*Tropical Biomedicine* 36(4): 1027–1037 (2019)

# IL-8 as a potential *in-vitro* severity biomarker for dengue disease

Soo, K.-M.[1], Tham, C.L.[2], Khalid, B.[3], Basir, R.[4] and Chee, H.-Y.[1*]
[1]Department of Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia
[2]Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia
[3]Department of Medicine, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia
[4]Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia
[*]Corresponding author e-mail: cheehy@upm.edu.my
Received 16 October 2018; received in revised form 30 May 2019; accepted 3 June 2019

**Abstract.** Dengue is a common infection, caused by dengue virus. There are four different dengue serotypes, with different capacity to cause severe dengue infections. Besides, secondary infections with heterologous serotypes, concurrent infections of multiple dengue serotypes may alter the severity of dengue infection. This study aims to compare the severity of single infection and concurrent infections of different combinations of dengue serotypes *in-vitro*. Human mast cells (HMC)-1.1 were infected with single and concurrent infections of multiple dengue serotypes. The infected HMC-1.1 supernatant was then added to human umbilical cord vascular endothelial cells (HUVEC) and severity of dengue infections was measured by the percentage of transendothelial electrical resistance (TEER). Levels of IL-10, CXCL10 and sTRAIL in HMC-1.1 and IL-8, IL-10 and CXCL10 in HUVEC culture supernatants were measured by the ELISA assays. The result showed that the percentage of TEER values were significantly lower in single infections ($p < 0.05$), compared to concurrent infections on day 2 and 3, indicating that single infection increase endothelial permeability greater than concurrent infections. IL-8 showed moderate correlation with endothelial permeability ($r > 0.4$), indicating that IL-8 may be suitable as an *in-vitro* severity biomarker. In conclusion, this *in-vitro* model presented few similarities with regards to the conditions in dengue patients, suggesting that it could serve as a severity model to test for severity and levels of severity biomarkers upon different dengue virus infections.

## INTRODUCTION

Dengue (DENV) is a common infection endemic in over 100 tropical and subtropical countries, causing 12 000 deaths worldwide every year (Maha *et al*., 2014). There are four antigenically distinct serotypes of the dengue virus, which are transmitted by *Aedes* mosquitoes, principally *Aedes aegypti*. The serotypes differ in their capacity to cause severe dengue infections, hence are responsible for various clinical outcomes of dengue illnesses (Holmes and Twiddy 2003; Suppiah *et al*., 2018).

The phenomena of secondary infection with heterologous dengue serotypes and concurrent infections of multiple dengue serotypes further complicate the association between the viral factor and severe dengue infection. The prevailing hypotheses antibody-dependent enhancement was proposed to explain the occurrence of severe dengue during secondary infections (Schlesinger, 1980). Meanwhile, concurrent infections are rare and have been detected, usually by RT-PCR since 1985 (Waterman *et al*., 1985). However, concurrent infections have drawn the attention of whether there is

any synergistic or competitive interaction present between the different dengue serotypes that simultaneously infect the patients and whether it causes altered clinical manifestations (Corwin *et al.*, 2001; Martins *et al.*, 2014; VinodKumar *et al.*, 2013). Moreover, cytokine storm occurs, in which soluble factors emanating from immune cells, platelets, stromal and endothelial cells in the form of cytokines and chemokines act as signalling molecules, modulating host responses to infections. These immune modulating proteins change with the clinical course of dengue, differing between dengue fever and severe dengue patients, and are believed to have a direct impact on the clinical manifestations such as increased vascular permeability, plasma leakage and thrombocytopenia (Lee, Leong, and Wilder-Smith, 2016).

However, limited human experimental data has hindered further understanding of the immunopathogenesis of DENV infection. This condition has urged the development of various *in-vitro* models that resemble the actual conditions (J.F. Kelley, Kaufusi, and Nerurkar, 2012; Raekiansyah, Espada-murao, and Okamoto, 2014). This study aims to use an *in-vitro* cell culture model to compare the severity of single infection and concurrent infections of different combinations of dengue serotypes at the same amount of virus titre. The *in-vitro* model in this study used mast cell as the target infected cell because this cell is among the first innate immune cells that encounter virus at the earlier stage of infection (St John *et al.*, 2011). Besides, mast cell products, histamine, tryptase and chymase were found to be higher in dengue patients with plasma leakage (Furuta *et al.*, 2012; Tuchinda, Dhorranintra, and Tuchinda 1977), implying that mast cells play a role in the pathogenesis of dengue. This study defined severity as the percentage of transendothelial electrical resistance (TEER) across endothelial cells. Levels of biomarkers secreted by HMC-1.1 and HUVEC upon infections of dengue virus were also measured. This *in-vitro* model measured levels of IL-8, IL-10, and CXCL10. These biomarkers were chosen based on the results of a previous meta-analysis (Soo *et al.*, 2017). In which, the meta-analysis presented the potential severity biomarkers (IL-7, IL-8, IL-10, IL-18, and VEGFR2) and biomarkers that showed significant differences between healthy control and DF patients (CXCL10 and TNF-α). Among the biomarkers, IL-7 and IL-18 were excluded because of weak evidence from only 1-3 studies, while VEGFR2 was excluded because of the need to carry out the measurement together with the measurement of VEGF and VEGF-VEGFR2 complexes (Srikiatkhachorn *et al.*, 2006), which will complicate the model. TNF-α was not selected as it displays a smaller mean difference compared to CXCL10. As such, after excluding the unsuitable biomarkers, IL-8, IL-10, and CXCL10 were selected. Furthermore, IL-10 and CXCL10 were able to be secreted by both HUVEC and HMC-1.1 (Burke *et al.*, 2012; Niu *et al.*, 2008; Secchiero *et al.*, 2005; Shin *et al.*, 2004). IL-8 was able to be secreted by HUVEC but not for HMC-1.1 (Niu *et al.*, 2008). Finally, this study analysed a correlation between the levels of biomarkers and the percentage of TEER, in order to identify the biomarkers that can act as an *in-vitro* severity biomarkers.

