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Growth characteristics and cytokine/chemokine induction profiles of dengue viruses in various cell lines

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Summary. – The dengue disease varies clinically from mild fever to hemorrhagic fever up to potentially fatal shock syndrome. Such differences in manifestation of dengue virus (DENV) infection have been ascribed to intrinsic differences in DENVs. Four DENV serotypes or four dengue viruses (DENV-1 to DENV-4) have been clearly distinguished; however, only limited information is available on their biological characteristics *in vitro*. To shed more light on this subject, replication kinetics of all four DENVs in various cell lines and induction of 26 cytokines/chemokines were investigated. The results showed that, (i) all DENVs replicated relatively similarly in various cell lines, (ii) DENV-1 was most effective in this regard, (iii) A549 cells showed the highest virus replication rate compared to other cells, and (iv) all DENVs induced in A549 cells similar levels of several cytokines/chemokines, namely eotaxin, granulocyte colony stimulating factor (G-CSF), interferon alpha 2 (IFN-α2), interleukins 6, 8 15 (IL-6, IL-8, IL-15), and IP-10. In conclusion, this study revealed a similarity in growth characteristics and cytokine/chemokine induction profile for all four DENV serotypes *in vitro*.

Keywords: dengue viruses; cell lines; growth characteristics; cytokines; chemokines

Introduction

Dengue viruses (DENVs, the genus *Flavivirus*, the family *Flaviviridae*) are the most common cause of arboviral disease with large global burden, contributing to an estimated 50 million infections annually and affecting more than 2.5 billion population living in areas with potential risk for dengue (Guzman *et al.,* 2010; Simmons *et al.,* 2012). There are four serotypes of the virus (DENV-1, -2, -3, -4) transmitted through the mosquito vectors *Aedes aegypti* and *A. albopictus*. Both viruses and mosquito vectors have expanded geographically throughout the tropical regions in the world (Gubler, 2011). The genome of DENV consists of approximately 10.7 kb positive-sense RNA which encodes three structural (C, prM/M, and E) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins (Guzman *et al.,* 2010). While infection with any of the DENV serotypes may be asymptomatic in the majority of cases, a wide spectrum of clinical symptoms may develop, ranging from a mild flu-like syndrome (dengue fever) to the most severe forms of disease, dengue hemorrhagic fever and dengue shock syndrome (Martina *et al*., 2009). Pathogenesis of DENV infection proved to be complex (Fink *et al*., 2006). DENV serotype and host immune status are thought to be factors contributing to dengue severity (Rico-Hesse *et al.,* 1997). The cellular tropism of DENV has been shown *in vivo* and many types of cell lines have been reported to support viral replication (Jessie *et al.,* 2004). However, DENV characteristics *in vitro* were reported to be influenced by cell types and viral serotypes (Diamond *et al.,* 2000; Marianneau *et al.,* 1996). The insect cell line C6/36 (*A. albopictus*) and the mammalian cell line Vero (*Cercopithecus aethiops,*

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Abbreviations: DENV(s) = dengue virus(es); DENV-1–4 = dengue virus 1–4; G-CSF = granulocyte colony stimulating factor; IFN-α2 = interferon alpha 2; IL = interleukin; p.i. = post infection

green monkey kidney) were reported to be the most sensitive cells to DENV isolation and widely used for DENV propagation (White, 1987; Gubler, 1998; Sydow *et al.,* 2000). Although *in vitro* research has been routinely used to study the underlying DENV pathogenesis mechanism, the limited information regarding viral growth characteristics in various cell models may become the limiting factor. We conducted a study to observe dengue viral growth characteristics in several cell lines, i.e. C6/36, Vero, MDCK (*Canis familiaris*, dog kidney), HEK-293 (*Homo sapiens*, embryonic kidney), HepG2 (*H. sapiens*, hepatocytes), and A549 (*H. sapiens*, epithelial alveolar). Growth characteristics were measured as replication kinetics of released viral particles quantified using plaque assay. Cytokine expression analysis was performed using Luminex™ platform. In this study, we investigated the growth characteristics of all four DENV serotypes in six different cell lines. In addition, we determined the cell-mediated response of A549 cells to the DENV infection by assaying 26 different cytokines. The results revealed a similarity in growth characteristics and cytokine induction profile for all four DENV serotypes *in vitro*.

