

Original Article

A PILOT STUDY ON THE IMPACT OF INFECTION ROUTES ON DISEASE PROGRESSION AND IMMUNE RESPONSE BY DENGUE INFECTION IN AG129 MICE

Nur Hana **Md Jelas**^{1*}, Nor Azrina **Norahmad**¹, Amirrudin **Muhammad**¹, Tiffany Chau Dee **Ho**¹, Bazilah **Jusoh**¹, Umi Rubiah **Sastu**¹, Murizal **Zainol**¹, Ravindran **Thayan**², Ami Fazlin **Syed Mohamed**¹ and Mohd Ridzuan **Mohd Abd Razak**¹

¹ Herbal Medicine Research Centre, Institute for Medical Research, National Institutes of Health, 40170 Shah Alam, Malaysia.

² Infectious Disease Research Centre, Institute for Medical Research, National Institutes of Health, 40170 Shah Alam, Malaysia.

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***Corresponding author:**
Nur Hana Md Jelas,
nurhana.mj@moh.gov.my,
my,
Tel: +603-33627984

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ABSTRACT

AG129 mouse is a mouse model commonly utilised in the study of dengue pathogenesis using various viral inoculation routes. Different viral infection routes may elicit distinct host responses. This pilot study compares the development of dengue virus infection in AG129 mice via intraperitoneal and subcutaneous routes. AG129 mice were infected with dengue virus type 2 (DENV-2) via intraperitoneal or subcutaneous injection. Disease progression was monitored through daily body weight and morbidity scores for 14 days. Plasma viral RNA levels were quantified by reverse transcription-quantitative PCR. Plasma non-structural protein 1 (NS1) level was determined by dengue NS1 antigen- ELISA method. Platelet and blood differential counts were counted. Plasma cytokines were assessed on day 1, 3, 5, and 7 post-infection using multiplex immunoassay. Body weight loss was similar between groups until day 4 post-infection, where intraperitoneally infected mice showed greater loss. Morbidity signs were more pronounced in intraperitoneally infected mice from day 2 to 6 post-infection. Viremia levels detected by NS1 protein and viral RNA were similar between both routes. Intraperitoneal infection triggered an earlier increase in white blood cells and neutrophil counts, as well as earlier lymphocyte reduction and thrombocytopenia. Cytokine analysis revealed early elevations of seven cytokines in intraperitoneally infected mice. In contrast, subcutaneously infected mice showed higher levels of interferon- γ and interleukin-10. Intraperitoneal infection in AG129 mice with DENV-2 induced thrombocytopenia, earlier immune responses and transiently higher morbidity as compared to the subcutaneous route. Therefore, different routes of virus inoculation should be carefully considered in future experimental designs using the AG129 mouse model.

KEYWORDS: Dengue virus type 2, AG129 mice, intraperitoneal, subcutaneous, viremia, immune response, cytokine

INTRODUCTION

Dengue viruses, which belong to the genus *Flavivirus* and family *Flaviviridae*, comprise four distinct serotypes: dengue virus type 1 (DENV-1), dengue virus type 2 (DENV-2), dengue virus type 3 (DENV-3), and dengue virus type 4 (DENV-4). Dengue virus is transmitted from an infected person to another person by a female *Aedes* mosquito. Dengue virus infection may present as a mild, flu-like illness known as febrile dengue fever (DF), or it can progress to a life-threatening complication called severe dengue haemorrhagic fever (DHF) (1).

AG129 mice are recognised as a small animal model that is sensitive to dengue virus infections. The lack of α , β , and γ interferon (IFN) receptors in these mice enables the mimicking of the inhibition of interferon signalling that occurred in humans after dengue virus infection (2). Interferons are important cytokines for viral clearance during innate and adaptive immune responses. In humans, the signalling cascade that expresses the interferons was interrupted by the NS4B protein (a non-structural dengue virus protein) produced by the host cells after dengue virus infection (3).

Several methods of inoculating dengue virus in mice have been reported, including the intraperitoneal (IP) route (4–6), subcutaneous (SC) route (7,8), intravenous route (9,10) and intracerebral route (11,12). All infection routes are able to produce effective dengue viral replication *in vivo* with clinical features associated with dengue infection. SC and IP injection are the least painful and allow a conscious mouse to be used during the infection process.

IP injection of dengue viruses is widely used in the dengue mouse model as the virus is directly introduced into the peritoneal cavity and rapidly enters the bloodstream, hence faster systemic infection. On the other hand, SC injection closely resembles the natural way of dengue virus infection through the skin via mosquito vector (13), although the rate of absorption is lower than intraperitoneal or intramuscular injection (14). Like the IP infection route, SC infection is able to induce systemic vascular leakage in AG129 mice (8,15). Furthermore, neutrophil influx to mosquito bites with the purpose of starting the localised innate immune response has been shown to enhance virus infection by allowing virus entry and infection of other cells (16).

This study aims to compare the morbidity level and immune responses elicited by the IP and SC infection route in AG129 mice infected with a Malaysian clinical DENV-2 isolate by characterising the viral RNA load, non-structural protein 1 (NS1) content, white blood cell (WBC) level, body weight, and cytokine levels.

MATERIALS AND METHODS

Cells and viruses

Vero cell line (CCL-81) was purchased from American Type Culture Collection (ATCC, USA) and maintained according to directions by ATCC. The cells were maintained at 37° in 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) growth media supplemented with 10% fetal bovine serum (FBS), 35mM sodium bicarbonate and 4mM L-glutamine. The DENV-2 DMOF015 in passage 2 were obtained from the Virology Unit, Institute for Medical Research, Kuala Lumpur, Malaysia. The virus was propagated to a high-count using Vero cells until passage 7. Briefly, 3 × 10⁶ of Vero cells were grown in a 75 cm² flask overnight. The media was discarded, and 3 mL of virus stock was added to the flask. The virus was incubated for one hour to allow virus adsorption. Then, 12 mL of DMEM growth media was

added to the flask. The virus stock was propagated for 5–7 days until visible cytopathic effect (CPE) was seen in cells. The virus suspension in the media was harvested and kept in -80°C until further use.