## MATERIALS AND METHODS

### Virus propagation, detection and quantification

DENV1-4 were isolated from confirmed dengue patients serum (Ethical approval number NMRR-15-923-25233). DENV 1-4 were propagated in Vero cells in MEM media. DENV were harvested as described by Tansey (Tansey, 2002). The presence of viral RNA was confirmed by RT-PCR following protocol by Seah *et al.* 1995 and viral protein was detected using immunofluorescence assays adapted from Chew *et al.* (Chew *et al.*, 2012). Following that, a virus titre in foci forming unit (ffu) was determined using focus-forming assay following a protocol described by Santos *et al.* (Santos *et al.*, 2013).

**Virus infection of HMC-1.1 and HUVEC**

The HMC-1.1 was provided by Dr J.H. Butterfield, Mayo Clinic. Rochester, MN. The cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) with HEPES with Glutamax (Gibco, Thermo Fisher Scientific, USA) at 37°C in tissue culture flasks. DENV infection of HMC-1.1 was carried out as described by Furuta *et al.* (2012). HMC-1.1 ($1 \times 10^5$ cells/ ml) were added with different combinations of dengue serotypes (DENV1, DENV2, DENV3, DENV4, DENV1-2, DENV1-3, DENV1-4. DENV2-3. DENV2-4 and DENV3-4, respectively) at a viral titre of 0.003 ffu/ cells and were incubated for 90 minutes at 4°C. The mock-infected group was added with non-infected Vero cells culture supernatant prepared similarly as dengue virus infected Vero cells. Cells were then centrifuged at 217 xg to remove the unabsorbed virus. Cells were grown with IMDM supplemented with 2% FBS for 7 days. The positive control group was added with non-infected Vero cell culture supernatant on day 0 and was added with 1 µg/ml of lipopolysaccharide (LPS) later, on day 6. HMC-1.1 culture supernatants of all treated groups were harvested at 7 days post infection and were used in TEER assays on HUVEC as well as ELISA assays of IL-8, IL-10, CXCL10 and sTRAIL.

Dengue infection of HUVEC was carried out as described by Brown *et al.* (Brown *et al.*, 2011) and Dewi *et al.* (Dewi, Takasaki, and Kurane, 2004). Type I Bovine Collagen (40 µg/ml) (07001, Stemcell Technologies, Canada) was coated on inserts (MCRP24H48, Merck Millipore, Germany) with a diameter of 0.6 cm and pore size of 1 µm and was left to incubate at room temperature for 1 hour. Uncoated collagen was then washed away with PBS. HUVEC ($2 \times 10^5$/ ml) were seeded on each insert. Inserts and lower chambers of plates were added with 200 µl and 500 µl of Endogro LS complete media (SCME001, Merck, Germany), respectively and media were changed every 24 hours. After the cells became 100% confluent, they were incubated with 200 µl of infected groups, mock-infected groups and positive control groups of HMC-1.1 culture supernatants for 1 hour at room temperature. The HMC-1.1 culture supernatant was removed and new culture media were added and subsequently changed every 24 hours for four days.

**HMC-1.1 viability**

The percentage of HMC-1.1 viability upon infections of DENV was measured different days post infection (Day 0, Day 4 and Day 7), according to the method adapted from Jurisic and Bumbasirevic (Jurišić and Bumbaširević, 2008).

**TEER assay on HUVEC**

Transendothelial electrical resistance (TEER) was measured on HUVEC using Evohmeter Millicell ERS-2 (Merck, Germany). TEER was measured on days 0, 1, 2, 3 and 4 post-infection. The percentage of TEER was calculated by using the following formula:

Percentage of TEER=(TEER after treatment (Day 1-4))/(TEER before treatment (Day 0))×100%

**ELISA assays**

The culture supernatant of HMC-1.1 (on day 7 post infection) and HUVEC (on day 1 post infection) were collected. ELISA assays of IL-10, CXCL10 and sTRAIL in the HMC-1.1 culture supernatant and IL-8, IL-10 and CXCL10 in the HUVEC culture supernatant were performed. ELISA assays were performed according to the protocol provided by the manufacturer (R&D systems, USA).

**Statistical analysis and interpretation of results**

Data were analysed using OpenMeta (Analyst) software (Brown School of Public Health, Providence, RI, USA) and GraphPad Prism® version 5.03. The combined mean and standard error of levels of biomarkers of each dengue serotypes of three individual experiments, each with two replicates, were calculated using a fixed-effect model. The combined mean and standard error of single infections and concurrent infections group were calculated using a random effect model, with the assumption that there are differences between different combinations of dengue

serotypes. The difference between each group was compared using the method described by Michael *et al.* (Michael *et al.*, 2009), in which statistical significance was set at $p < 0.05$. Pearson correlation was done to correlate between levels of biomarkers and TEER values. The correlation coefficient $r < 0.4$ was interpreted as weak or no correlation, $0.4 < r < 0.6$ as moderate correlation, and $r > 0.6$ as strong correlation. This study defined a dengue serotype as more severe than others when it caused a lower percentage of TEER. The synergistic interaction between two dengue serotypes was defined as when a significantly lower TEER ($p < 0.05$) or a significantly higher level biomarker ($p < 0.05$) was found in concurrent infections of the two dengue serotypes, compared to single infections of both of its component serotypes (i.e. DENV2 and DENV3 are component serotypes of concurrent infections of DENV2-3), and vice versa for competitive interactions. This is according to the definition used by a study done by Kelley *et al.* (J.F. Kelley *et al.*, 2012).

