Quantifying temporal differences in the induction of interferon-mediated signalling observed in a dengue virus 1 human infection model: insights from longitudinal proteome analysis

Caroline Struyfs,[a] Klaas Van den Heede,[b] Liesbeth Van Wesenbeeck,[a] Adam Tully Waickman,[c] Freya Rasschaert,[a] Guillermo Herrera-Taracena,[d] Stephen James Thomas,[c] Marnix Van Loock,[a] and Ole Lagatie[a,*]

[a]Johnson and Johnson, Beerse, Belgium
[b]D-Mine, Mechelen, Belgium
[c]State University of New York Upstate Global Health Institute, 5010 Campuswood Drive, Syracuse, NY, 13057, USA
[d]Johnson and Johnson, Horsham, PA, 19044, USA

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Summary

Background According to WHO, dengue is one of the top ten global health threats, with almost half of the world's population at risk of being infected. Most of the annual 400 million dengue virus (DENV) infections manifest asymptomatically or in a mild form, causing symptoms such as fever and headache. Nevertheless, every year 500,000 dengue cases require hospitalization and up to 25,000 patients die. Despite the high incidence, the DENV-elicited proteome response remains insufficiently understood.

Methods Therefore, we evaluated the proteome dynamics of nine dengue-naïve individuals experimentally infected with the underattenuated DENV-1 strain 45AZ5 via the Proximity Extension Assay technology of Olink®.

Findings Using Olink Explore, a total of ~3000 proteins were quantified simultaneously in serum samples at 8, 10, 14, and 28 days after the viral inoculation. We identified the top ten significant proteins via linear mixed effects models, i.e., interferons (IFNs), IFN-related proteins, and members of the CCL and CXCL chemokine family. In all participants, an increase in IFN-λ1 levels was observed after peak viral load, whereas in one participant an IFN-γ response was not detected. Interestingly, both the onset and peak viral load of this participant were, on average, delayed 4 days compared to other participants. To gain a detailed kinetic overview of the DENV-elicited proteome response, we designed a smaller, targeted Olink® panel to evaluate serum protein levels at multiple time points throughout the infection. Here, we revealed that type I/III IFN response precedes the type II IFN response.

Interpretation In conclusion, our analyses provided detailed insights into the temporal dynamics of the different IFN responses upon a primary DENV-1 infection. These insights might aid in better understanding dengue pathogenesis.

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Keywords: Dengue; Proteomics; Interferon response; Dengue human infection model (DHIM)

Introduction

The dengue viruses (DENV) are mosquito-borne positive-sense single-stranded RNA flaviviruses belonging to the *Flaviviridae* family. Four antigenically distinct DENV serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) are transmitted to humans through the bite of infected *Aedes* mosquitoes, i.e. *Aedes aegypti* and *Aedes albopictus*, in tropical and subtropical regions.[1,2] Annually, 400 million DENV infections occur globally, with around 500,000 individuals requiring hospitalization due to severe and life-threatening complications of the disease, resulting in up to 25,000 deaths.[3] From 2000 to 2019, the World Health Organization (WHO) documented a ten-fold increase in reported dengue cases globally,

*Corresponding author. Johnson & Johnson, Turnhoutseweg 30, 2340, Beerse, Belgium.
E-mail address: olagatie@its.jnj.com (O. Lagatie).

Research in context

Evidence before this study

According to WHO, dengue is one of the top ten global health threats, with almost half of the world's population at risk of being infected. Most of the annual 400 million dengue virus (DENV) infections manifest asymptomatically or in a mild form. Nevertheless, every year 500,000 dengue cases require hospitalization and up to 25,000 patients die.

Despite the high incidence, the DENV-elicited proteome response remains insufficiently understood. Biomarkers can potentially be used to predict dengue disease progression, which might reduce morbidity and mortality associated with dengue.

We searched the Pubmed database using the terms (("Dengue") AND ("Proteomics"). We identified 274 papers, of which 25 concerned biomarkers. One paper described relative levels of 368 inflammatory markers in plasma samples from patients with lab-confirmed dengue, but no proteome-wide data or detailed temporal proteome dynamics are currently available.

Added value of this study

In this manuscript, we report on the proteome dynamics of nine dengue-naïve individuals experimentally infected with the dengue virus 1 strain 45AZ5. From the 2941 assessed proteins, we identified the top ten of significantly increased proteins upon a primary DENV-1 infection as interferons (IFNs), IFN-related proteins, and members of the CCL and CXCL chemokine family. In addition, we gained a detailed kinetic understanding of the DENV-elicited proteome response by evaluating a subset of serum protein levels at multiple time points (at least 11 time points per subject) throughout the infection. Here, we revealed that type I/III IFN response precedes the type II IFN response.

