Dengue Virus (DENV)-1 Induces High Expression of Anti- and Pro-inflammatory Cytokines in Human Macrophages

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Keywords: Dengue Viruses, Cytokines, Monocyte-Derived Macrophages (MDMs).

Abstract: Dengue is caused by dengue virus (DENV) infection. The pathological mechanism of dengue infection is still poorly understood. Serotype variation and host's immune response are thought to have roles in disease severity. This study compared the ability of four DENV serotypes (DENV-1, -2,-3,-4) isolates from Indonesia to induce the expression of anti- and pro-inflammatory cytokines in Monocyte-Derived Macrophages (MDMs) differentiated from a healthy human. Monocytes were isolated from healthy human donors and differentiated into macrophages under the stimulation of Macrophage Colony Stimulating Factor (M-CSF). Mature MDMs were infected with four different serotypes of DENV isolates from Indonesia. The resulting expression kinetics of Interferon alpha (IFN-α2) and Interleukin-10 (IL-10) as anti-inflammatory cytokines and IL-1β as pro-inflammatory cytokine was measured at six different time points using Luminex immunoassay. The presence of DENV Non-Structural Protein 1 (NS1) as a marker of virus replication was also measured using ELISA. DENV-1 showed a tendency to induce the expression of IFNα2 and IL-10 more rapidly and at a higher level than other serotypes albeit of lower NS1 expression. The expression of IL-1β was down-regulated in response to all DENV serotypes infection. This study demonstrates the differences in the ability of four DENV serotypes in regulating the expression of cytokines in MDMs with DENV-1 showed a superior inducing capability compared to other serotypes. This data provides information of possible serotype-specific role on disease severity.

1 INTRODUCTION

Dengue is a febrile illness caused by dengue virus (DENV) infection.(Gubler, 1998) DENV is a member of the *Flaviviridae* family, which includes four serotypes identified as DENV-1, DENV-2, DENV-3, and DENV-4.(Lindenbach & Rice, 2003)(Kuno, Chang, Tsuchiya, & Karabatsos, 2014) The mechanisms that lead to clinical manifestations of dengue are believed to be multifactorial. There are two main factors which have been shown to contribute to disease severity, i.e., viral and host factors.(Halstead, 2008)

Viral factors (viral load and variations of viral serotypes) have been shown to play critical roles in the emergence of symptoms.(Halstead, 2008)(Clyde, Kyle, & Harris, 2006) Previous research has argued that the role of immune mediators such as anti- and pro-inflammatory cytokines may be as important as host immune factors.(Green & Rothman, 2006) In humans, macrophages have been determined as DENV targets.(Chen & Wang, 2002) The targeting of these cells by DENV may then lead to the immunological modulation,(Sun & Kochel, 2013) as evidenced by the expression of various cytokines.

This study aimed to compare the ability of the four serotypes of DENV in inducing the expression of anti- and pro-inflammatory cytokines in MDMs by looking at the kinetic patterns of cytokine expression *in vitro*.

Previously, we have described the cytokine/chemokine expression of four DENV serotypes in the human A549 cell line.(Yohan, Kendarsari, Mutia, Bowolaksono, & Harahap, 2014) Here, we study the expression profile of MDM cells, which more reflecting the natural pathogenesis mechanism of DENV infection in human.

Yulia, W., Yohan, B., Meutiawati, F., Harahap, A. and Sasmono, R.
Dengue Virus (DENV)-1 Induces High Expression of Anti- and Pro-inflammatory Cytokines in Human Macrophages.
DOI: 10.5220/0008790500440048
In *Proceedings of the 2nd Syiah Kuala International Conference on Medicine and Health Sciences (SKIC-MHS 2018)*, pages 44-48
ISBN: 978-989-758-438-1

2 METHODS

2.1 Monocyte Isolation and MDM **Differentiation**.

Monocytes were isolated from venous blood of healthy human volunteers. Ethical clearance was obtained from the Eijkman Institute Research Ethics committee.

Peripheral blood mononuclear cell (PBMCs) isolation was performed using Ficoll gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare). Monocytes fraction from PBMC were enriched by overnight cell adherence into the surface of cell culture flasks in RPMI-1640 medium (Gibco-Thermo Scientific) supplemented with 10% FBS (Gibco), 2 mM L-glutamine (Gibco), 100 U/ml Penicillin and 100 µg/ml streptomycin (Gibco).

