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Impaired production of immune mediators in dengue virus type 2-infected mononuclear cells of adults with end stage renal disease

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Chronic kidney disease is an epidemiologically identified risk factor for development of severe dengue in dengue-affected patients. However, available data on the immune pathogenesis in end stage renal disease (ESRD) patients affected by dengue is insufficient. We performed an *in vitro* study to evaluate the sequential immunological reactions and viral load in dengue virus type 2-infected mononuclear cells of patients with ESRD (n = 34) and in healthy controls (n = 30). The concentrations of interleukins (IL)-1 receptor antagonist (Ra), IL-2, IL-6, IL-8, IL-10, IL-12p40, granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1b (MIP-1b), vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF)- α and viral load cycle threshold (Ct) were measured in the dengue virus type 2-infected mononuclear cells at 6 h, 24 h, 48 h, and 72 h post-infection. We found in the ESRD group significantly higher GM-CSF and IL-2 levels at 6 h post-infection. However, IL-8, IL-10, IL-12p40, TNF- α , MCP-1, and MIP-1b levels were found significantly lower than in the control group. At 24 h, 48 h, and 72 h post-infection, significantly lower levels of IL-1Ra, IL-6, IL-8, IL-10, IL-12p40, TNF- α , MCP-1, and MIP-1b were detected in ESRD group. Concentration of VEGF at 24 h and 48 h, and of GM-CSF at 48 h and 72 h were also found to be lower in ESRD group than in control group. Compared with controls, the viral load Ct values were significantly lower in ESRD group at 6 h and 24 h post-infection. No significant difference in viral load Ct values between two groups was found at 48 h and 72 h post-infection. Our study discloses that the expression of immune mediators of dengue-infected mononuclear cells is impaired in ESRD patients.

Dengue is one of the most prevalent arthropod-borne viral diseases worldwide¹. It is widespread throughout the tropical and subtropical regions of the world^{1,2}. An estimate indicates that 390 million cases of dengue occur every year and 3.9 billion people are at a risk of infection². The spectrum of clinical manifestations of dengue ranges from mild nonspecific febrile illness and fever, to the potentially catastrophic complications of severe dengue, as stated in the 2009 World Health Organization guidelines on dengue¹. The complexity in dengue pathogenesis is an obstacle to the development of anti-viral agents. Thus, the cornerstone of management of dengue is early diagnosis and recognition of severe dengue cases, and timely fluid replacement^{1,3}. Severe dengue is characterized by increased capillary permeability leading to extensive plasma leakage, massive bleeding and/or organ involvement that can lead to shock and death¹. Antibody enhancement⁴, aberrant activation of immune response system along with cytokine production⁵, and host comorbidities⁶ are amongst the postulated pathogenesis for severe dengue. Cytokine cascade plays a major role in the pathogenesis of the grave form of dengue and involves complex interaction between pro- and anti-inflammatory cytokines and chemokines that cause increased vascular permeability, resulting in large fluid losses and subsequent shock^{5,7}.

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During the 1950's and 1960's, dengue was generally considered to be a pediatric disease^{8,9}. However, in recent decades, advancing age has been reported to be a risk factor for patients with severe dengue, as the comorbidities associated with ageing pose a substantial risk for mortality in elderly patients^{10–12}. Chronic kidney disease has been implicated in the development of severe dengue^{13–16}. Thein *et al.*, in a study of 108 patients with dengue, reported that renal disorder increased odds of death in adults¹⁷. Kuo *et al.* reported that patients with chronic kidney disease had a higher mortality rate than those without chronic kidney disease¹³. In a study of 304 adults with dengue, chronic renal disease was found to be a risk factor that contributes to the development of acute kidney injury, which is associated with high morbidity and mortality of the affected patients¹⁸. Wong *et al.* found that end stage renal disease (ESRD) is a risk factor for acute respiratory failure in dengue affected adults¹⁹. ESRD is also an important clinical predictor of gastrointestinal bleeding in adult patients with dengue²⁰. In fact, patients with ESRD are more susceptible to infections (such as bacterial sepsis) with poor clinical outcomes compared with the general population²¹. In spite of ESRD being an increasingly common disease, there is lack of enough data on the immune pathogenesis in ESRD patients affected by dengue. Thus, thorough investigation is urgently required for the better understanding of immune responses in dengue-affected ESRD patients. In the present study, we aim to explore the sequential immunological reactions and viral load *in vitro* by investigating dengue virus-infected mononuclear cells of adults with ESRD, which would eventually make it possible to target interventions for an improved treatment plan for dengue infection.