Dengue virus titre determination using plaque assay

The titre of the viruses was determined using plaque assay according to the protocol by (17) with some modifications. Briefly, 1.5 mL of 3×10^5 cells/ml Vero cells were grown in a 6-well plate overnight at 37°C, 5% CO₂. The growth media was aspirated, and 300 µL of DENV-2 DMOF015 virus stock that was serially diluted in the Hanks' Balanced Salt Solution (HBSS) supplemented with 0.4% bovine serum albumin was added to the wells. The cells and virus were incubated for one hour to allow virus adsorption onto the cells. Then, the viral media was aspirated, and 1.5 mL of DMEM-agarose overlay media (1.2% agarose, 10% FBS, 35mM sodium bicarbonate) was added to the wells. The agarose was allowed to harden, and the cells were incubated for 6 days at 37°C, 5% CO₂. One mL of 50% (w/v) trichloroacetic acid was added to the agarose overlay, and the plate was incubated at 4°C for 1 hour to fix the cells. The agarose overlay was carefully removed by gently flicking the gel, after which the cells were stained with 1% crystal violet prepared in 21% ethanol. Areas that remained clear and unstained within the cell monolayer indicated plaques formed by virus-infected cells. The plaques were counted, and the virus titre was expressed as plaque-forming units per mL (PFU/mL).

In vivo dengue virus propagation

Twelve male AG129 mice at four weeks old (129/Sv mice deficient in both alpha/beta and gamma IFN receptors) were purchased from Marshall BioResources, United Kingdom. Upon arrival, the mice were quarantined for two weeks and were further acclimatised for one week. The mice were randomly allocated into two experiments according to the route of infection, which were the SC infection route (n = 6) and intraperitoneal infection route (n = 6). In each experiment, the mice were divided further into two groups for infected (n = 3) and mock-infected (n = 3) mice. The dengue virus inoculum dose was prepared as mentioned previously (18). Infected mice were intraperitoneally or subcutaneously administered with 0.3 mL DENV2 DMOF015 virus (1×10^5 PFU/mouse). Media containing the virus was replaced with 0.3 mL plain media for mock-infected mice. The in vivo study was conducted under the guidelines and approval of the Animal Care and Use Committee, Ministry of Health Malaysia (ACUC-MOH), ACUC/KKM/02(9/2016).

Blood sample collection, clinical, and morbidity observation

Submandibular vein puncture technique (facial vein) was used to withdraw 200 µL of blood from the mice and was collected in an ethylenediaminetetraacetic acid EDTA tube on days 1, 3, 5, and 7 post-infections (p.i.). A blood smear was prepared using 10 µL of the blood collected and stained with Giemsa solution. Platelet and WBC differentiation counts were carried out. The balance of the blood was transferred to a 1.5 mL centrifuge tube and was spun for 15 minutes at $2000 \times g$ at 4 °C. The supernatant containing the plasma was transferred immediately to a clean 1.5 mL centrifuge tube and was used for analysing the content of NS1, cytokine screening and quantitative reverse transcription PCR (RT-qPCR).

Morbidity observations were conducted daily for 14 days using a scoring system on a scale of 1 to 5 as follows: 1=healthy; 2=ruffled fur and mild signs of lethargy; 3=hunched posture, ruffled fur, and intermediate signs of lethargy; 4=very lethargic, limited mobility, ruffled fur and hunched posture; and 5=moribund with limited to no mobility and inability to reach for food or water. Clinical observations were performed daily. Body weight of each mouse was recorded daily. Differences in body weight measured on day 0 (virus inoculation day) and other post-inoculation days were calculated, and on day 14 p.i., the mice were euthanised using the 5% isoflurane open-drop method.

Detection of NS1 antigen in plasma

The Platelia Dengue NS1 Ag Assay Kit (Bio-Rad, USA) was used to quantitate the NS1 antigen contained in the mice's plasma as previously described (19). The test was carried out according to the manufacturer's instructions with some modifications. Briefly, 5 μ L of the mice plasma was diluted ten times using 45 μ L phosphate-buffered saline, pH 7.2. Then, 10 μ L of the diluted plasma was mixed with 90 μ L sample diluent (R7) solution in the microplate. Next, 100 μ L of diluted peroxidase-conjugated NS1 monoclonal antibody solution was added to the plasma-R7 solution mixture in the wells. The mixture was incubated for 90 minutes at 37 °C. Then the plate was washed using washing solution (R2). When NS1 antigen is present in the sample, an immune complex of Mab-NS1-Mab-peroxidase was formed, and the immune complex was exposed to a chromogenic solution (tetramethylbenzidine and H₂O₂) that initiates colour development. Then, 160 μ L of Chromogen solution (R9) was added to the wells and incubated away from light. The plate was incubated in the dark for 30 minutes at room temperature. The reaction was stopped by adding 100 μ L of Stopping Solution (R10) containing 1N sulphuric acid into the wells. Optical density of the reaction was read at 450 nm using a microplate reader (FLUOstar® Omega microplate reader BMG Labtech, Germany).

Cytokine assay

Customised ProcartaPlex™ Immunoassay Mouse Cytokine 12-plex (ThermoFisher Scientific, USA) was used to detect and quantify cytokines present in the mouse plasma. The cytokine panel screened included colony-stimulating factor 3 (CSF3)/granulocyte colony-stimulating factor (G-CSF), colony-stimulating factor 2 (CSF2)/granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN- γ), interleukin (IL)-1 β , IL-4, IL-6, IL-10, IL-13, C-C motif chemokine ligand 2 (CCL2)/monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor alpha (TNF- α), C-C motif chemokine ligand 3 (CCL3)/macrophage inflammatory protein-1 alpha (MIP-1 α), and IL-18. The assay was carried out according to the manufacturer's instructions using 50 μ L of plasma. The reading was measured using xPONENT software version 4.2 (www.luminexcorp.com). The concentration (pg/ml) of protein was calculated using MasterPlex QT version 2.0.0.59 based on the standard curve of each cytokine and chemokine.

Viral RNA quantification

Dengue virus present in the plasma was quantified using real-time PCR (qPCR). First, viral RNA was extracted from the mouse plasma using QIAamp Viral RNA Mini Kit (Qiagen, USA), according to the manufacturer's instructions. Then, RT-qPCR of the purified viral RNA was carried out using Quantitect SYBR Green RT-PCR detection kit (Qiagen, USA) according to the manufacturer's instructions. The RT-qPCR was carried out in the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, USA). The primers and cycling temperatures used were previously described for dengue viral RNA with a known copy number used as a standard to determine the plasma virus titre. The primers and cycling temperatures for dengue viral RNA detection were previously described (19). The purified dengue viral RNA with known copy number was used as a standard for copy-number determination.

