THE REGULATIONS OF CYTOKINES AND CHEMOKINES IN DENGUE VIRUS-INFECTED PATIENTS

By

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ABSTRACT

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Chan Li Ching

Dengue virus (DV) infection affects millions of people and it is considered a major human arbovirosis. Although the clinical manifestations of dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) have already been described, the immunopathogenesis of these diseases is still not completely understood. The key pathological feature of DV infection is the increased levels of vasoactive cytokines that increase vascular permeability leading to plasma leakage into the interstitial space. In this study, the serum levels of eight vasoactive cytokines, namely interleukin (IL)-6, IL-8, IL-15, IL-16, interferon-gamma (IFN-γ), monocytes chemoattractant 1 (MCP-1), monokine induced by gamma interferon (MIG) and RANTES (regulated upon activation, normal T-cell expressed and secreted) in the DV-infected patients were measured by using enzyme-linked immunosorbent assay (ELISA). The results showed that the DV-infected patients had significant elevated serum levels of IL-6, IL-8, IL-15, IL-16, IFN-γ, MCP-1 and MIG as compared to the healthy controls ($p<0.05$). However, the serum level of RANTES was found to be significantly down-regulated as compared to the healthy controls ($P<0.05$). Therefore, these cytokines, except RANTES, play

a vital role in the immunopathogenesis of DV infection. Besides, a proposed cytokine network and mechanism was also suggested to represent the possible immunopathogenesis of DV infection.

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APPROVAL SHEET

This thesis entitled "**THE REGULATIONS OF CYTOKINES AND CHEMOKINES IN DENGUE VIRUS-INFECTED PATIENTS"** was prepared by CHAN LI CHING and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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SUBMISSION OF THESIS

It is hereby certified that CHAN LI CHING (07UEM08592) has completed this thesis entitled "**THE REGULATIONS OF CYTOKINES AND CHEMOKINES IN DENGUE VIRUS-INFECTED PATIENTS** under the supervision of Dr Alan Ong Han Kiat (Supervisor) from the Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, and Dr Chye Soi Moi (Co-Supervisor) from the Faculty of Pharmacy and Health Sciences, International Medical University.

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

CHAN LI CHING

17th August 2011

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
APC	Antigen-presenting cells
Bcl-2	B-cell lymphoma-2
C	Nucleocapsid
CCL	Chemokine (C-C motif) ligand
DC	Dendritic cells
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DV	Dengue virus
DSS	Dengue shock syndrome
E	Envelope
ENA-78	Epithelial neutrophil activating peptide 78
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
HCV	Hepatitis C virus
HepG2	Human liver carcinoma cell line
HIV	Human immunodeficiency virus
HMC-1	Human mast cells line-1
HMEC-1	Human dermal microvascular endothelial cells-1
HRP	Horseradish peroxidase
HUVEC	Human umbilical cord veins endothelial cells
H_2O_2	Hydrogen peroxide
IFN	Interferon

IL	Interleukin
IP-10	IFN-gamma inducible protein-10
I-TAC	Interferon-inducible T-cell alpha chemoattractant
JE	Japanese encephalitis
kb	kilo-base pair
kDa	kilo-Dalton
KU812	Human basophil cells line
LCF	Lymphocyte chemoattractant factor
M	Membrane-associated
MAb	Monoclonal antibody
MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility complex class
MIG	Monokine induced by interferon-gamma
MIP	Macrophage inflammatory protein
NK	Natural killer
NKT	Natural killer T
NS	Nonstructural
O.D.	Optical density
PBMC	Peripheral blood mononuclear cells
PDGF	Platelet-derived growth factor
RANTES	Regulated upon activation, normal T-cell expressed and secreted
SARS	Severe acute respiratory syndrome
TGF	Transforming growth factor
Th1	Type 1 helper T

Th2	Type 2 helper T
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor

CHAPTER 1

INTRODUCTION

Dengue is the most important mosquito-borne disease in the world in terms of morbidity, mortality, and economic costs, (Sang, Cuzzubbo, & Devine, 1998) with an estimation of up to 100 million of individuals infected annually (King, Anderson, & Marshall, 2002). About 0.5% of these infections result in potentially fatal dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Khanam, Khanna, & Swaminathan, 2006). Besides, this disease is also affecting more than 100 countries in the tropical and subtropical regions which include Africa, Americas, India, Southeast Asia and Western Pacific.

Dengue is transmitted from human to human mainly by the mosquito *Aedes aegypti* (McBride & Bielefeldt-Ohmann, 2000). This mosquito is a highly domesticated mosquito that lives in urban environments, breeding in small collections of clean water in and around human habitats (Jacobs, 2005). Only the females seek blood meals and they feed principally during the day, and feed repeatedly on different hosts, enhancing their role as vectors (Solomon & Mallewa, 2001). Besides, *Aedes albopictus* also plays an important role as a vector in the dengue virus (DV) transmission (Clyde, Kyle, & Harris, 2006). This vector prefers common forested habitats in suburban and rural areas. The microhabitats of *A. albopictus* larvae are mainly found in tree holes and a wide variety of containers including natural and artificial containers (Cecilio,

Campanelli, Souza, Figueiredo, & Resende, 2009). In addition, *Aedes polynesiensis* was found to be another *Aedes* mosquito that amplifies the spreading of dengue (Cao-Lormeau, 2009).

In the transmission of the DV, the female *Aedes* mosquito must bite an infected human during the viraemic phase of the illness. This viraemic phase of DF generally last 4 to 5 days but may also last up to 12 days (McBride & Bielefeldt-Ohmann, 2000). Then, this female *Aedes* mosquito becomes infective after an extrinsic incubation period of 7–14 days (Teo, Ng, & Lam, 2009). During this time, viral replication occurs in different mosquito tissues and the virus finally infects the salivary glands of the mosquitoes (Cao-Lormeau, 2009). DV is then transmitted back to humans by *Aedes* mosquito bites. The incubation period of 4-6 days occurred and is followed by viraemic phase of illness (Simasathien & Watanaveeradej, 2005). The cycle is the repeated and caused the spreading of this viral infection.

DV is a lipid-enveloped RNA virus that belongs to the family *Flaviviridae* (Chareonsirisuthigul, Kalayanarooj, & Ubol, 2007). The genomic RNA is approximately 11 kb in length and is composed of three structural protein genes, which includes nucleocapsid (C) protein, a membrane-associated (M) protein, an envelope (E) protein and seven nonstructural (NS) protein genes (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) (Kumarasamy et al., 2007). The largest structural protein is the E protein, which consists of 500 amino acids in three antigenic domains. It is thought to be important in viral entry into host cells, as well as being the major target of the humoral immune

response (Guzmán & Kourí, 2001) which involves the induction of neutralizing antibodies and development of protective immune response in the host (Pang, Cardosa, & Guzman, 2007). M protein is thought to be involved in virion release (Clyde et al., 2006) and the C protein is a structural protein involved in virion assembly (Solomon & Mallewa, 2001).

There are four antigenically distinct serotypes (dengue serotype-1, 2, 3 and 4) (King, Anderson, & Marshall, 2002) for DV. Any of the four DV can cause dengue infection (Azizan et al., 2006) and patients infected by DV may display DF, or a more severe form of dengue called DHF or DSS (Lee et al., 2007). DF is a self-limiting illness characterized by fever, headache, myalgia, arthralgia, nausea, mild thrombocytopenia, fatigue (V´azquez et al., 2005) and sometimes with the presence of rash (K. Huang et al., 2000). The higher viral level is DHF, and it is the potentially life-threatening form of DV infection (Pichyangkul et al., 2003). World Health Organization (WHO) categorizes DHF into four grades (WHO, 1997), from less severe form of illness in grade I to more severe form of illness in grade IV. Grade I is characterized by fluid leakage where there is an increment of haematocrit by 20%, presence of thrombocytopenia (<100 000/mL) and haemorrhage manifestation or positive tourniquet test. Frank bleeding (e.g. gum, nose or gastrointestinal bleeding) defines the occurrence of grade II. Plasma leakage in DHF grades III and IV, also known as DSS, can be so profound that shock (undetectable blood pressure) can occur (Lee et al., 2007). Cases of DHF/DSS can be fatal and have a case fatality rate as high as 44%, unless plasma leakage is corrected early (Azizan et al., 2006). Although infection with one serotype of DV

confers life-long protective immunity to that serotype, it does not protect the host from infection with other serotypes (Fink et al., 2007). Hence, infection with one serotype does not induce solid immunity to the others, and individuals may be infected with dengue more than once (Jacobs, 2005).

The immune system contributes to the maintenance of physiological integrity of the body mainly by eliminating foreign material and infectious pathogen (Chaturvedi, Shrivastava, & Upreti, 2004). As soon as viruses try to establish a site of infection, the host launches a complex defense system. Innate immunity is a non-specific response and serves as the first-line of defense where phagocytes, such as neutrophils and macrophages, and natural killer (NK) cells play central roles in neutralizing and clearing of pathogen (Matsukawa, Hogaboam, Lukacs, & Kunkel, 2000). Whilst the adaptive immune response, which provides long-lasting protection, takes days to develop and requires somatic mutations leading to the development of antigen-specific T cell receptor (cell-mediated immunity) and immunoglobulins (humoral immunity). Members of the cytokine and chemokine superfamily are crucially involved in both innate and adaptive immune responses (Esche, Stellato, & Beck, 2005).

Cytokines are proteins of low molecular weight and are locally acting tissue hormones that normally function as autocrine and paracrine regulators (Berczi & Szentivany, 2003). They comprise of interleukins (IL), tumor necrosis factors (TNF), chemokines, interferons (IFN), mesenchymal growth factors, transforming growth factor β and others (Sommer, 2006). Cytokines regulate

growth, differentiation, function, inhibition and apoptosis of cells in various tissues (Berczi & Szentivany, 2003). The subclass of cytokine are named chemokines, which comprised of a large family of small proteins consisting of 80-130 amino acids with molecular masses ranging from 6–14 kDa (Seet & McFadden, 2002). They have a homologous structure consisting of anti-parallel β-strands with connecting loops held together by disulfide bonds between cysteine residues (Melchjorsen, Sørensen, & Paludan, 2003). Chemokines are further subclassified on the basis of the arrangement of the cysteine residues located in the N-terminal region, as designated C, CC, CXC, and CX3C members, in which C represents the number of cysteine residues in the N-terminal region and X denotes the number of intervening amino acids in between the first two cysteines (Esche et al., 2005). The CXC subfamily is sometimes further classified into ELR+ and ELR- types based on the presence or absence of a triplet amino acid motif (Glutamic acid-Leucine-Arginine) that precedes the first cysteine residue in the primary structure of these chemokines (Chensue, 2001). The CXC ELR+ chemokines are predominantly neutrophil attractants and activators, while the CXC ELR- chemokines are mainly chemotactic for T cells (Le, Zhou, Iribarren, & Wang, 2004). Upon infection, viral products trigger the release of cytokines, which in turn are potent inducers of chemokines. Primary cytokines act as endogenous activators of the immune response, whereas inducible chemokines act as secondary mediators to attract leukocytes. Interaction between cytokines and chemokines will then enhance the antiviral response in the host (Gouwy, Struyf, Proost, & Damme, 2005).

There are a number of research that demonstrated the elevation levels of certain cytokines during DV infection (Medin, Fitzgerald, & Rothman, 2005) and thus, cytokines and chemokines have been thought to play an important role in the immunopathogenesis of DV infection (Chen & Wang, 2002). In this study, a number of cytokines and chemokines namely IL-6, IL-8, IL-15, IL-16, IFN-γ, MCP-1, MIG and RANTES, were chosen to study the immunopathogenesis of DV infection. IL-6, IL-8, IFN-γ and MCP-1 were chosen in this research since these cytokines and chemokines are associated with thrombocytopenia (Bozza et al., 2008; Rachman & Rinaldi, 2006) and plasma leakage (Lee et al., 2007; Medin et al., 2005; Schroder, Hertzog, Ravasi, & Hume, 2004; Yamada et al., 2003), where both are seen present in dengue patients. Thus, these cytokines and chemokines might have contributions towards the immunopathogensis of DV infection. In addition, IL-6 and IL-8 are endogenous pyrogen (Pela´ et al., 2000; Restrepo et al., 2008b), which might cause fever to dengue patients. Hence, these cytokine and chemokine were chosen in this research. Furthermore, cytokine rarely, if ever, act alone in an immune system. A combined effect of two or more cytokines is sometimes greater that the additive effects of the individual cytokines. This combined effect of the cytokines ensures the outcome where the magnitude of defense system is sufficient to clear invading virus.

Hence, the objective of this study is to highlight the point of which cytokines and chemokines contribute to the immune response of DV-infected patients. Also, this research was aimed to propose the possible correlation of the named cytokines and chemokines and the possible immunological pathways involved

by these cytokines and chemokines that leads to the pathogenesis of this disease.

CHAPTER 2

LITERATURE REVIEW

2.1 Epidemiology of Dengue

Dengue epidemics that are clinically compatible with dengue fever (DF) occurred as early as 1635 and 1699 in the West Indies and Central America respectively. Another literature also revealed an epidemic of knee fever both in Cairo, Egypt and Jakarta, Indonesia in 1979 (Simasathien & Watanaveeradej, 2005). However, the first accurate clinical description of DF was disclosed by Benjamin Rush from Philadelphia, USA in 1780 where he named this disease as "break bone fever" (Solomon & Mallewa, 2001). During the Second World War South-East Asia experienced the co-circulation of multiple DV serotypes and epidemic activity increased (Rigau-Pérez et al., 1998). Co-circulation of multiple serotypes resulted in the emergence of epidemic DHF in 1950s. The first outbreak of DHF in Asia was recognized in Manila, Philippines in 1953-1954 (Chaturvedi & Nagar, 2008) followed by an outbreak in Bangkok, Thailand, in 1958. Singapore, Malaysia and Vietnam experienced this DHF epidemic in the 1960s (Teo et al., 2009). Furthermore, the situation had worsened where there was a dramatic increase in frequency and in geographic extension of DF in Latin America and Brazil in the last two decades (Bozza et al., 2008). It is now the most widely distributed mosquito-borne virus disease and occurring in mostly every tropical and subtropical country (Figure 2.1).

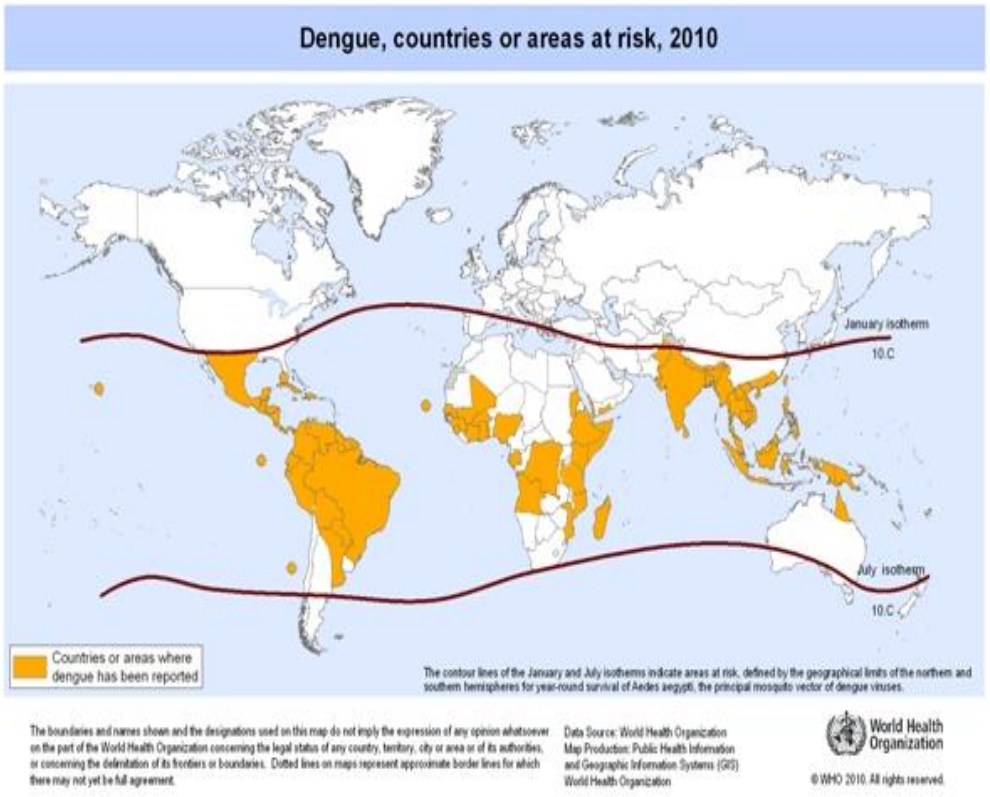

Figure 2.1: Countries or areas at risk of dengue transmission in 2010 (WHO, 2010)

In Malaysia, dengue continues to be a major health threat a century after its first reported outbreak in 1902 (Chua et al., 2006). Examination of the available outbreak data suggested that the major DF/DHF outbreak occurred in Malaysia in a cyclical pattern of approximately every 8 years (Abubakar & Shafee, 2002). However, the outbreak of disease had now changed to occur yearly (Senior, 2007). According to the data from Ministry of Health (MOH), Malaysia (2009), the incidence rate (per 100,000 population) of dengue in Malaysia had increased from 50.92 in the year 2004 to 167.76 in the year 2008. Also the mortality rate (per 100,000 population) of dengue was within 0.02

through out these 5 years (Figure 2.2). Thus, this disease had brought a big impact to health, social and even economy for this country.

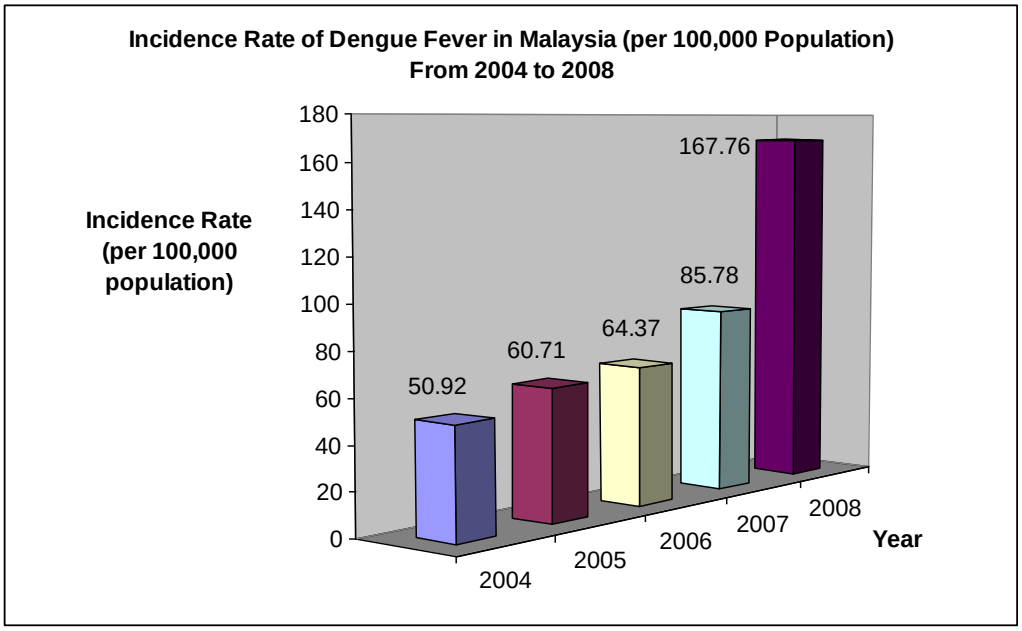

Figure 2.2: Incidence rate of dengue fever in Malaysia (per 100,000) population from 2004 to 2008 (MOH, 2009)

2.2 Pathophysiology of Dengue

The immune system plays an important role in the maintenance of physiological integrity of the body. It eliminates foreign materials and infectious pathogens that invade the host. This process is mediated through innate and adaptive immunity, which is a complicated process involving coordinated efforts of several types of cells and their secretory products (Chaturvedi et al., 2004). Cytokines are proteins secreted during innate and adaptive immunological responses, acting as inflammatory mediators or modulator molecules. During DV infections, cytokines are involved in the

disease onset and homeostatic regulation (Bozza et al., 2008). Also, they play an important role in the pathogenesis of DV infection (Chen & Wang, 2002).

There are numbers of research that investigated the regulations of cytokines in the virus infected cells (Cardier et al., 2006). King et al. (2002) showed that elevated levels of secreted macrophage inflammatory protein (MIP)-1 α , MIP-1 β, and regulated upon activation, normal T-Cell expressed and secreted (RANTES), but not IL-8 or epithelial neutrophil activating peptide (ENA)-78, were observed following infection of human basophil cell line (KU812) or human mast cells line (HMC-1) with DV. Chen and Wang (2002) further demonstrated the release of other cytokines and chemokines in DV-infected monocyte and macrophage, including TNF-α, IFN-α, IL-1β and IL-12, but not IL-6, IL-15, or nitric oxide. The up-regulation of TNF- α in DV-infected cell was confirmed by Lin et al. (2002) where they showed that infection by DV on B cells induced the production of TNF-α. However, Lin et al. (2002) found the infected B cells induced the production of IL-6, which was contradictory to the results obtained by Chen and Wang (2002). These contradictory results might be due to the difference in the cell line used in the respective research. On the other hand, a number of different cytokines was studied by Azizan et al. (2006). They reported that there was an increased level in IL-4, IL-10, INF-γ and granulocyte macrophage colony stimulating factor (GM-CSF), within DV-infected pulmonary endothelial cell line HPMEC-ST1.6R. Also, higher levels of IL-6 and RANTES were released from DV-infected human primary lung epithelial cells (Lee et al., 2007)

Serum levels of certain cytokines were also reported to be elevated during DV infection (Chen & Wang, 2002). Elevation of IL-12 level in dengue patients was demonstrated by Pacsa et al. in 2000. Mustafa, Elbishbishi, Agarwal, & Chaturvedi (2001) further demonstrated that IL-13 and IL-18 were up-regulated in DF patients and also have a higher magnitude in DHF patients. Increased levels of other cytokines and chemokines, including IL-2, IL-6, IL-8, IL-10, IFN-γ, TNF-α and monocyte chemoattractant protein (MCP)-1, were also observed in patients with DV infection (Lin et al., 2005). This result was confirmed by Chen et al. (2007) where they showed the presence of IFN- α, IFN- γ, and IL-10 in the plasma of dengue patients. However, IL-11 was not detected in DF and DHF patients (Cardier et al., 2006). Also, high concentrations of two chemokines named IFN-gamma inducible protein 10 (IP-10) and interferon-inducible T-cell alpha chemoattractant (I-TAC) were present in the serum of dengue patients (Fink et al., 2007).

However, the physiological roles of cytokines might be different (Salazar-Mather, Hamilton, & Biron, 2000); and thus it is important to investigate the regulation of different cytokines that are released from the DV-infected patients.

