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RESEARCH ARTICLE

A novel approach to assess human neurovirulence and neurotoxicity-related concerns in vaccine development

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ABSTRACT

Pre-clinical assessment of vaccines for neurotoxicity and neurovirulence is mandatory for safe human administration especially for vaccines developed to target neurotropic viruses. Several recent studies suggest that some vaccine candidates, for example yellow fever vaccine tested to be neuroattenuated in monkeys were later found to be neurovirulent in vaccinated population. In this study, we used a stem cell composed human microphysiological system configuration as an in vitro platform and infected them with four strains of DENV (Dengue Virus) followed by capturing the micrographs that were analyzed by AI/ML enabled software to generate neurovirulence score. Additionally, the host gene expression studies done with infected microphysiological system resulted in establishing the signatures specific to the host system against viral strains. Varying degrees of neurovirulence risk scores were recorded by the prediction model. Our approach is the first of its kind, showcasing the use of a healthy human Microphysiological System complemented with digital tools to generate neurovirulent gene signatures represented by HLA-B, C1QB, TIMP4, CD63 and RANTES, which may play a role in host protection or as a result of a pathological response against DENV infection.

Keywords: neurovirulence, vaccines, artificial intelligence, machine learning, digital solutions, alternatives to animal testing, human stem cells, dengue virus

Introduction

Vaccines are developed using a variety of different approaches and technologies, and their variety has grown with advances in the chemical, physical, biological sciences, and the computational sciences to match the expectations of efficacy[1]. Additionally, the safety and efficacy of the vaccines to healthy individuals to be vaccinated has impacted the varies approaches chosen. Among the different vaccines, those incorporating inactivated and live attenuated virus components as well as combinations developed as vaccines have proven largely effective. This effectiveness is achieved through the integration of key components that mimic either the structure or functions of the pathogen, thereby affecting the human body. For any vaccine designed to target neurotrophic pathogens (viruses), ensuring neuronal safety is of paramount importance at every stage of development.

The central nervous system (CNS) is a critical functional element for individual survival. Viral (neurotropic) infections can impair its functionality through the initiation of apoptotic or inflammatory processes, resulting in the loss of cognitive and motor skills. Despite the presence of an effective immune response and the multi-layered blood-brain barrier (BBB) within the CNS, various viruses are notorious for their secondary association with CNS diseases.

Neurovirulence like Guillain-Barré syndrome or GBS is a known adverse event after immunization which can be defined as the ability of the virus/vaccine to induce neurologic diseases[2-4]. The list of neurotropic viruses represents a definitive compilation of the most deleterious pathogens recognized by humanity, including West Nile virus, Cytomegalovirus, Japanese encephalitis virus, Influenza viruses, COVID-19 as well as the agents responsible for diseases such as polio, yellow fever, mumps, measles, rabies, herpes, HIV, smallpox, and monkeypox[5]. Therefore, a significant challenge in comprehending the pathogenesis of CNS viral infections lies in unraveling the specific host responses that either confer protection or contribute to pathology[6].

The recent development of the COVID-19 vaccine has helped us gain knowledge into the significance of the host system in protecting and pathological role in viral pathogenesis. It is important to gain deeper insights into the neuroinvasiveness and neurovirulence of the virus to understand the mechanism that contributes to the pathogenesis these the insights can suggest the risks of adverse effects caused by the vaccine with any residual effect of pathogen. Because neurovirulence targets the CNS, it often manifests clinically as encephalitis, including neurovascular injuries and lesions. Sometimes this manifestation is progressive and leads to acute conditions[7]. In the context of the viral vaccine development for neurotropic virus, it is under the regulatory guidelines mandate to evaluate the risk of neurovirulence concerns through the gold standard method - Monkey Neurovirulence Test (MNVT)[8]. The MNVT is used for lt release testing of viral vaccines especially for mumps, rubella and varicella vaccines. The test involves animal experimentation raising ethical concerns related to animal welfare, potential risks associated with extrapolating results to humans (as evidenced by documented instances of vaccines that passed MNVT causing severe neurovirulence, as reported on cdc.gov), lengthy testing durations, occupational hazards, and reliance on reference testing laboratories[9].