Implications of all the available evidence

This manuscript increases insights in the proteome dynamics upon a DENV infection. These insights are invaluable for enhancing our understanding of the complex immunopathogenesis of dengue, enabling us to leverage this knowledge in combating this infection more effectively. In the future, it will be of interest to explore these protein dynamics in mild and severe natural dengue infections with different DENV serotypes. Identifying biomarkers that can predict disease progression may enable healthcare providers to prioritize treatment for patients having a higher risk of developing disease complications, potentially reducing morbidity and mortality associated with dengue.

prompting them to consider dengue as a health priority.[4,5] In the first half of 2024, the Pan American Health Organization already reported 10,363,448 suspected dengue cases in the Americas—mainly in Brazil, which is an increase of 420% as compared to the average of the last five years.[6] Today, dengue is already endemic in more than 100 countries[7] and the spread of DENV continues to expedite due to multiple drivers, such as climate change, urbanization, population growth and increased global travel, to previously unaffected regions, encompassing southern regions of Europe and the US.[8,9] By 2080, the population at risk of a DENV infection is projected to increase to 6.1 billion.[8]

To date, no dengue-specific treatment is available, and the present focus is on pain, and/or fever control, and supportive fluid therapy.[10] A limited number of dengue-specific antivirals is currently in clinical trials, such as Johnson & Johnson's mosnodenvir (JNJ-1802) (Phase II), Novartis's EYU688 (Phase II), and Visterra's monoclonal antibody VIS513 (Phase II), currently licenced to the Serum Institute of India Pvt. Ltd (SIIPL).[10–13] Also, while there are two prophylactic DENV vaccines available, their indications and efficacy are limited.[14–17]

The clinical manifestations of dengue are classified into three categories: with or without warning signs, and severe dengue.[18] While the majority of the symptomatic DENV infections result in mild symptoms like fever and headache, severe dengue is characterized by vascular leakage, haemorrhage, and organ impairment, posing a significant risk of mortality. Interestingly, the progression to severe dengue coincides with a drop in viraemia and an exacerbated host immune response.[19,20] The immunopathogenesis of severe dengue is complex and multifactorial, involving interactions between the virus and the host immune response, and is not fully understood. Dysregulation of immune responses, including elevated levels of cytokines, chemokines, and lipid mediators, plays a crucial role in severe dengue complications.[21,22] Previous studies indicated that severe cases mostly occur during secondary DENV infections with a different serotype. Antibody-dependent enhancement represents one theory describing the immunopathogenesis of severe clinical outcomes,[23–25] but this paradigm was recently questioned by a study with 619 Indian children with febrile dengue-confirmed infection in which disease severity was not preferentially associated with secondary infections,[26] pointing to a knowledge gap in pathogenesis, anti-DENV immune response, and our understanding of who is at risk of developing severe dengue.

Insights in the host proteome dynamics upon a DENV infection are critical for enhancing our understanding of the host response, enabling us to leverage this knowledge in combating dengue more effectively. Studying the detailed DENV-elicited proteome responses longitudinally is challenging in natural infections as patients are only identified as soon as they

develop symptoms, and it is difficult to collect blood samples at sufficient time points for detailed kinetic studies. To overcome these difficulties, an experimental dengue human infection model (DHIM) can be used in which flavivirus-naïve participants are infected with a characterized and underattenuated DENV strain, administered in a controlled clinical setting.[27] The participants undergo extensive monitoring of clinical, virologic, and immunologic responses over time.[28]

In this study, we evaluated the DENV-elicited proteome dynamics of nine dengue-naïve individuals experimentally infected with the underattenuated DENV-1 strain 45AZ5. Using Olink® Explore, a total of 2941 proteins were quantified simultaneously in serum samples at key time points after the viral inoculation. Next, a detailed kinetic overview of the DENV-elicited proteome response was obtained by evaluating a smaller, targeted Olink® panel to study selected serum protein levels at numerous time points throughout the infection. Our analyses provide detailed insights in the temporal dynamics of the different responses upon a primary DENV-1 infection and reveal that type I/III IFN response, driven by IFN-α/β and IFNλ-l precedes the type II IFN response, driven by IFN-γ.

Methods

Ethical approval

The DENV-1 human infection model and associated analyses were approved by the State University of New York Upstate Medical University Institutional Review Board (SUNY-UMU; 1252992) and the Department of Defence's Human Research Protection Office. Human samples originate from a phase 1, open-label study (ClinicalTrials.gov identifier: NCT03869060), as described by Waickman and colleagues.[28] Written informed consent was obtained from all participants for this study.[28]