2.3 Interleukin-6 (IL-6)

In 1986, Kishimoto and collaborators first cloned a DNA encoding new human IL, T cell-derived B-cell differentiation factor (Streetz, Luedde, Manns, & Trautwein, 2000), which was later named IL-6 (Maggio, Guralnik, Longo,

& Ferrucci, 2006). IL-6 belongs to the IL-6 family of cytokines, including IL-11, oncostatin M, leukemia inhibitory factor, ciliary neurotrophic factor, cardiotrophin-1 and cardiotrophin-like cytokine. These cytokines are characterized by their common use of the gp130 receptor as a signaling subunit (Cronstein, 2007). The human IL-6 protein comprises 212 amino acids with a signal peptide of 27 amino acids and two potential NH2-linked glycosylation sites. The molecular weight ranges from 21 to 28 kDa (Kristiansen & Mandrup-Poulsen, 2005).

IL-6 is produced mainly by monocytes and macrophages (Cronstein, 2007) and in a smaller percentage by fibroblasts, endothelial cells, T cells, B cells, chondrocytes and keratinocytes (Łukaszewicz, Mroczko, & Szmitkowski, 2007). However, IL-6 is not constitutively expressed, but is readily induced by multiple stimuli, including DNA and RNA virus infection (Streetz et al., 2000). The production of IL-6 is also in response to many cytokines like TNF-α, IL-1, platelet-derived growth factor, IFN-γ (Yap & Lai, 2010) and IL-16 (Qi et al., 2002) but dampened by IL-4, IL-10, and IL-13 (Yap & Lai, 2010).

Because of its multiple activities, it has been suggested that IL-6 is the main factor involved in host response to a foreign pathogen. Its importance lies in the stimulation of B cells differentiation and induction of permanent differentiation of B cells into plasma cells which produce different classes of immunoglobulin (Łukaszewicz et al., 2007). Besides, IL-6 promotes inflammatory events through the T cells and NK proliferation, differentiation

and survival (Jones, 2005). It can also act as an endogenous pyrogen and interact with nervous and endocrine systems to modify host defense responses (Luheshi & Rothwell, 1996) as well as affect the permeability of the endothelium (Restrepo et al., 2008b).

IL-6 was shown to play important role in several virus infections. In the study done by Huang et al. (1999), they demonstrated that the serum level of IL-6 was significantly elevated in the patients with Hepatitis C, a disease infected from virus of *Flaviviridae* family, as compared with the controls. The level of IL-6 was also significantly increased in the microgial following Japanese Encephalitis (JE) Virus infection (Ghoshal et al., 2007). On the other hand, human umbilical cord veins endothelial cells (HUVEC) produced large amounts of IL-6 after DV infection (Y. Huang et al., 2000). Also, Pinto, Oliveira, Braga, Nogueira and Kubelka (1999) proved that IL-6 level was significantly higher in dengue patients than in the controls. In addition, Chen et al. (2006) confirmed that serum level of IL-6 was significantly increased in adult dengue patients. Similar results was obtained by Restrepo et al. (2008a) where they reported that there was significantly higher serum levels of IL-6 in children with dengue than without dengue. However, Chen and Wang (2002) failed to detect the presence of IL-6 in the DV-infected monocytes/macrophages. Moreover, the level of IL-6 in all dengue cases was not significantly higher than healthy controls (Priyadarshini et al., 2010). These opposing results had therefore brought about the aim of this cytokine to be studied in this research.

2.4 Interleukin-8 (IL-8)

Despite the tremendous interest in chemokines as a whole, the only chemokine with an IL designation is IL-8. According to the new chemokine nomenclature, IL-8 is now referred to as CXCL8 (Lin et al., 2005). In 1987, IL-8 was discovered as a neutrophil chemotactic factor (Yoshimura et al., 1987) and it was purified from supernatants of lipopolysaccharide stimulated human monocyte cultures (Payne & Cornelius, 2002). IL-8 is a member of ELR+ CXC chemokine family which contains the three amino acid sequence of glutamic acid-leucine-arginine that immediately precedes the first cysteine amino acid residue in the primary structure of the protein. This ELR+ CXC chemokine has its primary biological effect in promoting neutrophil recruitment and angiogenesis (Lin et al., 2005). Transcription of the IL-8 gene encodes for a protein of 99 amino acids that is subsequently processed to yield a 72 amino acids with molecular weight of 8400 Dalton (Waugh & Wilson, 2008), which is the major form secreted by monocytes and macrophages (Brat, Bellail, & Meir, 2005)

Normally, IL-8 protein is barely secreted from non induced cells, but its production is rapidly induced by a wide range of stimuli encompassing pro-inflammatory cytokines such as TNF, IL-1 (Taub, Anver, Oppenheim, Longo, & Murphy, 1996), IL-15 (Jabłońska et al., 2003), IL-6 and IFN-γ (Brat et al., 2005). The production of IL-8 is also in response to the stimuli like bacterial and viral products (Medin et al., 2005). IL-8 production had been observed *in vitro* in a wide variety of cells including monocytes, macrophages, neutrophils,

NK cells (Mukaida, 2003) and even non-leukocytic somatic cells like endothelial cells, fibroblasts and epithelial cells (Juffrie et al., 2000). The secreted IL-8 has numerous roles, including inflammation, cell recruitment, lymphoid trafficking, wound healing and angiogenesis. The diverse biological functions of IL-8 are vital in the recruitment of basophils, eosinophils, neutrophils (Y. Huang et al., 2000) and naïve T cells to the site of infection (Bosch et al., 2002). Also, IL-8 induces a respiratory burst of neutrophils, release of lytic enzymes, platelet-activating factor and leukotrienes (Medin et al., 2005), which are all inflammatory reactions to rid the host of invading pathogen.

Studies have shown that IL-8 is crucial in the defense system in several viral infections. In the study done by Juffrie et al. (2000) whey they illustrated that the level of IL-8 in dengue patients was increased as compared to the controls. *In vitro* studies were carried out to study the regulation of IL-8 in DV infection by a number of researchers to show the importance of this chemokine in pathogenesis of DV infection. Chen and Wang (2002) revealed that DV-infected monocytes/macrophages induced the production of IL-8. Also, a significant increased in IL-8 levels in the culture supernatant of primary human monocytes infected with DV was found in the study done by Bosch et al. (2002). Two yeara later, a similar result was obtained by Talavera, Castillo, Dominguez, Gutierrez and Meza (2004) where they showed that DV-infected human dermal microvascular endothelial cells (HMEC-1) induced the production of IL-8. Moreover, *in vitro* infection of human myeloid or endothelial cells with DV has been reported to induce secretion of IL-8

(Medin et al., 2005). All these results suggested the involvement of this chemokine in the host defense against the DV infection. However, a dissimilar result was obtained where the level of IL-8 was not significantly elevated in DF patients as compared to normal individuals (Y. Huang et al., 2000). King et al. (2002) also showed that level of IL-8 was not elevated in DV-infected human mast cell/basophil line.

2.5 Interleukin-15 (IL-15)

IL-15 is a 14–15 kDa protein with 114 amino acids (Alpdogan & Brink, 2005) in its structure. It is a member of the four-helix bundle cytokine family, which includes IL-2, IL-3, IL-6 and IL-7 and granulocyte colony-stimulating factor (G-CSF) (Ahmad, Ennaciri, Cordeiro, Bassam, & Menezes, 2007). It was first isolated from a simian kidney epithelial cell line CV-1/EBNA in 1994 (Budagian, Bulanova, Paus, & Bulfone-Paus, 2006). IL-15 is a novel cytokine related to type 1 helper T (Th1) response (Ueda et al., 2007) and was originally discovered due to its ability to promote T cell proliferation (Wang et al., 2005), a biological activity that is similar to IL-2 (Alpdogan & Brink, 2005). It has low secretion potential and soluble IL-15 is hardly detected in normal physiological conditions (Hocke et al., 2007). This pro-inflammatory cytokine is produced by macrophages (Ahmad et al., 2007), monocytes, dendritic cells (DC) (Hu, Wang, Wang, Huang, & Dong, 2007), neutrophils (Jabłońska et al., 2003) and epithelial cells (Hocke et al., 2007), but not by T cells (Ramsborg & Papoutsakis, 2007), in response to the environmental stimuli and infectious agent (Ueda et al., 2007).

IL-15 has been shown to play a vital role in the homeostasis and activation of natural killer T (NKT) cells (Alpdogan & Brink, 2005), neutrophils and eosinophils (Budagian et al., 2006). It activates NK cells proliferation and regulates its cytotoxicity (D'Ettorre et al., 2006). Moreover, IL-15 increases the production of IFN-γ from NK cells and T cells in enhancing Th1 response (Azeredo et al., 2005). It is also needed for the maintenance and renewal of viral-specific memory CD8+ T cells (Berard, Brandt, Paus, & Tough, 2003). Besides, it is important for the growth and differentiation in T cells (Ramsborg & Papoutsakis, 2007), B cells (Hu et al., 2007) and monocytes/macrophage (Ueda et al., 2007).

According to Fawaz, Sharif-Askari, and Menezes (1999), higher concentration of IL-15 was obtained after infection with influenza virus, a RNA virus, in cultured peripheral blood mononuclear cells (PBMC). Besides, D'Ettorre et al. (2005) also showed an enhancement in the production of IFN-γ by IL-15-stimulated NK cells, which was isolated from the human immunodeficiency virus (HIV)-infected patients. They also proved that IL-15 stimulated NK cells to produce CC chemokines which are able to inhibit HIV infection and replication. These results suggested that IL-15 is important in the immune response against various viral infections.

On the other hand, Chen and Wang (2002) infected blood monocytes and tissue macrophages with DV. They failed to detect the presence of IL-15 at any time after DV infection. However, there was another research done by Azeredo et al. in 2005 where they found that IL-15 was significantly elevated

in the plasma of most dengue patients during the early acute phase as compared to healthy donors. These contrary results brought the aim of this study to compare the level of IL-15 in the DV infected patients and healthy controls.

2.6 Interleukin-16 (IL-16)

IL-16 was initially identified in 1982 by Cruikshank & Center as a T cell chemoattractant factor that was secreted from mitogen- or antigen-stimulated human PBMC. It was one of the first characterized cytokines with chemoattractant activity for human T cells and therefore was originally designated as lymphocyte chemoattractant factor (LCF) (Cruikshank, Kornfeld, & Center, 2000).

This cytokine is synthesized by CD4+ T cells and CD8+ T cells and is released in response to antigen, mitogen and histamine (Reich et al., 2001). IL-16 is also produced in the circulation by activated monocytes and DC (Hu et al., 2007). Production of IL-16 was also demonstrated in eosinophils (Gianoukakis, Martino, Horst, Cruikshank, & Smith, 2003), mast cells (Desnues, Raoult, & Mege, 2005) and B cells (Tsai et al., 2005). Besides, non-immune cells like epithelial cells (Ferland, Flamand, Davoine, Chakir, & Laviolette, 2004), fibroblasts (Gianoukakis et al., 2003), neuronal cells (Cruikshank et al., 2000), microglial cells (Suzuki, Ishiguro, & Shimbo, 2003) have been found capable of IL-16 production. IL-16 is synthesized as a non-bioactive precursor protein (pro-IL-16) (Reich et al., 2001) which has 631

amino acids (Center, Kornfeld, Ryan, & Cruikshank, 2000). To generate active IL-16, the 631 amino acid pro-protein is cleaved by caspase-3 to generate a 121 amino acid protein that has biological activity after stimulation with specific antigen or other cytokines like IL-4, GM-CSF, IL-1β or transforming growth factor (TGF)- β (Liu et al., 2007).

Interleukin-16 is a T cell chemoattractant factor (Skundric, Zhou, Cruikshank, & Dai, 2005) that contributes to the regulatory process of T cell recruitment (Guo, Mittelbronn, Brabeck, Mueller, & Schluesener, 2004). The recruited T cells then synthesize and secrete more IL-16 that may serve to function as a positive feedback mechanism for further cell recruitment and activation (Wu et al., 1999). Besides induction of T cells chemotaxis, IL-16 also induces the migratory response in monocytes, eosinophils (Koike et al., 2002) and DC (Lynch, Heijens, Horst, Center, & Cruikshank, 2003). Furthermore, IL-16 stimulate monocytes to produce pro-inflammatory cytokines like IL-6, TNFα, IL-1α and IL-15 (Qi et al., 2002) and induce eosinophils to generate RANTES (Bandeira-Melo et al., 2002), which then further evokes the immune response towards the antigen. Besides, IL-16 up-regulates IL-2 receptor alpha on T cells (Hu et al., 2007) which in turn expanding the activated T cells (Reich et al., 2001). Although IL-16 has chemotactic properties, it is not a chemokine as it lacks the structural motifs of this family of proteins (Liu et al., 2007). On the other hand, IL-16 is also known to be preferentially induces a migratory response in Th1 cells (Jin et al., 2009). It inhibits the production of IL-4 and IL-5 from T cells and thus, impairs the type 2 helper T (Th2) immunity while induces a greater migratory response in the Th1 subset (Hu et al., 2007).

There was one research done by Message and Johnston in 2004 where they used the Rhinovirus as the model. Rhinovirus carries a similarity with DV as both of the viruses are single stranded RNA. They demonstrated that Rhinoviruses infection in bronchial epithelial cell lines and primary human epithelial cells increased the production of IL-16. Pinto et al. (2000) also found that the supernatant of influenza A virus-stimulated PBMC isolated from HIV patients, contained higher level of IL-16 as compared to the control.

To our knowledge however, no findings exist concerning IL-16 levels in DV infected patients. Therefore, the levels of IL-16 between the DV-infected patients and healthy controls was determined and compared.

2.7 Interferon-Gamma (IFN-γ)

IFN was discovered as an antiviral agent during studies on virus interference by Isaacs and Lindenmann in 1957. They reported that influenza virus-infected chick cells produced a secreted factor, which later named as IFN, mediated the transfer of a virus-resistant state against viruses. Although the IFN were first identified and named for their potent ability to interfere with and inhibit viral infections, it soon became apparent that they could be divided into two categories (Ellis & Beaman, 2004). The first category, named type I IFN is consisting of the IFN-α and IFN-β molecules. They are the classical IFN induced in response to viral infections (Samuel, 2001). The second class is solely composed of IFN-γ, which is also termed type II or immune IFN. IFN-γ is not related to the type I IFN at both the genetic and the protein levels.

Although it displays most of the biologic activities that have been described to the other IFN, it has a lower specific antiviral activity, but presents more immuno-modulatory properties than the type I IFN (Teixeira, Fonseca, Barboza, & Viola, 2005). Both types of IFN are crucial in the immediate cellular response to viral infection, but immuno-modulatory activities of IFN-γ, however, become important later in coordinating the immune response and establishing an antiviral state for longer term of control (Schroder et al., 2004).

IFN-γ with the molecular weight of 34 kDa (Teixeira et al., 2005) is mainly produced by Th1 cells, activated NK cells (Restrepo et al., 2008b) and macrophages (Antoniou, Ferdoutsis, & Bouros, 2003). Additionally, CD8+ T cells also produce IFN-γ after being activated (Yang, Wang, Asavaroengchai1, & Dey, 2005). There is now evidence that other cells, such as B cells, NKT cells, DC, and antigen-presenting cells (APC) secrete IFN-γ upon activation (Schroder et al., 2004). IFN-γ secretion by NK cells and possibly APC is likely to be important in early host defense against infection, whereas T cells become the major source of IFN-γ in the adaptive immune response (Yang et al., 2005). The production of IFN-γ is also controlled by other cytokine. For example, macrophage recognition of many pathogens induces secretion of chemokines which then in turn to attract NK cells to the site of inflammation. This chemotaxis promotes IFN-γ synthesis in these cells. Macrophages and NK cells in combination of chemokine stimulation will then further increases the IFN-γ production (Schroder et al., 2004).

IFN-γ is a major product of Th1 cells (Chen et al., 2005) and it stimulates the differentiation of naive T cells toward Th1 phenotype and inhibits Th2 cell differentiation by suppressing the release of Th2 cytokine from activated T cells (Teixeira et al., 2005). The inhibition of Th2 response further skews the immune response toward a Th1 phenotype (Gattoni, Parlato, Vangieri, Bresciani, & Derna, 2006). It also enhances the antigen presentation and stimulates the development of CD8+ T cells (Antoniou et al., 2003). In addition, it is crucial in the activation and differentiation of B cells, NK cells, macrophages, and others (Gattoni et al., 2006). Exposure to IFN-γ greatly enhances the activity of macrophages and induces them to secrete reactive oxidants like nitric oxide and cytokines such as IL-1, IL-6, IL-8, and TNF-α in the killing of intracellular and some extracellular infections. On the other hand, there has been increasing evidence that IFN-γ also down-regulates or restricts immune responses. IFN-γ plays an important role in the maintenance of T cell homeostasis and eliminates activated CD4+ T cells and CD8+ T cells by inducing apoptosis (Yang et al., 2005).

There are number of research showing the importance of IFN-γ in DV infection. Chen et al. (2005) found that IFN-γ was higher in the DV-infected patients than those healthy controls. Similar results were obtained by Chakravarti and Kumaria (2006) and Chen et al. (2006) where they found that the serum levels of IFN-γ was significantly increased in DF patients than in healthy controls. This result was proven again two years later by Bozza et al. (2008) where IFN-γ was significantly increased in dengue patients as compared to control samples. While in the research done by Restrepo and co-

workers (2008a), they showed a higher cytokine level in children with dengue than without dengue, with statistically significant difference for IFN-γ. Additionally, a parallel result was obtained by Priyadarshini et al. (2010) where level of IFN-γ was higher in dengue patients than in the controls. The involvement of IFN-γ suggested that this cytokine is critical for the immune response against DV infection and that IFN-γ -mediated immune responses are necessary for both the early and late clearance of the virus (Hsieh et al., 2006). However, the exact role of IFN-γ in DV infection is still controversial. Although some studies suggested that it protects the host against DV infection, others argue that it contributes to the pathogenesis of DHF, the more severe form of the illness (Diamond et al., 2000).

2.8 Monocyte Chemoattractant Protein-1 (MCP-1)

MCP-1, which is known as chemokine (C-C motif) ligand 2 (CCL2) (Dhillon et al., 2009), consists of 76 amino acid residues (Coillie, Damme, & Opdenakker, 1999). It is a member of the C-C chemokine family (Mori et al., 2000). MCP-1 can be produced by several cells like monocytes, macrophages, epithelial cells, endothelial cells, fibroblasts (Han, Han, Grenn, & Quehenberger, 1999) and smooth muscle cells (Cleanthis, Bhattacharya, Smout, Ashour, & Standby, 2007) but not T cells (Mori et al., 2000). It is secreted in response to lipopolysaccharide, or cytokines including IL-1, IL-4, IFN-γ, TNF-α (Zhou et al., 2001) and platelet-derived growth factor (PDGF) (Yamamoto et al., 1999).

MCP-1 plays a pivotal role in anti-viral immune responses, such as the recruitment of immune cells (Deauvieau et al., 2007). It recruits and activates monocytes to the site of inflammation (Cai et al., 2009) after exposed to activation stimuli, such as virus infection. MCP-1 then increases the endothelial permeability by disrupting tight junctions among cells (Bozza et al., 2008) to cause vascular leakage (Yamada et al., 2003) which is the necessary action for leukocytes to leave the circulation and infiltrate tissues (Mori et al., 2000) in clearing the invading virus. Also, MCP-1 actively takes part in the T-cell-mediated immune response against intracellular pathogens by controlling leukocyte recruitment to the site of infection and clearance of the pathogen (Dessing, Sluijs, Florquin, & Poll, 2007). Besides, MCP-1 also recruits NK cells (Han et al., 1999), macrophages, basophils, mast cells, DC (Dhillon et al., 2009) and neutrophils (Dessing et al., 2007) to the sites of inflammation.

Jin et al. (2009) showed that the plasma level of MCP-1 in adult acquired immunodeficiency syndrome (AIDS) patients were significantly higher than in their healthy controls. This showed that MCP-1 plays an important role in the immune response of the patients infected with single stranded RNA virus. The level of MCP-1 was significantly elevated in the JE virus-infected microglial (Ghoshal et al., 2007). Again it showed that MCP-1 is not only playing a vital role in the immune response of single stranded RNA virus-infected patients, but also the immune response of the patients who are infected with flavivirus. Lin et al. (2005) also showed that an increased of MCP-1 was observed in patients with DV infections. These results lead the

objective in this study to compare the level of MCP-1 in DV-infected patients and healthy controls in Malaysia.

2.9 Monokine Induced by Interferon-Gamma (MIG)

MIG is one of the members of ELR- (lacking a Glutamic acid-Leucine-Arginine motif in the N-terminal region) CXC chemokines that are associated with inflammatory reactions (Park et al., 2002). Monocytes and macrophages are thought to comprise the majority of MIG secreting cells (Brice, Graber, Hoffman, & Doolan, 2001). Besides, MIG is also secreted by eosinophils (Dajotoy et al., 2004), neutrophils, APC, B cells (Berthoud, Dunachie, Todryk, Hill, & Fletcher, 2009). The production of this chemokine is induced by IFN-γ and thus, has the potential to amplify the IFN-γ signal. In consequence, MIG is an important regulator in Th1 responses (Colvin, Campanella, Sun, & Luster, 2004). In mediating leukocyte migration to sites of infection (Hsieh et al., 2006), MIG mainly recruits T cells and NK cells in the immune response upon activation (Dajotoy et al., 2004). MIG also influences the behavior of non-immune cells where it inhibits endothelial cell proliferation resulting in the inhibition of angiogenesis (Gorbachev et al., 2007).

Elevation in MIG was observed in Hepatitis C virus (HCV)-infected patients in the study done by Bièche et al. (2005). On the other hand, Brainard et al. (2007) also demonstrated that MIG level was significantly increased in HIV-infected patients as compared to the levels in uninfected controls. Furthermore, production of MIG was induced from DV-infected DC in the

study done by Dejnirattisai et al. (2008). These results suggest that MIG has an essential role in the host defense system against viral infection.

MIG was selected for examination because its induction is reported to have a uniquely stringent and specific requirement for IFN-γ (Salazar-Mather et al., 2000). This allows it to provide a functional measure of IFN-γ activity in addition of detecting the presence of IFN-γ in DV-infected patients directly. In addition, the production of MIG is followed by the amplification of the IFN-γ signal. This may be a more sensitive measure and an easier way to detect the presence of bio-active IFN-γ (Berthoud et al., 2009) in DV-infected patients.

2.10 Regulated upon Activation, Normal T-Cell Expressed and Secreted (RANTES)

CCL5 or RANTES (regulated upon activation, normal T-cell expressed and secreted) belongs to a family of immuno-regulatory cytokines called chemokines and it is classified as C-C chemokines (Gianoukakis et al., 2003). This chemokine is secreted by many different cell types, such as endothelial cells, smooth muscle cells, macrophages (Cavusoglu et al., 2007), monocytes (Gianoukakis et al., 2003), lympohcytes (Gleissner, Hundelshausen, & Ley, 2008) and platelets (Tang, Yeaman, & Selsted, 2002).

RANTES is a chemoattractant factor for monocytes and T cells (Gleissner et al., 2008). It is found preferentially attracts Th1 cells rather than Th2 cells

(Kanda & Watanabe, 2003), which is important in the control of intracellular pathogen. Also, it is crucial in the induction of NK cell activation, chemotaxis, adhesion, and trans-endothelial migration (Chen & Wang, 2002).