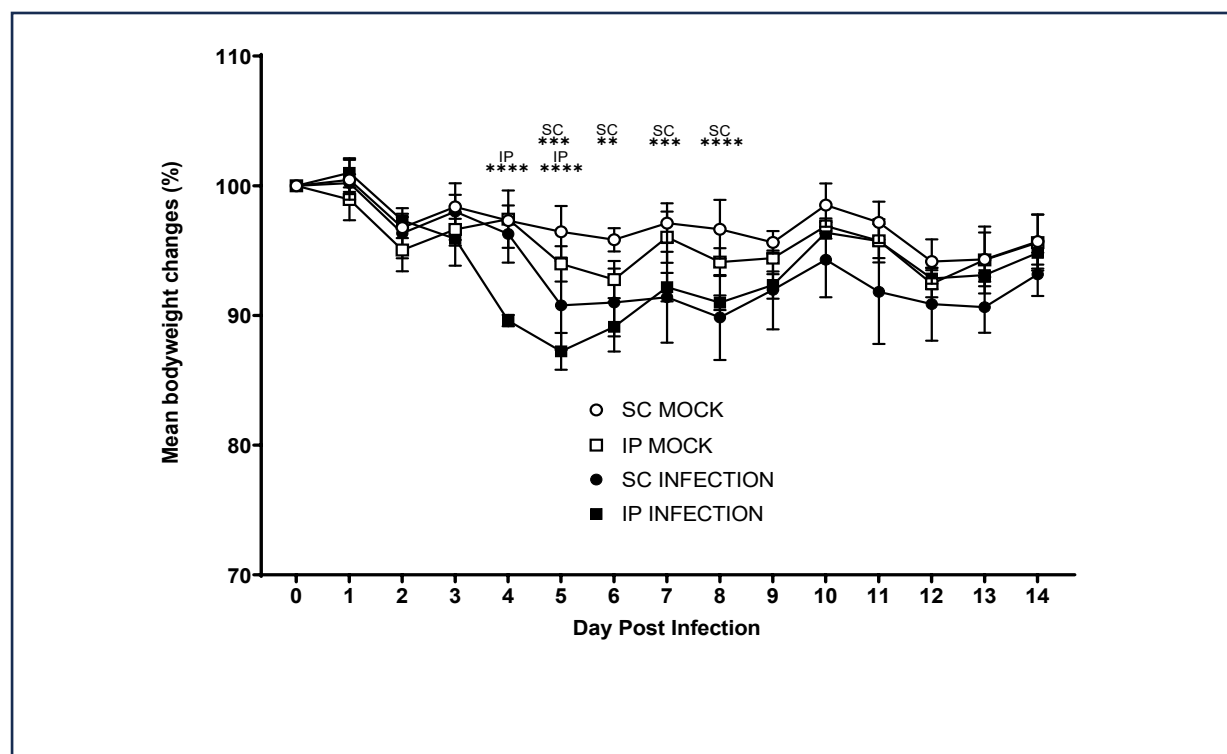
Statistics

Data were analysed using GraphPad Prism Version 8.4.3. Statistical significance was assessed via two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. In this pilot study (n = 3 per group), results were exploratory, which indicate the magnitude of differences between the groups with *P* value <0.05 was considered statistically significant.

RESULTS

Effect of DENV-2 infection on body weight and morbidity level

IP-infected mice exhibited significant body weight reduction on day 4 and 5 p.i., as shown in Figure 1(a). By day 6 p.i., IP-infected mice had begun to recover. SC-infected mice showed delayed body weight loss, starting on day 5 p.i., with recovery beginning later, on day 9 p.i. In contrast, the body weight of IP and SC mock-infected mice did not show any significant changes compared to day 0 throughout the 14-day observation period. Mice from IP and SC infection routes were visually scored for morbidity on a scale of 1 to 5. The morbidity scoring system scale is as follows: 1=healthy; 2=ruffled fur and mild signs of lethargy; 3=hunched posture, ruffled fur, and intermediate level of lethargy and failure to groom; 4=very lethargic, limited mobility, ruffled fur and hunched posture; and 5=moribund with limited to no mobility and inability to reach food or water (20). Morbidity scores were recorded as in Figure 1(b-c).



(a)