Study design

In this DENV-1 Live Virus Human Challenge open-label study, nine healthy, flavivirus-naïve individuals between 18 and 45 years old received a single subcutaneous inoculation of 0.5 mL of a suspension containing 6.5×10^3 plaque-forming units per mL of the underattenuated DENV-1 45AZ5 virus manufactured at the Walter Reed Army Institute of Research (WRAIR) Pilot Bioproduction Facility, Silver Spring, MD (U.S. Food and Drug Administration Investigational New Drug 16,332).[27] This virus strain originated from a Chinese patient with mild dengue fever and was serially passaged in foetal rhesus lung cells and mutagenized with 5-azacytidine to increase the likelihood of producing attenuated variants.[28,29] All enrolled participants completed the study. To evaluate the DENV-1 elicited proteome responses and DENV-1 RNA content, serum samples were collected daily from day 0 to 14 after viral inoculation, followed by alternate-day collections until day 28. Hospitalized participants were assessed daily during hospitalization, extended to 3 days post-discharge and then every other day until day 28. Quantitative DENV-1 RT-PCR was performed by Waickman and colleagues, as previously described.[28,30]

Proteomic analysis

The concentration of 2941 serum proteins was measured simultaneously at 0, 8, 10, 14, and 28 days after viral inoculation using the Olink® Explore 3072 library. This exploratory proteomics analysis was performed by Olink® Proteomics AB (Uppsala, Sweden). Olink®'s high-throughput protein biomarker discovery platform is based on Proximity Extension Assay (PEA) technology in which pairs of oligonucleotide-coupled antibody probes bind to their protein targets, enabling hybridization of complementary oligonucleotides.[31] DNA polymerase extends the hybridized oligonucleotides, forming a unique protein identification "barcode". Sample identification indexes and necessary nucleotides for Illumina® sequencing are included during the library preparation. Libraries undergo bead-based purification and quality assessment using the Agilent 2100 Bioanalyzer before sequencing on Illumina® NovaSeq™ 6000. Raw data was quality controlled, normalized, and converted into Normalized Protein eXpression (NPX), which is an arbitrary unit in Log2 scale.

Follow-up experiments were performed by Arcadia, UMC Utrecht (The Netherlands) with serum samples collected at 0, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 24, and 25 days after viral inoculation (as available) using Olink® Flex. Herein, 21 proteins of interest were selected based on Olink® Explore data and combined into one novel biomarker panel. In contrast to the Olink® Explore 3072 library, the DNA barcodes are detected and quantified using a microfluidic RT-PCR instrument (Biomark HD, Fluidigm) and the NPX values are calculated from normalized Ct values. Protein concentrations are reported both as absolute (pg/mL) and relative (NPX) units.

Statistical analysis

Differences in the abundance of a protein over time were evaluated by fitting a linear mixed effects model for every protein using the olink_lmer function of the Olink® Analyse package in R. The explanatory variable was the sampling day, and the participant ID was utilized as a random variable to take the paired nature of the data into account. Comparison of the protein abundance on two different sampling days was done using the olink_lmer_post-hoc function of the Olink® Analyse package in R for every protein. PCA plots were created using the prcomp function of the Stats package in R. For the K-means clustering analysis, the Cluster package in R was used. Data were visualized in RStudio version 2023 12.0 (RStudio, Boston, MA, USA),

GraphPad Prism version v9.5.1 (GraphPad Software, La Jolla, CA, USA), Tableau version 2022.3 (Tableau Software, Seattle, Washington, USA), and Ingenuity Pathway Analysis version 111,725,566 (QIAGEN, Germany). Adjusted p-values, according to the Benjamini-Hochberg procedure, <0.01 were considered statistically significant.

The primary objective of this clinical study was to assess the safety of the DENV-1-LVHC viral strain intended for use in a DHIM as defined by clinical and laboratory parameters. With 9 subjects, the probability of observing at least 1 adverse event (AE) is approximately 95% if the true incidence rate is 28%. Characterizing the clinical, immunologic and virologic responses following exposure to the DENV-1-LVHC viral strain as a DHIM was the secondary objective of this clinical study and focus of this manuscript.

Role of funders

This study was funded by Johnson and Johnson, the State of New York, and the Congressionally Directed Medical Research Program. Funders were not involved in the study design, data collection, data analyses, interpretation, or writing of the manuscript.

Results

Study design and clinical characterization

Clinical and virological characteristics of participants were previously described in detail by Waickman and colleagues.[28] Briefly, all nine participants had well-detectable viral RNA in serum and experienced at least one solicited systemic adverse event, such as headache (eight of nine), myalgia (seven of nine), eye pain (six of nine), fever (seven of nine), leukopenia (six of nine), rash (five of nine), elevated liver enzymes (three of nine) and weakness/fatigue (three of nine), within 28 days after viral inoculation.