Materials and Methods

Ethics statement. This study was approved by the Institution Review Board of Kaohsiung Chang Gung Memorial Hospital Medical Center, Taiwan (Document no. 102-5046B). Written informed consent from participants was obtained. This research adhered to the principles of the Declaration of Helsinki.

Study period and participants. The study was conducted at Kaohsiung Chang Gung Memorial Hospital from January 1, 2017 to December 30, 2017. Volunteers were recruited from those suffering from ESRD, and from healthy controls. Age is controlled in the study. ESRD affected volunteers refer to individuals with chronic kidney disease undergoing hemodialysis therapy thrice a week. A 10 ml sample of blood was obtained from each participant (ESRD and healthy individuals). To avoid the influence of dialyzer on the expression of immune mediators, blood samples from ESRD volunteers were collected before starting the dialysis session. The blood samples of all participants were tested for dengue virus-specific immunoglobulin IgM and IgG antibodies using a dengue blot detection kit (Gene Labs Diagnostics, Singapore), to determine any previous dengue infection²². Blood samples were anonymized after completion of demographic data collection.

Preparation of mononuclear cells. The whole blood from all participants (ESRD and healthy individuals) was separated into plasma and blood cells (i.e., leukocytes and erythrocytes) by centrifugation at 2,500 rpm (150 × g) for 20 minutes. Erythrocytes were removed from blood cells by 4.5% dextran sedimentation. After removal of erythrocytes, leukocytes were further separated into mononuclear cells and neutrophils using a density gradient centrifugation (350 g/30 min in Ficoll-Paque PLUS, Amersham Biosciences Corp.) in accordance with the procedures described elsewhere²³. After this, mononuclear cells from ESRD and healthy individuals were suspended in RPMI medium (Gibco) and seeded into a 24-well culture plate at a density of 1.0×10^6 cells per well for 1 day at 37 °C.

Preparation of dengue virus serotype 2 (DENV2). Three large dengue outbreaks occurred in 2002, 2014 and 2015 in southern Taiwan in which the DENV2 has been the predominant serotype in these outbreaks²⁴. In our series, an *in vitro* model with mononuclear cells infected with DENV2 was designed. DENV2 (New Guinea C strain, ATCC) was obtained from the Institute of Preventive Medicine, National Defense Medical Center, Taipei. The virus was propagated in *Aedes albopictus* C6/36 cells in Eagle's minimal essential medium (Gibco BRL, Grand Island, N.Y., USA) at 28 °C for 5 days. Virus titers were determined based on a standard plaque forming unit (PFU) assay on Baby hamster kidney-21 cells as described previously²⁵. The virus titers were adjusted to 5.0×10^6 PFU/mL in RPMI 1640 (Gibco BRL) medium. To achieve sufficient virus-infected mononuclear cells and avoid excessive cellular apoptosis, we used the multiplicity of infection (MOI) of 5, which has been proven appropriate previously²⁶.

DENV2 infection of mononuclear cells from ESRD and healthy individuals. The mononuclear cells from the 24-well culture plates (at a density of 1.0×10^6 cells per well) were inoculated with DENV2 having MOI of 5, and incubated at 37 °C for 2 hours. The infected mononuclear cells were washed to remove extracellular viruses and cultured in RPMI 1640 medium at 37 °C in a 5% CO₂ incubator. The supernatants and cells were harvested and analyzed at various time points after infection (6 h, 24 h, 48 h, and 72 h post-infection) for measurement of responsive immune mediators and viral loads.