Several studies proved that RANTES plays an important role in the immune system during viral infection. Jin et al. (2009) showed that the levels of RANTES in the adult AIDS patients were significantly higher than in their healthy controls, as RANTES was found to be able to suppress HIV replication (Appay & Rowland-Jones, 2001). In the study done by Sládková and Kostolanský (2006) where they found that RANTES was produced after epithelial cells and leukocytes infected by influenza A virus. Also, the level of RANTES was being measured in the JE patients. Results showed that there was an up-regulation of RANTES level in these flavivirus infected patients as compared to the healthy controls (Winter et al., 2004).

In addition, there were a number of studies done to investigate the level of RANTES in the supernatant of DV-infected cell line. An elevated level of secreted RANTES was observed following DV infection of KU812 or HMC-1 (King et al., 2002). Medin et al. (2005) infected both of the human liver carcinoma cell line (HepG2) and primary DC with DV. They also showed that infection of both cells induced RANTES production. Interestingly, there was a contrast result obtained by Pérez et al. (2004) where they reported that serum levels of RANTES detected in Cuban patients hospitalized with dengue infection were lower than those observed in the controls. Also, this is the only research that detects the presence of RANTES in the human biological sample.

Thus, the levels of RANTES in the serum of DV-infected patients and healthy controls were chosen to be studied.

2.11 Interrelation between the Cytokines and Chemokines

The cytokines and chemokines studied in this research are interrelated among each other. This interrelation among cytokines and chemokines might enable the host to have a desired magnitude of defense system to clear the invading virus. Thus, interrelation involving IL-6, IL-8, IL-15, IL-16, IFN-γ, MCP-1, MIG and RANTES, was revealed to figure out the immunopathogenesis of DV infection (Figure 2.3).

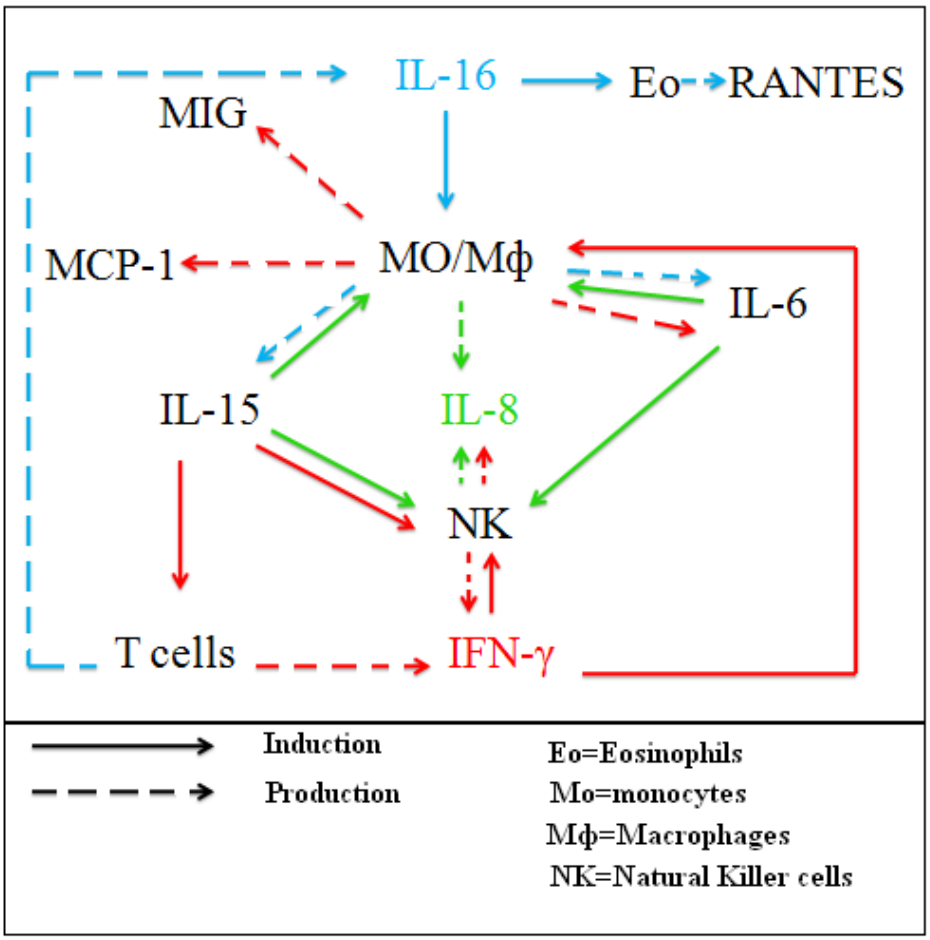

Figure 2.3: Interrelation among the cytokines and chemokines

Upon DV infection, IL-6, IL-8, IL-15, IL-16, IFN-γ, MCP-1, MIG and RANTES will be produced by a number of different leucocytes in magnifying the immune response. Besides the induction of viral particles, the production of these cytokines also takes place in response to other stimuli like cytokines. In response to viral particles, IL-16 is synthesized by T cells (Reich et al., 2001) and this cytokines might then stimulate the production of RANTES from eosinophils (Bandeira-Melo et al., 2002), IL-6 and IL-15 from monocytes (Qi et al., 2002). Production of IL-6 and IL-15 from monocytes might then further stimulate the synthesis of IL-8 (Jabłońska et al., 2003; Brat et al., 2005) from monocytes, macrophages, neutrophils and NK cells. Besides, IL-15 will enhance the Th1 response by increasing the production of IFN-γ from NK cells and T cells (Azeredo et al., 2005). Interferon-gamma will in turn act as the inducer for the additional production of IL-6 and IL-8 (Gattoni et al., 2006). In addition, the production of MCP-1 (Zhou et al., 2001) and MIG (Colvin et al., 2004) from monocytes or macrophages could be induced by IFN-γ upon DV infection in order to recruit more leucocytes to the site of infection for viral clearance.

CHAPTER 3

MATERIALS AND METHODS

3.1 Ethics Statement

This study was approved by the Medical Research and Ethics Committee, Ministry of Health Malaysia. Informed consent was obtained from each individual before inclusion in the study. The privacy and the confidentiality of the patients were taken care by using indirect identifiers and anonymous specimens without making any reference to identifying information of the participants.

3.2 Clinical Samples

In this research, patients presenting dengue-like illness and were admitted in Hospital Besar Kuala Lumpur, Malaysia from Sept 2007 to June 2009 were included. Blood of the patients (n=25) who showed clinical symptoms like fever, headache, myalgia, joint pain, and thrombocytopenia were collected. For each patient, the day of onset of fever was designated as first day of illness. The blood from the patients was collected after 6 days in average of onset of fever. Patients with other parasitic infection, respiratory infection and/or other diseases were excluded by the clinicians. As controls, bloods from healthy volunteers (n=25) with no signs of infection, and who were not taking any medications at the time of this study, were included.

Clinical symptoms presented by the patients were recorded and estimations of leucocytes, lymphocytes, neutrophils, monocytes, eosinophils and basophils counts for each patient were carried out at Hospital Besar Kuala Lumpur, Malaysia. Data were collected from patients' profiles.

3.3 Serum Preparation

Blood of the DV-infected patients and the healthy donors were clotted in the serum collection tube (BD Vacutainer plus, U.S.A.). After that, the serum collection tube was centrifugated (Hettich ZENTRIGUGEN, U.K.) at 9000rpm for 5 minutes. Serum was then collected and stored at -80°C (SANYO, Japan) for future assay.

3.4 Laboratory Diagnosis

In all cases, dengue infection was confirmed by the serological test with the detection of dengue NS1 antigen (Panbio Dengue Early ELISA) (Panbio, Australia) and detection of DV IgM (CALBIOTECH, U.S.A.).

3.4.1 Detection of Dengue NS1 Antigen by Panbio Dengue Early ELISA

3.4.1.1 Samples and Reagents Preparation

Serum of DV-infected patients and healthy controls, Positive Controls, Negative Controls and Calibrator were diluted 10X by mixing the sample with

Serum Diluent. Also Wash Buffer (1X) was prepared by adding Wash Buffer Concentrate to ultra pure water (ELGA, UK).

3.4.1.2 Assay Procedures

Diluted serum, Positive Controls, Negative Controls and Calibrators were pipetted (RAININ, U.S.A.) into the Anti-NS1 Antibody pre-coated microwells and was then covered and incubated at 37°C ± 1°C for 1 hour. After that, the wells were washed 6 times with 1X Wash Buffer and followed by the addition of Horseradish Peroxidase (HRP) Conjugated Anti-NS1 Monoclonal Antibody (MAb) into each well. Again, the wells were covered and incubated at 37°C ± 1°C for 1 hour. Then, the wells were washed 6 times with 1X Wash Buffer. Tetramethylbenzidine (TMB) was then added into each well and was subsequently incubated at room temperature for 10 minutes. The optical density (O.D.) (TECAN, Switzerland) of each well at wavelength of 450nm with a reference filter of 650nm was taken after the addition of Stop Solution. Calculation was done as follows to obtain the index value in determining the presence of dengue specific NS1.

Cut-off value	=	Calibrator mean O.D. X Calibrator Factor (in this case, 0.51)
Index Value	=	(Sample O.D. / Cut-off value)

A positive result with more than 1.1 Index Value indicated the presence of dengue NS1 antigen while sample with Index Value less than 0.9 showed the

negative results where there was absence of dengue NS1 antigen. Samples with Index Value within 0.9-1.1 were repeated with the test.

3.4.2 Detection of DV IgM by Dengue Virus IgM ELISA

3.4.2.1 Samples and Reagents Preparation

Dilution factor of 21X were prepared for the serum of DV-infected patients and healthy controls, Negative Controls, Positive Controls, and Calibrators by mixing the samples with Sample Diluent. Wash Buffer (1X) was also prepared by adding Wash Concentrate 20X with ultra pure water prior to assay procedure.

3.4.2.2 Assay Procedures

Diluted serum, Positive Controls, Negative Controls and Calibrators were dispensed into the Dengue Antigen pre-coated microwells and the plate was incubated for 20 minutes at room temperature. After incubation, the wells were washed 3 times with 1X Wash Buffer. Next, Enzyme Conjugated Anti-IgM MAb was added into each well and the plate was incubated at room temperature for 20 minutes. After that, the wells were washed 3 times with 1X Wash Buffer and TMB Substrate was added into each well. Wells were then incubated for 10 minutes at room temperature and followed by the addition of Stop Solution into each well. The O.D. of each well at wavelength

of 450nm with reference filter of 650nm was finally taken. Calculation was done as follows in order to obtain the Index Value of each sample.

Cut-off value	=	Calibrator mean O.D. X Calibrator Factor (in this case, 0.4)
Index Value	=	Sample O.D. / Cut-off value

Sample with Index Value more than 1.1 indicated the positive result where there was presence of DV IgM while sample with Index Value less than 0.9 showed that there was no detectable DV IgM. This test was repeated if the sample's Index Value fell within the range of 0.9 to 1.1.

3.5 Detection of Cytokines and Chemokines in Serum of DV-Infected Patients and Healthy Controls

Concentrations of IL-6, IL-8, IL-15, IL-16**,** IFN-γ, MCP-1, MIG and RANTES were measured in the serum of 25 confirmed DV-infected patients and 25 apparent healthy controls. Standards were included in each assay and the standard curves were used for the estimation of cytokine and chemokines concentrations (in pg/ml).

3.5.1 Detection of IL-6 by Human IL-6 ELISA Kit II (BD OptEIA™, U.S.A.)

3.5.1.1 IL-6 Standard Preparation

In the preparation of standard, lyophilized recombinant human IL-6 was reconstituted with required volume (noted on vial label) of standard/sample diluent to prepare a 300pg/mL stock standard. The stock standard was allowed to equilibrate for 15 minutes before making dilution. After that, standard/sample diluent was added into six tubes labeled with 150 pg/mL, 75 pg/mL, 37.5 pg/mL, 18.8 pg/mL, 9.4 pg/mL and 4.7 pg/mL. Serial dilution was then performed by adding fixed volume of each standard to the next tube and vortexing between each transfer. The undiluted standard served as the high standard (300pg/mL) while the standard/sample diluent served as the zero Standard (0 pg/mL).

3.5.1.2 Working Detector Preparation

Working detector with dilution factor of 250X was prepared within 15 minutes prior to use. In this preparation, required volume of biotinylated anti-human IL-6 MAb was mixed well with required quantity of concentrated streptavidin-HRP conjugate (250X) in a clean tube.

3.5.1.3 Wash Buffer Preparation

Wash buffer with dilution factor of 20X was prepared by mixing the wash concentrate (20X) with ultra pure water.

3.5.1.4 Assay Procedures

First, ELISA diluent was added into anti-human IL-6 MAb pre-coated microwells. Prepared standards and serum were then pipetted into each well. The plate was gently tapped to ensure complete mixing. After that, plate was covered with a plate sealer and was incubated for 2 hours at room temperature. Next, the plate was washed 5 times with diluted wash buffer and followed by the addition of working detector (was prepared within 15 minutes prior to use) into each well. Again, the plate was covered with a plate sealer and was incubated for 1 hour at room temperature. The plate was washed 7 times with diluted wash buffer after the incubation. TMB one-step substrate reagent was subsequently dispensed into each well and the plate was incubated without a plate sealer for 30 minutes at room temperature in the dark. Stop solution was then added into each well and the absorbance of each well at wavelength of 450nm with reference filter of 570nm was taken. Standard curve of IL-6 was finally plotted and the concentrations of IL-6 in the serum of DV-infected patients and healthy controls were determined (Appendix A).

3.5.2 Detection of IL-8 by Human IL-8 ELISA Kit II (BD OptEIA™, U.S.A.)

3.5.2.1 IL-8 Standard Preparation

Lyophilized recombinant human IL-8 was reconstituted with required volume (noted on vial label) of standard/sample diluent to prepare a 200pg/mL stock standard. The stock standard was then allowed to equilibrate for 15 minutes before making dilution. Standard/sample diluent was subsequently added into six tubes labeled with 100 pg/mL, 50 pg/mL, 25 pg/mL, 12.5 pg/mL, 6.3 pg/mL and 3.1 pg/mL. Serial dilution was then performed by adding fixed volume of each standard to the next tube and vortexing between each transfer. The undiluted standard served as the high standard (200pg/mL) while the standard/sample diluent served as the zero standard (0 pg/mL).

3.5.2.2 Working Detector Preparation

Working detector with dilution factor of 250X was prepared within 15 minutes prior to use. In this preparation, required volume of biotinylated anti-human IL-8 MAb was mixed well with required quantity of concentrated streptavidin-HRP conjugate (250X) in a clean tube.

3.5.2.3 Wash Buffer Preparation

Wash buffer with dilution factor of 20X was prepared by mixing the wash concentrate (20X) with ultra pure water.

3.5.2.4 Assay Procedures

ELISA diluent was first added into anti-human IL-8 MAb pre-coated microwells. Prepared standards and serum were then dispensed into each well. This plate was covered with a plate sealer and was incubated at room temperature for 2 hours. After that, the plate was washed 5 times with diluted wash buffer. Plate was covered with a plate sealers and was incubated at room temperature for 1 hour following the addition of working detector (was prepared within 15 minutes prior to use) into each well. The plate was then washed 7 times with diluted wash buffer and followed by the addition of TMB one-step substrate reagent into each well. Again, the plate (without a plate sealer) was incubated for 30 minutes at room temperature in the dark. After that, stop solution was added into each well and absorbance of each well at wavelength of 450nm with reference filter of 570nm was taken. Standard curve of IL-8 was finally plotted and the concentrations of IL-8 in the serum of DV-infected patients and healthy controls were calculated (Appendix B).

3.5.3 Detection of IL-15 by Human IL-15 Immunoassay (R&D Systems, Minneapolis)

3.5.3.1 IL-15 Standard Preparation

Lyophilized recombinant human IL-15 was reconstituted with 1.0mL of ultra pure water producing a stock standard of 2500 pg/mL. The stock standard was mixed to ensure complete reconstitution and was agitated gently for 15 minutes prior to making dilution. Nine hundred micro liter of calibrator diluent RD6-10 was pipetted into the tube labeled 250 pg/mL. Also, 500 µL of calibrator diluent RD6-10 was pipetted into the remaining tubes which labeled with 125 pg/mL, 62.5 pg/mL, 31.2 pg/mL, 15.6 pg/mL, 7.8 pg/mL and 3.9 pg/mL. Stock standard was then used to produce a dilution series and each tube was mixed thoroughly before the next transfer. The 250 pg/mL standard served as the high standard. The calibrator diluent RD6-10 served as the zero standard (0 pg/mL).

3.5.3.2 Substrate Solution Preparation

Stabilized hydrogen peroxide (H_2O_2) (Colour Reagents A) and stabilized chromogen (TMB) (Colour Reagent B) were mixed together in equal volumes within 15 minutes of use and the resultant mixture was protected from light.

3.5.3.3 Wash Buffer Preparation

Wash buffer with dilution factor of 25X was prepared by mixing the wash buffer concentrate with ultra pure water.

3.5.3.4 Assay Procedures

Assay diluent RD1-19 was added into mircowells pre-coated with mouse MAb against IL-15. Prepared standards and serum were then added into each well. The plate was covered with the adhesive strip provided and was incubated for 3 hours at room temperature. After that, the plate was washed 4 times with diluted wash buffer and followed by the addition of anti-IL-15 conjugate (mouse MAb against IL-15 conjugated to HRP). Then, the plate was covered with a new adhesive strip and was incubated for 1 hour at room temperature. Washing steps were then repeated as previous. After washing, substrate solution (prepared within 15 minutes of use) was added into each well and the plate was incubated for 30 minutes at room temperature in the dark. Subsequently, stop solution was added into each well and O.D. of each well at wavelength of 450nm with reference filter of 570nm was taken. Finally, the standard curve of IL-15 was constructed and the concentrations of IL-15 in the serum of DV-infected patients and healthy controls were calculated (Appendix C).

3.5.4 Detection of IL-16 by Endogen® Human IL-16 ELISA Kit (Thermo Scientific, U.S.A.)

3.5.4.1 IL-16 Standard Preparation

Lyophilized *Escherichia coli (E.coli)*-derived recombinant human IL-16 standard was reconstituted with ultra pure water. Tubes were labeled with 2000 pg/mL, 800 pg/mL, 320 pg/mL, 128 pg/mL, 51 pg/mL, and 0 pg/mL. Serial dilution of 1:2.5 was carried out to prepare the above said standard concentration in constructing the standard curve of IL-16.

3.5.4.2 Streptavidin-HRP Solution Preparation

Streptavidin-HRP concentrate was mixed with streptavidin-HRP dilution buffer to obtain a diluted solution with 400X dilution factor. Streptavidin-HRP Solution was prepared immediately before use.

3.5.4.3 Wash Buffer Preparation

Wash buffer (1X) was prepared by diluting 30X wash buffer with ultra pure water.

3.5.4.4 Assay Procedures

Prepared standards and five-fold diluted serum were added into anti-human IL-16 pre-coated microwells. The plate was covered with an adhesive plate cover and incubated for 1 hour at room temperature. After that, without washing, biotinylated antibody reagent was added into each well. The plate was then covered with adhesive plate cover and incubated for 1 hour at room temperature. Next, the plate was washed 3 times with 1X wash buffer and streptavidin-HRP solution (was prepared immediately before use) was subsequently added into each well. The plate was covered with a new adhesive plate cover and incubated for 30 minutes at room temperature. Then, the plate was washed 3 times with 1X wash buffer and followed by the addition of TMB substrate solution into each well. After that, the plate (without plate cover) was incubated at room temperature in the dark for 30 minutes. Stop solution was subsequently added into each well. The absorbance of each well at wavelength of 450nm with reference filter of 550nm was taken. Standard curve for IL-16 was finally plotted and the concentrations of IL-16 in the serum of DV-infected patients and healthy controls were calculated (Appendix D).

3.5.5 Detection of IFN-γ by Human IFN-γ ELISA Kit II (BD OptEIA™, U.S.A.)

3.5.5.1 IFN-γ Standard Preparation

Lyophilized recombinant human IFN-γ was reconstituted with standard/sample diluent to prepare a 300pg/mL stock standard. The stock standard was allowed to equilibrate for 15 minutes before making dilutions. Standard/sample diluent was then added into six tubes labeled with 150 pg/mL, 75 pg/mL, 37.5 pg/mL, 18.8 pg/mL, 9.4 pg/mL and 4.7 pg/mL. After that, serial dilutions were performed by adding fixed volume of each standard to the next tube and vortexing between each transfer. The undiluted standard served as the high standard (300 pg/mL) whilst the standard/sample diluent served as the zero standard (0 pg/mL).

3.5.5.2 Working Detector Preparation

Working detector with dilution factor of 250X was prepared within 15 minutes prior to use. In this preparation, required volume of biotinylated anti-human IFN-γ MAb was mixed well with required quantity of concentrated streptavidin-HRP conjugate (250X) in a clean tube.

3.5.5.3 Wash Buffer Preparation

Wash buffer with dilution factor of 20X was prepared by mixing the wash concentrate (20X) with ultra pure water.

3.5.5.4 Assay Procedures

ELISA diluent was pipetted into each microwell pre-coated with anti-human IFN-γ MAb. Prepared standards and serum were then pipetted into each well. Next, the plate was covered with a plate sealer and incubated for 2 hours at room temperature. After incubation, the plate was washed 5 times with diluted wash buffer, followed by addition of working detector (was prepared within 15 minutes prior to use) into each well. The plate was again covered with a plate sealer and incubated for 1 hour at room temperature. After that, the plate was washed 7 times with diluted wash buffer and TMB one-step substrate reagent was subsequently added into each well. Then, the plate (without a plate sealer) was incubated for 30 minutes at room temperature in the dark. Stop solution was added into each well after the incubation. Absorbance of each well at wavelength of 450nm with reference filter of 570nm was taken. Standard curve of IFN-γ was plotted and the concentrations of IFN-γ in the serum of DV-infected patients and healthy controls were determined (Appendix E).

3.5.6 Detection of MCP-1 by Human MCP-1/CCL2 Immunoassay (R&D Systems, Minneapolis)

3.5.6.1 MCP-1 Standard Preparation

Standard of MCP-1 (lyophilized recombinant human MCP-1) was reconstituted with calibrator diluent RD6Q to produce a stock standard of 2000 pg/mL. The stock standard was allowed to sit for 15 minutes with gentle agitation prior to making dilutions. Calibrator diluent RD6Q was then added into tubes labeled with 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL and 31.2 pg/mL. Each tube was mixed thoroughly before the next transfer. The 1000pg/mL standard served as the high standard while the calibrator diluent RD6Q served as the zero standard (0 pg/mL).

3.5.6.2 Substrate Solution Preparation

Stabilized H_2O_2 (Colour Reagents A) and Stabilized chromogen (TMB) (Colour Reagent B) were mixed together in equal volumes within 15 minutes of use and the resultant mixture was protected from light.

3.5.6.3 Wash Buffer Preparation

Wash buffer with dilution factor of 25X was prepared by mixing the wash buffer concentrate with ultra pure water.