Proteome analysis on key time points

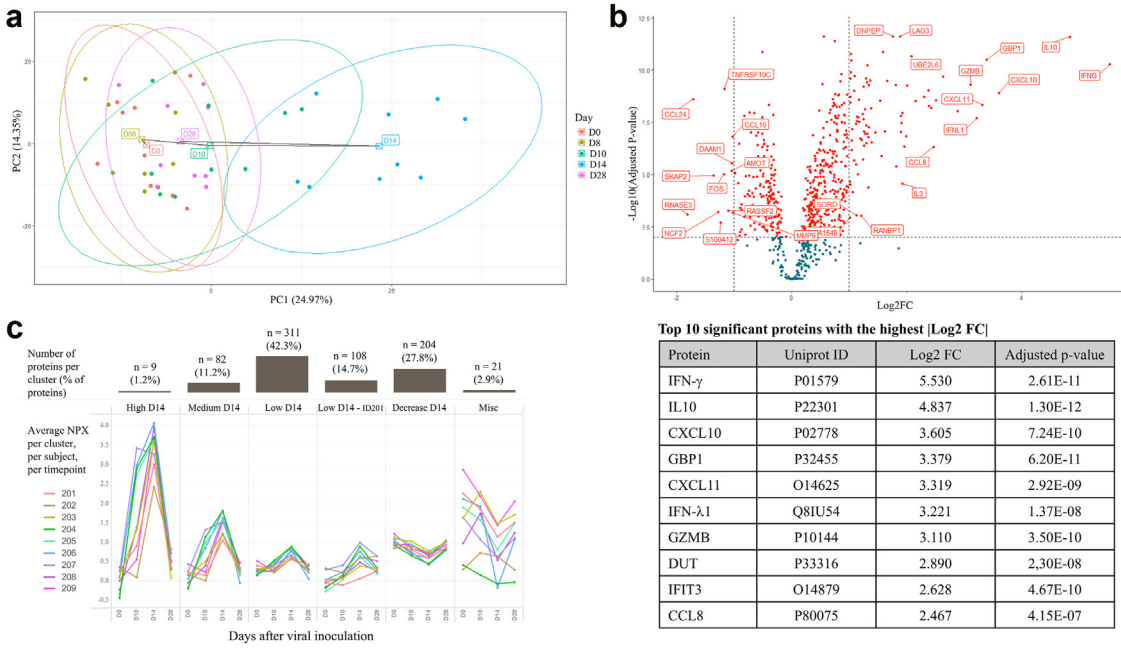
Based on the viral replication curves, key time points were selected on days 0, 8, 10, 14, and 28 for all nine study participants. These study days were selected to cover both the early innate/inflammatory response and the subsequent activation of DENV-reactive lymphocytes. Exploratory proteomics analysis was performed on the serum samples collected at these key time points using the Olink® Explore 3072 library.

Based on Olink®'s initial QC analysis, the Normalized Protein eXpression (NPX) values of 13 proteins were discarded as they did not meet the assay criteria (assay warnings), resulting in a total of 2928 proteins that were further investigated (Supplementary Table T1). Using linear mixed effects models ($\alpha = 0.01$), 736–out of 2928–serum proteins were identified of which the abundance changed significantly over the course of a primary DENV-1 infection (Supplementary Table T2). The principal component analysis of these significant proteins revealed a separation between samples collected on day 14 after viral inoculation and day 0, pointing to a distinct protein response on day 14 (Fig. 1a). Via post-hoc analyses, based on the linear mixed effects models ($\alpha = 0.01$), 3, 69, 578, and 62 proteins were identified, of which the concentration significantly differed on days 8, 10, 14, and 28, respectively, as compared to day 0 (Fig. 1b; Supplementary Tables T3–T6; Supplementary Figure S1). The top ten significant proteins with the highest absolute Log2FC from the comparison of day 0 to day 14, included interferons (IFNs), IFN-related proteins, and members of the CCL and CXCL chemokine family (Fig. 1b). Next, the significant proteins from the overall linear mixed effects models were clustered into six distinct groups via K-means clustering based on the temporal dynamics of protein expression levels (Fig. 1c; Supplementary Table T7).

We next assessed the group of nine proteins that were characterized by a high increase in their concentration on day 14 as compared to day 0, including IFN-γ, IFN-λ1, CXCL10, CXCL11, CCL8, IL10, IFIT3, GPB1, and ZBP1 (Supplementary Figure S2). All infected participants exhibited an IFN-λ1 and IFN-γ response following the peak viral load, except participant 202, who did not show an IFN-γ response (Fig. 2a–c). As both the onset and peak viral load of this participant were, on average, delayed by 4 days compared to other participants, we hypothesized that no IFN-γ response was detected for participant 202 as no samples, collected between day 14 and day 28 were included in this initial analysis.

In addition, we performed a secondary clustering on the proteins from the groups with high and medium increase in protein concentration on day 14 of the K-means clustering to select two different groups (groups 1 and 2 of Fig. 1c). In the first group, proteins were selected that showed at least doubling in their protein concentration on day 14 as compared to day 0 for all participants, thus following the IFN-λ1 kinetic profile (Fig. 2d; red), and in the second group, proteins were selected that showed at least doubling in their concentration on day 14 as compared to day 0 for all participants except 202, thus following the IFN-γ kinetic profile (Fig. 2d; purple).