Measurement of immune mediators. In this study, the pro-inflammatory mediators are demonstrated by interleukins (IL)-2, IL-6, IL-12p40, and tumor necrosis factor (TNF)- α . The anti-inflammatory mediators are demonstrated by IL-1 receptor antagonist (Ra), and IL-10. The leukocyte-derived growth and chemotactic factors are demonstrated by granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-8, whereas the monocyte and macrophage chemotactic factors are demonstrated by monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1b (MIP-1b). The vascular leakage mediator is demonstrated by vascular endothelial growth factor (VEGF). The concentrations of immune mediators in culture supernatants from the infected mononuclear cells were measured using the FlowMetrix System (Luminex Corporation, Austin, TX, USA), according to the manufacturer's instructions²⁷.

Determination of DENV2 cycle threshold (Ct) values by real-time reverse transcription-polymerase chain reaction (RT-PCR). Viral RNA was extracted from cultured mononuclear cells (ESRD and healthy individuals) to measure the Ct value by real-time RT-PCR, as previously described²⁸. The forward primer, reverse primer, and TaqMan probe sequences for detecting DENV2 were 5'-GGCTTAGCGCTCACATCCA-3', 5'-GCTGGCCACCTCTCTTCTT-3', and 5'-FAM-CGCCCACCACTATAGCTGCCGGA-TAMRA-3', respectively.

Data and statistical analyses. Measurements of immune mediators were expressed as mean value and standard deviation (pg/mL). Viral load was determined by Ct value of RT-PCR. Mann–Whitney U test was used to analyze differences in immune mediators and viral load at various time points (6 h, 24 h, 48 h, and 72 h post-infection) between the ESRD and control groups. A *P* value of <0.05 was considered statistically significant. All analyses were performed using SPSS 13.0 (Chicago, IL, USA).

Results

Demographic characteristics of the participants. The cells are obtained from 64 participants, including 34 ESRD patients (14 men and 20 women; median age 59.7 years) and 30 healthy controls (12 men and 18 women; median age, 58.8 years). Among the ESRD affected participants, hypertension (61.7%) was the most common comorbidity, followed by type 2 diabetes mellitus (41.2%). None of the participants in control group have underlying medical condition. All participants tested negative for dengue virus-specific immunoglobulins IgM and IgG antibodies, as determined by dengue blot detection kit²².

Immune profiles in overall ESRD affected participants and controls. Figure 1 demonstrates the immune profiles between ESRD group and control group at 6 h, 24 h, 48 h, and 72 h post-infection. Some immune mediators were not detected at a certain time point study. In control group, IL-1Ra, IL-2, IL-6, IL-8, IL-10, IL-12p40, GM-CSF, MCP-1, MIP-1b, VEGF, and TNF- α were not detected in 4 and 7 of the specimens at 24 h and 72 h post-infection, respectively. IL-1Ra, IL-2, IL-6, IL-8, IL-10, IL-12p40, GM-CSF, MCP-1, MIP-1b, VEGF, and TNF- α were not detected in 1 of the specimens in ESRD group at 48 h and 72 post-infection.

At 6 h post-infection, except GM-CSF and IL-2 were significantly higher in the ESRD group than in the control group, the levels of IL-8, IL-10, IL-12p40, TNF- α , MCP-1, and MIP-1b were found to be lower in the ESRD group. At 24 h post-infection, the ESRD group showed significantly lower levels of IL-1Ra, IL-6, IL-8, IL-10, IL-12p40, TNF- α , VEGF, MCP-1, and MIP-1b as compared to the control group. Again at 48 h post-infection, significantly lower levels of IL-1Ra, IL-6, IL-8, IL-10, IL-12p40, GM-CSF, TNF- α , VEGF, MCP-1, and MIP-1b were observed in the ESRD group. At the late phase of infection (72 h post-infection), levels of IL-1Ra, IL-6, IL-8, IL-10, IL-12p40, GM-CSF, TNF- α , MCP-1, and MIP-1b still remained significantly low for ESRD group, as compared to control group.

Immune profiles in ESRD affected participants without diabetes. Beyond viral factor, the current evidence shown diabetes is a potentially important co-factor that affects the outcome of dengue infection²⁹. To eliminate the impact of diabetes in production of immune mediators in ESRD patients, subgroup analysis was performed between ESRD participants without diabetes (*n* = 19) and control group (*n* = 30) (Fig. 2). At 6 h post-infection, IL-2 and GM-CSF were detected at higher concentrations, but there was a significant reduction of stimulated mononuclear cell production of IL-8, IL-10, IL-12p40, TNF- α , MCP-1, and MIP-1b in ESRD without diabetes group than in control group. At 24 h, 48 h and 72 h post-infection, the levels of IL-1Ra, IL-6, IL-8, IL-10, IL-12p40, TNF- α , MCP-1, and MIP-1b were still low in ESRD without diabetes group than in the control group. In addition, significantly lower concentration of VEGF was observed at 48 h post-infection in ESRD without diabetes group in comparison to controls.