Materials and Methods

Viruses. Four serotypes of laboratory-adapted DENV serotypes, i.e. DENV-1 WestPac, DENV-2 TSV01, DENV-3 H87, and DENV-4 H241 were used (gift from Novartis Institute for Tropical Diseases, Singapore). Virus stocks were prepared after a single passage on C6/36 cell line and titred using plaque assay method. Virus concentration was determined as plaque forming unit per milliliter (PFU/ml) and used to calculate the multiplicity of infection, MOI. Low MOI (0.01–0.1) was used to ensure that the resulting viral replication kinetics is not interfered by high number of starting virus amount.

Cell lines. The C6/36, Vero, MDCK, HEK-293, HepG2, A549, and BHK21 cell lines were obtained from ATCC. All cell lines were grown and maintained in RPMI 1640 medium supplemented with 10% of FBS, 2 mmol/l of L-glutamine, 100 U/ml of penicillin, and 100 µg/ml of streptomycin (all from Gibco-Life Technologies). C6/36 cells were cultured in 28°C incubator while mammalian cell lines in humidified 37°C incubator with 5% CO_2 supplementation.

Virus growth experiment. To determine DENV growth characteristics in six different cell lines, a viral kinetics assay was conducted. The assay was performed in duplicates. Cells were grown in T-75 flasks, detached using trypsin-0.25% EDTA (Gibco-Life Technologies), harvested, and counted using improved Neubauer hemacytometer (Superior Marienfeld, Germany). Cell viability was determined by Trypan blue exclusion and cells were seeded at 2 x 10^5 cells/well into 24-well plate. Plates were then incubated overnight at 28°C (insect cell line C6/36) or 37°C, 5% CO_2 (mammalian cell lines), to allow cells to form a monolayer. Viral stocks of four DENV serotypes were prepared in RPMI medium supplemented with 2% FBS and used to infect cell lines in designated wells using MOI of 0.01, in duplicates. Plates were then incubated for 1 hr to allow virus attachment. Inoculation media were then aspirated from wells and followed by three washes of PBS. Media were then replenished with 500 µl of fresh medium (2% FBS) and incubated for 12, 24, 36, 48, 60, and 72 hr. Supernatant was collected at designated time points, immediately stored in -80°C freezer, and titred using plaque assay.

Plaque assay. Plaque assay was performed using BHK 21 (*Mesocricetus auratus*, hamster kidney) cells according to protocol described elsewhere (Fink *et al.*, 2007). Briefly, 2x10^5 cells were seeded to 24-well plate and cultured overnight allowing to form a monolayer. Medium was removed and serial 10-fold dilutions of virus stock in RPMI medium (2% FBS) was added to individual wells in duplicates. Plates were incubated for 1 hr at 37°C in incubator with 5% CO2. Medium was aspirated from wells and replaced with 0.5 ml of 1% methyl cellulose overlay medium (2% FBS). Plates were then incubated for 5 days. Overlay was removed from wells and cells were fixed with 3.7% formaldehyde for 30 min. Cells were then stained using 1% crystal violet and rinsed in water. Plaques were counted manually.

Cytokine/chemokine induction experiment. Cytokine/chemokine induction experiment was performed in duplicates to study cytokine/chemokine profile of A549 cell line infected with DENV and the possibility of this cell line to be used as DENV cell infection model. A total number of 2 x 10^5 cells were seeded into 24-well plates. DENV infection was performed in duplicates using MOI of 0.1 and uninfected cells were used as control. Culture supernatants were harvested 72 hr post infection (p.i.) and directly stored in aliquots at -80°C freezer until use.