Next, Ingenuity Pathway Analysis (IPA) software was used to uncover biological patterns linked to a primary DENV-1 infection. Via the Canonical Pathway Analysis, pathways linked to a DENV infection were disclosed. The cut-off values for significance were set at p-value <0.01 and absolute z-score >3. Fourteen pathways were identified to be significantly affected by a primary DENV-1 infection. Most activated pathways were related to the immune response, such as pathogen-induced cytokine storm signalling pathway, macrophage classical activation signalling pathway, and IL10 signalling (Supplementary Figure S3a). To assess which regulators



Top 10 significant proteins with the highest |Log2 FC|

Protein	Uniprot ID	Log2 FC	Adjusted p-value
IFN-γ	P01579	5.530	2.61E-11
IL10	P22301	4.837	1.30E-12
CXCL10	P02778	3.605	7.24E-10
GBP1	P32455	3.379	6.20E-11
CXCL11	O14625	3.319	2.92E-09
IFN-λ1	Q8IU54	3.221	1.37E-08
GZMB	P10144	3.110	3.50E-10
DUT	P33316	2.890	2,30E-08
IFIT3	O14879	2.628	4.67E-10
CCL8	P80075	2.467	4.15E-07

***Fig. 1:* Protein profiling in dengue virus 1 human infection disease model. a)** Principal component analysis of 736 significant proteins (α = 0.01), identified via linear mixed effects models. Different colours indicate the different sampling days: red (D0), yellow (D8), green (D10), blue (D14), purple (D28). Averages of the samples per sampling day are marked with squares and the averages of consequent sampling days are connected by a black line **b)** Volcano plot comparing the protein abundance on day 14 versus day 0 (n = 9) assessed via post-hoc analyses of linear mixed effects models. Log2 Fold Change of the normalized protein abundance (NPX) is displayed on the X-axis and the -Log10 (adjusted p-value) is displayed on the Y-axis. Significant differences are indicated in red (adjusted p-value <0.01), non-significant differences are indicated in blue (adjusted p-value ≥0.01). The top ten significant proteins with the highest log2FC are depicted in the table below. **c)** K-means clustering into six groups based on the kinetic concentration profile of 735 significant proteins, identified via linear mixed effects models, excluding RABEPK (Rab9 Effector Protein with Kelch Motifs). RABEPK was excluded from this clustering analysis, since this protein completely drove the clustering resulting in a separate group only for RABEPK, and based on the post-hoc analyses it is only significant when comparing D0 vs D28. The number of proteins in each group is shown on top.

could drive the observed DENV-elicited proteome response, IPA's Upstream Regulator Analysis was performed. Here, IL27, TLR9, TNF, IFN-α2, and IFN-γ were identified as the top five predicted regulators, pointing to a role for IFN and IFN-related proteins to drive the observed proteome response upon a primary DENV-1 infection (Supplementary Figure S3b). Lastly, via the Graphical Summary a subset of the most significant entities, identified in the analysis, was highlighted by IPA and linked to generate an analysis summary. Herein, IFNs (IFN-γ, IFN-λ1, and IFN-α2), TNF, and IL1RN were included and linked to the inhibition of virus and of the replication of *Flaviviridae*, pointing to an important role for these proteins in the DENV-elicited proteome response (Supplementary Figure S3c).

In-depth analysis of selected proteins on multiple time points

To get more insights into what happens between day 14 and day 28 and to gain a more detailed quantitative kinetic view of the DENV-elicited proteome response, a smaller, targeted Olink® Flex panel of 21 proteins was designed to evaluate protein concentrations at multiple time points throughout the infection, based on the initial Olink® Explore output. At least 11 time points were evaluated per participant between day 0 and day 25 after DENV inoculation. Note that the proteins, available in Olink® Flex, are limited to a list of 197 pre-validated protein biomarkers. Hence, only a subset of the identified proteins of interest from the initial Olink® Explore analysis could be selected. E.g. only five of the nine proteins with a high increase in protein concentration on day 14 could be selected (Fig. 1c). The complete Olink® Flex panel consisted of five proteins from the K-means clustering analysis with the high protein concentrations on day 14, being IFN-λ1, IFN-γ, CXCL10, CXCL11, and CCL8. Four proteins from the second clustering were selected that follow the IFN-λ1 or IFN-γ kinetic profiles: IL12, LAG3, IL10, and CXCL9. From the overall linear mixed effects models, four proteins were included being CDCP1 (adjusted p-value: 3.19×10^{-9}), GZMB (adjusted p-value: 3.47×10^{-9}), CXCL5 (adjusted p-value: 1.98×10^{-8}), and IL18