In this study, we report a new approach that utilizes artificial intelligence (AI) and machine learning (ML) tools in probing human neurovirulence signals exhibited by clinically harvested Dengue virus (DENV) strains (neurotropic and Flaviviridae family) on human Microphysiological System (hMPS) partly mimicking host neuronal system. DENV-2 and DENV-3 serotypes are known to be causing secondary infections like encephalitis, meningitis and myelitis[10,11]. Given this known impact of DENV infections on neurological debilitation, we propose a strategy to profile human neurovirulent patterns of the viral strains and extrapolate the very technology application to predict the risks associated with human viral vaccines.

Materials & Methods:

Human MicroPhysiological System (In vitro Platform):

The hMPS in this study is a primary, processed stem cell configuration established as an in vitro platform coaxed to neuronal lineage that has previously been characterized[12].

Infecting human MicroPhysiological System with DENV strains

One day before infection, 0.2 million hMPS cells were seeded in a 35mm dish. Then, on the infection day, complete media was replaced with the media (without serum) containing four strains of DENV (0.5 moi) received from the collaborator's lab and incubated for 4 hr[13]. After the incubation, the media was replaced with complete media (with 2% FBS) and then the cells were collected on days 1, 3 and 5 for RNA isolation. Before collecting the cells for gene expression analysis, the cellular micrographs were captured at 20X magnification under a phase contrast microscope and passed through the AI-enabled software to generate the score on the percentage of affected cells. Cells treated with Phosphate Buffer Saline (PBS) was used as the negative control panel (mock) for both digital phenotype and RNA analysis.

AI analyzing Micrographs

The phase contrast images generated from DENV infection were analyzed using a trained neural network prediction model on convolutional neural networks (CNN)[14]. AI-based image analysis was performed on the concept of pipeline of individual modules while each module processes the image in sequential order which is object identification followed by measurement. Image analysis was based on cells shape, speckles in the cells, nuclear shape and cell aggregation into the category of either affected cells or healthy cells. All the cellular morphologies that are non-healthy cell category were placed under affected cell category (AC). The AC percentage was quantified from the phase contrast micrographs as insights.

Infecting human MicroPhysiological System with Mycobacterium smegmatis

A day before infection, 0.2 million hMPS cells per well were seeded in a 6 well plate. On the same day, the bacterial culture number was recorded by measuring optical density at 600 nm to inoculate. Then, on the day of infection, bacteria were subsequently added to the well at a ratio of one cell for every 10, 50, or 100 bacteria. (1:10, 1:50, 1:100) and incubated for 4 hours[15]. After the specified incubation time, the bacteria-containing used medium was

discarded and then fresh medium was added and culture was maintained for another 6 hours. Within the span of 6 hours, the infected cells were observed and micrographs were captured for every hour to assess any engulfment activity towards bacteria.

RNA isolation followed by q-RTPCR

Total RNA from the treated hMPS was extracted using the RNeasy (Qiagen) method. The final RNA concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific). First strand cDNA was generated using Prime Script 1st Strand cDNA kit (Takara) according to the manufacturer's instructions. Real-time PCR was performed using CFX96 PCR system with SYBR Premix Ex Taq II kit (Takara). The primers used for qRT-PCR analysis are listed in Table 1.

Table 1: List of primers used in the study.