## RESULTS

### HMC-1.1 viability

Figure 2 shows the percentage of viable HMC-1.1 at day 0, 4 and 7 post single and concurrent infections of different combinations of dengue serotypes. The percentage of viable cells in the negative control group is significantly lower than the concurrent infections groups ($p < 0.05$) on day 7. There is no significant difference found when other pairwise comparisons of the percentage of viable cells were carried out.

### The severity of single infections and concurrent infections

Figure 3 shows the results of the percentage of TEER values of single infections and concurrent infections of different combinations of dengue serotypes, on different days post infections. The result showed that the percentage of TEER values were significantly lower in single infections, compared to concurrent infections, on day 2 and 3, indicating that single infections

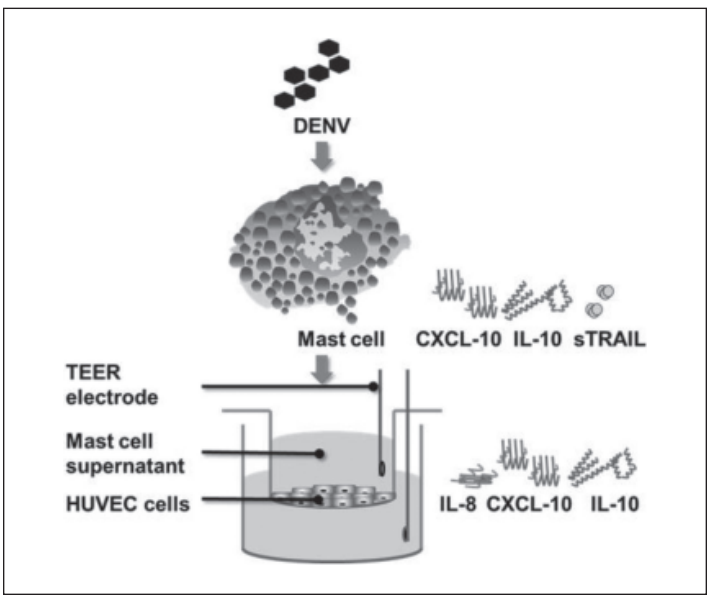


Figure 1. Schematic drawing of the *in-vitro* model used in this study. TEER assays were carried out to measure the change of endothelial permeability across the endothelial cells. ELISA assays were carried out to measure the amount of IL-10, CXCL10 and sTRAIL in HMC-1.1 culture supernatant at day 7 post infection as well as IL-8, IL-10 and CXCL10 in HUVEC culture supernatant at day 1 post infection.

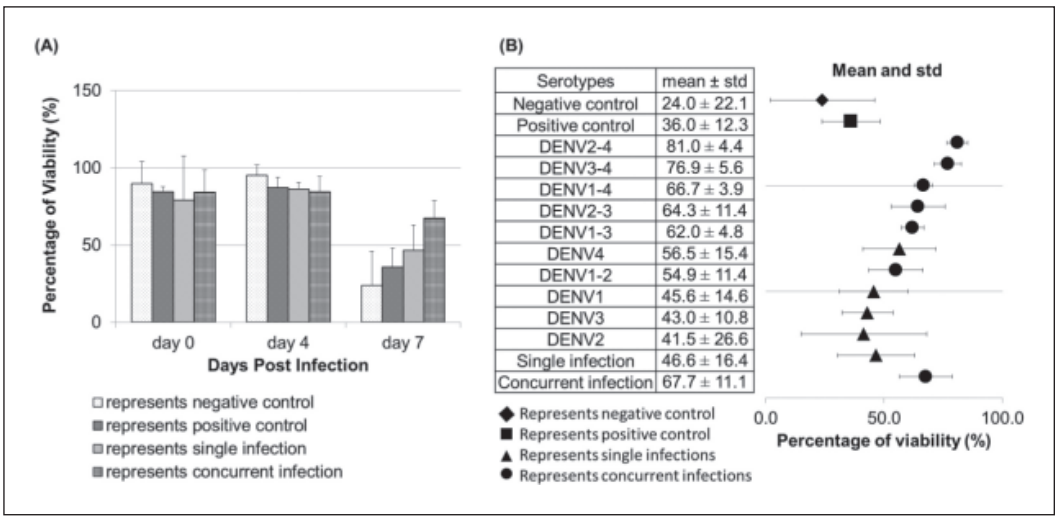


Figure 2. Percentage of viable HMC-1.1 at day 0, 4 and 7 days post single infections and concurrent infections of different combination of dengue serotypes.
Results were presented collectively as a single infection group and concurrent infection group (A) and separated into different combinations of dengue serotypes (B). Data were obtained from three independent experiments (n = 3) with duplicate readings.

