein encoded by pcNS1 from previous study is able to induce secretion of TNF- α cytokines from healthy people's PBMC in vitro that can be used as a reference for further DENV research.

MATERIALS AND METHODS

Recombinant pcNS1 Plasmid and Recombinant NS1 Protein. Recombinant pcNS1 plasmid contains NS1 gene insertion and recombinant NS1 protein was expressed in CHO-K1 mammalian cell line and also tend to be released outside the transfected CHO-K1 cells, as confirmed by immunostaining, rapid NS1 detection kit, and NS1 ELISA kit as "previously described" (Sjatha *et al.* 2019).

Recombinant pcNS1 Transfection. CHO-K1 mammalian cell used for plasmid transfection were obtained from Kobe University, Japan. Cell propagation and Recombinant pcNS1 transfection procedure accordance to previous studies (Sjatha *et al.* 2019).

Recombinant NS1 Protein Detection. Recombinant NS1 protein detected from pcNS1 transfected CHO-K1 cells supernatant at 48 hours post co-culture with PBMC using NS1 ELISA kit. The component preparation and the detection procedure in accordance with the step on product manual protocol (Abnova). The existence of recombinant NS1 protein was detected as absorbance value that higher than the negative control.

PBMC (Peripheral Blood Mononuclear Cells). PBMC were isolated from a healthy patient (no fever shows for the past 1 week) and confirmed as NS1 dengue negative, IgG and IgM dengue negative, and PRNT negative. Blood used for PBMC isolation were collected with ethical approval from Research Ethical Committee Faculty of Medicine, Universitas Indonesia No. 10901/UN2.F1/ETIK/2018. PBMC isolation conducted by mixing 2:2 ml of blood and PBS in a Falcon tube, then the mixture was put into a Falcon tube containing 5 ml of Ficoll-Paque slowly in an oblique position 45°C. Thereafter, the mixture was centrifuged without brake off with 2500 rpm of speed for 30 minutes at room temperature to collect the PBMC (shows as white ring circle). The PBMC with complete RPMI 1640 medium (10% FBS and 1% pen-strep) then incubated for 2 hours in 37° C incubator with 5% CO₂. Afterward, the medium removed to discard the non-adherent PBMC, then the flask was washed using RPMI 1640 medium without FBS twice with spray suction technique to collect the adherent

PBMC from the bottom of the flask. Hereafter, centrifuge was conducted at 1200 rpm for 5 minutes for one last time washes the PBMC, then PBMC was ready to co-culture. The number of adherent cells was counted by haemocytometer, then suspended in RPMI 1640 (Sigma) with 10% FBS and 1% penstrep (Gibco) to prepare the cells with a concentration of 1×10^6 cells/ml.

PBMC Co-Culture. 150 μ l of adherent PBMC with concentration of 10^6 cells/ml were added to each well contains transfected CHO-K1 cells at 48 hours post-transfection. 48 hours after PBMC co-culture, the supernatant was collected to measure the level of TNF- α . There were three treatment groups which were done in triplicate. The groups were co-cultured PBMC with pcNS1 transfected CHO-K1 cell (CHO-K1+pcNS1+PBMC), co-cultured PBMC with pcDNA transfected CHO-K1 cell (CHO-K1+pcDNA+PBMC), and co-cultured PBMC with PHA induced CHO-K1 cell (CHO-K1+PHA+PBMC).

TNF- α Measurement using ELISA. TNF- α levels were measured from transfected cell supernatant after co-cultured with PBMC using TNF- α Human in vitro ELISA kit (Antibodies-online, Germany). The component preparation and the measuring procedure in accordance with the step on product manual protocol (Abcam). The ELISA result were showed as absorbance value which then converted to levels using standard curve.

RESULTS

Extracellular NS1 Detection. Extracellular recombinant NS1 protein was detected using NS1 ELISA kit. The CHO-K1 cell co-cultured with PBMC at 48 hours post transfection with recombinant pcNS1 plasmid. At 48 hours after PBMC co-culture, the cells supernatant then collected and were used as samples in ELISA assay to detect the extracellular recombinant NS1 protein. The result come out as absorbance value which was detected right after the ELISA assay protocol was finished using ELISA reader at 450 nm wavelength. These extracellular NS1 protein detection was conducted to reconfirm that the recombinant NS1 protein was successfully expressed in PBMC co-culture. Render by figure 1, the absorbance value of pcNS1 transfected cell supernatant (0.239) was higher than negative control (0.176) and also higher than pcDNA transfected cell supernatant (0.166) whereas pcDNA transfected cell supernatant was lower than negative control. These indicated that recombinant

pcNS1 was able to express recombinant NS1 protein out of the CHO-K1 mammalian cells line.