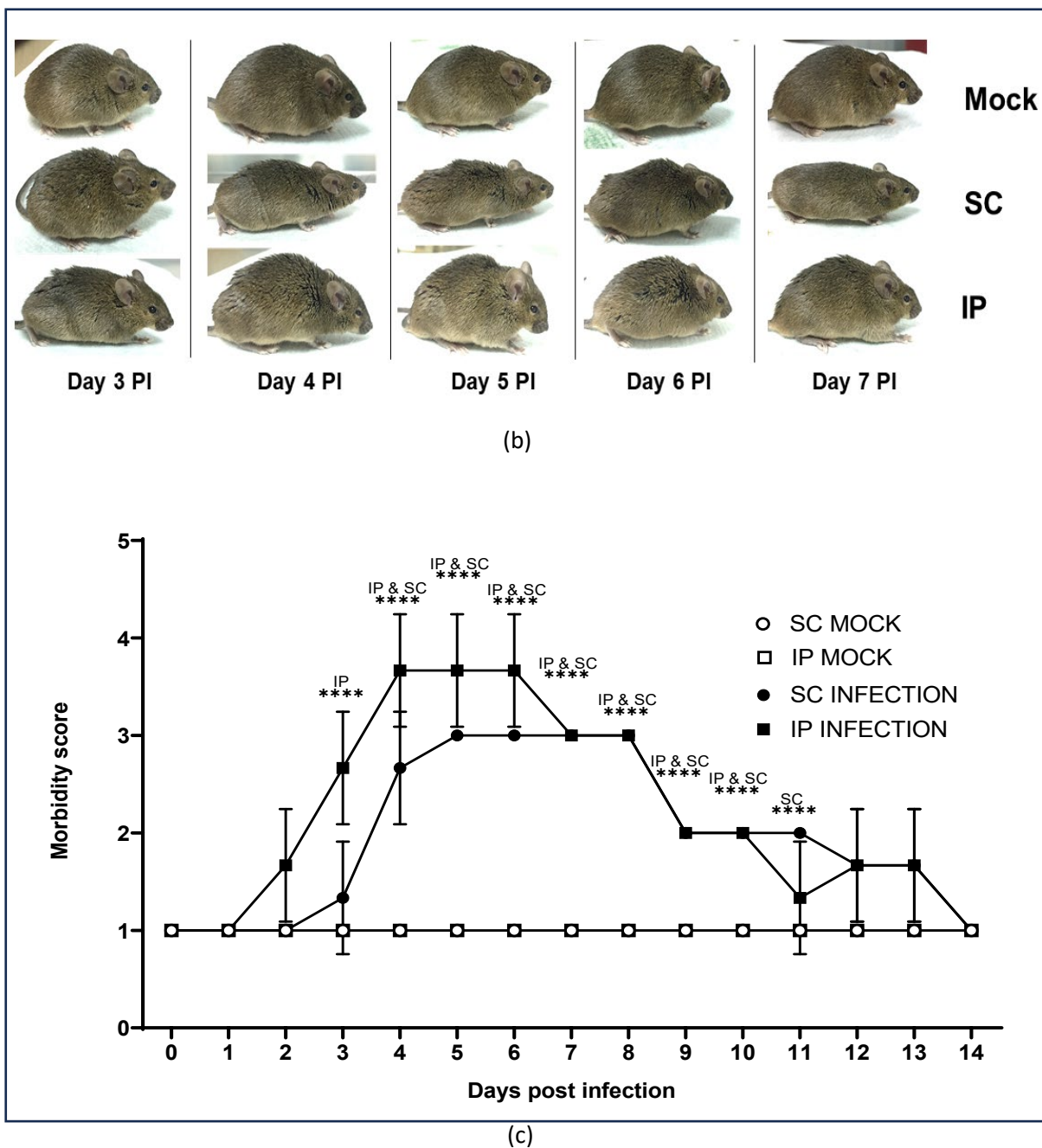


Figure 1. AG129 physical changes monitoring. (a) showed body weight changes of IP and SC-infected mice against their mock infection recorded for 14 days post-infection. Mock-infected mice received media without virus. (b) showed the actual observations of the mice for each infection route on day 3, 4, 5, 6, and 7 p.i.. (c) observed morbidity scoring of IP and SC infection routes following IP and SC infection compared to mock controls over 14 days post-infection. Data are presented as mean $\pm$ SD ($n = 3$). $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$. Differences were analysed using two-way ANOVA repeated measures followed by Tukey's multiple comparison test.

IP-infected mice began to show signs of morbidity from day 3 p.i., including ruffled fur, hunched posture, mild to intermediate lethargy, and failure to groom. Morbidity peaked between days 4 to 6 p.i., when symptoms progressed to intermediate to severe lethargy and limited mobility. SC-infected mice developed morbidity later, beginning on day 4 p.i., with similar signs of ruffled fur, hunched

posture, mild to intermediate lethargy, and failure to groom. SC-infected mice morbidity peaked between days 5 to 8 p.i. IP-infected mice consistently showed higher morbidity compared to SC-infected mice from day 2 to day 6 p.i. ($p < 0.001$). Recovery in IP-infected mice began 4 days after the first signs of morbidity, whereas SC-infected mice showed recovery 5 days after onset. Both infection routes were free of morbidity signs by day 14 p.i.

Viremia and NS1 levels in DENV-2-infected AG129 mice

Viremia levels in AG129 mice infected with the DENV-2 DMOF015 virus were detected using NS1 protein immunoassay and viral RNA quantification by RT-qPCR. Virus NS1 presence in the blood withdrawn from infected mice was detected at a similar level as shown in Figure 2(a) in the mice from both infection routes as early as day 1 p.i. The NS1 level remained high compared to the control from day 1 p.i. until day 5 p.i. On day 7 p.i., the NS1 level of both IP- and SC-infected mice dropped to zero.

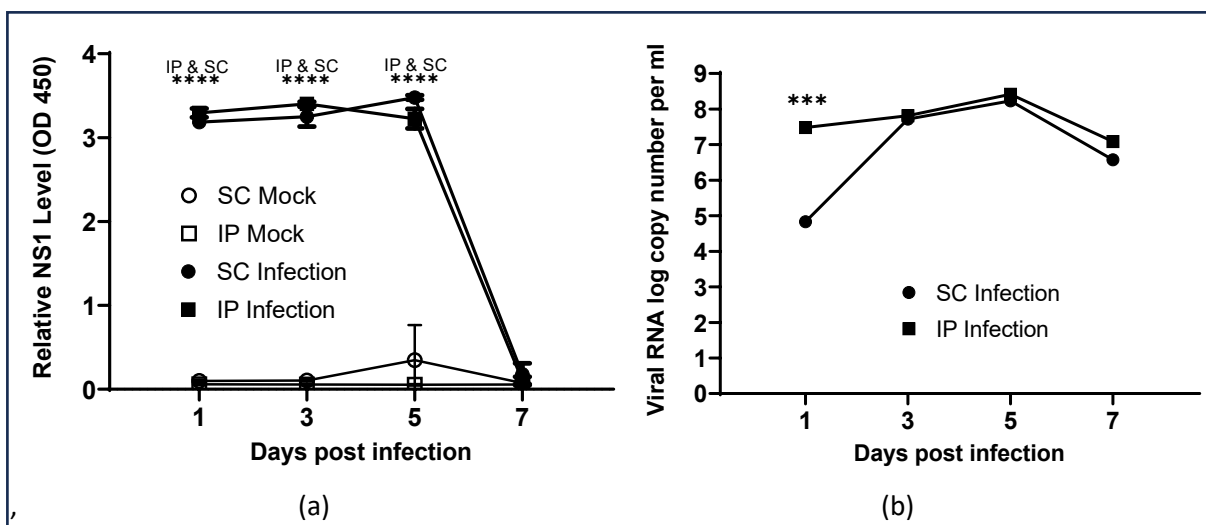


Figure 2. Viremia level of AG129 mice infected with DENV-2 DMOF015 and mock-infected mice. (a) showed viral NS1 levels in IP- and SC-infected mice compared to respective mock-infected mice and (b) showed comparison of viral RNA copy number of both infection routes on day 1, 3, 5, and 7 p.i. Data are presented as mean $\pm$ SD ($n = 3$). *** $P \leq 0.001$ and **** $P \leq 0.0001$. Differences were analysed using two-way ANOVA repeated measures followed by Tukey's multiple comparison test.