3.5.6.4 Assay Procedures

Assay diluent RD1-83 was added to mouse MAb against MCP-1 pre-coated microwells. Prepared standards and two-fold diluted serum was then added into each wells. After that, the plate was covered with the adhesive strip provided and was incubated for 2 hours at room temperature. Next, the plate was washed 3 times with diluted wash buffer. Then, anti-MCP-1 conjugate (polyclonal antibody against MCP-1 conjugated to HRP) was dispensed into each well. The plate was covered with a new adhesive strip and was incubated for 2 hours at room temperature. Washing steps was then repeated as previous. Substrate solution (was prepared within 15 minutes of use) was subsequently added into each well and the plate was incubated for 30 minutes at room temperature in the dark. After incubation, stop solution was added into each well. Finally, the O. D. of each well at wavelength of 450nm with reference filter 570nm was determined. The standard curve for MCP-1 was plotted and the concentration of MCP-1 in the serum of DV-infected patients and healthy controls was calculated (Appendix F).

3.5.7 Detection of MIG by Human CXCL9/MIG Immunoassay (R&D Systems, Minneapolis)

3.5.7.1 MIG Standard Preparation

MIG standard (lyophilized recombinant human MIG) was reconstituted with ultra pure water producing a stock standard of 20 000 pg/mL. The Stock

Standard was mixed to ensure complete reconstitution and allowed to sit for 15 minutes with gentle agitation prior to making dilutions. Nine hundred micro liter of calibrator diluent RD5P was pipetted into the tube labeled with 2000 pg/mL. Then, 500µL of calibrator diluent RD5P was pipetted into the remaining tubes which labeled with 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL and 31.2 pg/mL. After that, the stock standard was used to produce the dilution series. Each tube was mixed thoroughly before the next transfer. The 2000 pg/mL standard served as the high standard while the calibrator diluent RD5P served as the zero standard (0 pg/mL).

3.5.7.2 Substrate Solution Preparation

Stabilized H_2O_2 (Colour Reagents A) and Stabilized chromogen (TMB) (Colour Reagent B) were mixed together in equal volumes within 15 minutes of use and the resultant mixture was protected from light.

3.5.7.3 Wash Buffer Preparation

Wash buffer with dilution factor of 25X was prepared by mixing the wash buffer concentrate with ultra pure water.

3.5.7.4 Assay Procedures

Assay diluent RD1W was pipetted into the microwells pre-coated with mouse MAb against MIG. Prepared standards and serum was then added into each

well. Plate was covered with adhesive strip provided and was incubated for 2 hours at room temperature. After that, the plate was washed 4 times with diluted wash buffer, followed by the addition of anti-MIG conjugate (polyclonal antibody against MIG conjugated to HRP) into each well. Again, the plate was covered with a new adhesive strip and was incubated for 2 hours at room temperature. Washing steps was then repeated as previous. Substrate solution (was prepared within 15 minutes of use) was subsequently added into each wells and the plate was incubated for 30 minutes at room temperature in the dark. Stop solution was later added into each well and O.D. of each well at wavelength of 450nm with reference filter of 570nm was taken. Standard curve for MIG was finally plotted and the concentrations of MIG in the serum of DV-infected patients and healthy controls were determined (Appendix G).

3.5.8 Detection of RANTES by Endogen® Human RANTES ELISA Kit (Thermo Scientific, U.S.A.)

3.5.8.1 RANTES Standard Preparation

Lyophilized *E.coli*-derived recombinant human RANTES standard was reconstituted with ultra pure water. Tubes were then labeled with 2000 pg/mL, 800 pg/mL, 320 pg/mL, 128 pg/mL, 51.2 pg/mL, and 0 pg/mL. Serial dilution of 1:2.5 was carried out to prepare the above said standard concentration in constructing the standard curve of RANTES.

3.5.8.2 Streptavidin-HRP Solution Preparation

Streptavidin-HRP concentrate was mixed with Streptavidin-HRP dilution buffer to obtain a diluted solution with 400X dilution factor. Streptavidin-HRP solution was prepared immediately before use.

3.5.8.3 Wash Buffer Preparation

Wash buffer (1X) was prepared by diluting 30X wash buffer with ultra pure water.

3.5.8.4 Assay Procedures

Prepared standards and 100-fold diluted serum were added into the well pre-coated with anti-human RANTES. The plate was then covered with a new adhesive plate cover and was incubated for 1 hour at room temperature. After that, the plate was washed 3 times with 1X wash buffer. Following this, biotinylated antibody reagent was added into each well. Again, the plate was covered with a new adhesive plate cover and was incubated for 1 hour at room temperature. Washing steps was repeated as previous after the incubation. Streptavidin-HRP solution (was prepared immediately before use) was dispended into each well. Next, the plate was cover with adhesive plate cover and was incubated for 30 minutes at room temperature. The plate was washed 3 times again with 1X wash buffer and followed by the addition of TMB substrate solution into each well. The plate (without the adhesive plate cover)

was then incubated for 30 minutes at room temperature in the dark. Stop solution was subsequently added into each well. Absorbance of each well at wavelength of 450nm with reference filter at 550nm was taken. Standard curve of RANTES was finally constructed and the concentration of RANTES in the serum of DV-infected patients and healthy controls was determined (Appendix H).

3.6 Statistical Analysis

Differences between the groups were analyzed using the Mann-Whitney U test. Spearman rank order correlation was used to study the correlation between the cytokines and chemokines levels and clinical findings. Significance was set at $p < 0.05$. All the statistical analyses of the data were done using SPSS Statistics 19.0.

CHAPTER 4

RESULTS

4.1 Demographic Data of DV-Infected Patients

The demographic data of DV-infected patients was collected and summarized in Table 4.1.

Table 4.1: Demographic data of DV-infected patients

Category	Subcategory	
Total number of dengue cases		25
Dengue (number of cases)	Dengue Fever	24
	Dengue haemorrhagic fever	1
Mean age (years ± SD)		28 ± 9
Average day of infection		6
Gender	Males	17
	Females	8

4.2 Clinical Signs and Symptoms of DV-Infected Patients

Clinical signs and symptoms of DV-infected patients are tabulated in Table 4.2.

Table 4.2: Clinical signs and symptoms of DV-infected patients

Clinical sign/symptom	Number of cases in DF (%)
Fever	100
Myalgia	87.8
Joint pain	95.9
Headache	91.9
Nausea/vomiting	39.2
Rash	59.5
Thrombocytopenia	100

4.3 Laboratory Findings in DV-Infected Patients

Laboratory findings in DV-infected patients are shown in Table 4.3.

Table 4.3: Laboratory findings in DF

Variable	Normal range	DV-infected patients
Leukocyte count, mean cells ($X\ 10^3 \pm SD$)	4-11	3.419 ± 1.442
No. of cases with leucopenia (%)	-	73.5
Neutrophils count, mean cells ($X\ 10^3 \pm SD$)	2-7	1.325 ± 0.727
No. of cases with neutropenia (%)	-	85.4
Lymphocytes count, mean cells ($X\ 10^3 \pm SD$)	1.5-4	1.370 ± 0.864
Lymphocytopenia (%)	-	60.8
Monocytes count, mean cells ($X\ 10^3 \pm SD$)	0.2-0.8	0.902 ± 2.155
Monocytopenia (%)	-	9.1
Eosinophils counts, mean cells ($X\ 10^3 \pm SD$)	0.04-0.4	0.0580 ± 0.1
Eosinopenia (%)	-	65.5
Basophils counts, mean cells ($X\ 10^3 \pm SD$)	0.02-0.1	0.0833 ± 0.1
Basopenia (%)	-	43.1

4.4 Laboratory Diagnosis for Dengue

Blood taken from DV-infected patients were diagnosed as “confirmed and/or positive dengue cases” by using NS1 antigen and DV IgM serological test detection. All DV-infected patients included in this study had index value more than 1.1, which is an indication of positive results for both NS1 antigen and DV IgM tests and hence confirmed and/or positive for dengue infection (Figure 4.1).

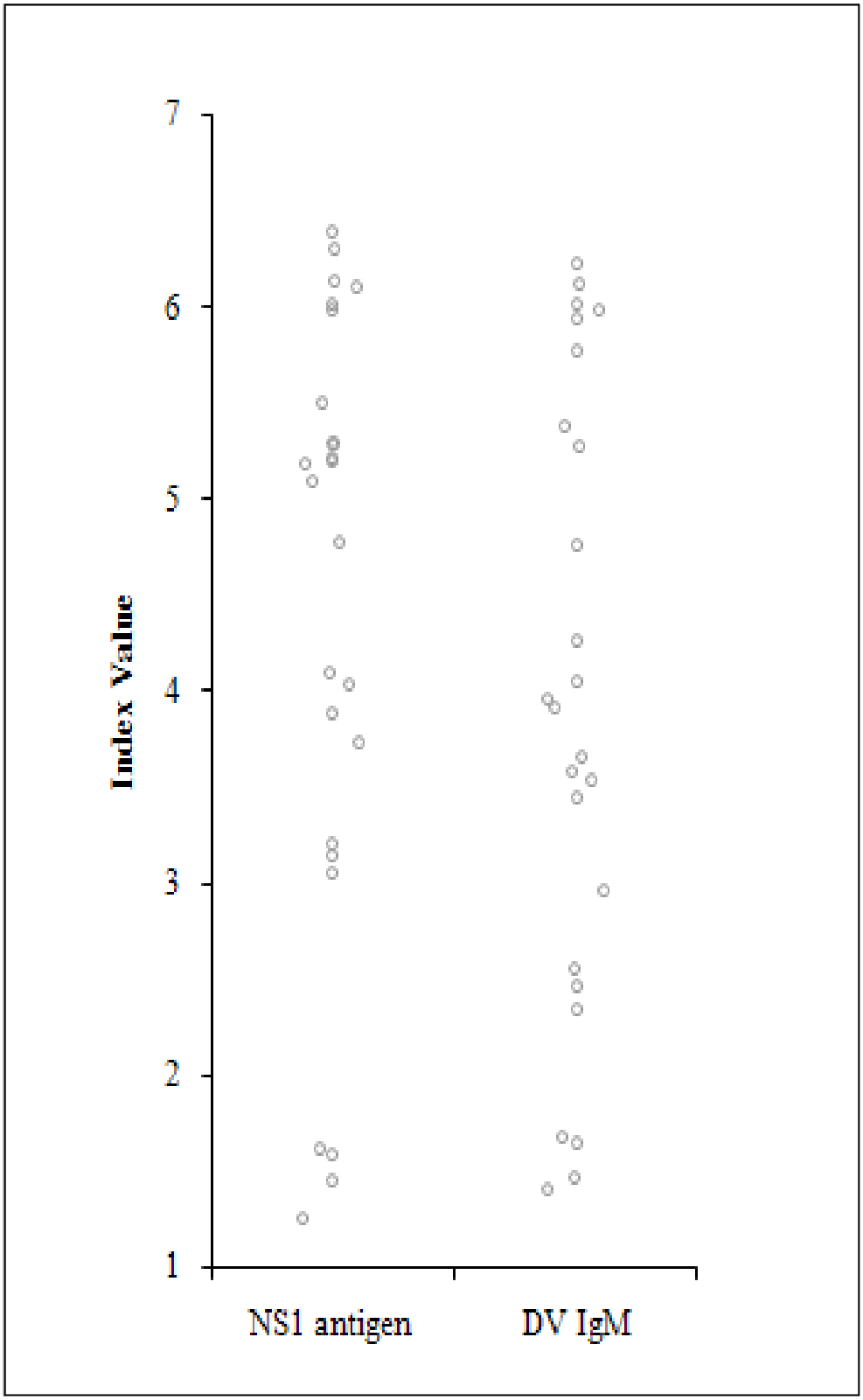

Figure 4.1: The results for NS1 antigen and DV IgM test for DV-infected patients.

4.5 Median Levels of IL-6 in Serum of DV-Infected Patients and Healthy Controls

Mann-Whitney *U* test reveals that there is a significant difference ($p<0.05$) in the levels of IL-6 in serum of DV-infected patients and healthy controls. The concentrations of IL-6 in serum of DV-infected patients and healthy controls are illustrated in Figure 4.2. The median level of IL-6 in healthy controls was approximately 3 times lower than the median level of IL-6 in DV-infected patients as tabulated in Table 4.4.

Table 4.4: The median concentrations of IL-6 (pg/mL) in the serum of DV-infected patients and healthy controls.

Cytokine	Groups	Median concentration (pg/mL)	Concentration range (pg/mL)	Mann-Whitney *U* test P value
IL-6	Healthy controls	5.03	1.31-10.13	$p<0.001$
	DV-infected patients	15.36	0.13-53.81	

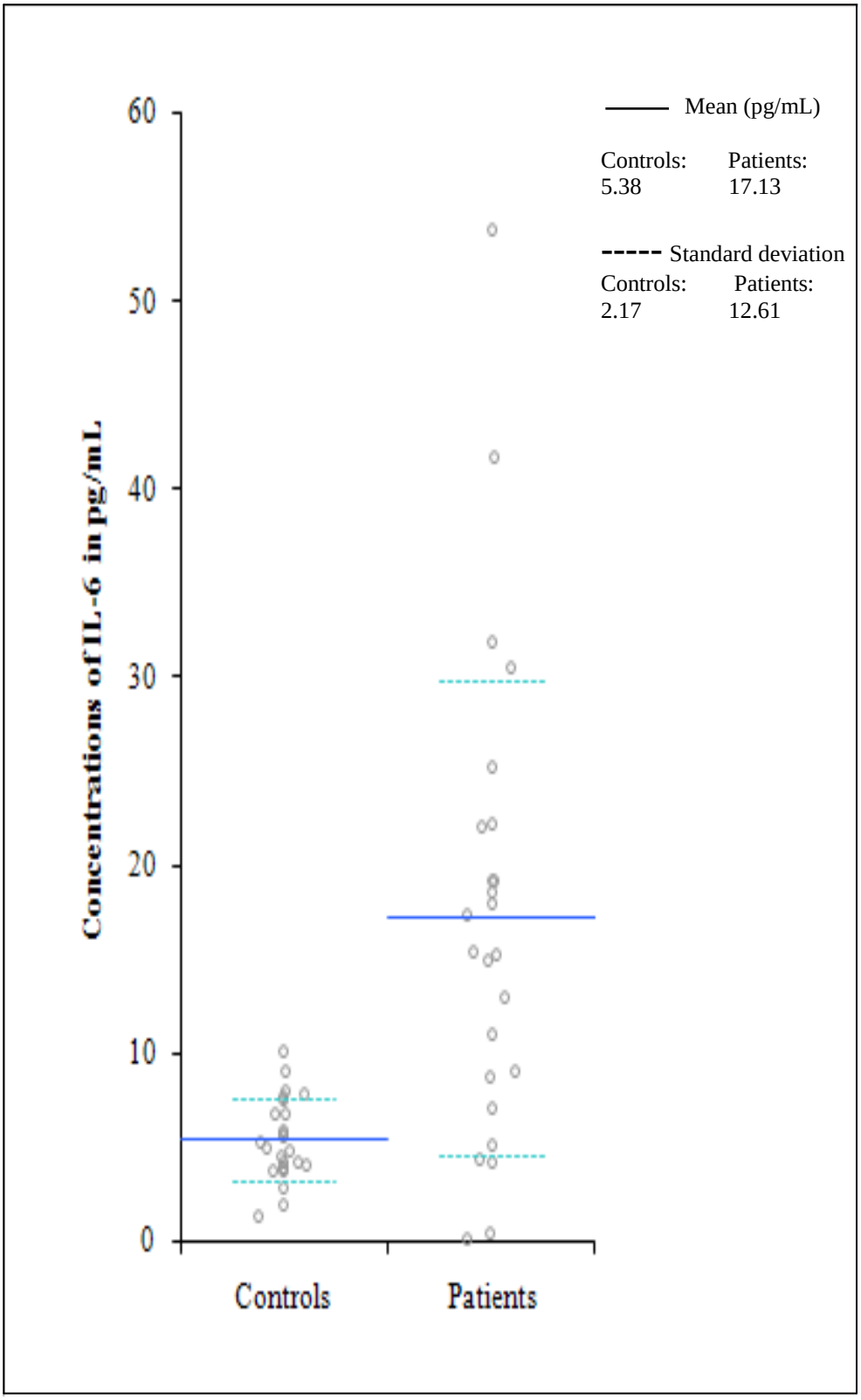

Figure 4.2: The concentrations of IL-6 (pg/mL) in the serum of DV-infected patients and healthy controls.

4.6 Median Levels of IL-8 in Serum of DV-Infected Patients and Healthy Controls

A significant difference ($p<0.05$) is found in levels of IL-8 in the serum of DV-infected patients and healthy controls using Mann-Whitney *U* test and IL-8 concentrations in serum of both groups are shown in Figure 4.3. The increment of median IL-8 level in the serum of DV-infected patients was about three times as compared to the median levels of IL-8 in serum of healthy controls (Table 4.5).

Table 4.5: The median concentrations of IL-8 (pg/mL) in the serum of DV-infected patients and healthy controls.

Chemokine	Groups	Median concentration (pg/mL)	Concentration range (pg/mL)	Mann-Whitney *U* test P value
IL-8	Healthy controls	30.92	15.41-60.58	$p<0.001$
	DV-infected patients	86.78	3.14-269.65	

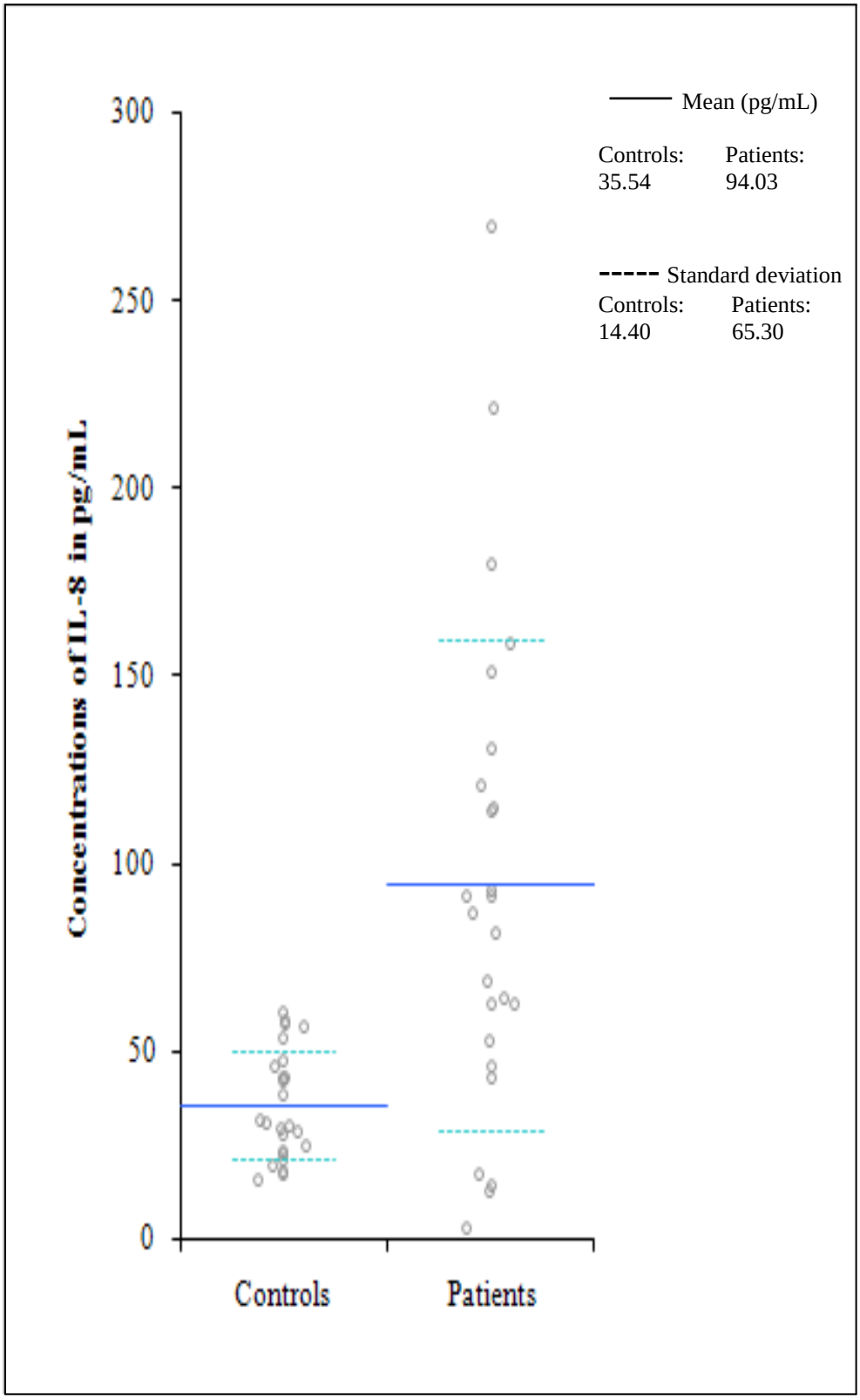

Figure 4.3: The concentrations of IL-8 (pg/mL) in the serum of DV-infected patients and healthy controls.

4.7 Median Levels of IL-15 in Serum of DV-Infected Patients and Healthy Controls

There is significant ($p < 0.05$) upregulated level of IL-15 in serum of DV-infected patients as compared to healthy controls. Figure 4.4 shows the concentrations of IL-15 in serum of DV-infected patients and healthy controls. Table 4.6 tabulates the approximately two times increment in the median level of IL-15 in serum of DV-infected patients as compared to the median level of IL-15 in serum of healthy controls.

Table 4.6: The median concentrations of IL-15 (pg/mL) in the serum of DV-infected patients and healthy controls.