S.No	Gene	Forward Primer	Reverse Primer
1	TAP2	ATGCCCTTCACAATAGCAGCGG	CCAAAACTGCGAACGGTCTGCA
2	HAM1	GCAGTCAACTCCTGGACCACTA	CAAGGTTCCCACTGCTTACAGC
3	HLA-B	CTGCTGTGATGTGTAGGAGGAAG	GCTGTGAGAGACACATCAGAGC
4	HLA-E	CGGCTACTACAATCAGAGCGAG	AATCCTTGCCGTCGTAGGCGAA
5	B2M	CCACTGAAAAAGATGAGTATGCCT	CCAATCCAAATGCGGCATCTTCA
6	EDB	TATGACCCAGAATGTGGAGCGG	ATTTCCGAGCCACCTTCTGCGA
7	GFAP	CTGGAGAGGAAGATTGAGTCGC	ACGTCAAGCTCCACATGGACCT
8	ITGB2	AGTCACCTACGACTCCTTCTGC	CAAACGACTGCTCCTGGATGCA
9	C1QB	CCCAGTACTGATGTTGCTCC	GGATACCCGGGATGCCAG
10	TIMP4	CACTACCATCTGAACTGTGGCTG	GCTTTCGTTCCAACAGCCAGTC
11	MGP	CCTCAGCAGAGATGGAGAGCTA	ATGGCGTAGCGTTCGCAAAGTC
12	GZMB	CGACAGTACCATTGAGTTGTGCG	TTCGTCCATAGGAGACAATGCCC
13	CD63	CAACCACACTGCTTCGATCCTG	GACTCGGTTCTTCGACATGGAAG
14	LILRB4	TGTTACTATCGCAGCCCTGT	AGGGTCACGCTCTTTCCTG
15	MCL-1	CCAAGAAAGCTGCATCGAACCAT	CAGCACATTCCTGATGCCACCT
16	CSTB	CGTGTCATTCAAGAGCCAGGTG	GCTTGGCTTTGTTGGTCTGGTAG
17	RANTES	CCTGCTGCTTTGCCTACATTGC	ACACACTTGGCGGTTCTTTCGG
18	IP-10	GGTGAGAAGAGATGTCTGAATCC	GTCCATCCTTGGAAGCACTGCA
19	GADD45B	GCCAGGATCGCCTCACAGTGG	GGATTTGCAGGGCGATGTCATC
20	MX1	GGCTGTTTACCAGACTCCGACA	CACAAAGCCTGGCAGCTCTCTA
21	CTSZ	GGATGGTGTCAACTATGCCAGC	CACGCTCCCTTCCTCTTGATGT
22	HMOX1	CCAGGCAGAGAATGCTGAGTTC	AAGACTGGGCTCTCCTTGTTGC
23	PLIN2	GATGGCAGAGAACGGTGTGAAG	CAGGCATAGGTATTGGCAACTGC

Results:

Host response recorded as human neurovirulent phenotype signatures in DENV infections

The AI-enabled software has an embedded prediction model (inspired by WHO SOP for neurovirulence test of types 1, 2 or 3 live poliomyelitis vaccines (oral) in monkeys, version 2012) that utilizes the percentage of AC and the percentage of infiltrated cells in the grading method (Figure 1). The percentage of AC for DENV-2 infections was 47% as analyzed by the AI-enabled software, which suggests that the strain may be more neurovirulent compared to the other strains as well as with the mock treated cells (Table 2). The percentages of AC increased proportionately with the number of days incubated (data not shown).