The viral RNA copy number of IP- and SC-infected mice was detected using real-time PCR and is shown in Figure 2(b). On day 1 p.i., 3.05×10^7 viral RNA copies/mL were observed in IP-infected mice, which was significantly higher compared to SC-infected mice ($P < 0.001$). SC-infected mice exhibited a 1000-fold increase in viral RNA copy number, reaching levels comparable to IP-infected mice by day 3 p.i. The high viral load was maintained until day 5 p.i., after which viral RNA copy number began to decline in both infection routes by day 7 p.i.

Effect of DENV-2 DMOF015 infection on WBC, platelet, lymphocyte, and neutrophil count in AG129 mice

Total WBC (Figure 3a) in the IP-infected mice increased significantly on day 5 p.i. ($P < 0.01$) followed by SC-infected mice on day 7 p.i. ($P < 0.0001$). All five WBC subsets—neutrophils, lymphocytes, monocytes, eosinophils, and basophils—were enumerated via differential WBC analysis. Given their predominance and established roles in dengue immunopathogenesis, only neutrophil and lymphocyte percentages are presented. Basophils were consistently undetectable across all samples, while monocytes and

eosinophils remained below 4% of total leukocytes and showed no statistically significant variation between experimental groups.

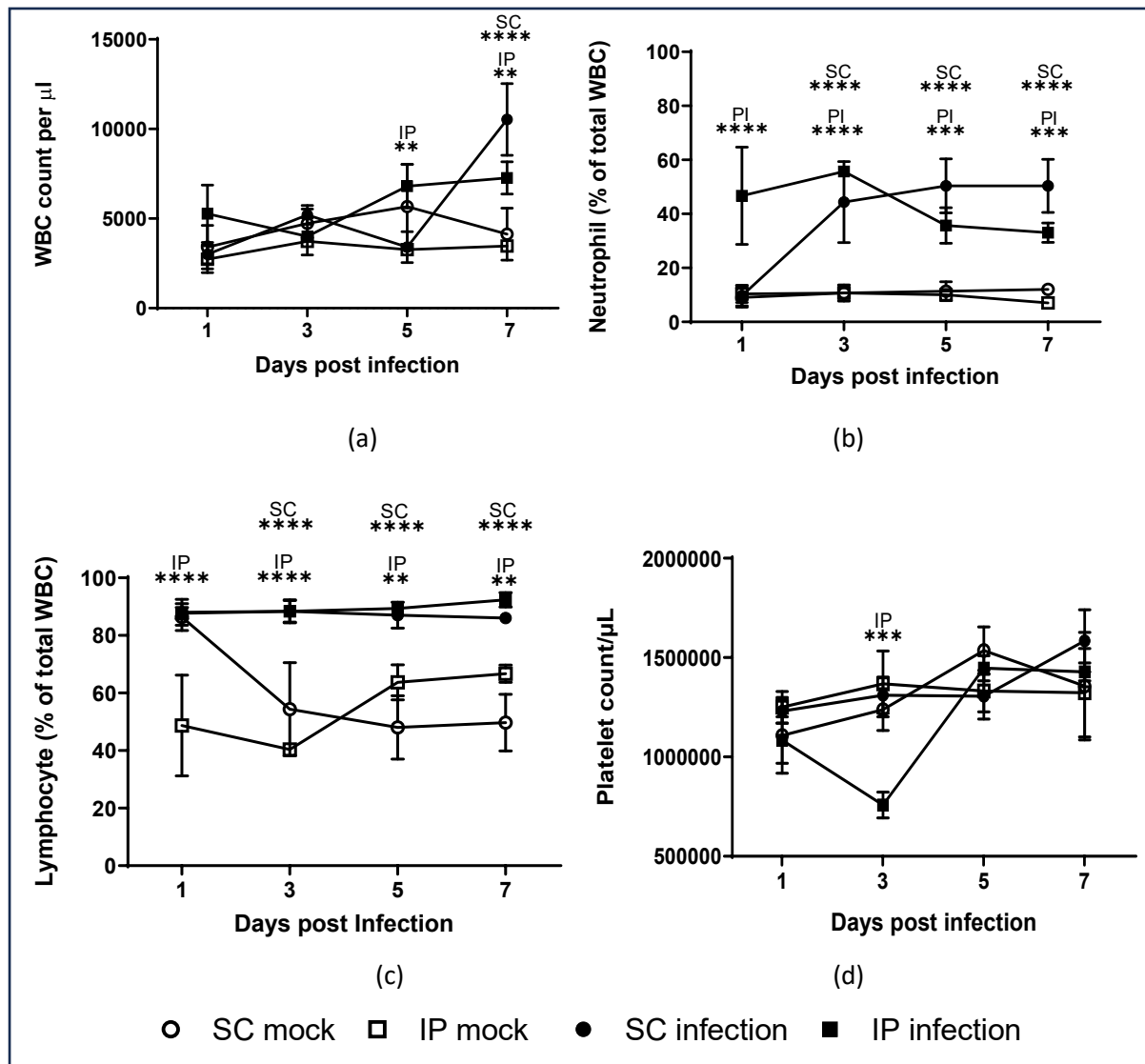
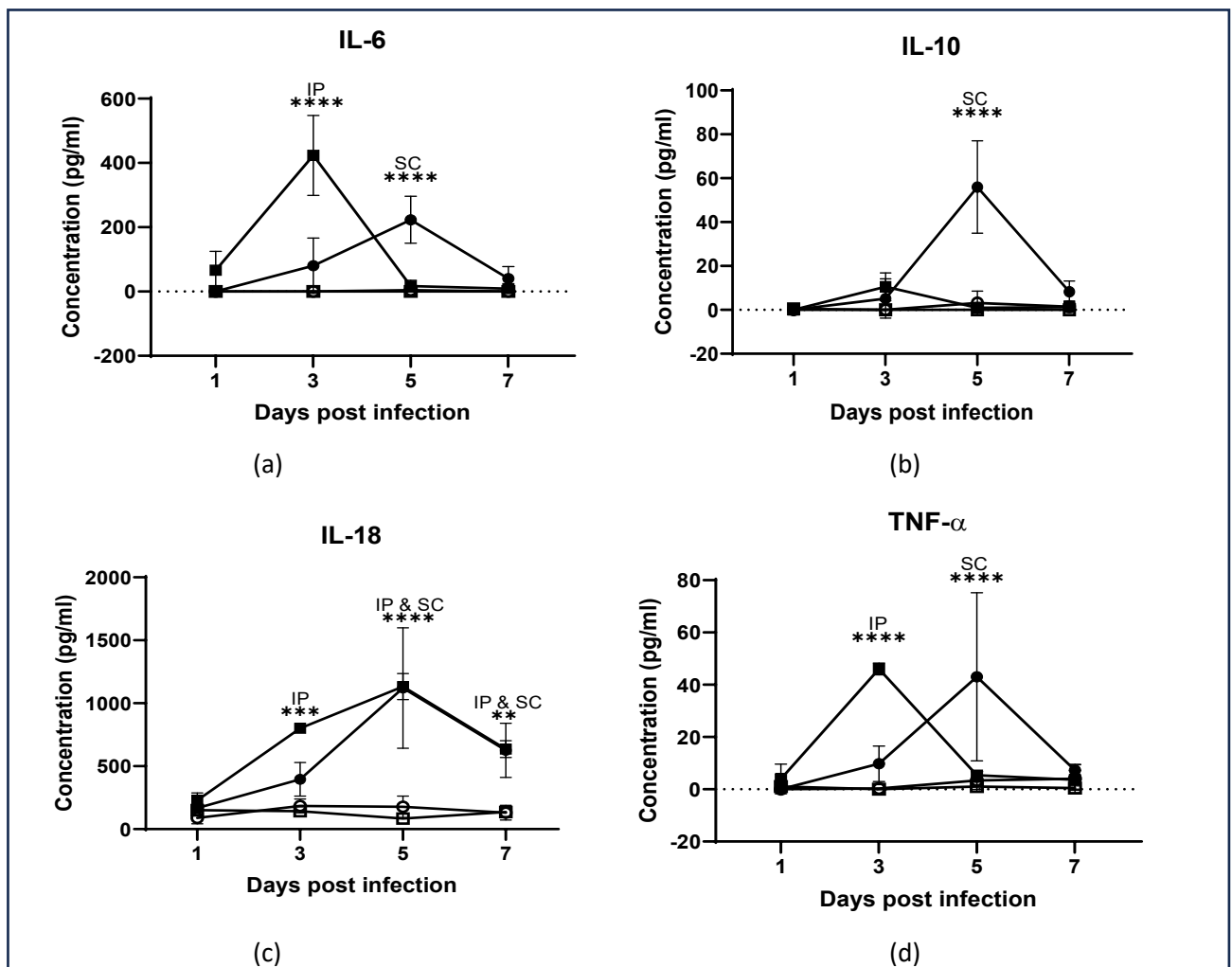