Cytokine/chemokine assay. A multiplex fluorescent microbead immunoassay containing fluorescent-dyed microspheres conjugated with monoclonal antibody specific to a target protein (Kellar *et al.,* 2001) was used to simultaneously detect and quantify 26 human cytokines and chemokines from culture supernatant. Milliplex MAP Human Cytokine/Chemokine Premixed 26-plex (Merck Millipore) was used according to the manufacturer's instructions to detect and quantify the level of eotaxin, granulocyte colony stimulation factor (G-CSF), granulocyte macrophage colony stimulation factor (GM-CSF), IFN-α2, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, CXCL10/IFN-inducible protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 alpha (MIP-1α), MIP-1β, tumor necrosis factor alpha (TNF-α), and TNF-β from a single sample. Briefly, 25 µl of sample was incubated with premixed beads conjugated with anti-cytokine/chemokine antibody. Complexes were washed then incubated with biotinylated detection antibody, and finally streptavidin-phycoerythrin. Concentrated human cytokine standard was provided by the vendor (Merck Millipore) and used in a range of 3.2–2,000 pg/ml of recombinant cytokines to generate standard curves. Analysis was performed using a multiplex array reader from Luminex™ Instrumentation System (LiquiChip 200 workstation from Qiagen) and Luminex™ Integrated System v.2.3 software.

Statistical analysis. Statistical analysis was done using SPSS Statistics software version 17.0 (SPSS Inc., USA). The non-parametric

Kruskal-Wallis analysis of variance by ranks test was used to compare growth characteristics between cell lines. The non-parametric Mann-Whitney U test was used to compare cytokine expression levels. The differences with $p < 0.05$ were considered statistically significant.

Results

Growth characteristics of DENVs in different cell lines

To assess the capability of various cell lines in supporting DENV replication and the replication rate of four DENV serotypes, we performed replication kinetics assays. Using the same number of MOI and experimental conditions, replication kinetics assay showed different growth characteristics of DENV serotypes in each cell line. In A549 cell line compared to other mammalian cell lines, DENVs infection had relatively highest growth (Fig. 1). Statistical analysis performed 72 hr p.i. showed significant difference in replication level of all DENV serotypes among six cell lines ($p < 0.05$). Growth kinetics of all DENV serotypes in A549 cells was comparable to or relatively higher than that in Vero cells. The higher DENV growth kinetics in A549 cells was observed when compared to virus growth in C6/36, only in DENV-1 infection while in other DENVs it was similar (DENV-3 and -4) or lower (DENV-2) (Fig. 1). Comparing the replication kinetics of all DENV serotypes in six cell lines *in vitro*, we observed that all serotypes of DENV replicate more efficiently in C6/36, Vero, and A549 cells (Fig. 1, solid lines). However, DENV replication was lower in the other 3 cell lines, i.e. MDCK, HEK-293, and HepG2 (Fig. 1, dotted