Figure 1

1a

1b

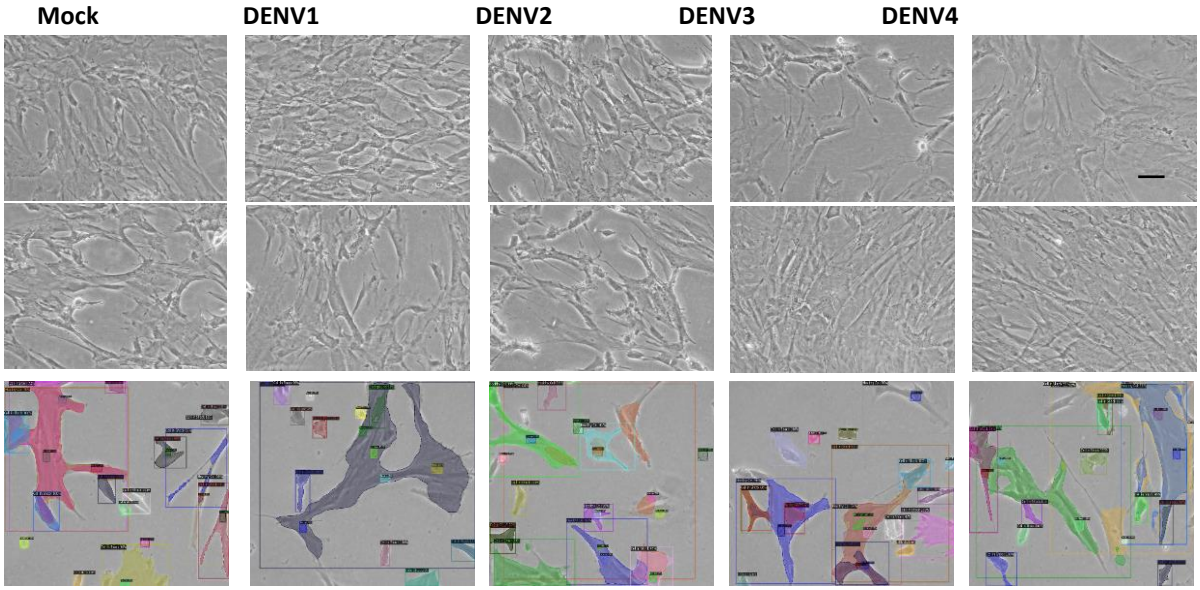

Figure 1: Digital Neurovirulence score assessment: 1a) Schematic for the assessment of neurovirulence upon viral infection on hMPS platform. 1b) The cells were treated with DENV strains and phase contrast microscopic images were captured at 20X magnification as shown in the 1st and 2nd row. The corresponding AI augmented images for the prediction of the affected cells were depicted in the 3rd row.

Table 2: Percentage of affected cells upon DENV infection with 4 different strains

	Control	DENV1	DENV2	DENV3	DENV4
Mean	**1**	**3.54**	**5.04**	**2.48**	**1.08**
STD	**0.8**	**2.48**	**4.1**	**1.9**	**0.9**
CV	**0.7**	**0.7**	**0.8**	**0.7**	**0.8**
% AC	**8.4**	**31.7**	**47.3**	**17.9**	**10.61**

Analysis done on 10 images per treatment as shown previously. Consolidated data mention the percentage of affected cells were given in the table.

We did not observe any significant correlation between the calculated neurovirulence score and the replication capability of the DENV viral strain[16]. In order to compare the result of the neurovirulence score generated in the software, the same hMPS was treated with an avirulent strain of *Mycobacterium smegmatis*. The micrographs generated after the bacterial infection were subjected to the AI analysis resulting in a similar AC percentage recorded as mock-treated hMPS (12%). This suggests that the increased percentage of AC noticed in the case of DENV was a real response to the infection (Fig 2).