Cytokine	Groups	Median concentration (pg/mL)	Concentration range (pg/mL)	Mann-Whitney *U* test P value
IL-15	Healthy controls	1.97	0.36-4.25	p<0.001
	DV-infected patients	4.58	1.76-17.42	

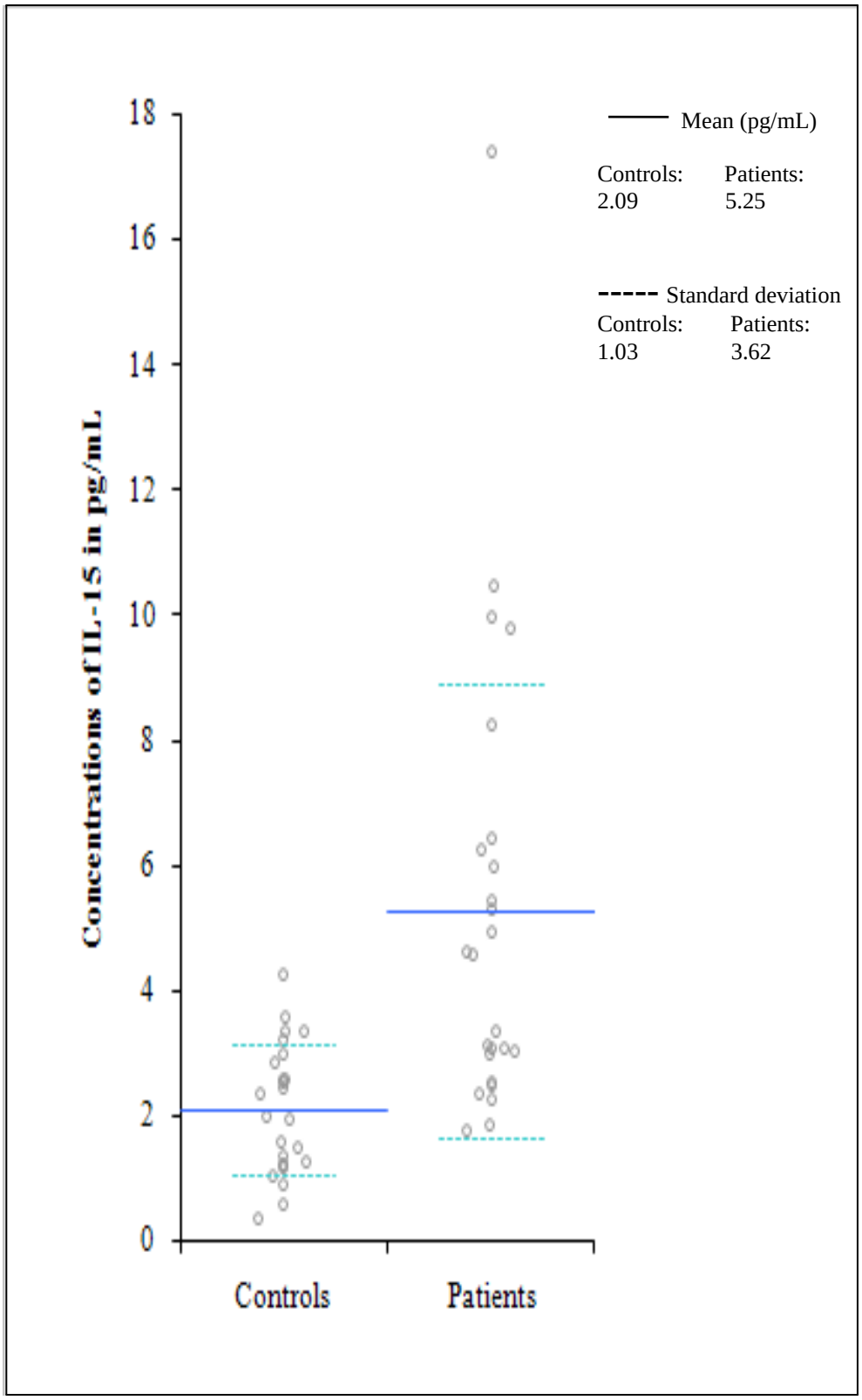

Figure 4.4: The concentrations of IL-15 (pg/mL) in the serum of DV-infected patients and healthy controls.

4.8 Median Levels of IL-16 in Serum of DV-Infected Patients and Healthy Controls

As analyzed by Mann-Whitney *U* test, a significant difference ($p<0.05$) is found in levels of IL-16 in serum of DV-infected patients and healthy controls (Table 4.7). The distribution of IL-16 levels for both dengue patients and healthy controls is shown in Figure 4.5. The result shows that there is an up-regulation of median level of IL-16 in serum of DV-infected patients as compared to the median level of IL-16 in serum of healthy controls. The up-regulation of median level of IL-16 in serum of DV-infected patients was about two times above those in the healthy controls.

Table 4.7: The median concentrations of IL-16 (pg/mL) in the serum of DV-infected patients and healthy controls.

Cytokine	Groups	Median concentration (pg/mL)	Concentration range (pg/mL)	Mann-Whitney *U* test P value
IL-16	Healthy controls	69.26	34.52-194.63	p<0.001
	DV-infected patients	142.26	17.78-352.04	

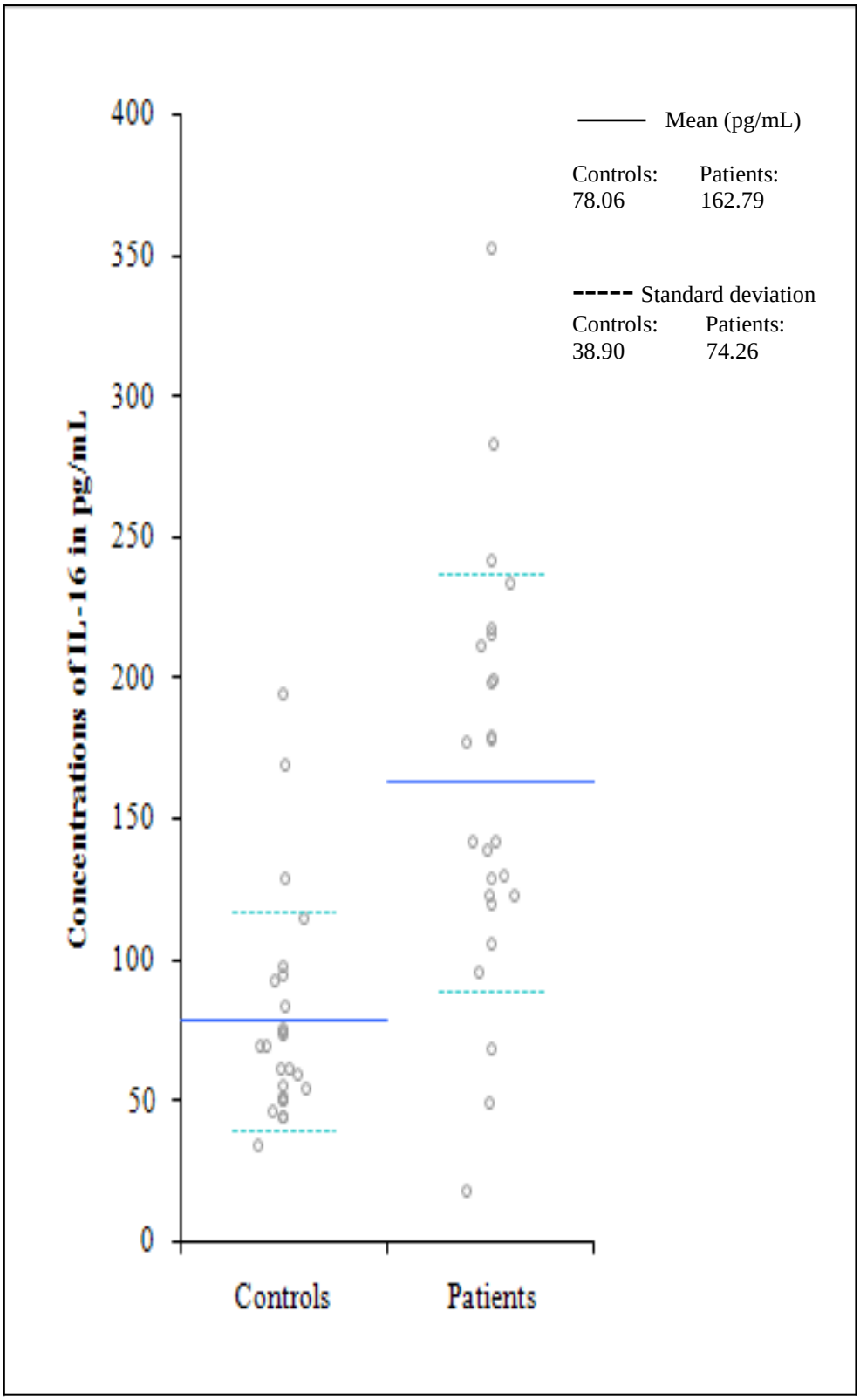

Figure 4.5: The concentrations of IL-16 (pg/mL) in the serum of DV-infected patients and healthy controls.

4.9 Median Levels of IFN-γ in Serum of DV-Infected Patients and Healthy Controls

Mann-Whitney *U* test demonstrates a significant difference ($p<0.05$) in levels of IFN-γ in serum of both DV-infected patients and healthy controls. Concentrations of IFN-γ in serum of patients with DV infection and healthy controls are plotted in Figure 4.6. As shown in Table 4.8, median level of IFN-γ in serum of DV-infected patients was around two times higher than those in the healthy controls.

Table 4.8: The median concentrations of IFN-γ (pg/mL) in the serum of DV-infected patients and healthy controls.

Cytokine	Groups	Median concentration (pg/mL)	Concentration range (pg/mL)	Mann-Whitney *U* test P value
IFN-γ	Healthy controls	7.82	4.20-69.44	p<0.01
	DV-infected patients	13.70	4.90-71.76	

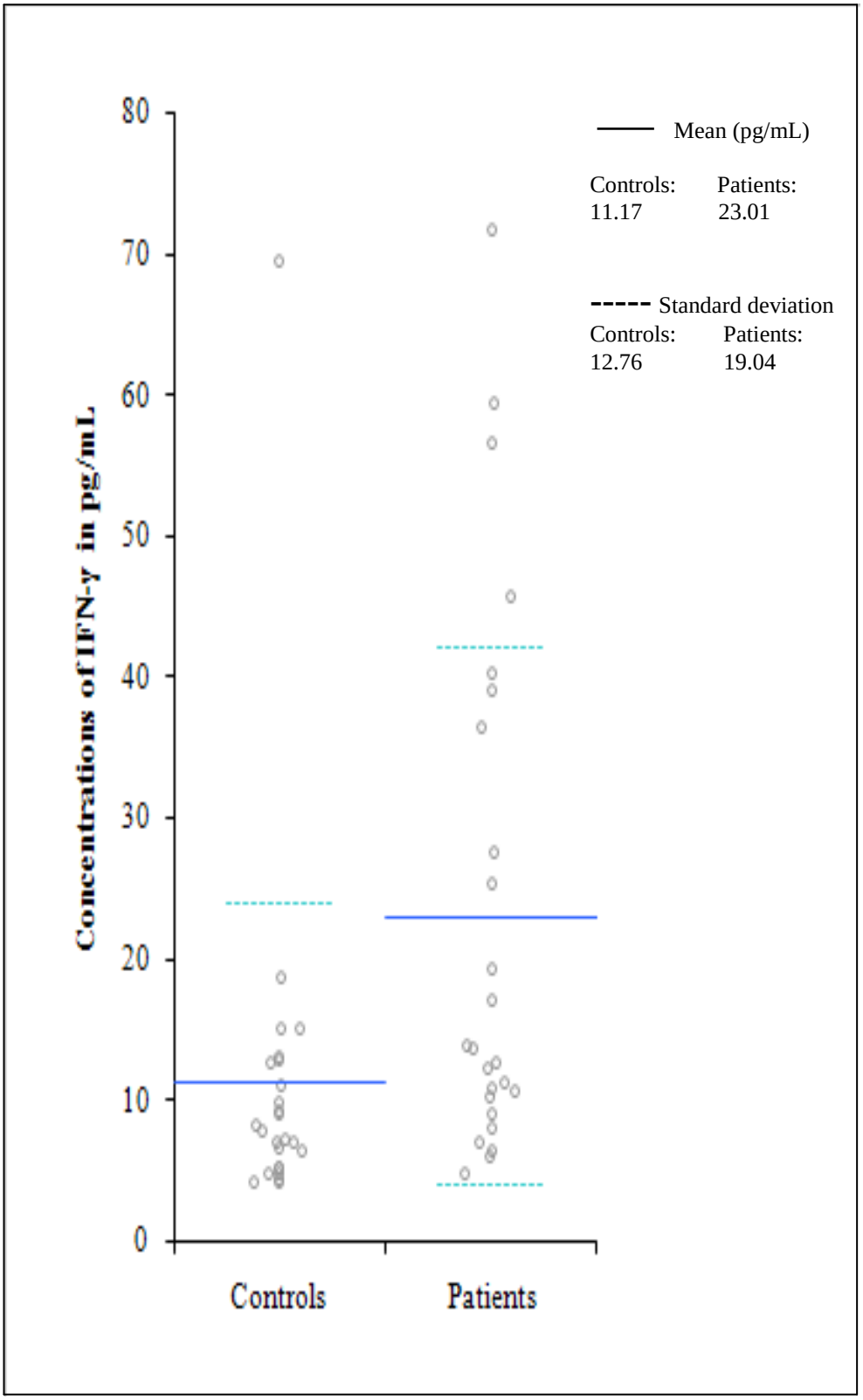

Figure 4.6: The concentrations of IFN-γ (pg/mL) in the serum of DV-infected patients and healthy controls.

4.10 Median Levels of MCP-1 in Serum of DV-Infected Patients and Healthy Controls

A significant difference ($p<0.05$) in levels of MCP-1 in serum of DV-infected patients and healthy controls is obtained by using Mann-Whitney *U* test. Figure 4.7 shows the MCP-1 levels in serum of both dengue patients and healthy controls. The results indicate that the median level of MCP-1 in serum of DV-infected patients was approximately two fold higher than those levels in healthy controls (Table 4.9).

Table 4.9: The median concentrations of MCP-1 (pg/mL) in the serum of DV-infected patients and healthy controls.

Chemokine	Groups	Median concentration (pg/mL)	Concentration range (pg/mL)	Mann-Whitney *U* test P value
MCP-1	Healthy controls	140.59	80.96-212.82	p<0.001
	DV-infected patients	248.29	117.82-625.73	

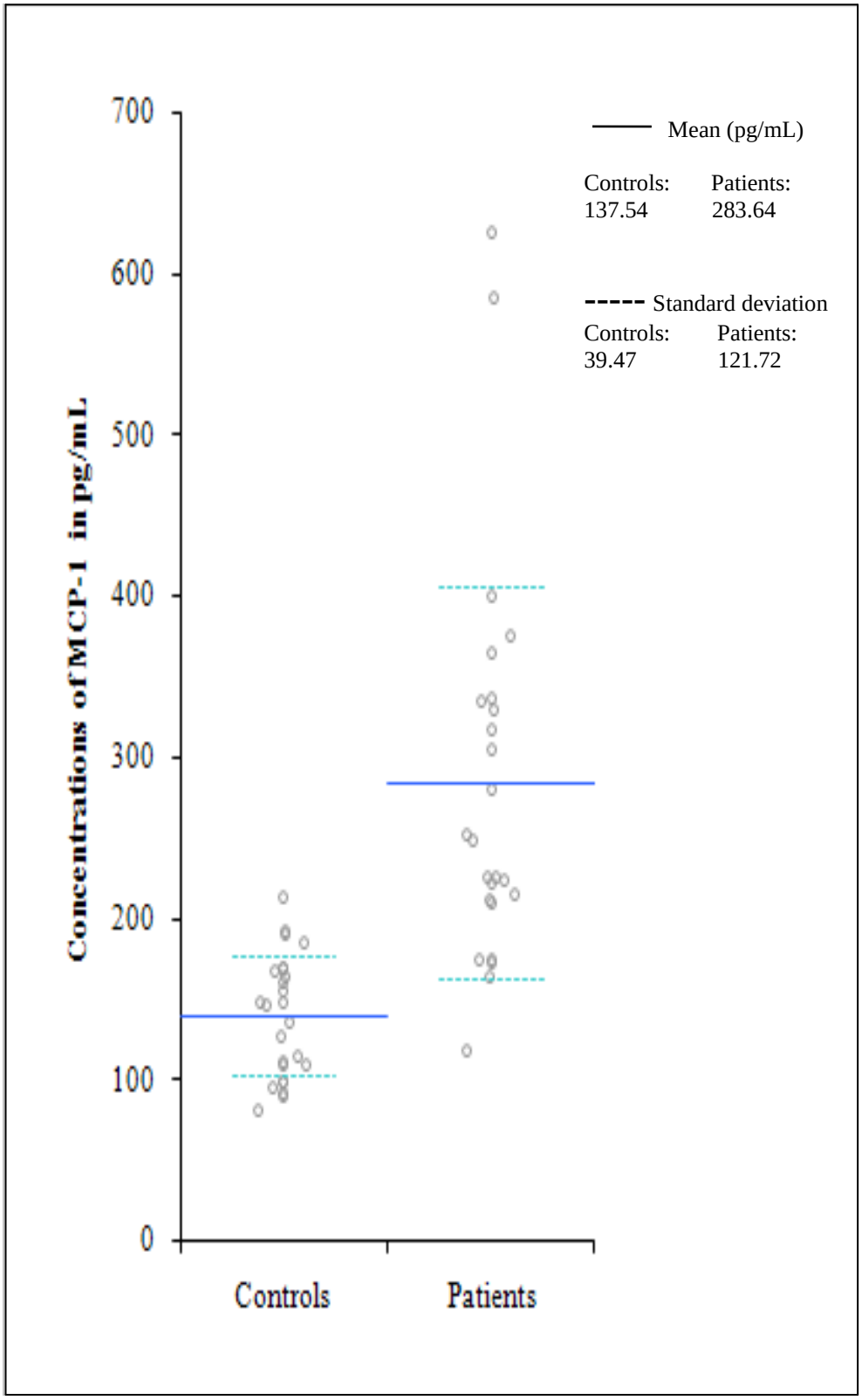

Figure 4.7: The concentrations of MCP-1 (pg/mL) in the serum of DV-infected patients and healthy controls.

4.11 Median Levels of MIG in Serum of DV-Infected Patients and Healthy Controls

The levels of MIG is seen to be significantly higher ($p<0.05$) in the serum of DV-infected patients as compared to healthy controls (Figure 4.8). There was a large increase in the median level of MIG in serum of DV-infected patients, where the median level of MIG in serum of DV-infected patients was around twelve times higher than those in the healthy controls (Table 4.10).

Table 4.10: The median concentrations of MIG (pg/mL) in the serum of DV-infected patients and healthy controls.

Chemokine	Groups	Median concentration (pg/mL)	Concentration range (pg/mL)	Mann-Whitney *U* test P value
MIG	Healthy controls	53.36	25.75-547.58	p<0.001
	DV-infected patients	631.92	119.50-3685.61	

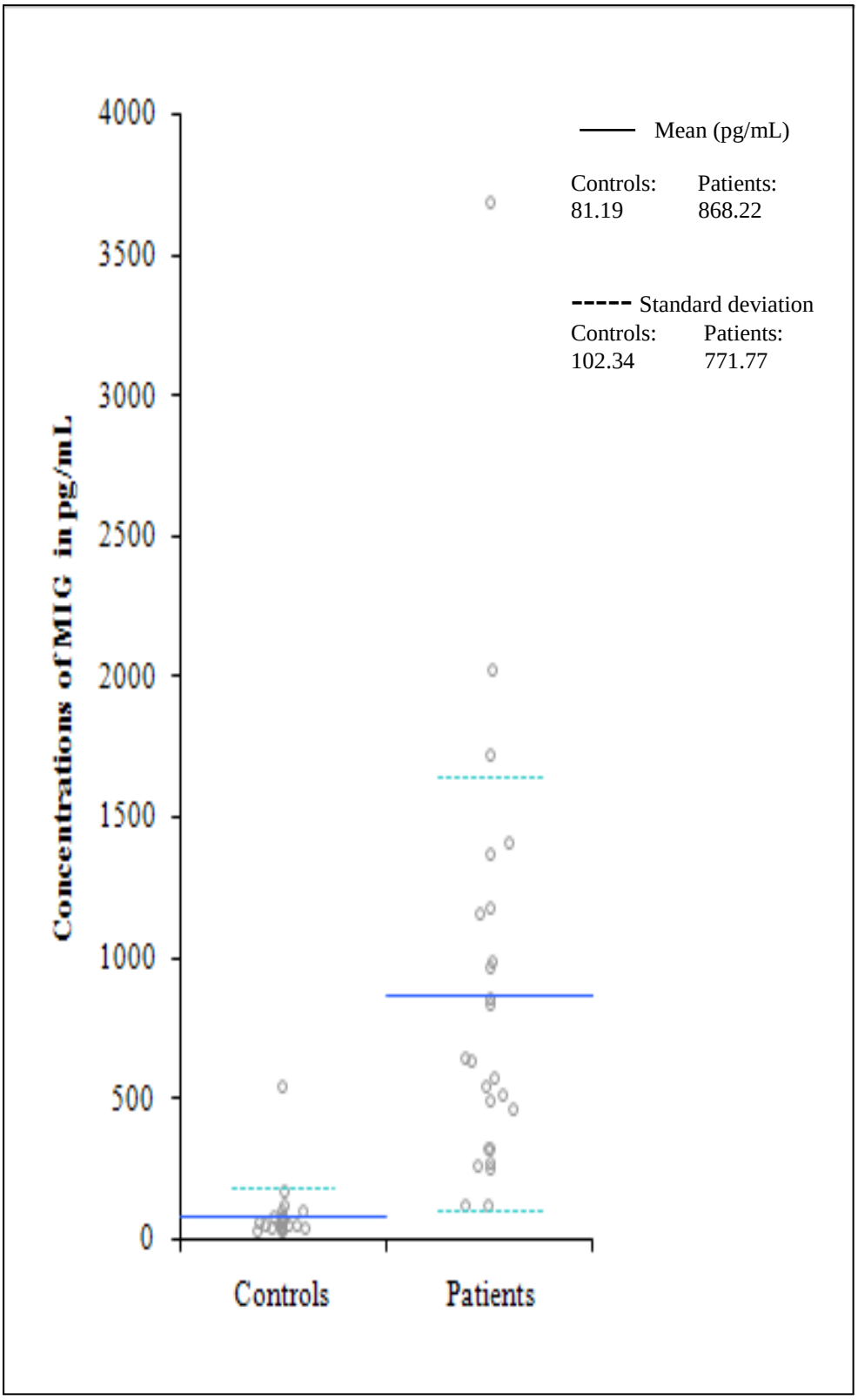

Figure 4.8: The concentrations of MIG (pg/mL) in the serum of DV-infected patients and healthy controls.

4.12 Median Levels of RANTES in Serum of DV-Infected Patients and Healthy Controls

The different concentrations of RANTES in serum of dengue patients and healthy controls are plotted in Figure 4.9. As illustrated by Mann-Whitney *U* test, a significant difference ($p<0.05$) was found in the levels of RANTES in serum of DV-infected patients and healthy controls. As shown in Table 4.11, the median level of RANTES in serum of DV-infected patients was down-regulated around eight folds as compared to the median level of RANTES in serum of healthy controls.

Table 4.11: The median concentrations of RANTES (pg/mL) in the serum of DV-infected patients and healthy controls.