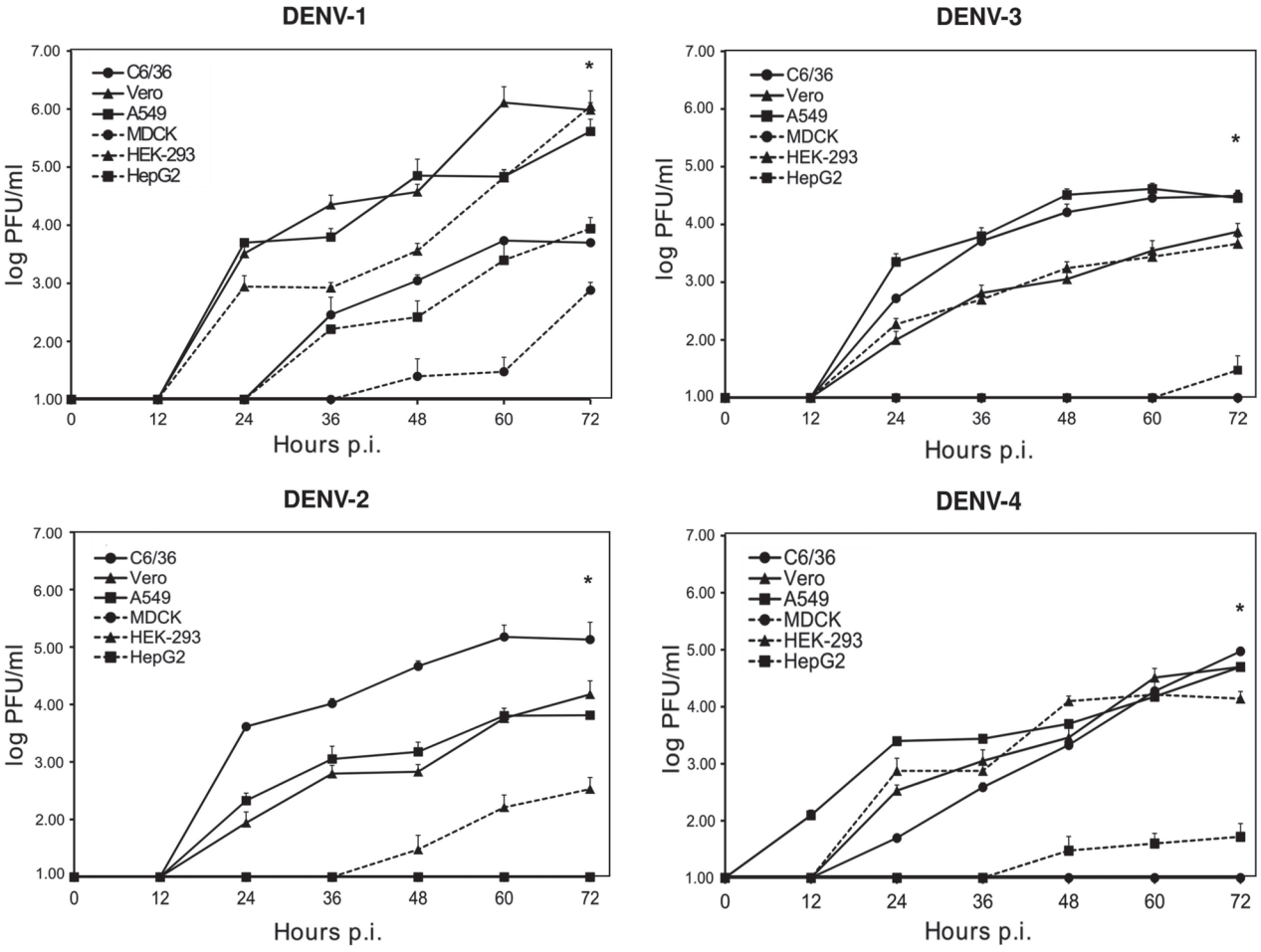

Fig. 1

Growth characteristics of DENVs in various cell lines
Bars show SD and asterisks indicate statistically significant differences.

lines). In addition, DENV-1 showed more efficient growth in all 6 cell lines compared to other serotypes, especially in MDCK, HEK-293, and HepG2 where other serotypes replicated in lower rates (Fig. 1).

Cytokine/chemokine induction profiles of DENVs in the A549 cell line

Based on the growth kinetics results, we further analyzed the ability of the A549 cell line to immunologically respond to the DENV infection. We also sought to determine whether different DENV serotypes induce different immunological responses. To answer the questions above, we performed cytokine/chemokine expression profiling of DENV-infected A549 cells using Luminex™ platform, measuring the expression of 26 cytokines/chemokines simultaneously. The expression profile of those cytokines/chemokines is shown in the Fig. 2. Among the 26 cytokines/chemokines assayed, we observed the significantly induced expression of seven proteins after 72 hrs p.i., compared to uninfected cells. Those induced cytokines/chemokines were eotaxin, G-CSF, IFN-α2, IL-6, IL-8, IL-15, and IP-10. In general, similar expression levels of those proteins were observed in all four serotypes (Fig. 2).