Figure 2

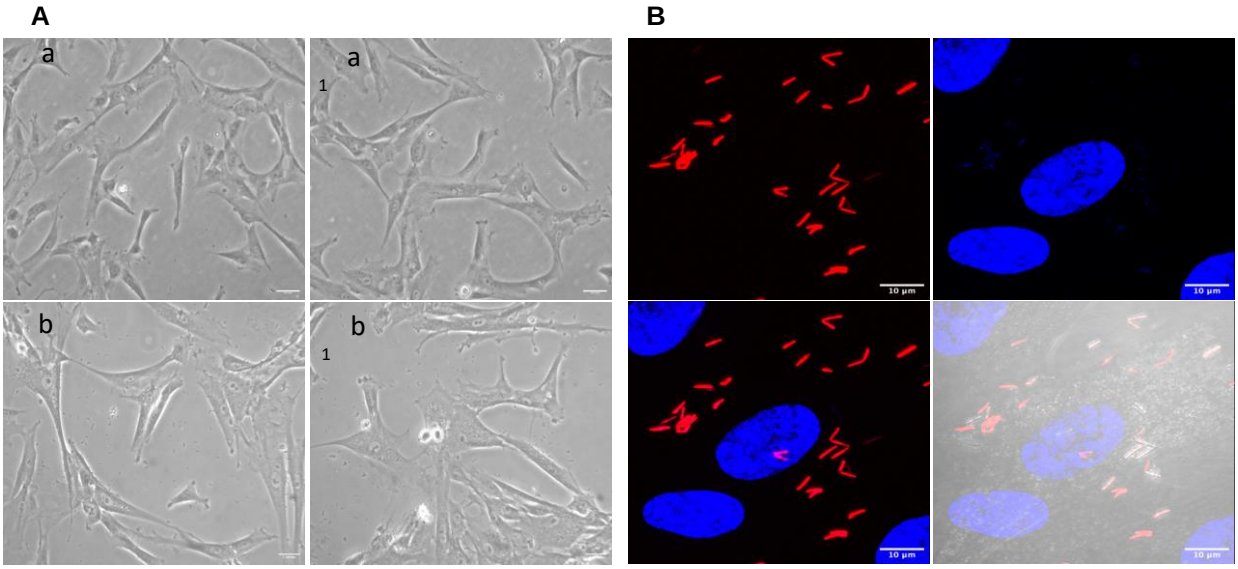

Figure 2: *Mycobacterium smegmatis* infected hMPS. A) Representative Phase contrast microscopic images of uninfected cells (a, a[1]) and *Mycobacterium smegmatis* infected hMPS (b, b[1]). B) Confocal microscopy images of the hMPS infected with mCherry tagged *Mycobacterium smegmatis*. Infected cells were stained with DAPI (blue) and the mCherry (red) label outlines the *Mycobacterium* strain. Localization of the strain inside the cell can be visualized.

Host response recorded as human neurovirulent genotype signatures in DENV infections

The Dengue viral infected hMPS was analyzed for transcriptomic signatures associated with the infections. RT-PCR analysis performed was to detect the expression of selected representative genes from the mouse study that systematically showed the host responsive genes towards the neurovirulent viral strain infections[17]. We relied upon the list of genes obtained from the extensive transcriptomic profile of the brain samples of mouse that were reported to be responsible for neurovirulence and are called neurovirulent gene signatures[17]. From the set of 75 genes tested for neurovirulent gene signatures, 23 genes showed indication of changes in fold expression upon DENV infecting the hMPS model. The selected genes fall under the following categories - Antigen presentation factors: TAP2, HAM1, HLA-B, HLA-E, B2M; Chemokines: IP-10, RANTES, MGP; CNS genes: EDB, GFAP; Complement factors: ITGB2 and C1QB; Matrix protein: TIMP4, MGP, Granule associated factor: GZMB; Interferon

related factor: MX1; Protein degradation factor: CTSZ; BCl2 family member: MCL-1; and Hematopoietic cell surface molecules: CD63, LILRB4 along with GADD45B, IFN related MX1, CTSZ, Hemoxygenase 1 (HMOX1) and Perilipin 2 (PLIN2) (Table 1). Among the tested genes, HLA-B, C1QB, TIMP4, CD63 and RANTES showed enrichment with all 4 strains of DENV (Table 3). Interestingly, infection with the DENV4 strain resulted in a higher degree of observable changes in the expression pattern. HAM1, HLA-B, B2M, EDB, TIMP4, GZMB, CD63, RANTES, MGP, MX1, HMOX1 (gene list) were found to be uniquely enriched in DENV4 infected hMPS model. The gene expression patterns are strain specific while the avirulent strain of *Mycobacterium smegmatis* infected hMPS model showed similar expression pattern to that of mock in the experiment.

Figure 3

Figure 3: Gene expression analysis: mRNA expression of genes involved in neurovirulence on the hMPS infected by the DENV strains. The expression of the genes was analyzed at 1, 3, 5 days of infection with DENV 1, 2, 3, 4 respectively. The data was normalized to GAPDH and fold enrichment of each individual gene was quantified by normalizing to the mock treated cells (served as baseline for comparison analysis).