DENV2 viral load of ESRD and control groups. Viral load Ct values were not detected in 1 of the specimens in ESRD group at 72 h post-infection, and in 1 of the specimens in the control group at 24 h, 48 h and 72 h post-infection, respectively. Compared with controls, the viral load Ct values were significantly lower in ESRD group at 6 h and 24 h post-infection. No significant difference in viral load Ct values between the cultured mononuclear cells from both groups was found at 48 h and 72 h post-infection (Fig. 3).

Discussion

Chronic kidney disease has been found to be an epidemiologically identified risk factor for development of dengue hemorrhagic fever among dengue patients^{13–20}. However, most of the previously published research on dengue immune pathogenesis involved a general population rather than a population with a specific underlying disease such as ESRD³⁰. In the present study, eleven immune mediators and viral load Ct values were measured in DENV2-infected mononuclear cells of adults with ESRD, and in healthy participants. Uremic itself presents a complex multidimensional inflammatory condition³¹. Previous studies have shown that T lymphocytes from ESRD patients are dysregulated and the plasma IL-18, IL-6, TNF- α and C-reactive protein concentrations were significantly higher in ESRD patients than those pre-dialysis controls³¹. In our series, apart from high levels of GM-CSF and IL-2 that were observed at 6 h post-infection, impaired production of immune mediators was observed at a later stage post-infection in the ESRD group. This finding is potentially valuable for understanding of the basis of immunopathology reactions and provides new insight in the management of ESRD patients with dengue virus infection.

Patients with dengue virus infection may progress through three clinical phases, namely, the febrile phase, the critical phase and the recovery phase¹. Our report describes an *in vitro* experiment for dengue virus infected mononuclear cells and analysis of immune mediators at different times after infection, in a setting that mimicked infection in human and the sequential immunological reactions.