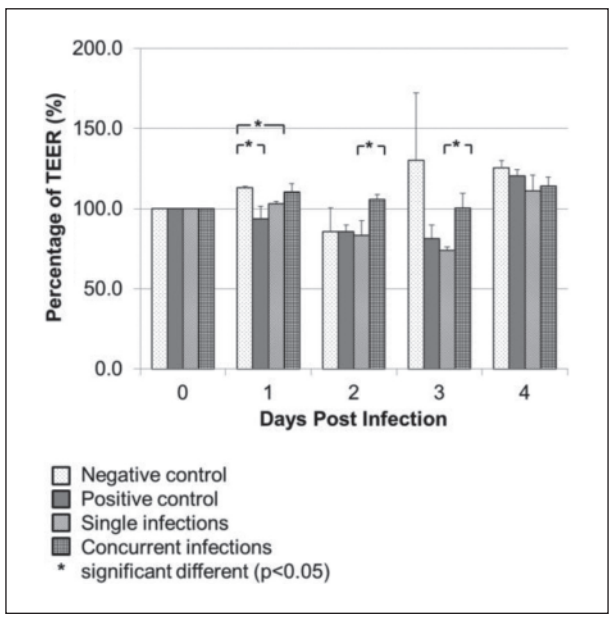


Figure 3. The percentage of TEER for negative control, positive control, single infection and concurrent infections.
Results were obtained from two individual experiments with triplicates.

increase endothelial permeability greater than concurrent infections. When data of single infection of different dengue serotypes were compared (sheet 1 of the supporting document), the percentage of TEER of DENV4 was significantly lower than DENV3 on day 2, and DENV3 and DENV2 were significantly lower than DENV1 on day 4; indicating that DENV2 and DENV3 cause higher endothelial permeability than DENV1.

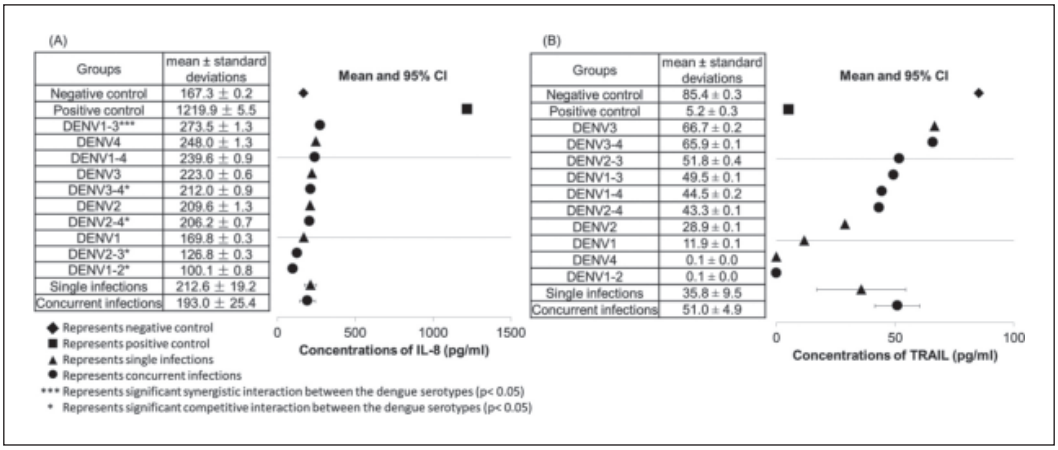


Figure 4. Effects of single infections and concurrent infections of different combinations of dengue serotypes towards levels of IL-8 (A) and sTRAIL (B).
Data was expressed as mean ± standard deviations, duplicate data from three individual experiments were used to obtain data.

There is no significant difference ($p< 0.05$) found when other pairwise comparisons of single infections were carried out. When comparing between single infections and concurrent infections, the percentage of TEER values of DENV2 and DENV3 were significantly lower than DENV2-3 on day 1, whereas DENV3 and DENV4 were significantly lower than DENV3-4 on day 3.

**Levels of biomarkers upon single infections and concurrent infections**

The result showed that levels of IL-10 in HMC-1.1 and HUVEC culture supernatants were lower than detection limits (31.3 pg/ml). Levels of CXCL10 in all infected HMC-1.1 culture supernatant were lower than the negative control, except for DENV2-4 infected culture supernatant. Levels of CXCL10 were not detected in HUVEC culture supernatant. Figure 4A shows the results of IL-8 levels in HUVEC culture supernatant, during single and concurrent infection of different combinations of dengue serotypes. Concurrent infections cause lower levels of IL-8, compared to single infections. Comparison of data of single infections of different dengue serotypes showed that DENV4 induced the highest levels of IL-8, followed by DENV3, DENV2 and DENV1. Furthermore, when single infections were compared with concurrent infections, concurrent infections of DENV1-2, DENV2-3, DENV2-4 and DENV3-4 caused lower levels of IL-8 than their respective component serotypes, whereas DENV1-3 stimulated a release of higher levels of IL-8 than component serotypes, DENV1 and DENV3. Moreover, correlation tests between levels of IL-8 with the percentage of TEER showed that there is a moderate correlation ($r> 0.4$, $p< 0.05$) between levels of IL-8 and percentage of TEER on day 3. On the other hand, results in Fig. 4B shows that the level of TRAIL secreted by negative control is higher than single infections and concurrent infections on HMC-1.1 on day 7.

