

# T HELPER (TH) 1 AND TH2 CYTOKINE EXPRESSION PROFILE IN DENGUE AND MALARIA INFECTION USING MAGNETIC BEAD-BASED BIO-PLEX ASSAY

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**Abstract.** Dengue and malaria infections are two very common vector-borne diseases annually affecting millions of people around the world. Both diseases show a variety of clinical presentations, ranging from mild symptoms of dengue fever (DF) to severe dengue hemorrhagic fever (DHF) in dengue infection, and low and high parasitemia in malaria infection. T helper (Th)1 and Th2 cytokine expressions in mild and severe forms of dengue virus type-2 (DENV-2) and *Plasmodium falciparum* infection, were compared to normal human sera using high throughput magnetic bead-based Bio-Plex assay. A significant elevation of Th1 and Th2 cytokines expression [interleukin (IL)-2, IL-4, IL-5, IL-10, IL-13, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)- $\gamma$ , and tumor necrosis factor (TNF)- $\alpha$ ] was detected in DENV-2 and *P. falciparum* malaria infections compared with normal controls ( $p < 0.05$ ). DENV-2 infection showed a slight higher expression of Th1 and Th2 cytokines in DHF than DF, except for IL-13. In *P. falciparum* infection, high parasitemia showed a significantly higher expression of IL-4, IL-10, GM-CSF, and TNF- $\alpha$  ( $p < 0.05$ ). Both DENV-2 and *P. falciparum* malaria infections manifested high IL-10 expression, greatest among the cytokines examined, and in the severe forms of infection. The results of this study should lead to a better understanding of pathogenesis of dengue infection and *P. falciparum* malaria.

**Keywords:** dengue, malaria, T helper, cytokines, Bio-Plex

## INTRODUCTION

Mosquito-borne diseases such as dengue and malaria are the most common diseases in tropical countries. Global warming has increased the risk of in-

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creasing mosquito populations and expansion of the problem into sub-tropical areas (SeiDel *et al*, 2008; BezirtZoglou *et al*, 2011). Dengue virus (DENV) infection is an emerging disease already affecting more than 100 countries, with 2.5 billion people annually at risk (WHO, 2012). The spectrum of dengue disease is broad, ranging from mild symptoms similar to flu [dengue fever (DF)], to the severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Deen *et al*,

2006). Clinical signs and symptoms are used in diagnosing suspected cases, then confirmed by subjecting sera samples to laboratory techniques, such as cell culture, ELISA and PCR (CDC, 2012).

Malaria is another important mosquito-borne disease, caused by the *Plasmodium* protozoa group. Symptoms in the mild form are fever, nausea, vomiting, and myalgia; symptoms of the severe form are anemia and cerebral malaria due to *Plasmodium falciparum*. Malaria severity is closely related to parasitemia in the early stages of infection, with signs and symptoms of low to high severity appearing later on (Trampuz *et al*, 2003). The gold standard method of diagnosing malaria infection is light microscopy using thick or thin film technique (Wongsrichanalai *et al*, 2007).

Both dengue and malaria are transmitted by infected female mosquitoes. Upon being infected, immune cells of the human host are initiated to function as part of the innate immune system (Hoffmann *et al*, 1999). Cytokines are expressed to induce T cells in particular CD4+T cells into developing into T helper (Th) 1 and Th 2 cells. Th1 cell produces interleukin (IL)-2, interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , and IL-12; and Th2 cell produces IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (Constant and Bottomly, 1997; Agnello *et al*, 2003). Cross-regulation of expression between the two groups of cytokines is necessary to ensure normal homeostasis. However, in certain conditions there is excessive release of IL-4, IL-5, IL-6, IL-10, IL-13, and TNF- $\alpha$ , which lead to increase in vascular permeability and severe vascular leakage as seen in DHF (Chaturvedi *et al*, 2000; Basu and Chaturvedi, 2008); and in malaria infection, excessive expression of IFN- $\gamma$  and TNF- $\alpha$  along with nitric oxide (NO) pro-

notes enhancement of malarial anemia (Perkins *et al*, 2011) and increased TNF- $\alpha$  is associated with cerebral malaria (Angulo and Fresno, 2002).

Identification of these cytokines can potentially serve as biomarkers of infection, especially in the early stages of dengue and malarial infection. Traditionally, study of cytokine expression involves time-consuming procedures using a large amount of samples per experiment (Thorpe *et al*, 1999). Thus a high throughput technique has been established to identify the expressed cytokines, known as magnetic bead-based Bio-Plex (Bio-rad, 2012), which uses a series of color-coded magnetic beads, each coupled to a unique antibody specific for a biochemical marker (*viz* cytokine), allowing simultaneous detection of many cytokines at the same time.

This study aimed at finding biomarker(s) for early detection of DENV-2 infection (DF and DHF) and *P. falciparum* malaria infection (low and high parasitemia) through the immune response of Th1 and Th2 cytokine expression, compared with normal human control.