TNF- α Levels. TNF- α levels were measured to perceive the ability of recombinant NS1 protein in inducing the secretion of cytokine TNF- α from PBMC. As seen in figure 2, the TNF- α levels in pcNS1 transfected cell supernatant after co-culture with PBMC (Mean \pm SD: 1561.778 \pm 122.217) was higher than the TNF- α levels in pcDNA transfected cell supernatant after co-culture with PBMC (Mean \pm SD: 1524 \pm 228.351) and also higher than TNF- α levels in PBMC supernatant post PHA induction (Mean \pm SD: 818.111 \pm 626.535). Significant differences in TNF- α levels ($P < 0.05$) were shown between TNF- α levels in pcNS1 transfected cell supernatants after co-culture with PBMC and the TNF- α levels in PBMC supernatant post PHA induction, but not the others. These results suggested that recombinant NS1 protein expressed by CHO-K1 mammalian cells line post-transfected by recombinant pcNS1 plasmid was able to induce secretion of TNF- α from PBMC.

DISCUSSION

The non-structural protein I (NS1) DENV is a 46 kDa glycoprotein found in infected cells, both on the cell surface and also secreted out of cells that are found circulating in the bloodstream (Alayli & Scholle, 2017). It has been found that DENV NS1 protein with high levels is associated with severity of infection and levels >600 ng/ml in the first 72 hours of disease occurrence associated with the development of DHF (Libraty *et al.* 2000). In addition, it has been found that DENV NS1 antigens still circulate in the body of DHF patients and this may be used as a marker of the severity of DENV infection (Paranavitane *et al.* 2014). NS1 DENV is known to interact with the complement system, and activate innate immune cells to produce proinflammatory cytokines such as IL-6, IL-1 β , TNF- α from PBMC that act through TLR4, which is likely to contribute to dengue-related "cytokine storms" (Modhiran *et al.* 2015). Modhiran *et al.* 2015 found that doses dependent NS1 protein was able to induce the production of cytokine TNF- α mRNAs measured from NS1 treated murine BMMs. Inflammatory cytokines are a key factor in the pathogenesis of DENV (Valero *et al.* 2014). In the other hand, NS1 can also induce immunosuppressive cytokines such as IL-10 from monocytes, thus potentially contributing to the pathogenesis of the disease (Adikari *et al.* 2016).

Based on the explanations above, detection of

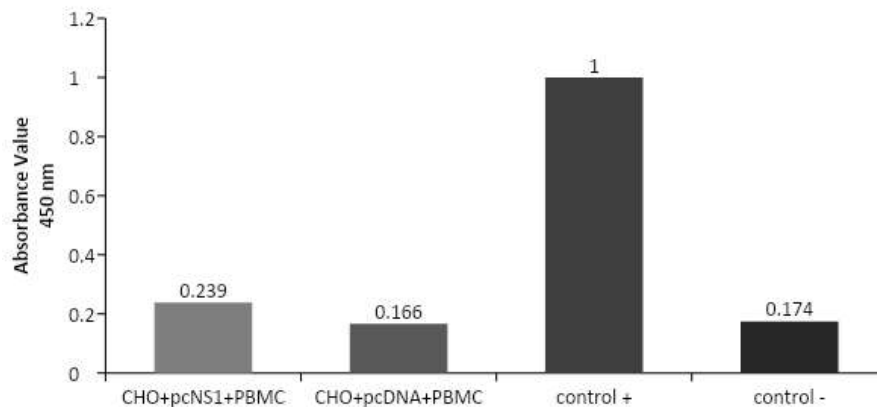


Fig 1 Results of recombinant NS1 protein detection from pcNS1 and pcDNA transfected CHO-K1 cells supernatant at 48 hours post co-culture with PBMC using ELISANS1 kit (Antibodies-online).