## DISCUSSION

The results showed that single infections of DENV2 and DENV3 were more severe than DENV1, which resembles the previous findings of meta-analysis (Soo *et al.*, 2016). Furthermore, single infections were more severe than concurrent infections. Previous experimental studies had demonstrated a reciprocal interference of one virus serotypes on the replication of the other virus serotypes (Loroño-Pino *et al.*, 1999; Pepin and Hanley, 2008). It was found that after a few tissue culture passages of concurrent infections, only one serotype was detected, in which another virus serotype was competitively excluded (Loroño-Pino *et al.*, 1999). Apart

from dengue infections, concurrent infections of HIV and GB virus C (a non-pathogenic virus) had shown less severe clinical symptoms and lower mortality than mono-infection of HIV. It was hypothesized that GB virus C induces the secretion of chemokines that reduces HIV replication (Xiang *et al.*, 2004, 2006). Furthermore, HBV/HCV co-infected patients demonstrated a condition where the viral antigen was not detected due to the suppression between viruses (Cho *et al.*, 2011). Nevertheless, previous studies have not conducted concurrent infections of dengue serotypes in a severity model. Hence, this study further suggests that suppression between viruses during concurrent infections may occur thereby lowering the severity as evidenced by the higher percentage of TEER.

Among the three selected biomarkers, only IL-8 was able to be secreted by HUVEC, after being treated with single or concurrently infected HMC-1.1 culture supernatant. Results of the IL-8 level upon a single infection resembles previous studies in dengue patients (Soo *et al.*, 2017), with DENV3 and DENV4 stimulated higher levels of IL-8.

To date, this is the first cytokine study of concurrent infection of dengue serotypes. Results showed that single infections, which caused a lower percentage of TEER than concurrent infections, also caused higher levels of IL-8. Levels of IL-8 correlate with endothelial permeability moderately, suggesting that IL-8 may be suitable as an *in-vitro* severity biomarker. IL-8 may not reflect the endothelial permeability immediately but instead takes two days to cause the increase of endothelial permeability in the model. This could be due to the low concentration of IL-8 being secreted at day 1 post-infection. Previous studies reported that decreased barrier function induced by IL-8 is dose and time dependence. A study that used IL-8 in the concentrations range of 100-200 ng/ml caused decreased barrier function at 4 hours, whereas another study that used IL-8 at a lower concentration range of 50-200 pg/ml caused decreased barrier function at 72 hours (Talavera 2004; Yu *et al.*, 2013). Moreover, another study on concurrent infections of *B. burgdorferi* and *Anaplasma phagocytophilum* bacteria also demonstrated that IL-8 levels correspond to TEER results, which supports the role of IL-8 as *in-vitro* severity biomarker (Grab *et al.*, 2007). IL-8 was associated with disruption of tight junction proteins and enhanced endothelial permeability (Bozza *et al.*, 2008; J. Kelley, Kaufusi, and Nerurkar 2012). Hence, higher IL-8 could cause higher severity as indicated by increased endothelial permeability in single infections than in concurrent infections. Furthermore, a previous study on HIV infection showed that higher IL-8 could stimulate viral replication (Grønborg *et al.*, 2017; Lane *et al.*, 2001) and the higher viral titre was associated with increased endothelial permeability (Dewi *et al.*, 2004), which further supports the observation seen in the current study. The current study suggests that dengue virus is unable to stimulate HMC-1.1 to secrete IL-10 and CXCL10. Thus, IL-10 and CXCL10 were unable to act as severity biomarkers in this model.

The negative control groups of HMC-1.1 showed a lower percentage of viable cells and higher secretions of TRAIL than the infected group. TRAIL is a molecule pre-formed in the granules of cells, that mediates programmed cell death (Simons *et al.*, 2008). In response to stress, cells could secrete TRAIL, which binds to death receptors and causes a cytotoxic effect (Roy *et al.*, 2014). Therefore, starvation in 2% IMDM media for 7 days could have caused stress to negative control groups HMC-1.1, causing them to lyze and degranulate to release TRAIL which in turn causes cell death (Lim *et al.*, 2012; Quast *et al.*, 2015). Whereas in a single infection group, the decrease of viable cells was observed; yet levels of TRAIL was low. A possible explanation for that is the single infection group may cause cell death via different mechanisms or different apoptosis-related molecules such as TNF-α and FasL (Roy *et al.*, 2014). Several studies supported this possibility and have shown that levels of TNF-α were higher in dengue patients compared to healthy control (Senaratne, Carr, and Noordeen, 2016; Soundravally *et al.*, 2014; Zhao *et al.*, 2016) thus causing no secretion of TRAIL. Conversely, concurrent infections induced the release of TRAIL but

caused less cell death compared to single infections and negative control groups. As the dengue-infected cells could release FasL which inhibit TRAIL receptor-induced apoptosis, which may explain the reason why the release of TRAIL did not increase cell death (Liao, Xu, and Huang, 2010).

## CONCLUSIONS

In conclusion, *in-vitro* results presented few similarities with regards to the conditions in dengue patients: (1) DENV2 and DENV3 being more severe than DENV1; (2) IL-8 correlate with severity of infection; and (3) DENV3 and DENV4 stimulated secretion of more IL-8 than other dengue serotypes. Hence, it suggests that the *in-vitro* model could serve as a severity model to test for severity and levels of severity biomarkers upon different dengue virus infection. This is crucial to understand the immuno-pathogenesis of severe dengue infection. In addition, this model may act as the model to test for potential drugs that mediate anti-permeability or immunomodulatory effects.