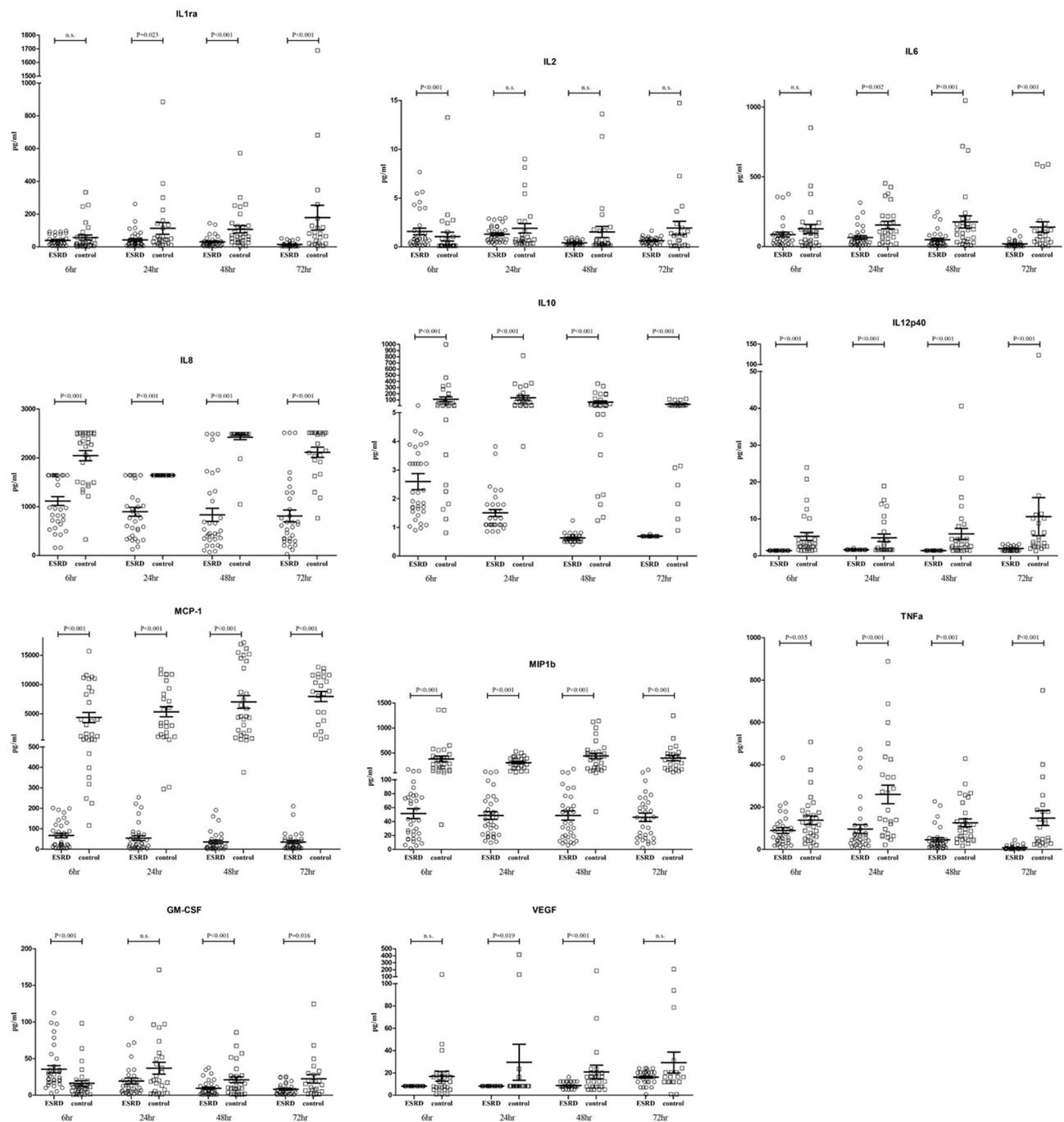


Figure 1. The immune profiles of DENV2-infected mononuclear cells at 6 h, 24 h, 48 h and 72 h post-infection in end stage renal disease (ESRD) group and control group. Data were expressed as mean value and standard deviation (pg/mL).

The levels of GM-CSF and IL-2 were significantly raised in overall ESRD population as well as those non-diabetic ESRD participants at 6 h post-infection. GM-CSF plays an important role in mediating the innate immune response that stimulates stem cells to produce granulocytes and facilitates development of the immune system to fight against infections³². GM-CSF has been reported to increase significantly in patients with severe dengue as compared mild dengue³³. IL-2 is an important pro-inflammatory cytokine in determining the activation of T-helper 1 response to stimulate cell-mediated immunity³⁴. Kurane *et al.* in a study of 69 children showed that the level of IL-2 was highest one day before defervescence in patients with dengue hemorrhagic fever³⁵. Although GM-CSF and IL-2 were expressed in higher concentrations at 6 h post-infection in ESRD group, the production of a large number of pro- and anti-inflammatory cytokines as well as chemokines, including IL-8, IL-12p40, TNF- α , MCP-1, MIP-1b, and IL-10 was markedly decreased. These results suggest derangements of