Chemokine	Groups	Median concentration (pg/mL)	Concentration range (pg/mL)	Mann-Whitney *U* test P value
RANTES	Healthy controls	457.93	57.47-1030.87	p<0.001
	DV-infected patients	54.93	5.00-644.27	

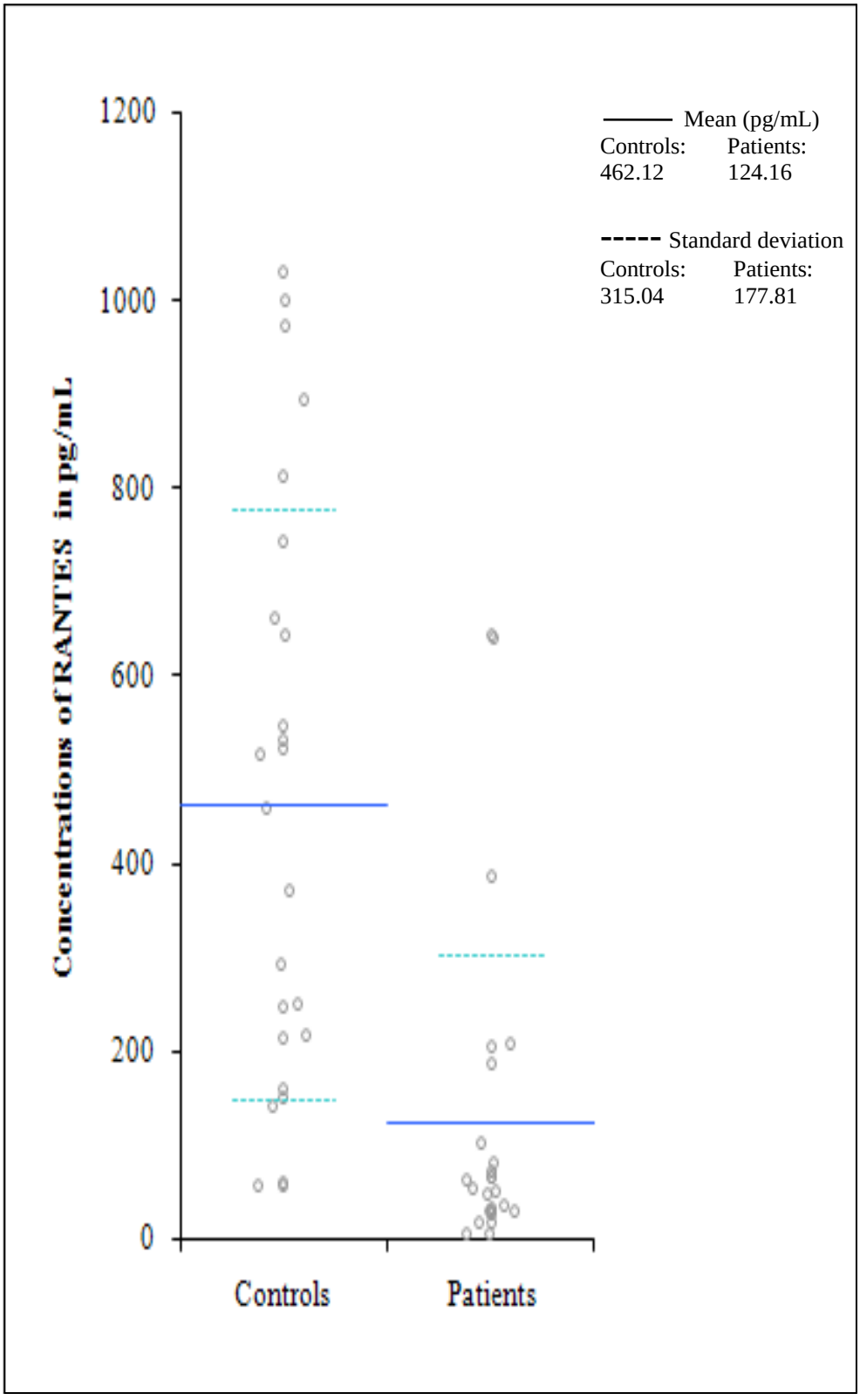

Figure 4.9: The concentrations of RANTES (pg/mL) in the serum of DV-infected patients and healthy controls.

4.13 Differences and Relationships in Cytokines and Chemokines Levels between Gender, Age, Day of Infection and Clinical Signs of Disease.

As shown by Mann-Whitney *U* test, there is no significant difference in cytokines and chemokines levels between female and male ($p>0.05$) (Table 4.12). Besides, Mann-Whitney *U* test also disclosed that there was no significant difference ($p>0.05$) between cytokines and chemokines levels as compared to different age groups (age ≤ 35 and >35) (Table 4.13), presence or absence of myalgia (Table 4.14) and presence or absence of joint pain (Table 4.15). On the other hand, in Table 4.16, the Spearman rank order correlation shows that there was no significant relationship ($p>0.05$) between cytokines and chemokines levels with days of infection.

Table 4.12: Median concentration (pg/mL) of cytokines and chemokines in DV infected patients for different gender.

Category	Median concentration (pg/mL) of cytokine or chemokine in DV-infected patients				Mann-Whitney *U* test
	Gender and standard deviation				
	Male	Standard deviation	Female	Standard deviation	P value
IL-6	17.66	10.77	12.99	16.09	0.61
IL-8	91.24	68.47	75.58	58.60	0.45
IL-15	5.19	4.02	3.02	1.12	0.12
IL-16	158.07	78.10	142.26	65.39	0.72
IFN-γ	13.79	18.02	11.41	21.98	0.32
MCP-1	278.56	143.72	223.94	56.56	0.34
MIG	612.07	507.05	631.92	1107.65	0.78
RANTES	33.97	197.18	63.73	127.58	0.20

Table 4.13: Median concentration (pg/mL) of cytokines and chemokines in DV infected patients for different age category (≤35 and >35 years).

Category	Median concentration (pg/mL) of cytokine or chemokine in DV-infected patients				Mann-Whitney *U* test
	Age (years) and standard deviation				
	≤35	Standard deviation	>35	Standard deviation	P value
IL-6	17.92	14.07	15.11	8.48	0.45
IL-8	81.71	70.19	125.71	52.87	0.22
IL-15	5.32	3.91	3.01	0.84	0.06
IL-16	177.52	74.03	132.35	72.58	0.31
IFN-γ	15.23	20.44	18.28	6.71	0.12
MCP-1	236.99	126.30	328.94	93.11	0.40
MIG	738.61	867.56	542.50	303.82	0.30
RANTES	66.23	163.67	50.87	224.68	0.90

Table 4.14: Median concentration (pg/mL) of cytokines and chemokines in DV infected patients in the absent or present of myalgia.

Category	Median concentration (pg/mL) of cytokine or chemokine in DV-infected patients				Mann-Whitney *U* test
	Myalgia and standard deviation				
	Absent	Standard deviation	Present	Standard deviation	P value
IL-6	17.92	8.64	15.11	13.55	0.89
IL-8	114.78	30.96	66.13	70.95	0.15
IL-15	3.24	0.87	4.93	3.85	0.46
IL-16	130.76	67.18	177.52	77.56	0.61
IFN-γ	12.72	12.67	13.74	20.37	0.73
MCP-1	183.05	92.26	252.39	121.55	0.23
MIG	639.49	404.04	577.08	851.94	0.66
RANTES	65.27	265.95	50.90	156.39	0.79

Table 4.15: Median concentration (pg/mL) of cytokines and chemokines in DV infected patients in the absent or present of joint pain.

Caterogy	Median concentration (pg/mL) of cytokine or chemokine in DV-infected patients				Mann-Whitney *U* test
	Joint pain and standard deviation				
	Absent	Standard deviation	Present	Standard deviation	P value
IL-6	15.46	18.33	16.39	12.38	0.13
IL-8	76.51	15.46	89.00	64.41	0.13
IL-15	2.24	3.63	3.98	3.64	0.27
IL-16	172.41	61.49	142.26	76.38	0.76
IFN-γ	13.79	20.84	13.21	19.35	0.89
MCP-1	174.14	153.67	250.34	122.13	0.27
MIG	576.00	403.57	631.92	771.77	0.37
RANTES	54.17	39.55	54.93	184.21	0.76

Table 4.16: Spearman rank order correlation between the cytokines and chemokines levels and days of infection.

Category	Infection day	
	Spearman rho value (r)	P value
IL-6	0.027	0.897
IL-8	-0.056	0.792
IL-15	0.103	0.625
IL-16	0.008	0.956
IFN-γ	-0.075	0.723
MCP-1	0.096	0.648
MIG	0.074	0.726
RANTES	0.083	0.682

4.14 Correlations among the Cytokines and Chemokines Levels and Clinical Findings.

As shown in Table 4.17, Spearman rank order correlation reveals that the levels of IL-15 were positively and moderately correlated with the levels of IFN-γ ($r = 0.343$, $p<0.05$) in DV-infected patients, where high levels of IFN-γ were associated with high levels of IL-15. Also, levels of IL-15 were negatively and moderately correlated with the levels of basophils ($r = -0.395$, $p<0.05$) in dengue patients. In addition, IL-15 levels showed a strong, negative associations with white blood cells count ($r = -0.557$, $p<0.01$), lymphocytes count ($r = -0.755$, $p<0.01$), monocytes count ($r = -0.757$, $p<0.01$) and eosinophils count ($r = -0.640$, $p<0.01$) in DV-infected patients. These results indicated that high levels of IL-15 were correlated with low levels of white blood cells like lymphocytes, monocytes, eosinophils and basophils. On the other hand, levels of IL-16 were positively and averagely correlated with MIG levels ($r = 0.482$, $p<0.05$) in DV-infected patients, where high MIG levels were associated with high levels of IL-16. Moreover, there was a strong, positive association between levels of MCP-1 and IL-8 ($r = 0.531$, $p<0.01$) in DV-infected patients, with high levels of MCP-1 associated with high levels of IL-8 in the patients. In contrast, a strong, negative association between MCP-1 levels and monocytes counts ($r = -0.575$, $p<0.01$) was obtained in DV-infected patients. Additionally, levels of MCP-1 was negatively and moderately associated with basophils count ($r = -0.485$, $p<0.01$) in patients with DV infection. These results suggested that low levels of monocytes and basophils were correlated with high levels of MCP-1. Furthermore, high

levels of IL-15 were associated with low levels of RANTES since a medium, negative relationship was found between RANTES and IL-15 levels ($r = -0.388$, $p<0.05$) in DV-infected patients. Besides, a large, positive relationship was obtained between levels of RANTES and lymphocytes counts ($r = 0.548$, $p<0.01$) in dengue patients whilst the correlation between levels of RANTES and eosinophils count in dengue patients was positively, medium associated ($r = 0.385$, $p<0.05$). Also, a positive, medium association was demonstrated between RANTES levels and platelets counts ($r = 0.474$, $p<0.01$) in patients with DV infection. These results again proved that low levels of RANTES were associated with low levels of lymphocytes, eosinophils and platelets.

Table 4.17: Spearman rank order correlation between the cytokines and chemokines levels and clinical findings.

Scale	Spearman rho value (r)			
	IL-15	IL-16	MCP-1	RANTES
IL-8	-0.261	0.073	0.531**	0.219
IFN-γ	0.343*	0.301	0.081	-0.256
MIG	-0.226	0.482*	0.205	0.030
RANTES	-0.388*	0.130	0.067	-
WBC	-0.557**	-0.085	-0.240	0.215
Neutrophils	-0.114	-0.109	0.261	0.076
Lymphocytes	-0.755**	-0.066	-0.305	0.548**
Monocytes	-0.757**	0.115	-0.575**	0.222
Eosinophils	-0.640**	0.038	-0.293	0.385*
Basophils	-0.395*	0.084	-0.485**	0.052
Platelets	0.059	-0.049	0.303	0.474**

*. Correlation is significant at the 0.05 level

**. Correlation is significant at the 0.01 level

CHAPTER 5

DISCUSSION

DV infection is an acute infectious viral disease that presents a broad clinical spectrum from asymptomatic to more severe manifestations (McBride & Bielefeldt-Ohmann, 2000). It is believed that host immune response plays a significant role in pathogenesis of DV infection (Pichyangkul et al., 2003). Studies have shown the elevation of certain cytokines and chemokines in the serum, blood or pleural fluid of patients with DV infection (Chen & Wang, 2002; Medin et al., 2005), as cytokines and chemokines are involved in the disease onset and homeostatic regulation (Bozza et al., 2008). Although the clinical manifestations of dengue had already been described, the immunopathogenesis of this disease is still not completely understood. Thus, a number of cytokines and chemokines that contributed to the immune response of DV-infected patients were highlighted and a possible cytokine network and mechanism was proposed in this study in order to figure out the immunopathogenesis of DV infection.

In this study, regulations of IL-6, IL-8, IL-15, IL-16, IFN-γ, MCP-1, MIG and RANTES showed no significant relationship with both age and gender. These results suggested that age and gender had no influence in the production of the mentioned cytokines and chemokines. Also, the days of DV infection had no effect in regulating the cytokines and chemokines levels in DV-infected patients as no significant correlation was observed between these two groups.

Besides, the levels of these cytokines and chemokines did not contribute to myalgia or joint pain that were observed in dengue patients since no significant relationship was obtained between this two groups.

5.1 IL-6

Levels of IL-6 in serum of DV-infected patients were significantly higher than the healthy controls. The up-regulation of IL-6 in dengue patients in this study was supported by Chen et al. (2006). They demonstrated that serum levels of IL-6 were significantly increased in dengue patients than in healthy controls. Similar result was obtained years later by Restrepo et al. (2008a) where they found that serum levels of IL-6 in children with dengue was higher than those without dengue. In addition, the degree of increment of IL-6 in DV-infected patients in this present research was approximately the same as the research done by Chen et al. (2006). In contrast, the mean levels of IL-6 in dengue patients in this present study were about three times higher than the mean levels of IL-6 in dengue patients in a study done by Restrepo et al. (2008a) (5.65 pg/mL). This might be due to a difference in the age group as the levels of IL-6 in this research were obtained from the adult patients whilst the levels of IL-6 done by Restrepo et al. (2008a) was obtained from the children patients.

Increased levels of IL-6 in serum of dengue patients were induced by DV infection. This is because IL-6 is not a cytokine that is constitutively expressed, but it is readily induced by multiple stimuli, which includes viral

infection (Streetz et al., 2007). Interleukin-6 is synthesized predominantly by monocytes and macrophages (Yap & Lai, 2010) upon viral infection. Besides, IL-6 is also produced by T cells and B cells in response to stimuli such as RNA virus (Yap & Lai, 2010), which lead to the up-regulation of IL-6 in DV-infected patients. Interleukin-16 also plays an important role in stimulating the production of IL-6 from monocytes (Qi et al., 2002). However it was found to have no or limited effect on IL-6 production in DV-infected patients in this present study as levels of IL-6 were not significantly associated with levels of IL-16 in DV-infected patients.

The elevation of IL-6 levels in the DV-infected patients showed that this cytokine played an essential role in the host defense against DV infection. IL-6 is thought to be involved in the transition from an innate immune response to an adaptive immune response (Ellis & Beaman, 2004) as it is essential in the normal development and function of both T cells and B cells (Yap & Lai, 2010). IL-6 activates T cells, which recognizes antigens and stimulates the proliferation and differentiation of CD8+ T cells (Łukaszewicz et al., 2007), which subsequently take part in the viral clearance. Also, it stimulates B cells differentiation and induces the maturation of B cells into plasma cells, which produces different classes of immunoglobulin (Yap & Lai, 2010) that might play an important role in the clearance of DV and viral products.

The up-regulation of IL-6 levels in the DV-infected patients might also contribute to several manifestations of this disease. IL-6 is an endogenous pyrogen (Restrepo et al., 2008b) where it increases the body temperature by

inducing the synthesis of prostaglandins (Łukaszewicz et al., 2007) and thus caused fever in the DV-infected patients. Elevated body temperature is beneficial to host defense as viruses grow less efficiently at raised temperature (Coico, Sunshine, & Benjamini, 2003). This might control the virus proliferation in the host system. Besides, up-regulation of IL-6 levels probably also cause anorexia and lethargy (Maggio et al., 2006) in DV-infected patients, as these manifestations are normally seen to occur in patients with DV infection. In addition, high levels of IL-6 in DV-infected patients might increase vessel permeability of the endothelial cells (Restrepo et al., 2008b) through induction of gap formation between adjacent endothelial cells, which subsequently caused the pleural effusion in the DV-infected patients (Lee et al., 2007). Priyadarshini et al. (2010) supported this idea where they found that there was an increased levels of IL-6 in 65% of the DV-infected patients who showed pleural effusion. This cytokine was also reported to play a crucial role in the enhancement of production of anti-platelet auto antibodies during DV infection (Rachman & Rinaldi, 2006) that possibly caused thrombocytopenia in the DV-infected patients. Therefore, IL-6 is a main factor involved in the immune response of DV-infected patients due to its multiple activities against the viral infection.

5.2 IL-8

Normally, IL-8 protein is barely secreted from non-induced cells (Hoffmann, Dittrich-Breiholz, Holtmann, & Kracht, 2002). However, levels of IL-8 in the serum of DV-infected patients, in this study, were significantly elevated as

compared to the serum levels of IL-8 in the healthy controls. This might be due to the production of IL-8 that was rapidly induced by DV or viral products (Medin et al., 2005) from monocytes. Macrophages and neutrophils might also produce IL-8 (Mukaida, 2003) upon DV infection. Interleukin-15 which was also found to act as an inducer in the production of IL-8 (Jabłońska et al., 2003), had no or limited effect in the production of IL-8 from DV-infected patients in this present study as levels of IL-8 were not in significant correlation with the levels of IL-15 in DV-infected patients.

Increased levels of IL-8 might subsequently chemoattracted neutrophils to the site of infection (Bosch et al., 2002) and induced a respiratory burst of neutrophils which consequently caused the release of lytic enzymes to rid the host of invading virus (Medin et al., 2005). Surprisingly, there was no significant relationship between levels of IL-8 and neutrophils counts. This result suggested that IL-8 had no or limited effect in chemoattracting neutrophils to infiltrate the tissue to reach the site of infection. However, this chemokine might have a role in degranulation of neutrophils to release the lytic enzyme to clear the viral particles.

Additionally, high concentrations of IL-8 could possibly alter the cytoskeleton and tight junctions of microvascular endothelium and changed the permeability of the endothelial monolayer (Medin et al., 2005). This leads to the occurrence of plasma leakage, which is normally seen in the more severe form of dengue illness. Priyadarshini et al. (2010) also showed that the increased levels of IL-8 were associated with plasma leakage.

Besides, high levels of IL-8 in the serum of DV-infected patients may also induce the synthesis and release of platelet-activating factor in neutrophils (Mukaida, 2003). Platelet-activating factor might then participate in platelet activation, which led to platelet consumption (Bozza et al., 2008) in DV-infected patients. This later could cause the platelet number to decrease and hence, thrombocytopenia was seen in patients with DV infection. Priyadarshini et al. (2010) supported the fact that IL-8 were associated with thrombocytopenia where they reported that the levels of IL-8 in dengue patients with thrombocytopenia were significantly higher than in the patients that showed normal platelet count. Furthermore, high levels of IL-8 may also contribute to fever in DV-infected patients since IL-8 is an endogenous pyrogen (Pela´ et al., 2000).

Moreover, elevation of IL-8 levels in DV-infected patients might indirectly cause T cell chemotaxis by inducing neutrophils to release several factors from their granules with chemotactic activities for T cells (Mukaida, 2003). Chemoattracted T cells might then produce more IL-8 as T cells are producers of this chemokine. Again, a positive feedback mechanism took place and this process amplified the T cells pool at the site of infection in helping the clearance of invading virus. As a result, this positive feedback mechanism led to high concentrations of IL-8 in the serum of DV-infected patients. This is supported by the research done by Y. Huang et al. (2000) where they found that IL-8 were up-regulated in DV-infected patients as compared to the healthy controls. The mean IL-8 concentrations in dengue patients of their study were also found to not deviate much from the mean IL-8 concentrations

in dengue patients conducted in this study. On the other hand, although Juffrie et al., (2000) found that the levels of IL-8 in DV-infected children were up-regulated as compared to the healthy controls, but the median IL-8 level obtained in that research was about three times lower than the median IL-8 level obtained in this study. This deviation may be due to the different age group that was included in their study, where they used children as their target group as compared to adults. Concentrations of IL-8 were determined in DV-infected children by Juffrie et al. (2000) while the concentrations of this chemokine were determined in DV-infected adults in this present research.

5.3 IL-15

Serum levels of IL-15 in DV-infected patients were significantly elevated as compared to the healthy controls. Azeredo et al. (2005) also demonstrated a similar result where they found that IL-15 was significantly elevated in the plasma of most dengue patients during the early acute phase as compared to healthy donors. The mean IL-15 concentrations shown by Azeredo et al. (2005) was approximately two times higher than mean IL-15 concentrations in this study. The measurement of IL-15 was done during the early acute phase (1-5 days after disease onset) by Azeredo et al. (2005) whilst the measurement of IL-15 was done 6 days in average after disease onset in this present study. The difference in the day of infection probably led to the difference seen in the levels of IL-15 in both studies.

The production of IL-15 in dengue patient was in response to DV infection. This is because IL-15 has low secretion potential (Hocke et al., 2007), and soluble IL-15 is hardly detected in normal physiological condition (Alpdogan & Brink, 2005). In fact, the production of IL-15 is in response to the danger signal such as RNA virus infection (Meier et al., 2005), in this case, DV infection. During DV infection, IL-15 might be produced by macrophages, monocytes and DC (Jabłońska et al., 2003) where these cells are critical in the first line of defense against viral infection. Moreover, the up-regulation of serum levels of IL-15 in DV-infected patients was not due to the release of IL-15 from monocytes by the stimulation of IL-16 as there was no significant correlation between these two cytokines.

The increased levels of IL-15 in serum of DV-infected patients suggested that this cytokine played an important role in the viral clearance during DV infection. IL-15 is involved in the activation and homeostatic maintenance of cells in the innate immune systems (Wang et al., 2005) as it is a NK cell regulator. It probably regulated NK-cell development, homeostasis and activity (Alpdogan & Brink, 2005) during the DV infection in this present study. This regulation is vital in the host defense against the viral infection since numerous studies had demonstrated that NK cells can selectively lyse virus-infected target cells while sparing uninfected cells (Fawaz et al., 1999). Azeredo et al. (2005) also proved the above statements where it was shown that IL-15 was seen to have an NK cell-dependent antiviral activity against HIV. In addition, high levels of IL-15 could possibly drive T-cell proliferation and induced effector T cells along with the stimulation of development of

memory T cells (Ramsborg & Papoutsakis, 2007) in the immune system of DV-infected patients. Also, the levels of IL-15 were significantly moderately correlated with the levels of IFN-γ in dengue patients. This proved that the levels of IFN-γ were in response to the levels of IL-15 in dengue patients. The high levels of IL-15 in DV-infected patients caused the up-regulation of IFN-γ as IL-15 appeared to enhance Th1 responses by increasing IFN-γ production from NK cells and T cells (D'Ettorre et al., 2006; Milano et al., 2002), which in turn contributed to an antiviral state in DV-infected patients.

High levels of IL-15 were also significantly correlated with low levels of white blood cells, which include lymphocytes, monocytes eosinophils and basophils in the circulation of dengue patients. This is because, IL-15 served as an important cytokine for the differentiation of lymphocytes (Hu et al., 2007; Ramsborg & Papoutsakis, 2007) and monocytes (Ueda et al., 2007) and this cytokine caused differentiation of these leukocytes and subsequently infiltrate through tissues to reach the site of infection in the defense system. Thus, lower levels of leukocytes were found in the circulation of dengue patients in this present study. Also, this cytokines could be regulating the activities for both eosinophils and basophils in establishing the barrier against the viral attack.