## MATERIALS AND METHODS

### Serum samples

Dengue-infected sera were DENV-positive (using NS1 strip; Biorad, Hercules, CA). Stored specimens were kindly provided by Professor Krisana Pengsaa, Department of Tropical Pediatrics, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. The NS1-positive sera were identified as DENV-2-positive by RT-PCR and nested PCR (Lanciotti *et al*, 1992).

Sera from malaria infected subjects were stored specimens, and were diagnosed as low or high *P. falciparum* parasitemia by thick and thin film technique

(Murray *et al*, 2008). A blood smear with *P. falciparum* parasite density of 8,000-10,000 asexual parasites/ $\mu$ l was identified as being low parasitemia (Lo Pf), while *P. falciparum* parasite density > 200,000 asexual parasites/ $\mu$ l was considered as high parasitemia (Hi Pf). These sera were kindly provided by Professor Srivicha Krudsood, Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University.

Sera from healthy subjects, with normal complete blood count examination and no evidence of fever during three days before collection were obtained from 10 healthy volunteers. Ethical approval of the project was obtained from the Ethics Committee of Faculty of Tropical Medicine, Mahidol University (MUTM 2012-017-01).

The stored sera from DENV-2 infection (DF and DHF) and from *P. falciparum* malaria infection were quick-thawed and centrifuged at 800g for 10 minutes, then kept on ice. Healthy human sera were similarly treated. Aliquots of 20  $\mu$ l from each sample were stored at -70°C until used.

#### Quantitation of cytokines expression level

The Th1 and Th2 Bioplex cytokine kit (Bio-Rad, Hercules, CA) used is a multiplex biometric immunoassay, containing fluorescent microspheres conjugated with monoclonal antibodies specific to Th1 and Th2 series: IL-2, IL-4, IL-5, IL-10, IL-13, GM-CSF, IFN- $\gamma$ , and TNF- $\alpha$ . DENV-2 serum samples of dengue fever, dengue hemorrhagic fever, Lo Pf, and Hi Pf, as well as normal human sera (NHS) were analyzed according to the manufacturer's instructions. In brief, 20  $\mu$ l aliquot of sample was diluted 1:4 with sample diluent, incubated with antibody-coupled beads, washed, incubated with biotinylated secondary antibodies, and finally incubated

with streptavidin-phycoerythrin. Data were analyzed using Bioplex Manager Software.

#### Statistical analysis

SPSS statistical analysis was used for nonparametric Kruskal-Wallis one-way ANOVA, and Wilcoxon rank-sum test.

## RESULTS

Cytokine profiles were generated from 50 stored sera of Thai patients infected with DENV-2 (DF and DHF) or *P. falciparum* malaria (Lo Pf and Hi Pf) and from NHS using multiplex bead-based Bio-Plex assay. The results (Table 1) reveal that the overall expression level of each cytokine tested among the two patients' groups (DENV-2 infection and *P. falciparum* malaria infection) are significantly higher than normal human sera. Between dengue and malaria infection, IL-10, IL-2 and TNF- $\alpha$  levels are statistically higher in the malaria group than the dengue group (Fig 1). IL-10 had the highest levels in DHF and in Hi Pf groups. In addition, Hi Pf infection group shows statistically higher expression of IL-10, GM-CSF, and TNF- $\alpha$  than Lo Pf.

## DISCUSSION

In infection, cell-mediated immune response (CMIR) predominates and affects pathogenesis earlier than humoral mediated immune response (HMIR). Antigen presenting cells, such as activated dendritic cell, together with macrophage, soluble mediators such as growth factors and cytokines activate T cells to become cytotoxic T cells, which then destroy infected cells. IL-12 induces CD4+ T cells to differentiate to Th1 cells, while IL-4 induces CD4+ T cells to become Th2 cells. Th1 cells express cytokines (*viz.* IL-2, IFN- $\gamma$ ,

Table 1  
Expression of Th1 and Th2 cytokines.

Cytokine	Median level (min, max) (ng/μl)			p-value
	Dengue (n = 20)	Malaria (n = 20)	NHS (n = 10)	
IL-2	14.5 (8.5,169.5)	29.0 (6.0, 59.1)	7.9 (7.5, 8.8)	< 0.001
IL-4	10.0 (6.0, 173.0)	9.4 (6.0, 19.7)	6.7 (6.5, 7.5)	0.001
IL-5	6.8 (2.5, 249.0)	4.1 (3, 6.4)	3.4 (2.5, 4.0)	0.006
IL-10	396 (31.0, 1,882)	3,170 (1,600, 8,172)	37.0 (32.5, 39)	< 0.001
IL-13	11.0 (7.0, 64.5)	8.9 (7.4, 11.7)	8.0 (6.8, 9.8)	0.007
GM-CSF	14.5 (7.5, 226)	12.0 (6.5, 21.0)	8.0 (7.5, 8.7)	0.005
IFN-γ	15.8 (6.0, 273)	10.7 (6.0, 19.0)	6.8 (5.8, 7.4)	< 0.001
TNF-α	7.0 (5.0, 54.8)	16.5 (6.5, 44.7)	5.9 (5.0, 7.0)	0.001