The enriched monocytes were further differentiated into macrophages by stimulation with 10 ng/ml Macrophage Colony Stimulating Factor (M-CSF) (Sigma) for eight days. Differentiation of monocytes into macrophages was monitored based on morphological observation and the expression of *c-fms* gene as macrophage marker by RT-PCR. β-actin gene was also amplified as RNA loading control. The primer pairs used were as follows: *c-fms* forward, 5-ACACTAAGCTCGCAATCCC-3, and
revese 5'-GTATCGAAGGGTGAGCTCAAA-3'; β-actin forward,
5'-CATCTCTTGCTCGAAGTCCA-3', and reverse,5'-ATCATGTTTGAGACCTTCAACA-3'.(Jia et al., 2010)

2.2 DENV Infections

Four DENV clinical strains representing all four serotypes were isolated from Indonesian dengue patients' sera. Viruses were cultured in Vero cells. Viral titers were measured in plaque forming units/ml (PFU/ml), using a modified plaque assay method.(Lambeth, White, Johnston, & Silva, 2005) MDMs were infected by four serotypes of DENV using 0.1 multiplicity of infection (moi). Controls included non-infected and lipopolysaccharide (LPS)-stimulated cells. For cytokines and NS1 antigen measurement, the cell culture supernatant was collected in 12-hour intervals for a total of 72 hours and immediately stored at -80°C. DENV NS1 antigen expression was measured in cell culture supernatants by using Panbio Dengue Early NS1 ELISA kit (Alere, USA).

2.3 Cytokines Assay

The level of IFN-α2, IL-10 and IL-1β were measured using Milliplex MAP Human Cytokine kit (Merck Millipore, MPXHCYTO-60K, Germany). Multiplex fluorescent microbead immunoassay containing fluorescent microspheres, conjugated with specific monoclonal antibodies for the target protein were used to detect and quantify the cytokines from 25 µl of culture supernatant, simultaneously, as described elsewhere.(Yohan et al., 2014) Results were obtained in Median Fluorescent Intensity (MFI) and further analyzed using MasterPlex QT software to measure the cytokines concentration in pg/ml.(www.ReaderFit.com)

3 RESULTS

3.1 Isolation and Differentiation of MDMs

In the early isolation of PBMC, the monocytes were round upon microscopic inspection. Monocytes were observed as small and uniformly distributed on the tissue culture flask. Morphological changes were observed during the eight days of differentiation and can be seen in FIGURE 1. Cell development was observed to have accelerated rapidly from day five onward. Morphological observation of MDMs on day eight indicated that the cells were fully differentiated into macrophages, demonstrating cell adherent, fibroblast-like morphology, and the appearance of pseudopodia.(Abbas, Lichtman, & Pillai, 2012)(Sasmono & Hume, 2004) Detection of the *c-fms* gene, a marker of macrophage differentiation(Sasmono & Hume, 2004), was prominent at day 8 using RT-PCR. This gene was not detected in fibroblasts control cells.

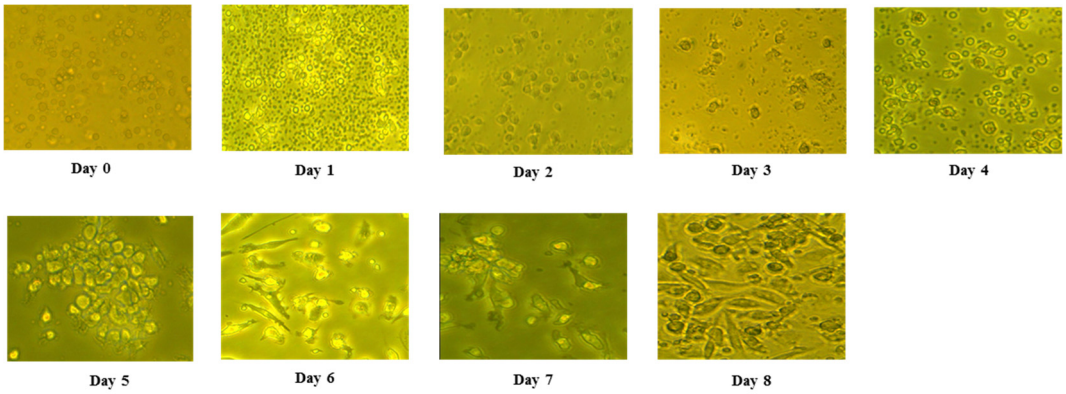

Figure 1. Morphology MDMs isolation and differentiation on day 0 to day 8. Cell growth appears slow from day 0 to 4 of isolation, and rapid growth seen up to day 8. *20 x magnification*.