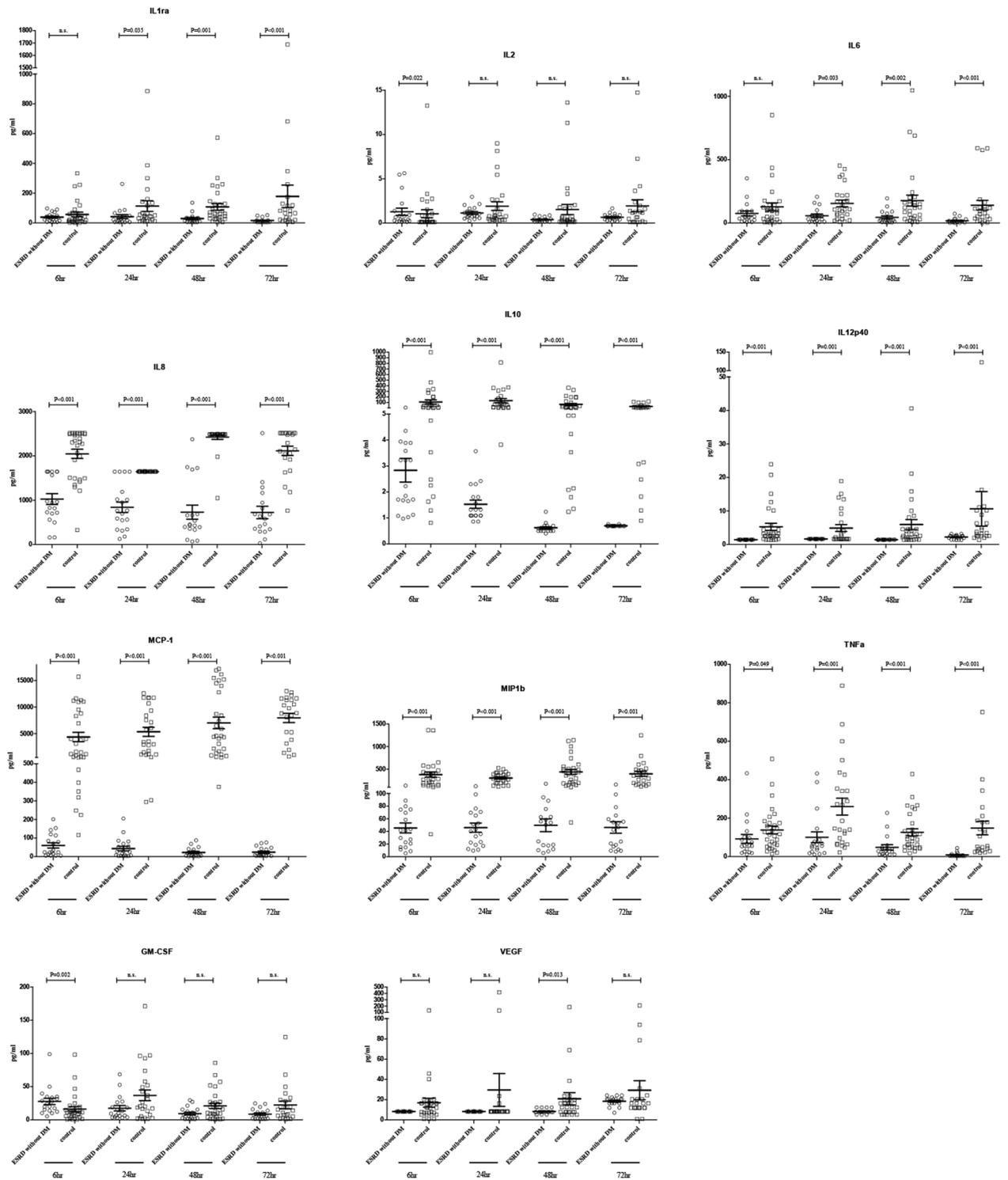


Figure 2. Cytokines/chemokines produced by DENV2-infected mononuclear cells of end stage renal disease (ESRD) without diabetes group and control group at various time points post-infection. Data were expressed as mean value and standard deviation (pg/mL).

immune reaction between pro- and anti-inflammatory cytokines/chemokines in ESRD patients during the early phase of dengue illness.

The hallmark of severe dengue is a transient perturbation in blood vessel integrity, leading to an increase in vascular permeability that results in plasma leakage^{1,3}. This change is most likely due to effects of biological mediators as a consequence of complex interactions between dengue virus and host innate and adaptive immune responses^{4,5,7}. For example, increased levels of TNF- α and soluble TNF- α receptors as well as VEGF have been

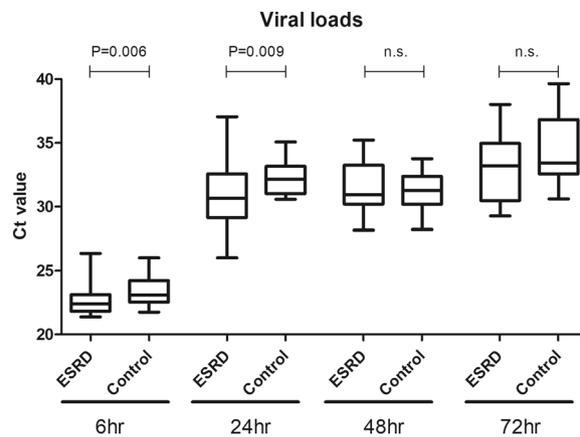


Figure 3. Dengue viral load threshold cycle values in the DENV2-infected mononuclear cells of end stage renal disease (ESRD) group and control group at various time points post-infection.