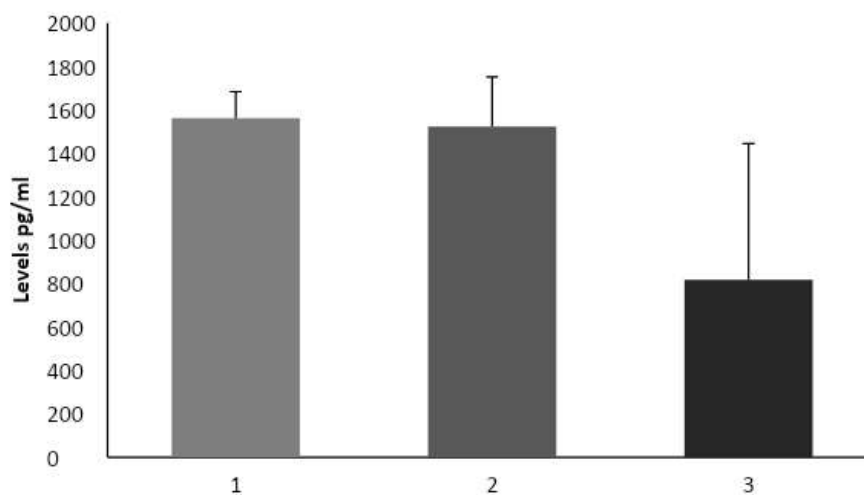


Fig 2 TNF- α levels from CHO-K1 cells supernatant post transfected with pcNS1, pcDNA, and PHA induction at 48 hours after PBMC co-culture. TNF- α levels was detected using TNF- α human in vitro ELISA kit *P<0.05 (abcam).

cytokine levels secreted by PBMC cells after co-culture with pcNS1 transfected CHO-K1 cells was carried out to determine the relationship of NS1 protein to the pathogenesis of DENV infection by secreting the inflammatory cytokines and to know the ability of recombinant NS1 expressed by pcNS1 transfected CHO-K1 cells to induce cytokine secretion, especially TNF- α from PBMC cells in vitro.

Detection of TNF- α selected in this study because it is known that TNF- α and other inflammatory cytokines play important roles during DENV infection, and the low response for those cytokines by DENV-infected monocytes from neonates and elderly people could be important in the development of the disease (Valero *et al.* 2014). TNF- α and other inflammatory cytokines also known involved in DENV hemorrhagic manifestations. In this regard, a rapid increase in the levels of cytokines, especially TNF- α , plays a key role

in inducing unique clinical manifestations of DHF such as plasma leakage, shock, and hemorrhagic manifestation (Kurane 2007).

Several studies have found that DENV NS1 protein is able to induce the secretion of TNF- α based on time and dosage dependent, where low doses of NS1 will induce the low levels of TNF- α secretion and the longer incubation time will decrease the secretion of TNF- α (Modhiran *et al.* 2015; Chen *et al.* 2015). This finding is consistent with what was obtained in this study. TNF- α levels secreted by PBMC co-cultures with pcNS1 transfected CHO-K1 cells are not significantly different from TNF- α levels secreted by PBMC co-cultures with pcDNA transfected CHO-K1 cells, possibly caused by the incubation time has passed the peak hours of TNF- α secretion which is 24 hours after NS1 induction (Chen *et al.* 2015). To induce TNF- α secretion in this study was conducted differently from

others study existed. Chen *et al.* 2015 have been measured TNF- α levels from PBMC supernatant after treated with individual DENV NS1 commercial protein, whereas this study used mNS1 and sNS1 expressed by pcNS1 transfected CHO-K1 cells to induce TNF- α secretion from PBMC. Measuring the levels of NS1 secreted by pcNS1 transfected CHO-K1 cells was not conducted in this study prior PBMC co-culture. Therefore, it is unknown yet whether the low doses of NS1 affected the low levels of TNF- α secreted in this study. However, previous studies have proven that pcNS1 transfected CHO-K1 cells are able to secrete NS1 protein that are detected using NS1 rapid test kit and immunostaining (Sjatha *et al.* 2019). This study also measured the NS1 at the same time as the TNF- α levels, and the Optical Density (OD) value of NS1 were low. The low of OD NS1 value potentially causing the TNF- α levels secreted by PBMC co-culture with pcNS1 transfected CHO-K1 cells were not significantly different with TNF- α levels secreted by PBMC co-culture with pcDNA transfected CHO-K1 cells. Suggested for further research to measure the NS1 levels prior PBMC co-culture to find out whether the NS1 doses affected the levels of TNF- α secretion. In addition, the management of incubation time also needs to be adjusted to the peak hours of TNF- α secretion to obtain the maximum levels of TNF- α .

In conclusion, this suggests that recombinant NS1 protein expressed by pcNS1 in mamalian CHO-K1 cells is able to induce secretion of TNF- α cytokines in PBMC.

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