Figure 3. Blood count of SC and IP infection route compared to mock infection on day 1, 3, 5, and 7 p.i. for (a) WBC and differential counts of (b) neutrophil, (c) lymphocyte and (d) platelet level in the blood was recorded. Data are presented as mean $\pm$ SD (n = 3). ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$. Differences were analysed using two-way ANOVA repeated measures followed by Tukey's multiple comparison test.

Neutrophils increased significantly on day 1 p.i. for IP-infected mice ($P < 0.0001$) and day 3 p.i. for SC-infected mice ($P < 0.0001$) compared to mock controls (Figure 3b). Lymphocyte counts decreased significantly on day 1 p.i. for IP-infected mice ($P < 0.0001$) and day 3 p.i. for SC-infected mice ($P < 0.0001$) compared to mock controls (Figure 3c). Neutrophil counts remained high while lymphocyte counts remained low until day 7 p.i. for both infection routes. Platelet levels (Figure 3d) in IP-infected mice decreased on day 3 p.i. ($P < 0.001$) but returned to normal by day 5 p.i., with no differences observed thereafter. SC-infected mice did not exhibit significant changes in platelet counts compared to controls throughout the seven days ($P > 0.05$).

Effect of DENV-2 DMOF015 infection on cytokine levels in AG129 mice

Out of the 26 cytokines screened, seven (IL-6, IL-10, IL-18, TNF- α , IFN- γ , G-CSF, and CCL2/MCP-1) showed significant increases compared to controls (Figure 4a–g). In IP-infected mice, IL-6, IL-18 and TNF- α cytokine levels rose earlier, with IL-6 and TNF- α subsiding 1 day sooner than in SC-infected mice, which exhibited delayed but comparable responses. Apart from differences in timing, overall cytokine levels were similar between infection routes, except for IFN- γ and IL-10, which were higher in SC-infected mice, and CCL2/MCP-1, which was elevated in IP-infected mice.



