Indonesian Journal of
Tropical and Infectious Disease
Vol. 7 No. 4 January-April 2019

Research Report

EXPRESSION OF FOUR CYTOKINE/CHEMOKINE GENES IN PERIPHERAL BLOOD MONONUCLEAR CELLS INFECTED WITH DENGUE VIRUS

Sri Masyeni[1a], Usman Hadi[2], Kuntaman[2], Benediktus Yohan[3], Nur Ita Margyaningsih[3], R. Tedjo Sasmono[3]
1 Faculty of Medicine and Health Science, Warmadewa University, Denpasar-Bali, Indonesia,
2 Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
3 Eijkman Institute for Molecular Biology, Jakarta, Indonesia
a Corresponding author: masyeniputu@yahoo.com

ABSTRACT

Overproduction of numerous pro-inflammatory cytokines, during dengue virus (DENV) infection, has been related to plasma leakage in the vascular endothelium and studied elsewhere with conflicting results. The current study objective is to evaluate the expression of four cytokine/chemokine genes following DENV-2 infection within peripheral blood mononuclear cells (PBMC) isolated from a healthy donor. Venous blood was drawn, and PBMCs were isolated using Ficoll density gradient centrifugation. Cells were maintained in culture medium and infected with Indonesian isolate of DENV-2. Cells were harvested and followed by total RNA extraction and reverse-transcription into cDNA using oligo d(T) primers and Reverse Transcriptase enzyme system. The SYBR Green-based quantitative qRT-PCR was used to calculate the relative expression of IL-6, IL-8, IP-10 and MIP-1ß- encoding genes during infection time points, compared to uninfected cell controls. The observation of the cytokine was on the 6 and 18 hours post-infection. The different expression profiles of cytokines/chemokines were observed. The up-regulation of gene expression was observed for IL-8 and IP-10. In contrast, the down-regulatory of IL-6 and MIP-1ß genes expression was documented during the infection period. The cytokine IL-8 and IP-10 are potent chemoattractants in the recruitment of neutrophil, basophil, and lymphocytes in response to an infection. The highlight of this study is on the up-regulation of IL-8 and IP-10 genes expression which may confirm the roles of these chemokines in the pathogenesis of dengue infection.

Keywords: *dengue, gene expression, cytokine, chemokine, PBMC*

ABSTRAK

Produksi sitokin pro-inflamasi berlebihan pada infeksi virus dengue yang dihubungkan dengan terjadinya kebocoran plasma pada endotel vaskular telah diteliti dengan hasil yang bervariasi. Penelitian ini bertujuan untuk mendeteksi ekspresi empat gen pengkode sitokin atau kemokin pada sel mononuklear darah tepi yang di-infeksi virus dengue serotipe-2. Sel mononuklear darah tepi (PBMC) diisolasi dari donor sehat dengan menggunakan metode sentrifugasi gradient Ficoll. Ekstraksi RNA dilakukan terhadap sel mononuklear darah tepi, kemudian sintesis cDNA dilakukan dengan menggunakan primer oligo d(T) dan sistem enzim reverse transcriptase. Dengan menggunakan quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) berbasis SYBR-Green, ekpresi gen penyandi IL-6, IL-8, IL-10 dan MIP-1ß dibandingkan antara sel yang terinfeksi dan tidak terinfeksi virus dengue serotipe-2. Observasi dilakukan pada waktu pengamatan jam ke-6 dan waktu pengamatan ke-18 dari infeksi. Ekspresi gen penyandi IL-8 dan IP-10 ditemukan lebih tinggi, sebaliknya ekspresi gen penyandi sitokin IL-6 dan MIP-1ß lebih rendah pada sel mononuklear darah tepi yang diinfeksi virus dengue serotipe-2 dibandingkan dengan kontrol Interleukin-8 dan IP-10 adalah sitokin yang bersifat sebagai kemotaktik untuk memicu kemotaksis dari sel netrofil, basophil dan limfosit sebagai respon dari suatu inflamasi. Hasil penelitian ini menunjukkan bahwa ekspresi kedua gen penyandi kemokin yang meningkat setelah infeksi virus dengue serotipe-2 mungkin berperan pada patogenesis terjadinya kebocoran plasma pada infeksi virus dengue.