*Acknowledgements*. We would like to thank Dr J. H. Butterfield, Mayo Clinic, Rochester, MN for providing HMC-1.1 used in this study. We would also like to thank Jeyanthi Suppiah in providing tittered dengue supernatant used in this study and Dr Malina Osman for helpful comments on the statistical analysis. Besides, we would like to acknowledge Ministry of Education, Malaysia for granting us the Long-term Research Grant Scheme (LRGS) LR001/2011A and Initiatif Putra Siswazah grant (IPS) 9478000. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## REFERENCES

Bozza, F.A., Cruz, O.G., Zagne, S.M.O., Azeredo, E.L., Nogueira, R.M.R., Assis, E.F., Bozza, P.T. & Kubelka, C.F. (2008). Multiplex Cytokine Profile from Dengue Patients: MIP-1beta and IFN-Gamma as Predictive Factors for Severity. *BioMed Central Infectious Diseases* **8**: 86.

Brown, M.G., Hermann, L.L., Issekutz, A.C., Marshall, J.S., Rowter, D., Al-Afif, A. & Anderson, R. (2011). Dengue Virus Infection of Mast Cells Triggers Endothelial Cell Activation. *Journal of Virology* **85**(2): 1145-1150.

Burke, S.M., Thomas, B.I., Karkada, M., Patrick, W.K.L., Maya, S. & Marshall, S. (2012). Human Mast Cell Activation with Virus-Associated Stimuli Leads to the Selective Chemotaxis of Natural Killer Cells by a CXCL8-Dependent Mechanism Human Mast Cell Activation with Virus-Associated Stimuli Leads to the Selective Chemotaxis of Natural Killer. *Blood* **111**(12): 5467-5476.

Chew, M.H., Rahman, M.M., Jelip, J., Hassan, M.R. & Isahak, I. (2012). All Serotypes of Dengue Viruses Circulating in Kuala Lumpur, Malaysia. *Current Research Journal of Biological Sciences* **4**(2): 229-234.

Cho, L.Y., Yang, J.J., Ko, K.-P., Park, B., Shin, A., Lim, M.K., Oh, J.-K., Park, S., Kim, Y.J., Shin, H.-R., Yoo, K.-Y. & Park, S.K. (2011). Coinfection of Hepatitis B and C Viruses and Risk of Hepatocellular Carcinoma: Systematic Review and Meta-Analysis. *International Journal of Cancer* **128**(1): 176-184.

Corwin, A.L., Larasati, R.P., Bangs, M.J., Wuryadi, S., Arjoso, S., Sukri, N., Listyaningsih, E., Hartati, S., Namursa, R., Anwar, Z., Chandra, S., Loho, B., Ahmad, H., Campbell, J.R. & Porter, K.R. (2001). Epidemic Dengue Transmission in Southern Sumatra, Indonesia. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **95**(3): 257-265.

Dewi, B.E., Tomohiko, T. & Ichiro, K. (2004). *In vitro* Assessment of Human Endothelial Cell Permeability: Effects of Inflammatory Cytokines and Dengue Virus Infection. *Journal of Virological Methods* **121**(2): 171-180.

Furuta, T., Murao, L.A., Lan, N.T.P., Huy, N.T., Huong, V.T.Q., Thuy, T.T., Tham, V.D., Nga, C.T.P., Ha, T.T.N., Ohmoto, Y., Kikuchi, M., Morita, K., Yasunami, M., Hirayama, K. & Watanabe, N. (2012). Association of

Mast Cell-Derived VEGF and Proteases in Dengue Shock Syndrome. *Public Library of Science Neglected Tropical Diseases* **6**(2): e1505.

Grab, D.J., Elvis, N., Nicole, C.B., Olga, V.N. & Dumler, J.S. (2007). Anaplasma Phagocytophilum-Borrelia Burgdorferi Coinfection Enhances Chemokine, Cytokine, and Matrix Metalloprotease Expression by Human Brain Microvascular Endothelial Cells. *Clinical and Vaccine Immunology* **14**(11): 1420-1424.

Grønborg, H.L., Sanne, J., Bo, L.H., Søren, J.-F. & Christian, W. (2017). Review of Cytomegalovirus Coinfection in HIV-Infected Individuals in Africa. *Reviews in Medical Virology* **27**(1): e1907.

Holmes, E.C. & Twiddy, S.S. (2003). The Origin, Emergence and Evolutionary Genetics of Dengue Virus. *Infection, Genetics and Evolution* **3**(1): 19-28.

Jurišić, V. & Vladimir, B. (2008). *In vitro* Assays for Cell Death Determination. *Archive of Oncology* **16**(3-4): 49-54.

Kelley, J.F., Kaufusi, P.H. & Nerurkar, V.R. (2012). Dengue Hemorrhagic Fever-Associated Immunomediators Induced via Maturation of Dengue Virus Nonstructural 4B Protein in Monocytes Modulate Endothelial Cell Adhesion Molecules and Human Microvascular Endothelial Cells Permeability. *Virology* **422**(2): 326-337.

Kelley, J.F., Pakieli, H.K., Esther, M.V. & Vivek, R.N. (2011). Maturation of Dengue Virus Nonstructural Protein 4B in Monocytes Enhances Production of Dengue Hemorrhagic Fever-Associated Chemokines and Cytokines. *Virology* **418**(1): 27-39.

Lane, B.R., Lore, K., Bock, P.J., Andersson, J., Coffey, M.J., Strieter, R.M. & Markovitz, D.M. (2001). Interleukin-8 Stimulates Human Immunodeficiency Virus Type 1 Replication and Is a Potential New Target for Antiretroviral Therapy. *Journal of Virology* **75**(17): 8195-8202.

Lee, Y.H., Leong, W.Y. & Wilder-Smith, A. (2016). Markers of Dengue Severity: Systematic Review of Cytokines and Chemokines. *Journal of General Virology* **97**(12): 3103-3119.