reported in dengue hemorrhagic fever cases^{36,37}. IL-10, having anti-inflammatory properties counter-regulates the cascade of pro-inflammatory cytokines and it has been reported to correlate with disease severity³⁸. Remarkable, the results of the present study showed a significant impairment of IL-1Ra, IL-6, IL-8, IL-10, IL-12p40, TNF- α , MCP-1, and MIP-1b expression at 24 h, 48 h, and 72 h post-infection. VEGF levels at 24 h and 48 h, and GM-CSF levels at 48 h and 72 h were also reported to be low in ESRD group. Impaired cytokines/chemokines expression also has been found in ESRD without diabetes group as well. Our results have been somewhat inconsistent with the reported overwhelming immune activation that is associated with dengue disease severity^{5,7}; however, our findings clearly reflect the impairment of immune responses *in vitro* study of DENV2-infected mononuclear cells in ESRD patients. Uremia per se can also alter the immune system in ESRD patients³⁹. The complete blood count is not performed in our series. Previous studies, however, have shown that monocytes and monocyte-derived dendritic cells of ESRD patients have impaired endocytosis and maturation⁴⁰. Lisowska *et al.* reported that ESRD patients have lower percentages of CD4+ and CD8+ T cells, as well as B cells in peripheral blood⁴¹. In fact, several factors influence immunity in ESRD patients, such as uremic toxin, malnutrition, chronic inflammation, and therapeutic dialysis^{39–42}. In this regard, our findings and previous reports indicate that ESRD patients have immune dysregulation with impaired expression of immune mediators in case of dengue virus infection.

Although the role of pro- and anti-inflammatory cytokines/chemokines in the pathogenesis of vascular permeability in dengue-affected ESRD patients is uncertain in our series, the impairment of immune responses indicate reduction of the activation of the immune system in dengue-affected ESRD patients that can lead to increased risk of morbidity and mortality in case of secondary bacterial infection. Notably, in a study of 22 severe dengue patients with concurrent bacteremia, 18.2% of them had ESRD⁴³. Of 25 adult dengue-affected patients with bacteremia, 16% were found to have ESRD⁴⁴.

Another finding recognized in this study is that significantly lower viral load Ct values at 6 h and 24 h post-infection in ESRD group than controls. However, no significant difference in viral load Ct values between both groups was found at 48 h and 72 h post-infection. The relationship between the dengue viral load and the severity of disease is still controversial^{45,46}. Murgue *et al.* had reported that high levels of viral load might contribute to the pathogenesis of dengue hemorrhagic fever⁴⁵. In another study of 128 confirmed adult dengue cases, Chen *et al.* found that viral load was not significantly different between dengue fever and dengue hemorrhagic fever patients⁴⁶. Further studies are needed to determine the role of viral load in the pathogenesis of severe dengue in ESRD patients.

A limitation of the present study is that it is unclear whether similar immunologic responses occur in secondary dengue infection in dengue-affected ESRD patients. Despite the limitation, this is the first study that provides a basis for understanding the role of immune mediators in ESRD population with dengue infection.

In conclusion, the results of our study disclose that impaired immunologic reactions of mononuclear cells to a DENV2 infection in ESRD individuals. This implies that caution needs to be exercised in the immune dysregulation of ESRD population and it might be a key determinant for clinical outcome when treating dengue-affected ESRD patients. Our study highlights that further clinical investigation beyond *in vitro* study is needed to confirm this important finding.

Data availability

All data generated or analyzed during this study are included in this published article.

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Author contributions

I.K.L. and Z.S.Y. performed the experiments, I.K.L. and C.T.L. analyzed the data. I.K.L. wrote the manuscript. The study was designed by I.K.L., H.Y.N., L.C.L., W.C.H., Y.C.C., C.Y.T. and C.T.L.

Competing interests

The authors declare no competing interests.

Additional information

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