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

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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, Dhorrinantra, 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 (Srikitakkhachorn 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 x 10^5 cells/ml) were added with different combinations of dengue serotypes (DENV1, DENV2, DENV3, DENV4, DENV1-2, DENV1-3, DENV1-4, 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 x 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 Bumbașirevic (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:

\[
\text{Percentage of TEER} = \left( \frac{\text{TEER after treatment (Day 1-4)}}{\text{TEER before treatment (Day 0)}} \right) \times 100\%
\]

**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 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.
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

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.
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 preformed 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 lyse 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 immunopathogenesis 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.

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REFERENCES


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### 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.