Besides the above, IL-15 is important in expansion, survival, homeostasis and functional maturation of NKT cells (Alpdogan & Brink, 2005). The functional status of NKT cells fall somewhere between innate and the adaptive immune system suggesting that the up-regulation of IL-15 in DV-infected

patients may aid the transition of host immune response in between both innate and the adaptive immune system. Also, increased level of IL-15 might enhance the activity of NK cells, T cells and NKT cells by delaying their apoptosis (Jabłońska et al., 2003) via induction of anti-apoptotic molecules like B-cell lymphoma-2 (Bcl-2) (Budagian et al., 2006).

5.4 IL-16

In this study, the levels of IL-16 in the serum of DV-infected patients were significantly higher as compared to the serum levels of IL-16 in healthy controls. A similar result was obtained where HIV infection up-regulated the production of microglial IL-16 (Zhao et al., 2004). Also, the mean concentrations of IL-16 in severe acute respiratory syndrome (SARS) patients also showed similar results where there were higher concentrations of IL-16 in SARS patients than that of the control group (Wang et al., 2003). On the other hand, serum levels of IL-16 in patients with systemic lupus erythematosus, which has the similar symptoms as dengue (Koike et al., 2002), were also up-regulated. These results suggested that IL-16 might play a crucial role in defense system of viral infection, in this case, the DV infection.

The induction of high levels of IL-16 in the DV-infected patients during the immunological response, might be due to the stimulation of cytokines production by the released viral products, in this case DV, as viruses are known to be the potent stimulators of cytokine expression *in vitro* and *in vivo* (Shen et al., 2004). Upon infection, APC internalize the viruses or viral

products and present the immunogenic viral peptides with major histocompatibility complex class (MHC)-I or class II (MHC-II) molecules for activation of virus-specific CD8+ or CD4+T cells, respectively (Abdel-Motal, Guay, Wigglesworth, Welsh, & Galili, 2007). IL-16 is then secreted by CD4+ or CD8+ T cells and response to antigen (Reich et al., 2001). Subsequently, IL-16 contributes to the recruitment of CD4+ T cells (McFadden et al., 2007) to the site of infection. The recruited T cells then synthesize and secrete more IL-16 that may serve to function as a positive feedback mechanism for further cell recruitment and activation (Wu et al., 1999). All this possibly brought about the up-regulation of IL-16 levels in DV-infected patients.

Besides induction of T cells chemotaxis, IL-16 also induces the migratory response in DC (Lynch et al., 2003). The chemotaxis of this APC to the site of infection might enhance their ability as APC and therefore, more IL-16 is being produced by CD4+ T cells and CD8+ T cells. Furthermore, DC are also the source of production of IL-16 (Hu et al., 2007), which might further up-regulated IL-16 levels in DV-infected patients in this present study.

5.5 IFN-γ

During viral infection, NK cells, which play an important role in the innate defense mechanisms of the host, has an aim to eliminate virus-infected cells (Fawaz et al., 1999). Upon infection, NK cells produce IFN-γ (Yang et al., 2005), which play a role in mediating protection against viral infection, especially long-term control of viral infections (Samuel, 2001). IFN-γ then

activates the NK cell effector functions (Schroder et al., 2004), where various cytotoxic molecules are released by NK cells to eliminate the invading virus. Since NK cells are the source of IFN-γ production, the up-regulation of IFN-γ in this present study might be produced by NK cells as IFN-γ is vital in enhancing the viral clearance during the innate immune response (Diamond et al., 2000) in DV-infected patients.

Besides, IFN-γ also mediates the differentiation of naive T cells toward Th1 phenotype (Teixeira et al., 2005) as the phenotype adopted by a naive T cell during T cell activation is heavily influenced by the cytokine (Schroder et al., 2004). Th1 cells then secrete large amount of IFN-γ (Chen et al., 2005) as Th1 cells are the main producers of this cytokine (Restrepo et al., 2008b). This process further skews the immune response toward a Th1 phenotype, and this process probably caused the up-regulation of IFN-γ levels in the serum of DV-infected patients. Th1 cells are important in the viral elimination as they are responsible for cell-mediated immunity with the activation of NK cells, macrophages and CD8+ T cells (Coico et al., 2003). Th1 cells are also essential in providing phagocyte-dependent protective responses (Romagnani, 2000) in the host immune response. In downstream process of Th1 response, cytokine and chemokine are produced in order to chemoattract specific immunocytes to the site of infection. As discussed earlier, IFN-γ secretion is the hallmark of Th1 response (Gattoni et al., 2006). The IFN-γ might amplify the production of chemokines that participate in a wide range of bioactivities. One of the most important roles of chemokine is to direct the recruitment or chemotaxis of immune cells to the site of infection, which ultimately lead to

destruction of virally infected cells (Le et al., 2004). In addition, IFN-γ may also stimulate the development of CD8+ T cells (Yang et al., 2005), which is important as the downstream effectors of Th1 cells. At the same time, CD8+ T cells synthesize IFN-γ (Samuel, 2001) and this might further caused the higher serum levels of IFN-γ in DV-infected patients than that in the healthy controls.

Apart from that, IFN-γ is an activator of macrophages (Ellis & Beaman, 2004) and it converts macrophages from a resting state to an active state and induces the synthesis of an array of receptors for binding to pathogens (Antoniou et al., 2003). Macrophage activation is very important in the viral clearance as they act as APC. They ingest the virus, digest them and present their antigens with MHC class molecules on their cell membranes to B cells or T cells to generate antigen-specific immune response (Chaturvedi, Nagar, & Shrivastava, 2006). This process is probably enhanced by IFN-γ as it is able to up-regulate antigen processing and presentation pathways (Diamond et al., 2000). Furthermore, activated macrophages acquire antiviral effector functions and secrete pro-inflammatory cytokines like IL-6 and IL-8 (Gattoni et al., 2006). All these events might cause further increase of the IL-6 and IL-8 concentrations in the DV-infected patients in this present study. As a result, different types of immune cells might be recruited to the site of infection followed by the elimination of the virus by phagocytosis or the release of toxic metabolites (Schroder et al., 2004). Besides, IFN-γ is shown to be produced by macrophages (Ellis & Beaman, 2004). The cycle of macrophage activation by IFN-γ and the production of IFN-γ from macrophages might again cause the

elevation of serum IFN-γ levels in the DV-infected patients in this present study.

In addition to NK cells, Th1 cells, CD8+ T cells and macrophages, IFN-γ is also produced by B cells (Teixeira et al., 2005). IFN-γ activates B cells and regulates its functions such as immunoglobulin production and class switching (Rosloniec, Latham, & Guedez, 2002). Positive feedback mechanism takes place again to ensure higher production of IFN-γ in order to activate more B cells in enhancing the elimination of invading virus. As a result of the above, a high concentration of IFN-γ was obtained in the serum of DV-infected patients in this present study. Similar results were reported where the productions of IFN-γ were induced in DV-infected patients and a similar mean IFN-γ concentrations were obtained between this present study and the other research (Chen et al., 2005; Chen et al., 2006; Hsieh et al., 2006; Priyadarshini et al., 2010; Restrepo et al., 2008b). This suggested that IFN-γ is critical for the early immune response to DV infection and IFN-γ-mediated immune responses are necessary for both the early and late clearance of the virus (Hsieh et al., 2006).

High levels of IFN-γ in serum of DV-infected patients might cause local dilation of the blood vessels and thereby decreasing the local blood flow rate which caused the gathering of blood in leaky vessels (Schroder et al., 2004). This cytokine may also regulate other chemokines like MIG (Dajotoy et al., 2004) and MCP-1 (Zhou et al., 2001) to chemoattract the specific leukocytes to extravasate into the tissue for viral clearance, and subsequently caused

plasma leakage in the DV-infected patients. Also, IFN-γ in DV-infected patient could have produced platelet activating factors, which participated in platelet and endothelial cell activation, leading to platelet consumption (Bozza et al., 2008) that led to thrombocytopenia in the DV-infected patients in the present study.

5.6 MCP-1

MCP-1 is produced by monoctyes (Mori et al., 2000) upon viral infection. The high levels of MCP-1 were significantly highly correlated to high levels of IL-8 in dengue patients as both of these cytokines are produced by monocytes after stimulation of virus infection (Bozza et al., 2008). Similar to IL-8, elevated MCP-1 in the DV-infected patients might cause the increase of endothelial permeability by disrupting tight junctions among cells (Lee et al., 2006), leading to plasma leakage and haemoconcentration (Yamada et al., 2003) in the DV-infected patients in the present study. Lee et al. (2006) found that the increased permeability and disrupted tight junctions of human vascular endothelium cells were affected through a mechanism partially dependent on MCP-1. Besides, high levels of MCP-1 might contribute to thrombocytopenia in DV-infected patients as they are able to cause platelet activation and platelet consumption (Bozza et al., 2008).

As the concentration of MCP-1 increased, the number of monocytes and basophils in serum of DV-infected patients decreased since the levels of MCP-1 was significantly negatively associated with monocytes and basophils count

in dengue patients. This is because MCP-1 is considered an important mediator that specifically stimulates directional migration of monocytes (Cleanthis et al., 2007) and basophils (Dhillon et al., 2009) to extravasate into the tissue for viral clearance and hence the lower amount of both leukocytes in the blood circulation. Monoctyes might then differentiate into macrophage, which is then responsible for the engulfment of the viral particles, in this case, DV, since they possess the phagocytic ability. Also, chemoattracted monocytes and macrophages amplify the antigen presenting pathway since they are APC (Glew et al., 2003). Additional virus will then be internalized and presented to T cells. This might further increase the production of IFN-γ in the host system and this cytokines might in turn stimulate the secretion of MCP-1 from monocytes in controlling the magnitude of cellular accumulation at the site of infection (Rahangdale et al., 2006). Hence, significant elevated levels of MCP-1 in the serum of DV-infected patients were observed in this study. High concentrations of MCP-1 in the DV-infected patients might also evoke T cells and NK cells chemotaxis (Han et al., 1999), which enhanced the host immune system in removing of viral product during the infection.

The up-regulation of MCP-1 in DV-infected patients in this study was parallel with the research done by Dejnirattisai et al., (2008) and Lin et al., (2005) where levels of MCP-1 in DV-infected patients were higher than that in the normal controls. Mean MCP-1 concentrations obtained in their research was higher than the mean MCP-1 concentrations obtained in this study. This might be due to the inclusive of the DHF group patients in the research done by Dejnirattisai et al., (2008) and Lin et al., (2005) whilst the patients included

in this present research were mostly from DF group. However, the increased levels of MCP-1 observed in dengue patients in this present study showed the importance of this chemokine in the host defense against DV infection.

5.7 MIG

Levels of MIG in the serum of patients with DV infection was found to be significantly elevated as compared to the serum levels of MIG in the healthy controls. Dejnirattisai et al. (2008) also demonstrated a similar result where it was shown that MIG was induced in the patients with DV infection. However, the mean MIG levels in dengue patients shown by Dejnirattisai et al. (2008) were three times lower than that in this present study. This was because different category of patients was included in the study. Dejnirattisai et al. (2008) determined the MIG levels in serum of DHF patients whereas MIG levels were determined in mostly DF patients in this present study.

MIG is secreted by monocytes, macrophages, APC, B cells (Whiting et al., 2004) and eosinophils (Dajotoy et al., 2004) upon viral infection. MIG was thought to attract NK cells to the site of infection (Hsieh et al., 2006) and subsequently mediate their protective effect by releasing the cytotoxic molecules in clearing the virus-infected cells. Also, NK cells may produce large amount of cytokine such as IFN-γ in the protective action during the viral infection (Makrigiannis & Anderson, 2003). Interferon-gamma, which is the inducer for MIG production (Colvin et al., 2004) would then induce extra secretion of MIG from different immune cells that consequently contributed to

the up-regulation of MIG concentration in the serum of DV-infected patients in this present study.

Additionally, MIG also promote the migratory response of activated T cells, especially Th1 cells to the site of infection (Takeuchi et al., 2006) in the host defense system. The induction could have enhanced the production of IL-16 from activated T cells. Since IL-16 levels were significantly correlated with MIG levels in DV-infected patients, the up-regulation of MIG could have caused an increased in the amount of IL-16 in DV-infected patients in this present study.

Furthermore, the determination of the MIG levels in the DV-infected patients is very important. This is because production of MIG is uniquely stringent and it is specifically induced by IFN-γ (Salazar-Mather et al., 2000), which may in turn allows a functional measure of IFN-γ activity. Also, MIG is produced following an amplification of the IFN-γ signal and detecting the concentration of MIG is a more sensitive measure of bio-active IFN-γ than detecting IFN-γ directly (Esche et al., 2005). Hence, the high concentration of MIG in the serum of DV-infected patients suggested the presence of bio-active IFN-γ in the immune system of DV-infected patients.

5.8 RANTES

RANTES is a chemokine secreted by activated T cells (Cavusoglu et al., 2007). It is also produced by monocytes and macrophages (Sun, Wang, Zhang, Zeng,

& Wang, 2005). NK cell is also another source of production of this chemokine. RANTES induces the migration of a variety of leukocyte types, but found to preferentially attract Th1 cells (Kanda & Watanabe, 2003). It is also a chemoattractant for monocytes (Gianoukakis et al., 2003), which play essential role in the host defense system. In addition, this chemokine also has a crucial role in trafficking and activating other immune cells such as DC (Carr, Ash, Lane, & Kuziel, 2006), mast cells (Fryer et al., 2000) and NK cells (Dorner et al., 2002) towards site of infection. The involvement of RANTES in viral infection is shown in various *in vitro* studies, where they showed that it is secreted in the supernatant of DV-infected cell lines (Avirutnan, Malasit, Seliger, Bhakdi, & Husmann, 1998; Chen, Ng, & Chu, 2008; Chen & Wang, 2002; King et al., 2002; Medin et al., 2005).

Interestingly, in contrast with other *in vitro* studies, the RANTES levels in the serum of DV-infected patients in this present study were significantly down-regulated as compared to healthy controls. This may be because the RANTES levels were determined from the serum of DV-infected patients in this present study. In other studies, the determination of RANTES levels was obtained from the supernatant of DV-infected cell lines. Other soluble factors that are naturally present in DV-infected patients but not in DV-infected cell line may influence the production of RANTES and hence the contrast results obtained.

Given that the activated immune cells are among the stimuli that elicit the production of RANTES, it would have been expected that there would be an elevated levels of RANTES in the serum of patients with DV infection in this

study. However, the observed reduction in the serum RANTES levels might be due to the decreased amount of platelets in DV-infected patients since there was a significant correlation between the levels of RANTES and platelet count in dengue patients. This is because in addition to T cells, monocytes, macrophages and NK cells, platelets are also considered one of the major source of RANTES (Eissa, Zaki, El-maghraby, & Kadry, 2005; Gleissner et al., 2008; Niwa et al., 2001). Since the DV-infected patients involved in this study all suffered from thrombocytopenia, the low amount of platelets in these DV-infected patients might contribute to the low levels of RANTES in these patients. Ellis and co-workers (2005) also supported this result where they found that platelet count changed in a parallel fashion to the RANTES concentrations and the correlation between platelets and RANTES was significant. Down-regulation of RANTES in the individual with severe malaria was also found to correspond to the thrombocytopenic in the malaria patients (John, Opika-Opoka, Byarugaba, Idro, & Boivin, 2006). Moreover, one single report from Cuba supported the result obtained for DV infection, where they found that serum RANTES levels detected in patients with DV infection were also lower than those observed in the healthy controls (Pérez et al., 2004). Also, lymphocytes are one of the producers of RANTES (Gleissner et al., 2008). Low levels of lymphocytes contributed to the low levels of RANTES in patients with DV infection as there was a strong significant correlation between the levels of RANTES and lymphocytes counts. Low RANTES concentrations then caused the decreased in the eosinophils count in DV-infected patients as RANTES levels had medium, significant relationship with eosinophils count. Moreover, a negative, medium significant correlation

between levels of RANTES and IL-15 showed that the low RANTES levels contributed to high levels of IL-15 in DV-infected patients. This might be because both RANTES and IL-15 have important role in regulating the activities of NK cells (Alpdogan & Brink, 2005; Chen & Wang, 2002) and T cells (Appay et al., 2000; Ramsborg & Papoutsakis, 2007). The low levels of RANTES were compensated with high levels of IL-15 in order to regulate the activities of both NK cells and T cells to ensure the efficiency of viral clearance.

5.9 Cytokine Network and Mechanism

As mentioned earlier, cytokines rarely, if ever, act alone in an immune system. A combined effect of two or more cytokines is sometimes greater that the additive effects of the individual cytokines. The combined effects of the cytokines ensure the outcome where the magnitude of defense system is sufficient to clear the invading virus. Hence, a proposed cytokine network and mechanism is suggested to represent the immunopathogenesis of DV infection (Figure 5.1).

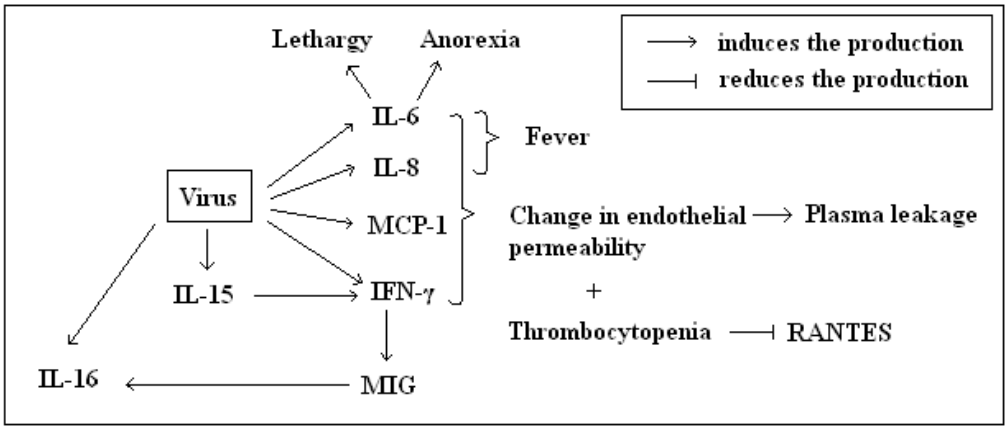

Figure 5.1: Proposed cytokine network and mechanisam for immunopathogenesis of dengue

Upon DV infection, productions of IL-6, IL-8, IL-15, IL-16, IFN-γ and MCP-1 from monocytes might be induced by the viral particles. Occurrence of anorexia and lethargy in DV-infected patients might be due to the up-regulation of IL-6. Also, as endogenous pyrogens, IL-6 and IL-8 would then induce fever in dengue patients by raising the temperature in the host defense system to reduce the activities of viral particles in the host. At the same time, IL-6, IL-8, MCP-1 and IFN-γ then induce the change in endothelial permeability by disrupting tight junctions among cells to cause vascular leakage, which is deemed to be a necessary action for leukocytes to leave the circulation and infiltrate tissue to site of infection in clearing the invading virus. If the vascular leakage is too serious and is not corrected early, a more severe form of illness named plasma leakage might take place in severe dengue patient.

Secreted IL-8 might recruit naïve T cells to the site of infection (Bosch et al., 2002) to expose this naïve T cells to encounter the viral particles in DV

infection. The naïve T cells bounded with viral particles are necessary to initiate the immune response. IFN-γ might stimulate the differentiation of naïve T cells toward Th1 phenotype (Teixeira et al., 2005) as the phenotype adopted by a naïve T cell during T cell activation is heavily influenced by the cytokine (Schroder et al., 2004). Th1 cells might then take part in cell-mediated immunity and also provides phagocytes-dependent protective response in the host immune system.

Besides inducing the development of Th1 cells, IFN-γ also activated macrophages (Ellis & Beaman, 2004), which act as APC to present the viral peptides to T cells for further degradation (Abdel-Motal et al., 2007). Interferon-gamma might then indirectly enhance the antigen processing and presentation pathways (Diamond et al., 2000). Also, as phagocytes, macrophages play this crucial role in engulfing those viral particles in clearing foreign pathogens from the host. Furthermore, activated macrophages might secrete pro-inflammatory cytokines like IL-6 and IL-8 (Gattoni et al., 2006) which led to further increase of the IL-6 and IL-8 concentrations in the DV-infected patients.

A raise in the IL-6 levels in DV-infected patients might then stimulate the proliferation and differentiation of CD8+ T cells (Łukaszewicz et al., 2007), which are the important effector cells in the downstream of Th1 immune response as CD8+ T cells (Łukaszewicz et al., 2007) take part in the viral clearance by releasing the cytotoxic substances from their granule and caused apoptosis of the viral infected cells. Also, high levels of IL-6 in dengue

patients might stimulate differentiation and maturations of B cells to produce different classes of immunoglobulin (Yap & Lai, 2010) that subsequently take part in the clearance of DV products. On the other hand, increased levels of IL-8 might induce a respiratory burst of neutrophils which consequently caused the release of lytic enzymes to rid the host of invading virus (Medin et al., 2005) in enhancing the immune status in the dengue patients.

High levels of MCP-1 were significantly highly correlated with high levels of IL-8 in dengue patients as both of these cytokines are produced by monocytes after stimulation of virus infection (Bozza et al., 2008). Also, the significant negative correlations between MCP-1 levels and both monocytes and basophils counts suggested that MCP-1 stimulated directional migration of monocytes (Cleanthis et al., 2007) and basophils (Dhillon et al., 2009) to extravasate into the tissue through the disturbed tight junctions among cells that caused by high levels of IL-6, IL-8, MCP-1 and IFN-γ. Therefore, this phenomenon caused the significant lower amount of both moncotyes and basophils in the blood circulation. Accordingly, monoctyes were then differentiated into macrophages in the tissue, which engulfed the DV, since they possess the phagocytic ability. Also, chemoattracted macrophages amplified the antigen presenting pathway since they are APC (Glew et al., 2003). Additional virus will then be internalized and presented to T cells and this increased the efficacy in viral clearance. High concentrations of MCP-1 in the DV-infected patients might also evoke T cells and NK cells chemotaxis (Han et al., 1999) to the site of DV infection, which further enhanced the host immune system.

MIG, on the other hand, is specially induced by IFN-γ (Salazar-Mather et al., 2000). The presence of high concentrations of MIG demonstrated that IFN-γ were bioactively present in the DV-infected patients. High levels of MIG were significantly associated with high levels of IL-16. This showed that high levels of MIG in dengue patients might chemoattract extra NK cells and activated T cells to the site of infection (Takeuchi et al., 2006) by infiltrating themselves through the disrupted tight junctions among cells. The chemoattracted T cells at the site of infection might enhance the production of IL-16 from activated T cells (Reich et al., 2001) that subsequently significantly up-regulated IL-16 levels in dengue patients and also formed a T cells pool at the site of infection. Interleukin-16 might then induce the migratory response in DC where chemotaxis of this APC to the site of infection might cause extra viral antigens to be presented to T cells for degradation.

On the other hand, IL-15 might regulate cytotoxicity of the chemoattrated NK cells (D'Ettorre et al., 2006). This regulation is important in host defense system as NK cells are found to be selectively able to lyse virus-infected target cells but at the same time sparing the uninfected cells. This is done by destroying the viral infected cells not by phagocytosis but by releasing biologically potent molecules that, within a very short time, kill the viral infected cells (Coico et al., 2003). In addition, high levels of IL-15 might stimulate the development of memory T cells (Ramsborg & Papoutsakis, 2007), which later play a vital role in secondary infection in order to boost up the immune response in a faster pace within a short time against the virus.