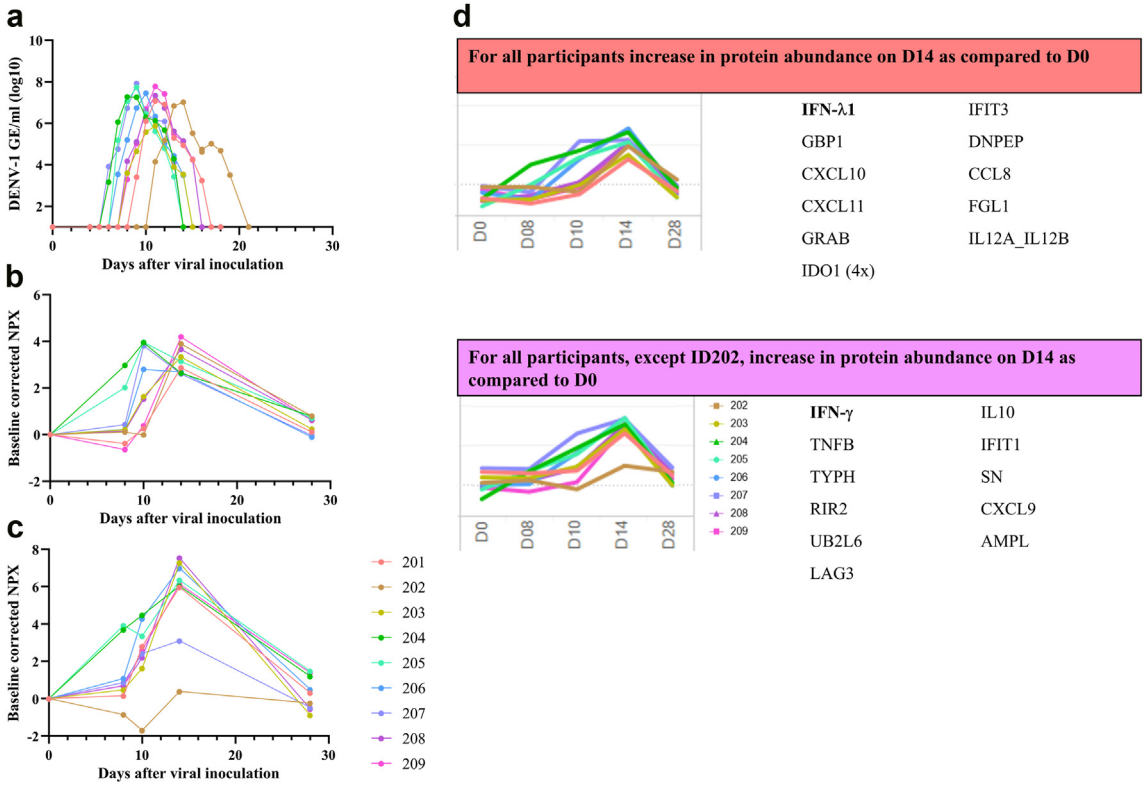


Fig. 2: **a)** Kinetics of DENV-1 RNA content in serum of nine participants, **b and c)** IFN responses of nine participants **b)** IFN-λ1, **c)** IFN-γ) expressed in baseline corrected NPX values, at key time points using Olink® Explore **d)** Clustering of proteins based on their profile in participant 202. The first group (red) includes proteins with a doubling in their concentration on day 14 as compared to day 0 for all participants, following the IFN-λ1 profile. In the second group (purple), proteins were selected that showed a doubling in their concentration on day 14 as compared to day 0 for all participants except participant 202, following the IFN-γ profile.

(adjusted p-value: 2.40×10^{-8}). Note that CXCL5 was included as a representative for the proteins that showed a reduced serum concentration at day 14 (Fig. 1c). Four proteins were selected from the post-hoc analyses of the linear mixed effects models: two proteins from the comparison between day 0 and 10, IL1RN (adjusted p-value: 8.13×10^{-3}) and IL15 (adjusted p-value: 1.80×10^{-3}), and two proteins from the comparison between day 0 and 14, CCL19 (adjusted p-value: 2.58×10^{-8}) and LAMP3 (adjusted p-value: 1.22×10^{-8}). In addition, one protein known to be fever-related, being TNF-α, and one negative control, MMP7 were included. Lastly, IFN-α2 and IFN-β1 were included (Supplementary Tables T8 and T9). These proteins were not part of the initial Olink® Explore panel, but to gain a comprehensive overview of the IFN responses in a primary DENV-1 infection, IFN-α2 and IFN-β1 were included in the Olink® Flex panel.