Kata kunci: *dengue, ekspresi gen, sitokin, kemokin, PBMC*

INTRODUCTION

Infection caused by dengue virus is still the major cause of acute febrile illness in the world, particularly in the tropical and subtropical area, including Southeast Asian countries.[1] The widely-spread dengue virus (DENV) and the *Aedes* mosquitoes vector have now become a major problem, and more than 125 countries are known to be dengue-endemic regions, including Indonesia.[2] The high DENV expansion is related to climate change, globalization effect, traveling communities, socioeconomics, settlement and viral evolution.[2] Dengue is a complex disease, caused by an RNA virus, entailing of four antigenically similar but with immunologically distinct serotypes (DENV-1 to DENV-4).[3,4] A serotype-specific DENV infection confers life-long immunity with only partial protective immunity for the other serotypes hence people in endemic countries can be infected up to four times by different DENVs.[5] The clinical manifestation varies from asymptomatic to the severe, life-threatening manifestation.[6] The majority of dengue clinical manifestation is mild, asymptomatic dengue or mild fever. In the lesser incidence, the more severe form of dengue is Dengue Hemorrhagic Fever (DHF) with various degrees and Dengue Shock Syndrome (DSS), in which the fatality rate may exceed more than 5% in special populations.[4,5] Expanded dengue syndrome or unusual dengue syndrome with a high mortality rate can be appeared without any sign of plasma leakage, the hallmark of severe dengue.[7]

The detailed pathogenesis of dengue is not yet entirely understood. The existence of cytokine storm-induced endothelial dysfunction in DENV infection has been published over the past decades.[8] The excessive release of various cytokines recognized as cytokine storm has been regarded as the underlying mechanism of plasma leakage in DENV infection.[9] The role of diverse cytokines and chemokines were observed during the more severe manifestation of dengue might be considered to be correlated with the infecting DENV serotype.[10] At the different phase of illness, cytokines/chemokines profiles were found to be increased in patients at the febrile phase.[11] Several studies were also reported the increased expression of cytokines/chemokines within *in vitro* in human monocytes[12] or epithelial[13] cells or *in vivo* in dengue patients' serum.[14] The dual role of both innate and inflammatory pathways were activated during dengue disease and revealed the involvement of immune mediators.[14] Utilizing dengue human cell line infection model, the up-regulated cytokines/chemokines gene expression profiles during DENV serotypes infection have been described and among them were IL-6, IL-8, and IP-10.[13] Other report is highlights the induction of MIP-1β by dengue virus.[15] In this study, we are reported the expression profiles of four genes encoding cytokines and chemokines in the peripheral blood mononuclear cells (PBMCs), infected with Indonesian isolate of DENV-2.

MATERIAL AND METHOD

Ethical Considerations

The ethical considerations of this study have been reviewed and approved by the Institutional Review Board of Udayana University, Bali, Indonesia (Document No. 2072/UN.14.2/KEP/2017).

Blood Collection and Peripheral Blood Mononuclear Cells (PBMCS) Isolation

Thirty mL of venous blood was drawn from a healthy donor and subjected to PBMC isolation using Ficoll Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation. Isolated PBMCs were maintained in 1 RPMI medium supplemented with 10% of fetal bovine serum (FBS), 1% of antibiotic/antimycotic, and 2 mM of L-glutamine (all from Gibco-Thermo Fisher Scientific, Carlsbad, CA). Cells were seeded at 1 10^6 cells per well of 24-well plates (Corning, NY). The seeded cells were allowed to rest during overnight incubation at 37°C incubator with 5% CO_2 supplementation.

DENV Infection

The DENV-2 virus strain SMG-SE001 was isolated from a severe dengue patient from Semarang in 2012[16] (Eijkman's collection). Cells were infected with DENV-2 using the multiplicity of infection (MOI) of 1 (theoretical calculation of one virus PFU per cell), prepared in 1 RPMI medium-2% FBS, performed in duplicate. To achieve the MOI of 1 (theoretically one virus particle infecting one cell), a number of 10^6 Plaque Forming Unit (PFU) of DENV-2 was inoculated into 10^6 PBMC cells. The DENV PFU titre was measured using a standard plaque assay method, as described elsewhere, [16] meanwhile uninfected cells controls were inoculated using the addition of medium only. Plates were then incubated for 1 hour at 37°C, 5% CO_2 to facilitate virus infection. The incubation period was continued and cells were harvested at 0, 6, and 18 hours post-infection to represent the early phase of dengue illness.