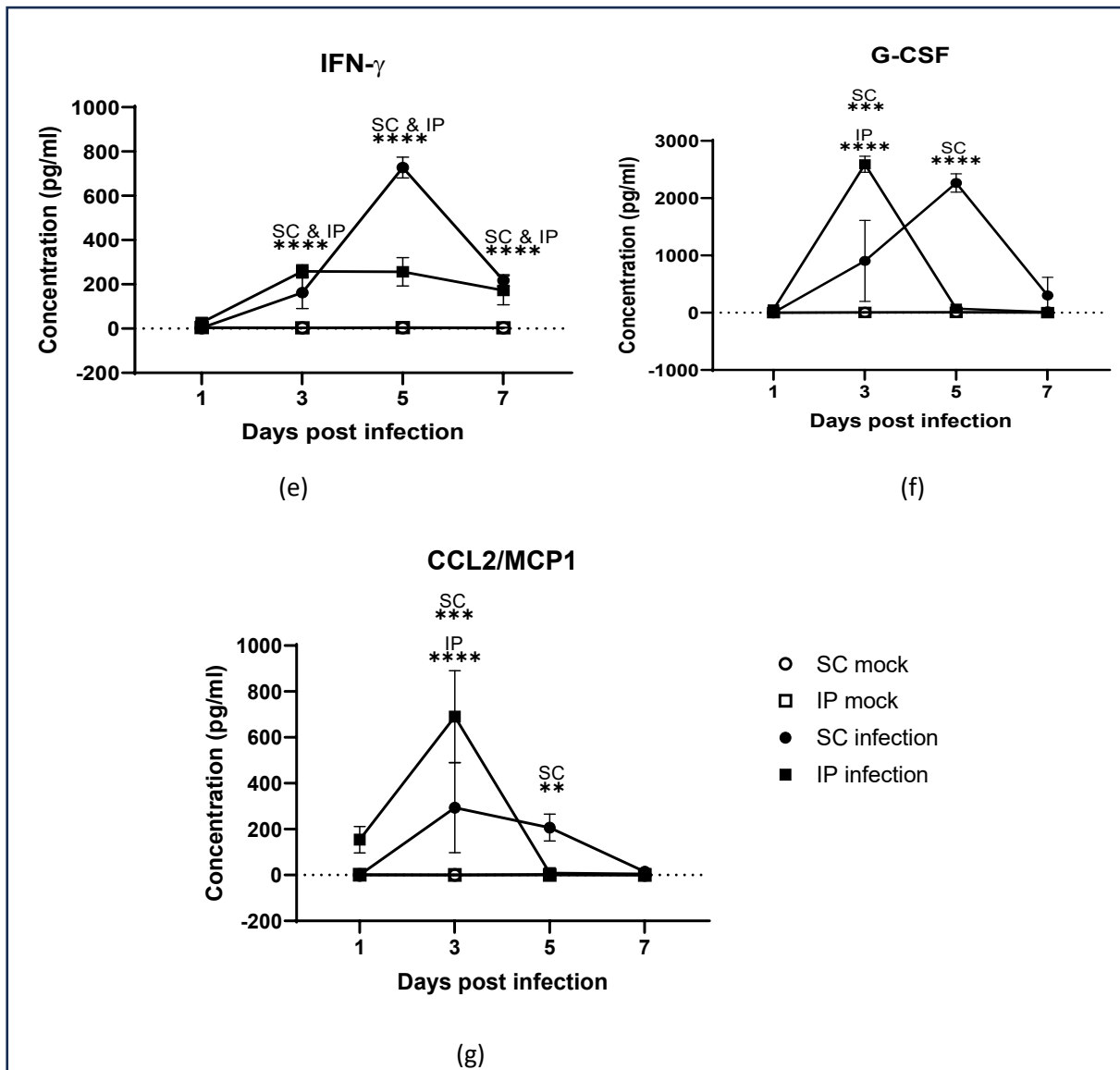


Figure 4. Cytokine levels of IL-6, IL-10, IL-18, TNF- α , INF- γ , G-CSF and CCL/MCP-1 are shown in Figure 4 (a to g). Plasma was collected from IP and SC mock-infected, and IP SC-infected AG129 mice groups at day 1, 3, 5, and day 7 p.i. Detection and quantitation of cytokines were carried out using the ProcartaPlex immunoassay. Data are presented as mean $\pm$ SD ($n = 3$). $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$. Differences were analysed using two-way ANOVA repeated measures followed by Tukey's multiple comparison test.

DISCUSSION

The effects of DENV-2 DMOF015 infection through IP and SC infection were compared in this study through body weight and morbidity measurement, NS1 and viral RNA detection, WBC and platelet levels, as well as cytokine expression. Minimal body weight loss was observed in the mock-infected mice, possibly due to the stress after the intervention (21). The periodic blood collection through the submandibular vein could cause discomfort, although insignificant. Weight loss in mice infected with dengue is a sign of successful dengue infection (22). The weight loss was reflected in the morbidity scoring, whereby morbidity was observed in both the IP and SC infection routes. An increasing

morbidity score indicates worsening conditions of the mice. This clinical event is seen in dengue infection in humans during the febrile phase, which lasts 3 to 7 days post-infection (23). Dengue patients develop sudden onset of high fever, often accompanied by headache and myalgia, as shown by the increase in the infected mice morbidity level. Dengue patients also develop gastrointestinal symptoms, which in infected mice, it is shown by weight loss and a leaner condition in physical observation.

Early virological and haematological differences may explain the higher morbidity scores observed in IP-infected mice compared to SC-infected mice. IP infection showed faster viral replication kinetics as early as day 1 p.i., four out of seven distinctively earlier cytokine responses, and thrombocytopenia on day 3 p.i., all of which are hallmarks of severe dengue pathogenesis. Although morbidity was monitored until day 14 and our mechanistic measurements were limited to day 7, these early alterations are plausible contributors to the subsequent morbidity trajectory. Extended profiling of immune and haematological parameters beyond day 7 would be required to establish direct correlations.

The presence of dengue virus was detected by quantification of viral RNA and measurement of NS1 levels. Dengue virus replication kinetics in its hosts are strain- and host-specific (24). The dengue virus strain that causes the highest mortality in mice is typically the one that replicates more efficiently (25). In this study, we found that apart from strain and host factors, different infection routes also affect dengue virus replication in mice, but only during the first 3 days post-infection. Detected NS1 levels of both infection routes are the same from day 1 to day 7 p.i. A decrease in NS1 secretion does not affect virion release (26). It is seen in this study where the NS1 level was reduced to almost zero on day 7 p.i., however, the number of viral RNA detected remained high. The function of dengue NS1 protein has long remained inconclusive but recently, it was put into perspective that the dengue virus NS1 protein is secreted in high concentrations during infection, mimics high density lipoprotein (HDL) structurally and functionally by binding to the HDL receptor, the Scavenger Receptor class B, member 1 (SRB1), interacts directly with HDL and Apo-A1 to potentially enhance viral replication and inflammation, alters lipid profiles in patients by competing with the HDL, and activates platelets, contributing to thrombocytopenia and vascular pathology (27). Nevertheless, detection of NS1 in dengue infection makes it an important biomarker for early diagnosis of the disease (28,29).