By evaluating protein concentrations at multiple time points throughout the infection, an IFN-γ response for participant 202 was observed, peaking 5 days after the peak viral load, which is comparable with what was observed for other participants (Supplementary Figure S4). Why the peak viral load of participant 202 was delayed, remains unclear. As the detected concentrations of IL12 were below the LLOQ value, we excluded IL12 from further analyses. All individual protein responses can be found in the Supplementary Information (Supplementary Figure S4). The onset of IFN responses varied highly among participants. The earliest time point for induction above LLOQ after viral inoculation was day 8 for IFN-α2, IFN-λ1, and IFN-γ, and day 9 for IFN-β1 (Supplementary Figure S4). The timing of the peak protein concentration differs largely between the 21 selected proteins, ranging from 1 to 7 days after peak viral load. The first protein peaks observed were IL1RN, IFN-α2, and IFN-β1. The IFN-λ1 response also follows shortly after the peak viral load. This type I/III IFN response is accompanied by an increase of chemokines, i.e. CXCL10, CCL8, CCL19, CXCL11, that induce the directional migration of immune cells, e.g. leukocytes. Then, a sharp IFN-γ response is observed which is followed by peaks of other cytokines/chemokines, such as CXCL9, IL10, IL18, and TNFα (Fig. 3).

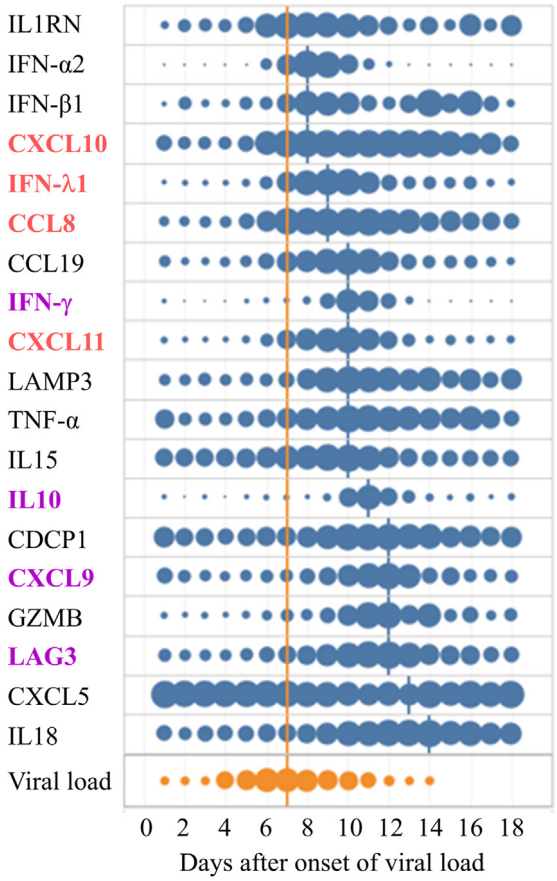


Fig. 3: Kinetic overview of the average viraemia (orange) and average protein concentrations of the Olink® Flex panel (blue) for all nine participants. Peaks are indicated by vertical lines. The timing of a participant's viraemia and protein concentrations were corrected to that participant's onset of viral replication before averages were calculated. The size of the dots gives an indication of the magnitude of the relative viraemia and protein concentration. Protein concentrations above the upper limit of quantification (ULOQ) were replaced by the protein's ULOQ value. IL12 is not included in this graph as the observed protein concentrations were below the lower limit of quantification (LLOQ) (Supplementary Figure S4). Colouring of the protein names reflect the clustering of proteins based on their profile in participant 202. Proteins with a doubling in their concentration on day 14 as compared to day 0 for all participants, following the IFN-λ1 profile, are marked in red. Proteins that showed a doubling in their concentration on day 14 as compared to day 0 for all participants except participant 202, following the IFN-γ profile, are marked in purple.

Protein responses correlating with clinical outcomes and covariates

Lastly, protein responses that correlate with clinical outcomes, being fever and leukopenia, and covariates, being age and sex, were identified. Using linear mixed effects models including interaction effects ($\alpha = 0.01$), 3, 3, 9, and 1 proteins were identified that differentiate participants on day 14 with a log2FC > |2|, comparing fever vs no fever, leukopenia vs no leukopenia, male vs female sex and age, respectively (Supplementary Tables T10–T13). Nevertheless, no clear differentiation between participants with different clinical outcomes, could be made (Supplementary Figure S5), only baseline MICB_MICA levels were observed to be clearly associated to dengue induced leukopenia (Supplementary Figure S5f). Baseline levels of 5 out of 9 proteins clearly differentiated male vs female sex (SPINT3, KLK3, INSL3, ACRV1, and EDDM3B), but their responses were unrelated to the effect of a primary DENV-1 infection (Supplementary Figure S5). Note that PSMC3 was identified as differentiator of development of leukopenia, female vs male sex, and age, thus interpreting these results must be done with caution taking the limited sample size and multicollinearity into account (Supplementary Figure S5d, m, p).