Discussion

The understanding of dengue pathogenesis is hampered by the lack of appropriate animal model. Numerous primary cell lineages and established cell lines have been studied for their relative permissiveness for DENV and *in vitro* cellular responses have been used to elucidate virus-host interactions (Noisakran *et al.,* 2010, Warke *et al.,* 2003). The pathogenesis of dengue is also found to be complex, especially with the presence of four DENV serotypes which have been associated with disease severity and different virulence (Balmaseda *et al.,* 2006; Nisalak *et al.,* 2003; Vaughn *et al.,* 2000). One way to estimate the virulence is by assessing the replication kinetics of the virus (Rico-Hesse, 2010). In this paper, we sought to compare the replication kinetics of four DENV serotypes *in vitro* using various cell lines as a model. This study was also intended to explore the best alternative mammalian cell lines to support the growth of four DENV serotypes, and the immune response against DENV infection.

We used plaque assay that has been regularly used to measure the amount of DENV infectious particles in samples and widely utilized to quantify viral growth or infectivity (Eckels *et al.,* 1976; Russell *et al.,* 1967; Sydow *et al.,* 2000). Comparing the growth kinetics of four DENV serotypes in six different cell lines, we observed the differing viral growths which reflect the different cell susceptibility to DENV infection. DENV-1 showed relatively more efficient growth in all six cell lines compared to other serotypes. Noticeable difference can be observed in DENV-1 growth in MDCK, HEK-293, and HepG2 cells, while other serotypes apparently were not growing well in those cells. Four serotypes of laboratory-adapted DENV serotypes were used in this study. The laboratory-adapted serotypes are widely used in dengue research among others because of their ability to grow better in tissue culture conditions compared to clinical isolates. However, without knowing their biological characteristics, the use of these serotypes may introduce bias when comparing each serotype in laboratory experiments. The replication kinetics data obtained in this study offer the information about the growth characteristics of four DENV serotypes, in which similar replication rates were observed in particular cell lines.

In parallel with the DENV serotypes growth characteristics assessment described above, our study also compared the ability of various cell lines to support DENVs growth. We compared the growth of DENVs in various cell lines to the C6/36 insect cell line and Vero mammalian cell line. The C6/36 and Vero cells were known to be the most sensitive cells for DENVs isolation and widely used for DENVs propagation (Desmyter *et al*., 1968; White, 1987). In addition, Vero has been the standard mammalian cell line for manipulation and the study of DENV due to its high susceptibility to all DENV serotypes (Sydow *et al.,* 2000). However, the use of this non-human primate-origin cell line in *in vitro* DENV infection might yield different responses compared to *in vivo* settings due to defect in its interferon system (Emeny and Morgan, 1979). In this study, we observed the ability of human epithelial alveolar A549 cell line to support DENV infection and replication much better compared to other cell lines. Our results confirm previous findings by Lee *et al.* (2007) who compared the susceptibility of six lung cancer cell lines to DENV-2 infection. We observed that replication of four serotypes of DENVs show a similar or higher kinetics in A549 cells compared to Vero cells. The establishment of A549 cell line was first described by Giard *et al.* (1973). The cell line proved to be an efficient, practical, and economical alternative cell system for the isolation of clinically significant viruses (Smith *et al.,* 1986; Woods and Young, 1988). Our data therefore further provide more information for the use of A549 cell line in dengue research.

Given the ability of A549 cells in supporting dengue growth, we further explored whether the cells are able to respond immunologically to DENV infection. We measured the expression of various cytokines/chemokines using Luminex™ assay. Among 26 cytokines/chemokines assayed, increased expressions of eotaxin, IP-10, G-CSF, IFN-α2, IL-6, IL-8, and IL-15 were observed after infection with four serotypes of DENV. The roles of the cytokines/chemokines described above have been well documented.