Table 3: Consolidated table for gene expression levels

Gene family name	Gene name	DENV1	DENV2	DENV3	DENV4
Antigen presentation	TAP2	X	X	☑	☑
	HAM1	X	X	X	☑
	HLA-B	☑	☑	☑	☑
	HLA-E	X	X	X	X
	B2M	X	X	☑	☑
CNS Specific	EDB	X	X	X	X
	GFAP	☑	X	☑	X
Complement	ITGB2	☑	X	☑	☑
	C1QB	☑	☑	☑	☑
Extracellular matrix and cell adhesion	TIMP4	☑	☑	☑	☑
	MGP	X	X	☑	☑
Granule associated	GZMB	☑	X	☑	☑
Hematopoietic cell surface molecule	CD63	☑	☑	☑	☑
	LILRB4	☑	X	☑	☑
Bcl-2 family members	MCL-1	X	X	X	X
Cysteine protease inhibitor	CSTB	X	X	☑	☑
Chemokines	RANTES	☑	☑	☑	☑
	IP-10	X	X	X	X
Growth arrest and DNA damage inducible	GADD45B	X	X	X	X
IFN related	MX1	X	X	X	X
Protein degradation	CTSZ	X	☑	☑	☑
Hemeoxygenase 1	HMOX1	☑	☑	X	X
Perilipin 2	PLIN2	☑	☑	X	☑

Cumulated data for three days of expression

☑ Signifies >1.3 fold enrichment

'X' signifies no change in expression compared to the mock treated sample

Discussion

The gold standard method used to test/screen for neurovirulence is traditionally MNVT, both at the preclinical stage and for any periodic assessments to be undertaken for viral vaccines[18]. *In vitro,* cell culture methods[7] to study the mechanisms of neurovirulence is a known science, but lacked translatability, as the human nervous system is a complex arrangement with several types of cells that respond differently to different viruses. *In vitro* cell based models produced from human pluripotent stem cell technology were used to study the neurotropism of Zika virus, HIV, HCMV, and measles virus[19]. When Dengue type 4 virus (DEN4) cDNA was used as a vector to express genes of the distantly related tick-

borne encephalitis virus (TBEV), the vector conferred neurovirulence in the mice model[20] conveying it as the phenomenon. In vivo and in vitro models have been employed to study these questions that have revealed important differences among pathogen variants in their neuroinvasive, neurotropic, and neurovirulent potential. Determining the mechanism of viral infections on the human surrogate systems can facilitate efficient development of vaccine design, further ensuring the adequate neuroattenuation and usage of hMPS configurations with primary and progenitor cellular moieties (pheno-geno barcoded neuronal cells) can serve as a better non-animal model for scalability and flexibility, throughput screening, mimicking the healthy human CNS microenvironment in its native status. We have utilized a well characterized hMPS in vitro platform as the host model in this study that attempted to generate human neurovirulent phenomic signatures[21] for DENV strains. We propose that the generated signatures may be utilized as reliable set of benchmark signals in screening dengue vaccine neurovirulence risks within the developmental workflows.

The assessment of cells undergoing apoptosis, necrosis, cells in shock and dead cells were the preliminary signals used to calculate the percentage of AC in grading neurovirulence score while the composition of the hMPS utilized influenced the authenticity of insights captured to analyze. The shown AI based neurovirulence scoring system utilizes significant machine training, in detecting the subtle morphological features exhibited by the hMPS acquired micrographs. One of the important features in the trained prediction model of the software is detecting the dead cells and cells in stressed condition as categories that are summated for measuring AC. This feature can be further exploited in detecting the varying degrees of neurovirulent strains from mild to strong as shown by alpha viruses[22]. The phenotype data in the micrographs format fed into the AI-enabled and ML-trained software picked signals to measure AC and report the neurovirulence score on the prediction model embedded. This shows that using an ML-based neural network approach can be effectively used to distinguish the subtle changes in the cell morphologies just by phenotyping the cells[23]. Katarzyna reported a deep learning model that distinguishes between pluripotent stem cells and differentiated hepatocytes based on the cell morphologies[24]. This further suggests that the minor changes in the morphological features that may contain crucial information can be detected by a well-trained prediction model that attains an accuracy rate near 1. In this study, the AI enabled ML-trained software, which was trained on ~1000 images achieved a predictive accuracy rate of 0.924.