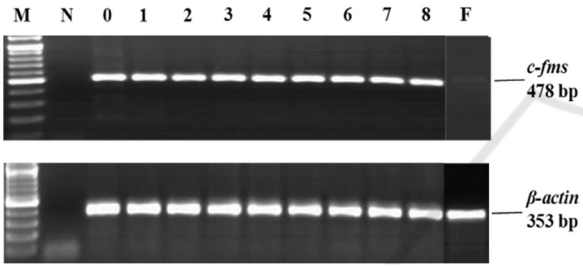

Figure 2. Expression of c-fms gene in the MDMs. Comparison of the macrophage colony-stimulating factor receptor (*c-fms* gene) and human actin (β-actin gene) as an RNA loading control using RT-PCR from isolation day one to day eight. M, DNA Marker (100 bp) (Invitrogen); N, negative control; F, Fibroblast.

3.2 DENV NS1 Antigen Detection

NS1 protein secretion is one marker of dengue virus replication in the host cell. The ability of dengue virus to replicate in infected MDMs was evidenced by the detection of DENV NS1 antigen in all four serotypes, observed at 72 hours post-infection. NS1 antigen of DENV-4 appeared to predominate, whereas DENV-1 NS1 expression was the lowest among all serotypes.

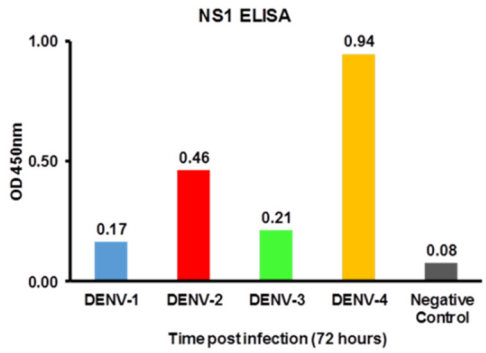

Figure 3. Detection of DENV 1-4 NS1 antigen in the MDMs. DENV NS1 antigen was measured using commercial NS1 ELISA tested to culture supernatant collected 72 hours post-infections.

The absorbance levels were considered as Positive using the Panbio Unit calculation. Non-infected cell control showed no detection of NS1 (FIGURE 3).

3.3 Kinetics Profile of Cytokines Expression in the MDMs

Our study demonstrated that IFN-α2, IL-10 and IL-1β were expressed as a result of the exposure of MDM to the four serotypes of DENV. The kinetics of the expression patterns of cytokines is shown in FIGURE 4. The intensity of IFN-α2 expression on MDMs exposed by DENV-1 was significantly different in comparison with the other serotypes.

IFN-α2 expression is seen to rise sharply after 24 hours, then decreased sharply after 60 hours. The pattern of cytokine expression seen in DENV-4 appears to increase at a slower rate, which then starts to increase sharply at 60 and 72 hours post-infection. In DENV serotype -2 and -3, the increase in IFN-α2 was overall not as high as in the two other serotypes.

The induction of IL-10 expression against DENV-1 infection tended to be higher than the other serotypes, despite lower levels of DENV NS1 antigen being detected. The IL-10 expression

patterns showed a sharp increase at about 36 hours post-infection in all serotypes, and then plateaued or declined.

Expression of IL-1β slightly increased and then gradually decreased after 36 hours. All serotypes showed decreasing patterns until 60 hours and then continue to slightly increase after 72 hours, except for DENV-1. In those two cytokines, there was a tendency that DENV-1 induces relatively highest expression of cytokines among the DENV serotypes.