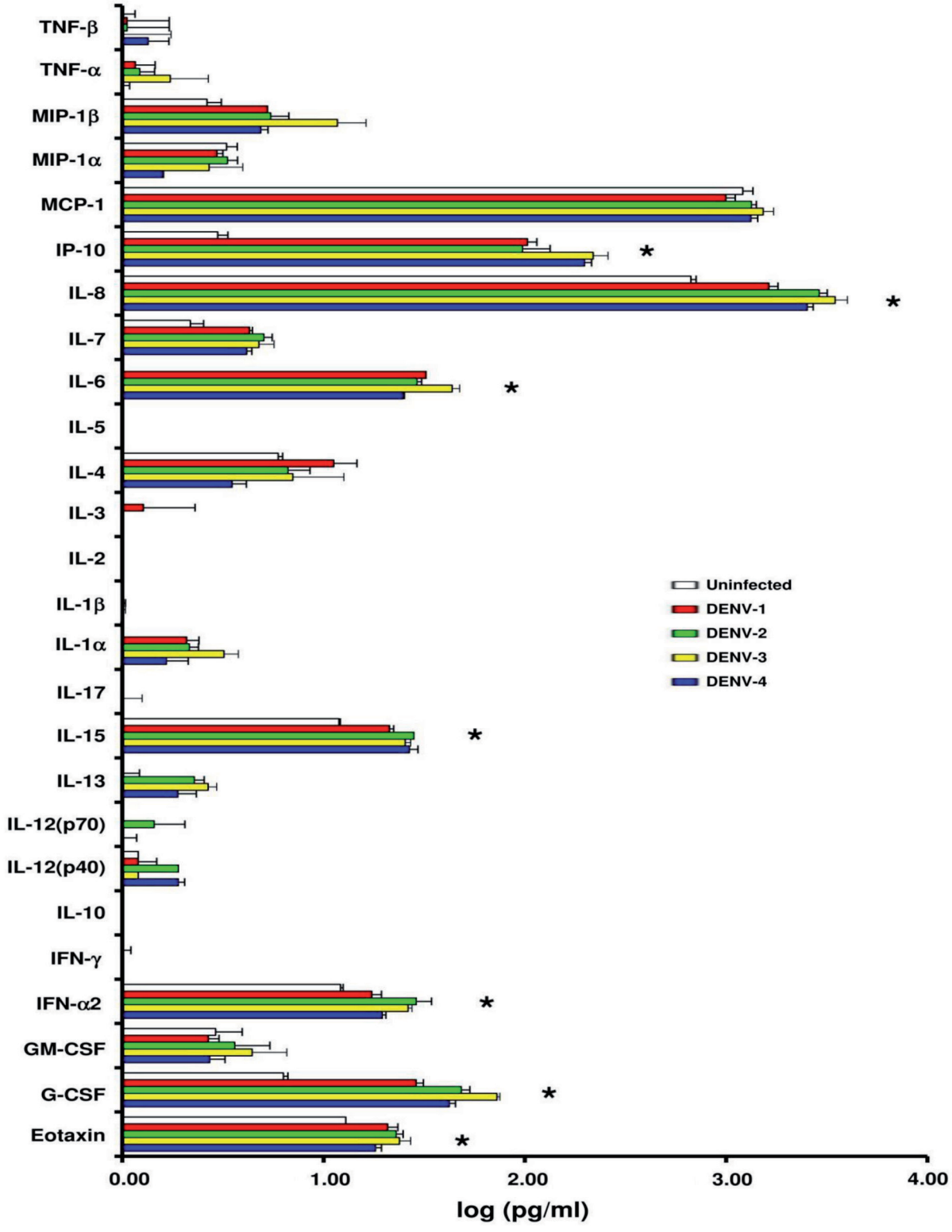

Fig. 2

Cytokine/chemokine induction profiles of DENVs in the A549 cell line
Bars show SD and asterisks indicate statistically significant differences.