Liao, H., Juan, X. & Junqi, H. (2010). FasL/Fas Pathway Is Involved in Dengue Virus Induced Apoptosis of the Vascular Endothelial Cells. *Journal of Medical Virology* **82**(8): 1392-1399.

Lim, E.-J., Park, D.-W., Jeong, T.-W., Chin, B.-R., Bae, Y.-S. & Baek, S.-H. (2012). TRAIL Is Involved in CpG ODN-Mediated Anti-Apoptotic Signals. *Oncology Reports* **27**(4): 1213-1218.

Loroño-Pino, M.A., Cropp, C.B., Farfán, J.A., Vorndam, A.V., Rodríguez-Angulo, E.M., Rosado-Paredes, E.P., Flores-Flores, L.F., Beaty, B.J. & Gubler, D.J. (1999). Common Occurrence of Concurrent Infections by Multiple Dengue Virus Serotypes. *American Journal of Tropical Medicine and Hygiene* **61**(5): 725-730.

Maha, B., Felipe, J.C., Tobias, L., Iain, R.L. & Paul, R.H. (2014). Climate Change and the Emergence of Vector-Borne Diseases in Europe: Case Study of Dengue Fever. *BioMed Central Public Health* **14**: 781.

Martins, V.D.C.A., De Bastos, M.S., Ramasawmy, R., De Figueiredo, R.P., Gimaque, J.B.L., Braga, W.S.M., Nogueira, M.L., Nozawa, S., Naveca, F.G., Figueiredo, L.T.M. & Mourão, M.P.G. (2014). Clinical and Virological Descriptive Study in the 2011 Outbreak of Dengue in the Amazonas, Brazil. *Public Library of Science One* **9**(6): e100535.

Michael, B., Hedges, L.V., Higgins, J.P.T. & Rothstein, H.R. (2009). *Subgroup Analyses. In: Introduction to Meta-Analysis*. John Wiley and Sons Ltd, 1-38.

Niu, Q.-X., Chen, H.-Q., Chen, Z.-Y., Fu, Y.-L., Lin, J.-L. & He, S.-H. (2008). Induction of Inflammatory Cytokine Release from Human Umbilical Vein Endothelial Cells by Agonists of Proteinase-Activated Receptor-2. *Clinical and Experimental Pharmacology and Physiology* **35**(1): 89-96.

Pepin, K.M. & Kathryn, A.H. (2008). Density-Dependent Competitive Suppression of Sylvatic Dengue Virus by Endemic Dengue Virus in Cultured Mosquito Cells. *Vector Borne and Zoonotic Diseases* **8**(6): 821-828.

Quast, S.A., Katja, S., Michael, P. & Jürgen, E. (2015). Sensitization of Melanoma Cells for Death Ligand TRAIL Is Based on Cell Cycle Arrest, ROS Production, and Activation of Proapoptotic Bcl-2 Proteins. *Journal of Investigative Dermatology* **135**(11): 2794-2804.

Raekiansyah, M., Lyre, A.E.-M. & Kenta, O. (2014). Dengue Virus Neither Directly Mediates Hyperpermeability nor Enhances Tumor Necrosis Factor- a -Induced Permeability In vitro. *Japanese Journal of Infectious Diseases* **67**(2): 86-94.

Roy, S.G., Beata, S., Emmanuel, D., Richard, A.L. & Zahra, Z. (2014). Regulation of Cell Survival and Death during Flavivirus Infections. *World Journal of Biological Chemistry* **5**(2): 93-105.

Santos, J.J., Da, S., Marli, T.C., Giovani, R.B., Ernesto, T.de.A.M. & Laura, H.V.G.G. (2013). Construction and Characterisation of a Complete Reverse Genetics System of Dengue Virus Type 3. *Memorias do Instituto Oswaldo Cruz* **108**(8): 983-991.

Schlesinger, R.W. (1980). *The Togaviruses: Biology Structure, Replication*. Academic Press, 339-341.

Seah, C.L.K., Chow, V.T.K., Tan, H.C. & Chan. Y.C. (1995). Rapid, Single-Step RT-PCR Typing of Dengue Viruses Using Five NS3 Gene Primers. *Journal of Virological Methods* **51**(2-3): 193-200.

Secchiero, P., Federica, C., Maria, G.di.L., Arianna, G., Elisa, B., Giorgio, Z. & Pober, J.S. (2005). TRAIL Counteracts the Proadhesive Activity of Inflammatory Cytokines in Endothelial Cells by down-Modulating CCL8 and CXCL10 Chemokine Expression and Release. *Blood* **105**(9): 3413-3419.

Senaratne, T., Jillian, C. & Faseeha, N. (2016). Elevation in Liver Enzymes Is Associated with Increased IL-2 and Predicts Severe Outcomes in Clinically Apparent Dengue Virus Infection. *Cytokine* **83**: 182-188.

Shin, H.-Y., Song, Y.-S., Kim, H.-M. & Shin, T.-Y. (2004). Inhibitory Effect of Inflammatory Cytokines Production from Activated Mast Cells by Gamisopoonghwanghyul-Tang. *Immunopharmacology and Immunotoxicology* **26**(4): 587-596.

Simons, M.P., William, M.N., Kevin, G.L. & Thomas, S.G. (2008). TNF-Related Apoptosis-Inducing Ligand (TRAIL) Is Expressed throughout Myeloid Development, Resulting in a Broad Distribution among Neutrophil Granules. *Journal of Leukocyte Biology* **83**(3): 621-629.