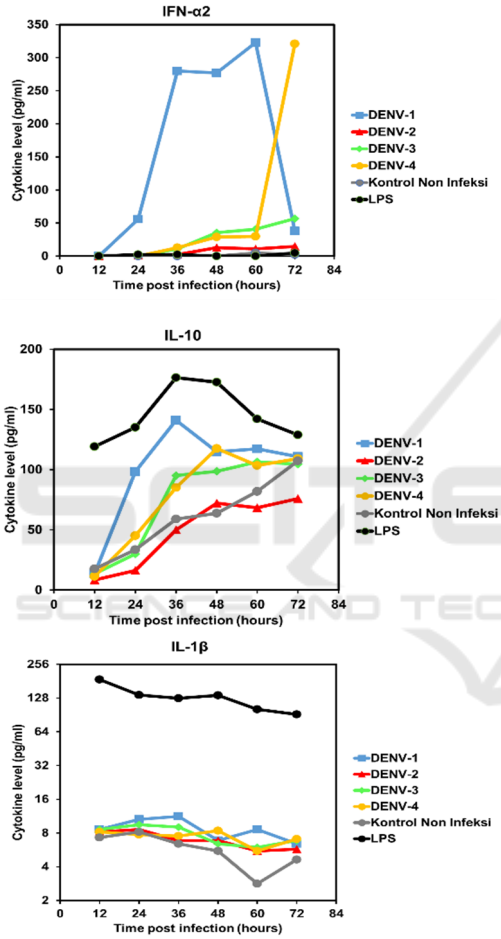

Figure 4. Cytokines expression kinetics in MDMs in response to infection of four DENVserotypes.

4 DISCUSSION

In this study, differences in the pattern of expression of cytokines in MDMs (FIGURE 4) was demonstrated, with DENV-1 exposed MDMs expressing the highest levels of cytokines. The increase in cytokine expression is inversely related to the detection of DENV NS1 antigen (FIGURE 3).

The response to DENV-4 was observed to be lower than most other serotypes and virus NS1 antigen was detected at highest levels than with exposure to DENV-1 and -3. This raises the question of whether there are intrinsic factors in DENV-1 virus titers which, although less prominent than other serotypes, triggers an increase in cytokine expression compared to other serotypes. Further study is needed to determine the possible existence of DENV-1 intrinsic factors, especially when associated with viral genetic traits that may be observed with the complete genome sequence of the virus.

Interferon (IFN) is a host defense system that is activated during the initial stages of infection. Previous in vitro studies showed that DENV infection in human cells could be inhibited by initial therapy using IFN-α which inhibits translation of viral RNA kinetics.(Diamond & Harris, 2001) The results of this study demonstrate that the expression pattern of IFN-α2 in MDMs infected by DENV-1 and DENV-4 may demonstrate a correlation between the concentrations of IFN-α2 and expression of DENV NS1 antigen ELISA (FIGURE 3 and 4). The high-level of IFN-α2 expression during the early phase of DENV-1-infected MDM (up to 60 hrs post-infection) resulted in the low level of virus titer, measured as NS1 level at 72 hrs. Inversely, the relatively low level of IFN-α2 expression in the initial stages of DENV-4 infection yielded increased level of NS1. However, the patterns were not clearly observed in DENV-2 and DENV-3-infected MDM. These figures indicate a possible role of IFN-α2 in inhibiting DENV replication in the host and the serotype-specific induction mechanisms.

In DENV pathogenesis, resistance against IFN can be caused by IL-10 led immunosuppression, followed by failure in achieving viral clearance by the immune system and persistent infection in acute viral infections.(Diamond & Harris, 2001) The results of this study demonstrate that the expression pattern of IL-10 was increasing during the progression of DENV infection. This may be due to the immunosuppression mechanisms intrinsic to IL-10 as the anti-inflammatory cytokine. The increase in IL-10 expression may be correlated to the role of the DENV in regulating the host's immune system.

IL-1β is a pro-inflammatory cytokine produced by macrophages. This cytokine plays a role in the cellular activity, including proliferation and differentiation kinetics of cytokine expression, and

apoptosis.(Abbas et al., 2012) In this study, most serotypes showed a gradually decreased expression at later stages, post-infection. The role of this cytokine in DENV infection may need to be explored more.

5 CONCLUSION

In conclusion, cytokines expression in MDM infected by various DENV serotypes showed a marked difference in expression. These findings are useful to assess the ability of serotypes in inducing the host immune response by demonstrating the variations in patterns of cytokines expression. In line with previous research showing that cytokine IFN-α2 has viral inhibition characteristic, our findings suggest that IFN-α2 may contribute to DENV inhibition. Further studies are needed to assess the roles of infecting DENV serotype in disease severity.

ACKNOWLEDGMENT

We thank the member of Dengue Laboratory Eijkman Institute for support and suggestion.

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