Total RNA Extraction and qRT-PCR

Infected cells were harvested at the designated time points and subjected to the total RNA extraction using miRCURY RNA Isolation kit – Cell and Plant (Exiqon, Vedbaek, Denmark), as described in the manufacturer's instructions. Total RNA quantity was measured using Qubit 3.0 fluorometer and Qubit RNA BR Assay Kit (Life Technologies-Thermo Fisher Scientific, Eugene, OR). An amount of 100 ng of total RNA was used in cDNA synthesis performed using oligo d(T) primer and GoScript Reverse Transcription System (Promega, Madison, WI). The resulting cDNA was then amplified by quantitative real-time PCR using GoTaq qPCR Master Mix (Promega) and primers as listed in Table 1. The relative gene expression analysis was performed using the equation of $2^{-\delta\delta Ct}$ of normalized Ct value to human β-actin.

Table 1. Primers Used in Cytokine/Chemokine Relative Gene-Expression Analysis Using qRT-PCR.

Target gene	Direction	Sequence (5'-3')
IL-6	Sense	GAGGATACCACTCCCAACAGACC
	Antisense	AAGTGCATCATCGTTGTTCATACA
IL-8	Sense	TGCCAAGGAGTGCTAAAG
	Antisense	CTCCACAACCCTCTGCAC
IP-10	Sense	TTCAAGGAGTACCTCTCTCTAG
	Antisense	CTGGATTCAGACATCTCTTCTC
MIP-1β	Sense	CTGTGCTGATCCCAGTGAATC
	Antisense	TCAGTTCAGTTCCAGGTCATACA
β-Actin	Sense	CATCTCTTGCTCGAAGTCCA
	Antisense	ATCATGTTTGAGACCTTCAACA

RESULT AND DISCUSSION

The increased expression of IL-8 and IP-10 was observed during infection of DENV-2, with the highest level seen at 18 hours post-infection (Figure 1). By contrast, the relative expression of IL-6 and MIP-1β genes was relatively decreased along the infection time. The up-regulation of IL-8 and IP-10 was reaching more than two-fold at 18 hours post-infection, relative to the uninfected

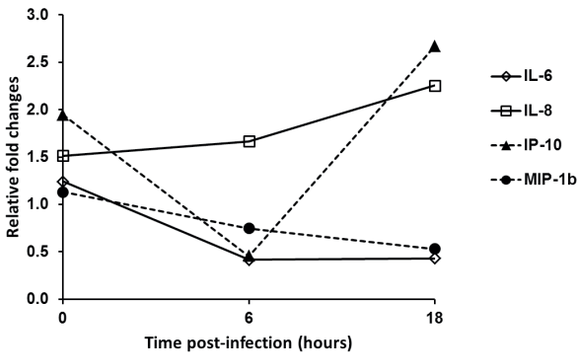

Figure 1. The Relative Cytokine/Chemokine Genes Expression of DENV-2 infected-PMBCs Compared to Uninfected Controls During Three Infection Time Points.

control. The reduction of IL-6 and MIP-1β genes expression to the level of nearly half-fold was apparent at the same time points.

We observed the up-regulation of IL-8 and IP-10 in PBMCs infected with DENV-2, relative to the uninfected controls. The IL-8 is the pro-inflammatory cytokines, a member of CXCL chemokine family and the factor of a neutrophil chemotactic factor, which have been widely investigated and found to increase at the protein level.[17] The cytokine IL-8 is a neutrophil chemoattractant produced by macrophages and other cell types, such as endothelial cells, fibroblasts, and synovial cells.[17] It has been reported that increased levels of IL-8 cytokine in the sera of dengue patients were correlated with the severe form of dengue.[18] The level of IL-8 was significantly higher in samples from severe dengue cases and lower in cases of dengue without warning signs than in healthy controls. Samples that were positive for anti-DV IgG antibody had higher levels of IL-8.[18]