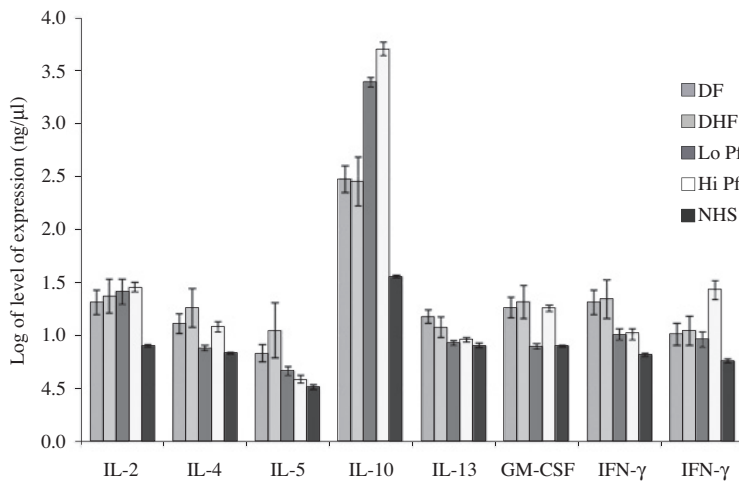


Fig 1—Cytokine levels in sera of dengue and malaria patients. Cytokines were determined using multiplex bead-based Bio-Plex assay. DF, dengue fever; DHF, dengue hemorrhagic fever; Lo Pf, low parasitemia of *P. falciparum*; Hi Pf, high parasitemia of *P. falciparum*; NHS, normal normal human sera.

ular leakage (Croft, 2009). Th2 cells express cytokines that are related to disease progression and are mostly co-factors which promote antibody production (Torre *et al*, 2002).

Cross-regulation of Th1 and Th2 cells is necessary to produce homeostasis. IL-4 and IL-10 from Th2 cells inhibit Th1 cytokines expression, and vice versa, IFN-γ from Th1 inhibits Th2 cytokines expression. However, disproportionate or uncontrolled cytokine expression in immune re-

sponse can occur, resulting in a state of severe disease. Th1 cytokines can also cause over-expression of Th2 cytokines (Roelant and Meirleir, 2012), some of which increase permeability of vascular endothelium cells lining the blood vessels and capillaries. In dengue infection, this condition results in the clinical manifestation of bleeding in DHF (Chaturvedi *et al*, 2000; Basu and Chaturvedi, 2008). In the case of malaria infection, an imbalance of

TNF-α) to resolve infections and clear pathogens, with IL-2 activating macrophage and IFN-γ controlling intracellular pathogens by promoting CMIR, which also depends on IL-12 (Agnello *et al*, 2003; Fagundes *et al*, 2011). TNF-α has been implicated in tumor regression and septic shock, as it stimulates production of IL-1 and IL-6, which increase accumulation of platelets with endothelial cells, increasing vascular permeability, and results in vas-

Th1 and Th2 cells is one of the immune responses that can occur in severe and fatal cerebral malaria disease syndrome (Schofield and Grau, 2005).

This study selected eight cytokines (IL-2, IL-4, IL-5, IL-10, IL-13, GM-CSF, IFN- $\gamma$ , and TNF- $\alpha$ ) of Th1 and Th2, and measured their expression level in DENV-2 and *P. falciparum* infections. A high throughput magnetic bead-based technique was used, which requires low volume (20  $\mu$ l) of sample allowing measurement of the cytokines in only one experiment. DENV-2 infection showed a higher expression of cytokines (IL-5, IL-10, and IFN- $\gamma$ ) in DHF than DF, similar to the studies of Bozzan *et al* (2008) and Espada-Murao and Morita (2011), which showed IFN- $\gamma$  to be a predictive factor for dengue severity. IL-10, GM-CSF, and TNF- $\alpha$  are significantly increased in Hi Pf infection, in agreement with Kern *et al* (1992), who showed TNF- $\alpha$  is correlated with parasitemia and disease severity. In malaria infection, Th1 cytokines expression is involved in the clearance of *P. falciparum* (Angulo and Fresno, 2002; Torre *et al*, 2002).

In summary, this study showed changes in Th1 and Th2 cytokine expressions profiles in severe DENV-2 and *P. falciparum* infections. Employment of the bead based multiplex, Bio-Plex assay allowed measurement of numerous cytokine expressions at the same time and in a short period of time (within 3 hours). The cytokine expression profile obtained in the current study will help to guide us in understanding pathogenesis, in improving treatment, and in developing vaccines against dengue and malaria.

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