Since the molecular mechanisms of neuropathogenesis, relevant infections are known to be specific to pathogen type, the majority of the genes tested in this study were classified into functional groups that are known to be involved in the host response. The genes belonging to the families like antigen presentation molecules, immune cell activation markers, chemokines, CNS related genes, complement factors are specifically cited for their role in neurotropism and inflammation. The attributed changes in the expression of genes are primarily linked to the role of host system as a defense mechanism in the host protective or pathologic response against neurovirulent viral infection [17].

The results highlight several host genes that are unique to DENV strain infections, while the expression of chemokines RANTES and IP-10, which are known to have a neuroprotective and proinflammatory role in CNS[25], were found to be enriched in DENV infections. The selective increase of RANTES/C-C Motif chemokine ligand 5 in DENV infected hMPS is noteworthy in light of its activity, as it functions as one of the natural ligands for the chemokine receptor and suppresses the *in vitro* replication of R5 strains of HIV-1[26], also involved in the pathogenesis of mouse hepatitis virus induced demyelination. Complement related proteins like ITGB2, C1QB were observed to be upregulated in DENV infected hMPS, connecting their role in the innate immune response to pathogen invasion and impairment of the regenerative process[27].

Granzyme B (GZMB) protein was cited as linked to an activation cascade of caspases responsible for apoptosis[28]. We noticed that the DENV strain 1, 3, 4 infected hMPS were associated with increased levels of GZMB expression detected, which must have promoted apoptosis as a mark of host defense mechanism that aligns with our observation of dead cell debris at day 10. TIMP4 and MGP genes are related with extracellular matrix and cell adhesion like tissue related pathways, involved in the inhibition of matrix metalloproteinases and ectopic tissue calcification respectively[29-31]. Interestingly, all the four strains of DENV infection yielded upregulated expression of TIMP4 while not MGP with DENV 1 or 2 treated hMPS. Hematopoietic cell surface molecules CD63 and LILRB4 were found to be over-expressed in DENV infected hMPS. These factors are surface receptors involved in various activities like antigen capture/ presentation and related pathways of the innate immune system, adhesion, and role in VEGFA signaling in the physiology[32,33]

Here, we report for the first time a human neurovirulent phenomics signature as a host response from the DENV infections in the specified non-animal characterized microphysiology with a potential to become sub set of biomarker panel in screening for vaccine neurovirulence as a case report of the revealed new approach that leverages hMPS model and AI/ML tools in assaying.

Conclusions

Despite rigor on the methods to predict adverse events like vaccine neurovirulence after immunization, the development of vaccines in promoting human health is an important topic as healthy humans are the recipients. Neurological complications that may arise after vaccination can sometimes be progressive and thus need stringency, relevance in not just predicting but acting on if detected under critical quality attribute reporting. Here, we have shown that upon infection with dengue viral strains, the phenomics signatures captured as responses from the hMPS can be quantified to generate neurovirulence scores in a non-animal setting by utilizing digital tools. This we submit as a new approach methodology that can be calibrated to neurotrophic virus strains (alternative to MNVT) to the community for exploring the adoption of the technology.

Conflict of Interest Statement:

None

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Author contributions:

Subhadra Dravida: Conceptualization, work review, manuscript editing; Vasanthi Dasari: experimental design, methodology and manuscript writing, Paparao Bolimera and Swati Shukla: Wet lab experiments and data collection, Rahul Ganar and Timothy Elwell: Software and validation, manuscript editing.

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