The IP-10 is a member of CC-motif chemokine in response to interferon-γ in infectious diseases.[18] The previous study result on the induced expression of IP-10 chemokine has been well-documented in dendritic cells and other primary cell lineages in response to *in vitro* dengue infection[19] as well as in PBMCs of dengue patients.[20] The level of IP-10 has been found to be increased in serum of dengue-infected patients in studies in Venezuela[19] and Singapore.[21] Moreover, the increased level of IL-8 and IP-10 cytokines/chemokine was also observed in A549 human lung epithelial cells infected with DENV.[13]

We observed the reduction trend in both IL-6 and MIP-1β gene expressions during DENV-2 infection of PBMCs. The increased level of IL-6 has been reported in dengue patients.22 In addition, the increased level of IL-6 has been correlated to disease severity with higher level observed in the more severe form of dengue manifestation, through the up-regulation of inflammatory responses in macrophages and induction of B cell maturation.23 Dengue NS1 protein was found to significantly increase the production of IL-6 thoroughly activation of TLR-2 and TLR-6 in PBMC infected with DENV.24 The higher expression of MIP-1β was reported in PBMC from DENV-infected patients.11 However, in this study we did not found the elevated level of gene expressions for IL-6 and MIP-1β within 18 hours of DENV infection. A systematic review on markers of dengue disease severity revealed that increased level of IL-6 and MIP-1β was observed in samples taken more than

48 hours after onset of fever.25 The results from this study may be related to the difference in the experimental setup of genes expressions analysis and the designated sample collection period to represent the early phase of dengue disease development during 18 hours of virus infection. However, the decreasing levels of IL-6 and MIP-1β have been observed in dengue with warning signs during disease progression from febrile to convalescence phase.11 The current study result concordant to another study result where increased serum levels of IL-6 and IL-8 were detected in patients with dengue haemorrhagic fever but not dengue fever.26 More in-depth research is needed to study the kinetics of these cytokines during dengue infection.

CONCLUSION

It has been reported here the dynamic of four cytokines related to an early phase of DENV infection. The up-regulation of IL-8 and IP-10, chemokines with profiles that highly correlated with dengue than other febrile diseases and have the potential to be used as a biomarker of dengue, since high expression of these cytokines at the earlier phase of observation in this study.