A sign of dengue infection is the presence of leukopenia, a condition where there is a reduced number of WBC (30). Total WBC is usually normal at the onset of fever, followed by leukopenia development with decreasing neutrophils and lasting throughout the febrile period. On the contrary, DENV-2 DMOF015 IP and SC infection in AG129 mice has failed to decrease neither WBC nor neutrophil. The number of WBC and neutrophils was observed to be increased gradually from day 1, until day 7 p.i., which represents the end stage of the febrile period. Failure to reproduce all clinical signs of dengue infection in animal models is a known factor that has hampered advances in dengue research (31). However, detecting leukopenia as an early diagnosis of dengue infection only has a positive predictive value of 70%–80% (30). The increase in WBC of DENV-2 DMOF015-infected mice is in tandem with increased T cell activation and increased cytokine production in patients with dengue haemorrhagic fever during both primary and secondary dengue virus infections (32).

The increase in neutrophil count from day 1 until day 7 p.i. in this study agreed to the findings where dengue patients had higher neutrophil percentage in the first five days of fever (33). An increase in WBC and neutrophil following dengue infection in AG129 mice has been reported previously (5,34), where a small but significant increase of neutrophil from day 1 p.i., that peaks on day 3 p.i. was observed. Furthermore, these studies reported a decrease in lymphocyte and platelet count. Early

infection onset caused by IP infection in mice was seen in increased neutrophil counts as early as day 1 p.i. and was followed by SC-infected mice on day 3 p.i. In this study, IP-infected mice showed a reduction in platelet count on day 3 p.i., indicating thrombocytopenia. However, there was no reduction in platelet count for SC-infected mice. The lack of platelet reduction in SC-infected mice did not agree with the thrombocytopenia that usually occurs after dengue virus infection (35). This absence of thrombocytopenia in SC-infected mice might explain the less severe acute weight loss and less severe morbidity level scoring of SC-infected mice during the first seven days p.i. compared to IP-infected mice. The reduction in platelet count is correlated with the severity of DHF and DF. Patients that showed a reduction in platelet count during the febrile phase later progressed to the more severe DHF (36). Corresponding to this, DF patients that did not progress to more severe DHF did not show a reduction in platelet count. While a study using different dengue virus strains suggested thrombocytopenia to be strain-specific (37), the lack of thrombocytopenia in SC-infected mice in this study shows that the development of thrombocytopenia is also related to the virus route of infection.

DENV-2 DMOF015 infection increased seven types of cytokines in plasma samples from both infection routes. IP-infected mice exhibited a general increase in cytokine levels at day 3 p.i. whereas SC-infected mice showed elevated cytokine levels at day 5 p.i. Notably, a heightened cytokine profile on day 3 after symptom onset is typically associated with severe dengue (38). The rise in cytokine levels during dengue infection is closely linked to the vascular damage seen in severe manifestations such as dengue DHF and dengue shock syndrome (8). The cytokine storm that occurs after a severe dengue virus infection is also linked to the severe multiple organ failure suffered by dengue patients (39). However, the delayed cytokine surge seen here in SC-infected mice is not indicative of less severe disease. Instead, it reflects a later onset of viral replication, which begins around day 5 p.i. compared to IP-infected mice, where viral multiplication starts as early as day 1 p.i. Moreover, the increased level of IFN- γ and IL-10 cytokines has been associated with antiviral and anti-inflammatory responses, respectively, in the recovering phase of symptomatic dengue patients (40).

In dengue infection, elevated IL-6 and IL-10 levels are correlated with low platelet counts, contributing to clinical manifestations (41). However, AG129 mice lack both type I and type II interferon receptors. That might skew the AG129 immune response toward uncontrolled viral replication and heightened pro-inflammatory cytokines (TNF- α , IL-6) but often blunts regulatory cytokines like IL-10. The balance is shifted toward inflammation rather than regulation. Serum IL-10 levels are reported to correlate inversely with total white cell and lymphocyte counts in dengue patients (42). The skewed immune response in AG129 mice in our study is also seen where lymphocyte counts decreased as expected, but the opposite trend was observed for total white cell counts. Instead of declining, WBC steadily increased from day 5 p.i. and reached higher levels by day 7 p.i. in both infection routes. This divergence is reflected in the absence of a consistent elevation of IL-10 across infection routes. Besides, IL-10 induction in dengue is usually delayed and transient. In AG129 mice, viral kinetics are accelerated, so IL-10 may rise only briefly during the sampling window, as seen in the sudden rise of IL-10 in SC infected mice on day 5 p.i. and abrupt decrease of IL-10 detection on day 7 p.i. Although the dengue infection effect is slower in SC infection, the increase of anti-inflammatory cytokines follows the same trend in dengue pathogenesis (43), where elevated cytokines such as MCP-1, TNF- α , IFN- γ and IL-10 are usually detected after three days p.i.

This study only focused on the Malaysian DENV-2 isolate (DMOF015), and the disease progression from other dengue virus strains may be different. This study is also limited to viremia and cytokine levels in the plasma. Therefore, tissue-specific viremia and cytokine levels could not be highlighted in this study. Throughout the duration of the study, no mice exhibited moribund behaviour or mortality. All animals remained viable until the scheduled endpoint, at which they were euthanised according to ACUC guidelines.

CONCLUSION

The effect of the IP virus infection is more immediate compared to the SC infection route. Both IP and SC infection routes are able to produce the same level of DMOF015 viremia in AG129 mice, where the virus multiplication and severity of both infection routes reflect the infection that occurred in humans. However, viruses inoculated through the IP infection route caused an earlier increment of cytokines level in AG129 mice compared to the SC infection route. It is also interesting to note that SC-infected mice in this study failed to produce the classic sign of thrombocytopenia in dengue infection. Therefore, the IP infection route is recommended as the preferred model because it produces faster disease onset, earlier cytokine response, and more representative clinical features of dengue infection compared to the SC route.

Supplementary Materials: Not applicable

Data Availability: The final published paper will include the information collected or studied during this assessment.

Author Contributions: N.H.M.J (Virus preparation; Data analysis; Writing—original draft), N.A.N (Data curation), A.M, T.C.D.H, B.J, and U.R.S (Performed animal and laboratory experiments; Raw data collection and analysis), M.Z (Project administration), R.T (Project guidance), A.F.S.M (Writing—review & editing), and M.R.M.A.R (Conceptualisation; Conceived and designed the project; Performed animal and laboratory experiments; Supervision; Writing—review & editing).

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Institutional Review Board Statement: The in vivo study was conducted under the guidelines and approval of the Animal Care and Use Committee, Ministry of Health Malaysia (ACUC-MOH), ACUC/KKM/02(9/2016).

Informed Consent Statement: Not applicable.

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CITATION

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