Eotaxin (CCL11) is a pro-inflammatory chemokine that has a critical role in regulating the tissue level of eosinophil and leukocyte recruitment during inflammation as well as in normal conditions (Matthews *et al.*, 1998). Another pro-inflammatory chemokine that is induced upon DENV infection is the IP-10 (CXCL10). This protein is a member of CXC subfamily of chemokine family that specifically target lymphocytes (Farber, 1997). The induced expression of IP-10 has been reported in dendritic cells and other primary cell lineages in response to dengue infection *in vitro* (Becerra *et al.*, 2009) as well as in peripheral blood mononuclear cells (PBMCs) of dengue patients (Ubol *et al.*, 2008). Increased level of IP-10 has been found in serum of dengue-infected patients in two different cohort studies in Venezuela (Becerra *et al.*, 2009) and Singapore (Fink *et al.*, 2007). It has also been proposed that IP-10 was able to inhibit viral binding to target cells *in vitro* through competition of binding to heparan sulfate on the cell surface (Chen *et al.*, 2006).

The cytokines that were induced upon DENV infection observed in this study were G-CSF, IFN-α2, IL-6, IL-8, and IL-15. The G-CSF plays role in the basal regulation of neutrophil production and its response to inflammatory stimuli (Demetri and Griffin, 1991). The production of G-CSF as neutrophil chemotactic activity in A549 cells has also been reported (Koyama *et al.,* 1998). The IFN-α2 is a member of interferon type I family considered as a major antiviral substance with effects on both cell-mediated and humoral immune response (Gresser, 1997). The increased level of IL-6 has been reported in dengue patients and considered to play role in dengue pathogenesis (Hober *et al.,* 1993; Nguyen *et al.,* 2004). In addition, the increased level of IL-6 has been correlated to disease severity with higher level observed in more severe form of dengue manifestation (Hober *et al.,* 1993; Juffrie *et al.,* 2001). IL-8 is a neutrophil chemoattractant produced by macrophages and other cell types, such as endothelial cells, fibroblasts, and synovial cells. The secretion of IL-8 by alveolar epithelial A549 cell line has been reported (Standiford *et al.,* 1990). It has also been reported that increased levels of IL-8 in the sera and IL-8 mRNA in PBMCs of dengue patients were correlated with severe form of dengue (Raghupathy *et al.,* 1998). Extremely high level of IL-8 was also found in plasma and pleural fluid of patients with DSS manifestation and the induced expression was observed in DENV-infected human endothelial cells *in vitro* (Avirutnan *et al.,* 1998). IL-15 has important effects on cells of innate and adaptive immune response and is crucial to development and survival of cells of innate immunity, including natural killer (NK) cells, dendritic cells, and macrophages (Ohteki, 2002).

Together, the increased expression of these 7 cytokines/chemokines may resemble early innate immune responses to DENV infection. Similar profiles were observed in all four serotypes and the level of expression may be correlated with viral replication profile. Our data support the use of A549 cell line as DENV cell infection model in previous studies in gene expression profiling and signaling pathways (Fink *et al.,* 2007; Umareddy *et al.,* 2008).

The use of A549 cell line in DENV research was based on the evidence of viral antigen detection in macrophages and vascular endothelial cells of the lung tissue (Jessie *et al.,* 2004). Despite the fact that A549 cells were derived from epithelial origin, infection of several human cell lines with DENV-2 showed the superiority of these cells to support viral replication when compared to several other human-derived cells i.e. HepG2, SK-Hep1, K562, HUV-EC-C, THP-1, and HeLa (Fink *et al.,* 2007). DENV-2 infection of A549 and other lung cancer cell lines showed susceptibility of A549 to DENV and highlighted the high level of IL-6 and RANTES expression which may be correlated with lung related immuno-pathogenesis (Lee *et al.,* 2007).

This is the first study, to the best of our knowledge, to assess the growth kinetics and cytokine expression of four DENV serotypes in various cell lines and highlights the similar growth characteristics and cytokine/chemokine induction capability of all laboratory-adapted DENV serotypes. In addition, we observed the comparable susceptibility of DENV infection of A549 cells to Vero cells. Since Vero cells has become standard cell model, this result may support the use of A549 cells as an alternative mammalian cell model of human origin for DENV infection *in vitro*. This cell line may be valuable for the study of replication kinetics, cytokine induction profiling of different virus serotypes such as clinical isolates, and other *in vitro* studies.

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