Soo, K.-M., Khalid, B., Ching, S.-M., Tham, C.L., Basir, R. & Chee, H.-Y. (2017). Meta-Analysis of Biomarkers for Severe Dengue Infections. *PeerJ* **5**: e3589.

Soo, K.-M., Khalid, B., Ching, S.-M. & Chee, H.-Y. (2016). Meta-Analysis of Dengue Severity during Infection by Different Dengue Virus Serotypes in Primary and Secondary Infections. *Public Library of Science One* **11**(5): e0154760.

Soundravally, R., Hoti, S.L., Patil, S.A., Cleetus, C.C., Zachariah, B., Kadhiravan, T., Narayanan, P. & Kumar, B.A. (2014). Association between Proinflammatory Cytokines and Lipid Peroxidation in Patients with Severe Dengue Disease around Defervescence. *International Journal of Infectious Diseases* **18**: 68-72.

Srikiatkhachorn, A., Ajariyakhajorn, C., Endy, T.P., Kalayanarooj, S., Libraty, D.H., Green, S., Ennis, F.A. & Rothman, A.L. (2006). Virus-Induced Decline in Soluble Vascular Endothelial Growth Receptor 2 Is Associated with Plasma Leakage in Dengue Hemorrhagic Fever. *Journal of Virology* **81**(4): 1592-1600.

St John, A.L., Rathore, A.P.S., Yap, H., Ng, M.-L., Metcalfe, D.D., Vasudevan, S.G. & Abraham, S.N. (2011). Immune Surveillance by Mast Cells during Dengue Infection Promotes Natural Killer (NK) and NKT-Cell Recruitment and Viral Clearance. *Proceedings of the National Academy of Sciences of the United States of America* **108**(22): 9190-9195.

Suppiah, J., Ching, S.-M., Amin-nordin, S., Low, G.K.-K., Thayan, R. & Chee, H.-Y. (2018). Clinical Manifestations of Dengue in Relation to Dengue Serotype and Genotype in Malaysia/: A Retrospective Observational Study. *Public Library of*

*Science Neglected Tropical Diseases* 1-20.

Talavera, D. (2004). IL8 Release, Tight Junction and Cytoskeleton Dynamic Reorganization Conducive to Permeability Increase Are Induced by Dengue Virus Infection of Microvascular Endothelial Monolayers. *Journal of General Virology* **85**(7): 1801-1813.

Tansey, W.P. (2002). Ultimate Freeze-Thaw Lysis for Mammalian Cells. *Cell* 1999-2000.

Tuchinda, M., Dhorranintra, B. & Tuchinda, P. (1977). Histamine Content in 24-Hour Urine in Patients with Dengue Haemorrhagic Fever. *The Southeast Asian Journal of Tropical Medicine and Public Health* **8**(1): 80-83.

VinodKumar, C.S., Kalapannavar, N.K., Basavarajappa, K.G., Sanjay, D., Gowli, C., Nadig, N.G. & Prasad, B.S. (2013). Episode of Coexisting Infections with Multiple Dengue Virus Serotypes in Central Karnataka, India. *Journal of Infection and Public Health* **6**(4): 302-306.

Waterman, S.H., Gubler, D.J., Sather, G.E. & Kuno, G. (1985). A Case of Natural Concurrent Human Infection with Two Dengue Viruses. *The American Journal of Tropical Medicine and Hygiene* **34**(1): 170-173.

Xiang, J., George, S.L., Wünschmann, S., Chang, Q., Klinzman, D. & Stapleton, J.T. (2004). Inhibition of HIV-1 Replication by GB Virus C Infection through Increases in RANTES, MlP-Lα, MIP-1β, and SDF-1. *The Lancet* **363**(9426): 2040-2046.

Xiang, J., James, H.M., Qing, C., Thomas, M.K. & Jack, T.S. (2006). An 85-Aa Segment of the GB Virus Type C NS5A Phosphoprotein Inhibits HIV-1 Replication in CD4+ Jurkat T Cells. *Proceedings of the National Academy of Sciences of the United States of America* **103**(42): 15570-15575.

Yu, H., Xianliang, H., Yunlong, M., Min, G., Ou, W., Ting, G., Yang, S. & Xiaoheng, L. (2013). Interleukin-8 Regulates Endothelial Permeability by Down-Regulation of Tight Junction but Not Dependent on Integrins Induced Focal Adhesions. *International Journal of Biological Sciences* **9**(9): 966.

Zhao, L., Huang, X., Hong, W., Qiu, S., Wang, J., Yu, L., Zeng, Y., Tan, X. & Zhang, F. (2016). Slow Resolution of Inflammation in Severe Adult Dengue Patients. *BioMed Central Infectious Diseases* **16**(1): 291.

**Funding**

Long-term Research Grant Scheme (LRGS) LR001/2011A granted by Ministry of Higher Education, Malaysia and Initiatif Putra Siswazah grant (IPS) 9478000 granted by Universiti Putra Malaysia supported this study in terms of purchasing of research materials. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests**

The authors declare there are no competing interests.

**Author Contributions**

- Kuan-Meng Soo conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables.
- Chau Ling Tham conceived and designed the experiments and reviewed drafts of the paper.
- Bahariah Khalid, Rusliza Basir and Hui-Yee Chee reviewed drafts of the paper.

**Supporting document**

Sheet 1 – Percentage of TEER of different combinations of dengue serotypes.

Sheet 2 – All raw data.