ACKNOWLEDGEMENT

This work was supported by the Faculty of Medicine and Health Science, University of Warmadewa. We would like to thank Prof. Aryati, Prof. Yoes Prijatna Dachlan, Dr. Sunaryo, Dr. H. Muchlis A. Udji, and Dr. Gondo Mastutik, for their critical inputs and suggestions to the study.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Ooi E-E, Gubler DJ. Dengue in Southeast Asia: epidemiological characteristics and strategic challenges in disease prevention. Cad Saude Publica. 2009;25:S115–24.
2. Murray NEA, Quam MB, Wilder-Smith A. Epidemiology of dengue: past, present and future prospects. Clin Epidemiol. 2013;5:299–309.
3. Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, Gubler DJ, et al. Dengue: A continuing global threat. Nat Rev Microbiol. 2010;8(12):S7–16.
4. Guzman MG, Harris E. Dengue. Lancet. 2015;385(9966):453–65.
5. Katzelnick LC, Harris E, Baric R, Coller B-A, Coloma J, Crowe Jr JE, et al. Immune correlates of protection for dengue: State of the art and research agenda. Vaccine. 2017;35(36):4659–69.
6. Martina BEE, Koraka P, Osterhaus ADME. Dengue virus pathogenesis: An integrated view. Vol. 22, Clinical Microbiology Reviews. 2009. p. 564–81.
7. WHO. Comprehensive guidelines for prevention and control of dengue and dengue haemorrhagic fever [Internet]. WHO Regional Publication SEARO. 2011. 159-168 p. Available from: http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Comprehensive+Guidelines+for+Prevention+and+Control+of+Dengue+and+Dengue+Haemorrhagic+Fever#1
8. Srikiatkhachorn A, Mathew A, Rothman AL. Immune-mediated cytokine storm and its role in severe dengue. Semin Immunopathol. 2017 Jul;39(5):563–74.
9. Basu A, Chaturvedi UC. Vascular endothelium: The battlefield of dengue viruses. Vol. 53, FEMS Immunology and Medical Microbiology. 2008. p. 287–99.
10. Mangione JNA, Huy NT, Lan NTP, Mbanefo EC, Ha TTN, Bao LQ, et al. The association of cytokines with severe dengue in children. Trop Med Health. 2014 Dec;42(4):137–44.
11. Rathakrishnan A, Wang SM, Hu Y, Khan AM, Ponnampalavanar S, Lum LCS, et al. Cytokine Expression Profile of Dengue Patients at Different Phases of Illness. PLoS One. 2012;7(12).
12. Puerta-Guardo H, Sandino AR, González-Mariscal L, Rosales VH, Ayala-Dávila J, Chávez-Mungía B, et al. The cytokine response of U937-derived macrophages infected through antibody dependent enhancent of dengue virus disrupts cell apical junctional complexes and increase vascular permeability. J Virol. 2013;JVI-00085.
13. Yohan B, Kendarsari RI, Mutia K, Bowolaksono A, Harahap AR, Sasmono RT. Growth characteristics and cytokine/chemokine induction profiles of dengue viruses in various cell lines. Acta Virol. 2014;58(1):20–7.
14. Costa VV, Fagundes CT, Souza DG, Teixeira MM. Inflammatory and innate immune responses in dengue infection: protection versus disease induction. Am J Pathol. 2013 Jun;182(6):1950–61.
15. Bozza FA, Cruz OG, Zagne SMO, Azeredo EL, Nogueira RMR, Assis EF, et al. Multiplex cytokine profile from dengue patients: MIP-1beta and IFN-gamma as predictive factors for severity. BMC Infect Dis. 2008;8:1–11.
16. Fahri S, Yohan B, Trimarsanto H, Sayono S, Hadisaputro S, Dharmana E, et al. Molecular surveillance of dengue in semarang, indonesia revealed the circulation of an old genotype of dengue virus serotype-1. PLoS Negl Trop Dis. 2013 Aug;7(8):e2354.
17. Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. Annu Rev Immunol. 2014;32:659–702.
18. Tsai YT, Chang SY, Lee CN, Kao CL. Human TLR3 recognizes dengue virus and modulates viral replication in vitro. Cell Microbiol. 2009;11(4):604–15.
19. Becerra A, Warke K, Xhaja K, de Bosch N, Rothman AL, Bosch I. Gene expression profiling of dengue infected human primary cells identifies secreted mediators in vivo. J Med Virol. 2009 Aug;81(8):1403–11.
20. Ubol S, Masrinoul P, Chaijaruwanich J, Kalayanarooj S, Charoensirisuthikul T, Kasisith J. Differences in global gene expression in peripheral blood mononuclear cells indicate a significant role of the innate responses in progression of dengue fever but not dengue hemorrhagic fever. J Infect Dis. 2008 May;197(10):1459–67.
21. Fink J, Gu F, Ling L, Tolfvenstam T, Olfat F, Chin KC, et al. Host gene expression profiling of dengue virus infection in cell lines and patients. PLoS Negl Trop Dis. 2007;1(2):e86.
22. Kanwal F, Lu C, Qadri I, Sohail M, Zia-ur-Rahman F. Interleukin-6; A promising disease severity index for dengue virus infection. Asian Pacific J Trop Dis. 2017;7(5):266–9.
23. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro-and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta (BBA)-Molecular Cell Res. 2011;1813(5):878–88.
24. Chen J, Ng MML, Chu JJH. Activation of TLR2 and TLR6 by Dengue NS1 Protein and Its Implications in the Immunopathogenesis of Dengue Virus Infection. PLoS Pathog. 2015;11(7).
25. Lee YH, Leong W-Y, Wilder-Smith A. Markers of dengue severity: a systematic review of cytokines and chemokines. J Gen Virol. 2016;97(12):3103–19.
26. Huang YH, Lei HY, Liu HS, Lin YS, Liu CC, Yeh TM. Dengue virus infects human endothelial cells and induces IL-6 and IL-8 production. Am J Trop Med Hyg. 2000;63:71–5.