Furthermore, the high concentrations of IL-15 were significantly correlated with high concentrations of IFN-γ in dengue patients. This showed that IL-15 might increase the production of IFN-γ from NK cells and T cells in enhancing Th1 response (Azeredo et al., 2005). Additionally, significant negative relationships between IL-15 and white blood cells were demonstrated. This is because IL-15 is important for the differentiation of lymphocytes (Hu et al., 2007; Ramsborg & Papoutsakis, 2007) and monocytes (Ueda et al., 2007). The differentiation of these leukocytes and the subsequent infiltration of these leukocytes through disrupted tight junctions between cells to reach the site of infection would then cause significant lower levels of these leukocytes found in the blood circulation of dengue patients. Also, these cytokines regulate the activities for eosinophils in establishing the barrier against the viral attack.

Moreover, IL-6 might enhance the production of anti-platelets antibodies which then contributed to low amount of platelet in the dengue patients. Also, IL-8, MCP-1 and IFN-γ might produce platelet-activating factor that participated in platelet activation. This led to platelet consumption and caused the platelet number to decrease in circulation and hence the occurrence of thrombocytopenia in DV-infected patients. Since the RANTES levels was parallel with the amount of platelets presence in the host, less RANTES is produced upon DV infection due to the presence of thrombocytopenia.

CHAPTER 6

CONCLUSION

As compared to the serum levels of IL-6, IL-8, IL-15, IL-16, IFN-γ, MCP-1 and MIG in healthy controls, serum levels of all mentioned cytokines and chemokines were up-regulated in the patients with DV infection. However, levels of RANTES in the serum of DV-infected patients were down-regulated as compared to those in the healthy controls. This suggested that IL-6, IL-8, IL-15, IL-16, IFN-γ, MCP-1 and MIG but not RANTES played a crucial role in the immunopathogenesis of DV infection. However, age, gender and day of DV infection had no significant effect in the regulations of the mentioned cytokines and chemokines. Productions of IL-6, IL-8, IL-15, IL-16, IFN-γ and MCP-1 might be induced by the viral particles upon infection. Besides induction by viral particles, synthesis of IFN-γ was found to be induced by IL-15 in enhancing the host defense system. Also, bioactive IFN-γ was proven by the presence of MIG because the production of MIG is uniquely stringent and it is specifically induced by IFN-γ. Induced MIG might then recruit more T cells to the site of infection and subsequently enhanced the production of IL-16 from activated T cells. The up-regulation and inter-relationship of these cytokines and chemokines led to the manifestations of this disease, which might evoke a more severe form of illness in these DV-infected patients. High concentrations of IL-6 possibly induce the occurrence of anorexia and lethargy in DV-infected patients. As an endogenous pyrogen, high levels of both IL-8 and IL-6 were probably playing a role in inducing fever in dengue patients by

raising the temperature in the host defense system. Also, elevated IL-6, IL-8, MCP-1 and IFN-γ then possibly induced a change in endothelial permeability to cause vascular leakage, which sometimes may subsequently cause the occurrence of plasma leakage in some of the severe form of dengue patients. Besides, the up-regulated IL-6, IL-8, MCP-1 and IFN-γ could possibly cause thrombocytopenia in dengue patients, which then caused the presence of low amount of RANTES in dengue patients. Last but not least, all these mentioned cytokines and chemokines did not contribute significantly to the occurrence of myalgia and joint pain in the dengue patients.

Future study should be carried out to study the precise inter-relationship and roles of these cytokines and chemokines. Also, other cytokines and chemokines shall be studied in future in order to map out the whole immune mechanism which is involved in patients with DV-infection. In addition, comparison of levels of cytokines and chemokines between DF patients and DHF patients should be studied in identifying the possible biological marker that leads to the development of more severe disease illness, and thus, a possible drug development to prevent the occurrence of DHF.

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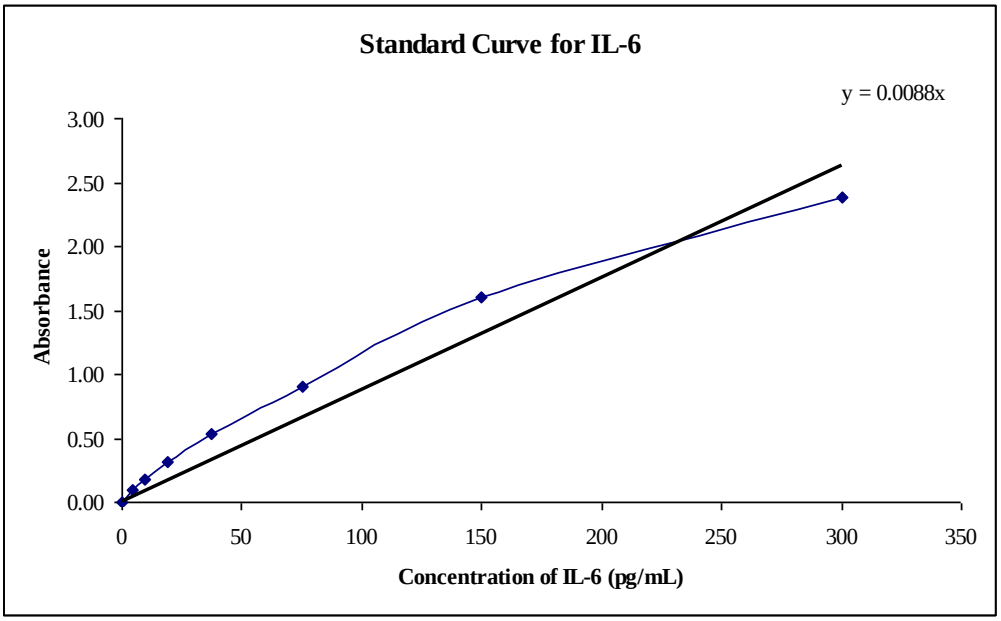
APPENDIX A

Standard Curve for IL-6:



Calculation of serum IL-6 concentration in a patient or healthy control:

The relationship between absorbance and concentration of IL-6 was obtained from the standard curve, which is

$y = 0.0088x$, where

y is the absorbance

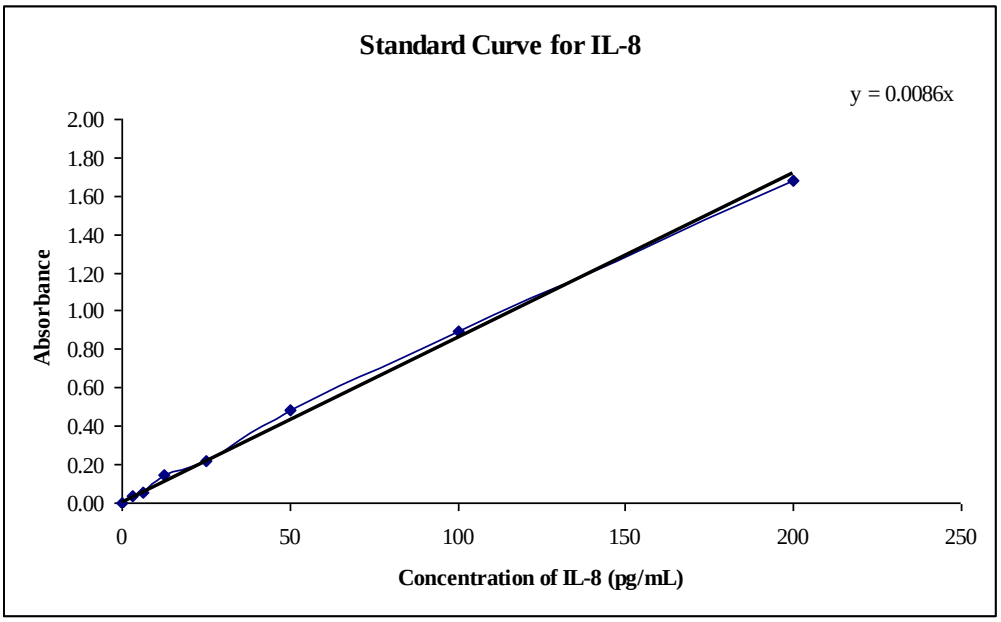
x is the concentration of IL-6

$\therefore$ The serum IL-6 concentration in a patient or healthy control

= (absorbance / 0.0088) X dilution factor of the serum

APPENDIX B

Standard Curve for IL-8:



Calculation of serum IL-8 concentration in a patient or healthy control:

The relationship between absorbance and concentration of IL-8 was obtained from the standard curve, which is

$y = 0.0086x$, where

y is the absorbance

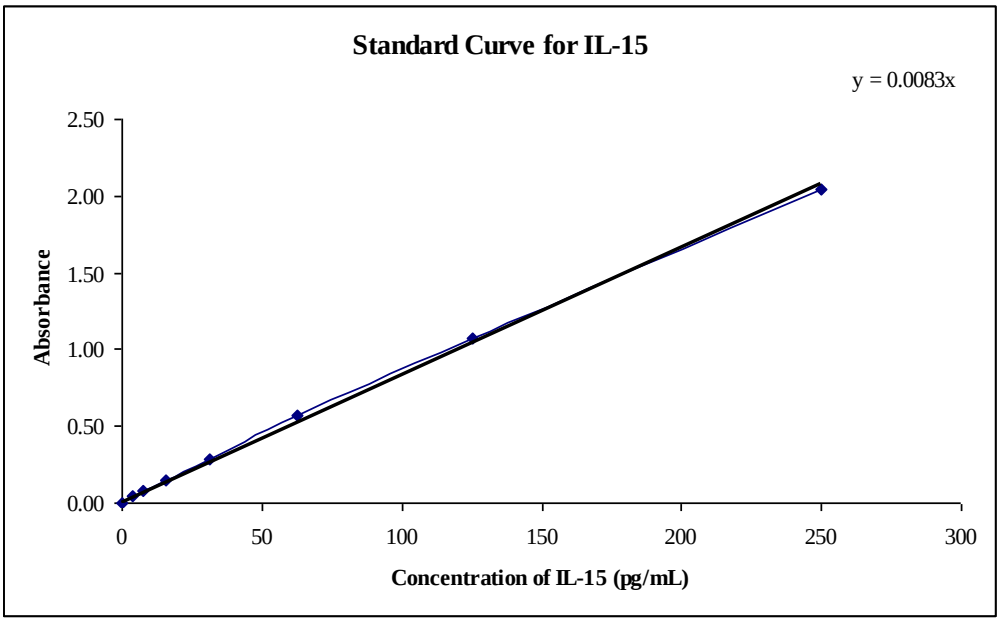
x is the concentration of IL-8

$\therefore$ The serum IL-8 concentration in a patient or healthy control

= (absorbance / 0.0086) X dilution factor of the serum

APPENDIX C

Standard Curve for IL-15:



Calculation of serum IL-15 concentration in a patient or healthy control:

The relationship between absorbance and concentration of IL-15 was obtained from the standard curve, which is

$y = 0.0083x$, where

y is the absorbance

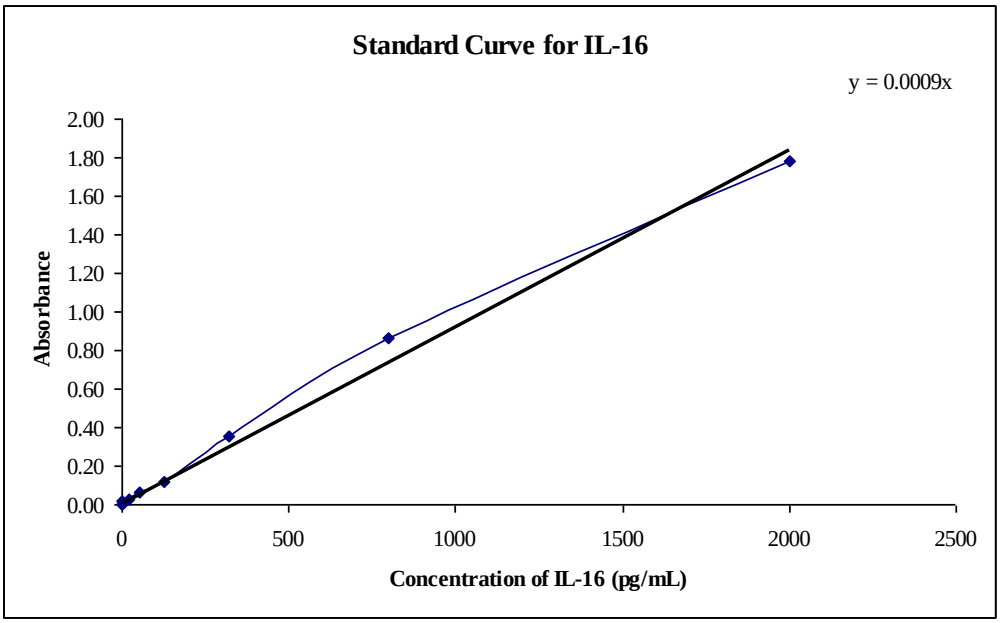
x is the concentration of IL-15

$\therefore$ The serum IL-15 concentration in a patient or healthy control

= (absorbance / 0.0083) X dilution factor of the serum

APPENDIX D

Standard Curve for IL-16:



Calculation of serum IL-16 concentration in a patient or healthy control:

The relationship between absorbance and concentration of IL-16 was obtained from the standard curve, which is

$y = 0.0009x$, where

y is the absorbance

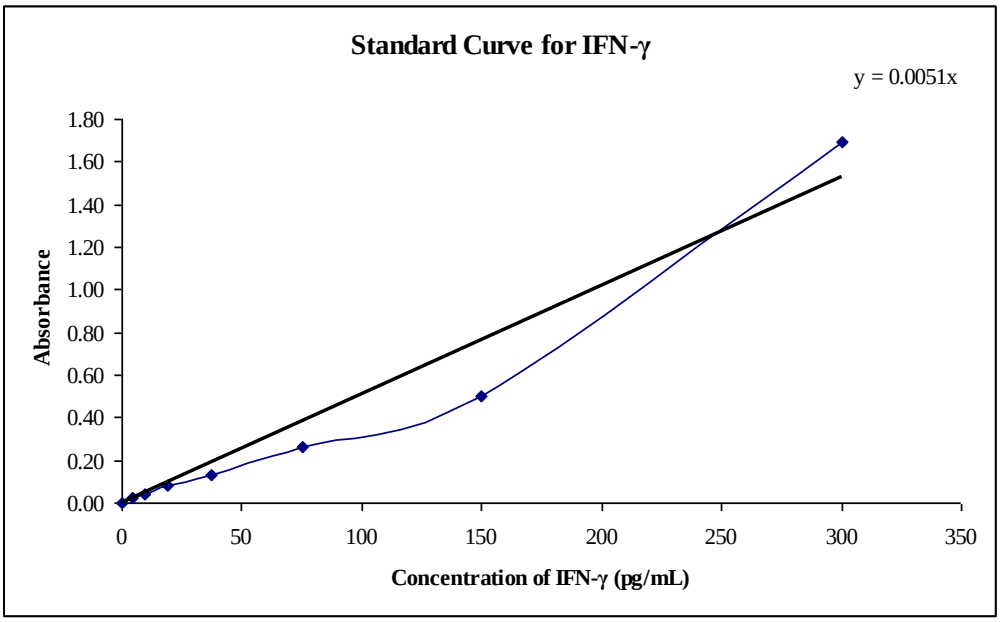
x is the concentration of IL-16

$\therefore$ The serum IL-16 concentration in a patient or healthy control

= (absorbance / 0.0009) X dilution factor of the serum

APPENDIX E

Standard Curve for IFN-γ:



Calculation of serum IFN-γ concentration in a patient or healthy control:

The relationship between absorbance and concentration of IFN-γ was obtained from the standard curve, which is

$y = 0.0051x$, where

y is the absorbance

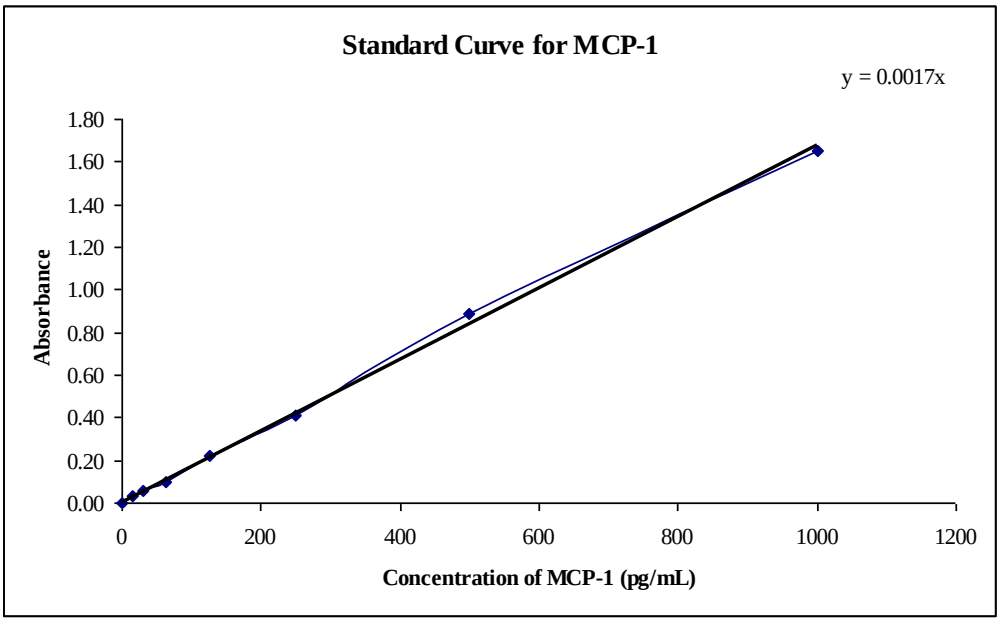
x is the concentration of IFN-γ

∴ The serum IFN-γ concentration in a patient or healthy control

= (absorbance / 0.0051) X dilution factor of the serum

APPENDIX F

Standard Curve for MCP-1:



Calculation of serum MCP-1concentration in a patient or healthy control:

The relationship between absorbance and concentration of MCP-1was obtained from the standard curve, which is

$y = 0.0017x$, where

y is the absorbance

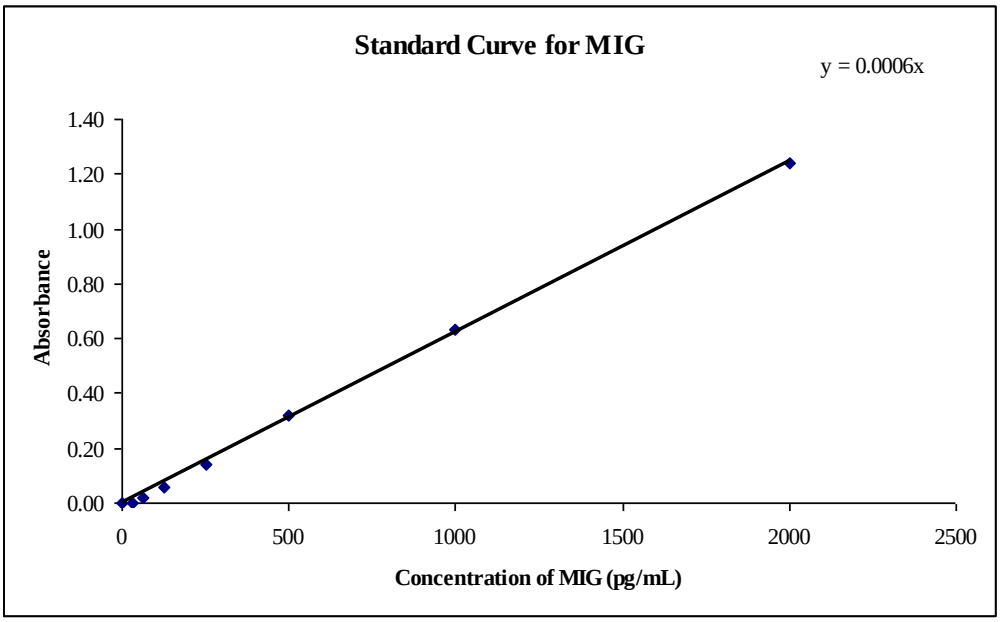
x is the concentration of MCP-1

$\therefore$ The serum MCP-1 concentration in a patient or healthy control

= (absorbance / 0.0017) X dilution factor of the serum

APPENDIX G

Standard Curve for MIG:



Calculation of serum MIG concentration in a patient or healthy control:

The relationship between absorbance and concentration of MIG was obtained from the standard curve, which is

$y = 0.0006x$, where

y is the absorbance

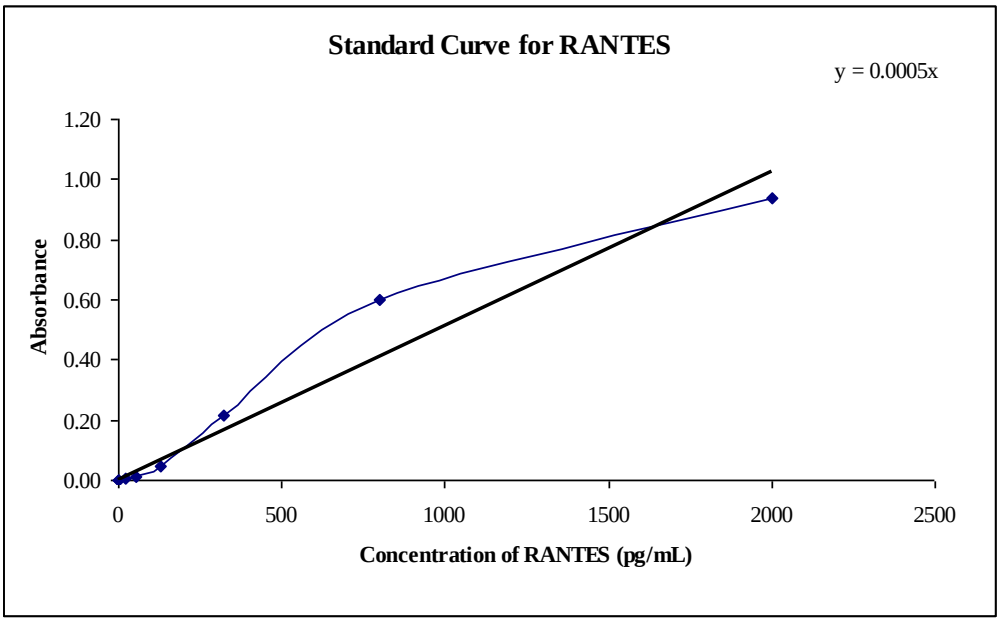
x is the concentration of MIG

$\therefore$ The serum MIG concentration in a patient or healthy control

= (absorbance / 0.0006) X dilution factor of the serum

APPENDIX H

Standard Curve for RANTES:



Calculation of serum RANTES concentration in a patient or healthy control:

The relationship between absorbance and concentration of RANTES was obtained from the standard curve, which is

$y = 0.0005x$, where

y is the absorbance

x is the concentration of RANTES

$\therefore$ The serum RANTES concentration in a patient or healthy control

= (absorbance / 0.0005) X dilution factor of the serum