Discussion

In this study, the serum proteome dynamics upon an experimentally induced, primary DENV-1 infection were investigated in nine flavivirus-naïve individuals. In contrast to most studies that only investigated a limited set of proteins via immunoassays,[32–34] here, 2941 proteins were analysed simultaneously in serum using the Olink® technology. The abundance of 736 proteins changed significantly over the course of a primary DENV-1 infection with a distinct protein response on day 14 after viral inoculation. Immune response-related proteins comprise most of these significant proteins, including IFNs (IFN-λ1 and IFN-γ), IFN-related proteins (e.g. GBP1 and IFIT3), and members of the CCL (e.g. CCL8) and CXCL (e.g. CXCL10 and CXCL11) chemokine family. Based on these results, more detailed kinetic profiles were obtained for 21 proteins, such as IFN-α2, IFN-β1, IFN-λ1, and IFN-γ.

Waickman and colleagues also studied the whole blood transcriptome in identical samples of the DENV-1 Live Virus Human Challenge open-label study investigated here, thereby observing a transcriptional shift away from the pre-infection profile on day 10 and 14 after viral inoculation.[28] Most of the differentially expressed gene products corresponded to pathways associated with acute/innate immune responses (day 10), lymphocyte activation (day 14), and the induction of the adaptive immune response (day 14).[28] More specifically, the differential expression of IFN-stimulated and antiviral gene products was observed from day 10 after viral inoculation onward (including GBP1 and IFIT3).[28] In our study, GBP1 and IFIT3 were among the 9 proteins that exhibited a high increase in their concentration on day 14 (Supplementary Figure S2), confirming the observations of Waickman and colleagues. Notably, we observed the increased GBP1 and IFIT3 concentration at a later time point as we evaluated the proteomic response upon a DENV-1 infection, whereas Waickman et al. evaluated the transcriptional response. This delay

in increased protein abundance compared to gene expression was also observed for many other proteins (GBP1, IFIT3, IL1RN, LAMP3, and ZBP1). Whereas gene expression might be more straight forward to assess, it is the gene product that confers its function. Thereby making proteome analysis as relevant. Using a DENV-2 human infection model, Hanley and colleagues evaluated the host immunotranscriptome response upon primary infection with a partially attenuated rDEN2Δ30 virus. Here, also pathways enriched in the type I/II IFN and antiviral responses were upregulated during viraemia as compared to baseline,[35] which corroborates the findings of Waickman et al.[28] Also in this study, GBP1 was identified as a viraemia-tracking gene.[35]

IFNs are potent antiviral cytokines secreted in response to viral infections that regulate both the innate and adaptive immune responses.[36] Upon viral infection, they activate JAK/STAT signalling pathways,[37] leading to the expression of numerous IFN-stimulated genes that block viral replication.[38,39] The interplay between IFNs and DENV is complex, as DENV can evade the innate immune response by blocking the production and signalling of type I IFNs (α/β).[38,40,41] Nevertheless, induction of all evaluated IFNs, being IFN-α2, IFN-β1, IFN-λ1 and IFN-γ, was observed upon a primary DENV-1 infection in all nine participants. Here, the type I IFN response (α/β) was first observed, shortly followed by the type III IFN response (IFN-λ1). These responses preceded a sharp type II response (IFN-γ). Although type I IFN responses were described as more rapid and potent than type III IFN responses,[42,43] we observed a minimal difference in timing upon a primary DENV-1 infection. The observed increase in IFN-α2 levels was higher than for IFN-λ1 levels in most participants, but, in turn, the increase in IFN-λ1 levels was higher than the increase in IFN-β1. The duration of type I and III IFN responses was found to be similar in this study. Note that the responses of IFN-λ2 and IFN-ω1 were also evaluated, but no statistically significant effect upon a primary DENV-1 infection was observed for both proteins (Supplementary Table T2). The type II IFN response was observed later and was more pronounced for some participants. In general, the intensity of IFN responses differed among participants and between different types of IFN responses (Supplementary Figure S4a–d). The difference in timing of the measured protein responses suggests that the induction of the initial chemokines, CXCL10, CCL8, CCL19, and CXCL11 might be driven by type I/III IFN response rather than by the type II IFN response. This also corresponds with the clustering of proteins based on their profile in participant 202. Herein, the kinetic profiles of CXCL10, CXCL11, and CCL8 coincide with the IFN-λ1 response (Fig. 2d; red). In contrast, the kinetic profiles of IL10, CXCL9, and LAG3 were similar to the IFN-γ response (Fig. 2d; purple). The protein peaks of IL10, CXCL9, and LAG3 occurred noticeably later as compared to the protein peaks of the aforementioned chemokines, and thus, might be driven by the type II IFN response, rather than by the type I/III IFN responses.

Type I and III IFNs are known to trigger similar antiviral transcriptional responses, albeit via distinct heterodimeric receptors.[42,43] As type III IFN receptors are mainly expressed on epithelial cells, type III IFNs potentially function as a frontline defence mechanism, combatting viral infections at epithelial barriers.[42] As their responses are less potent than type I IFN responses, type III IFN responses result in less damaging inflammatory responses and, thus, less collateral damage.[44] This might point to an interesting role for type III IFN responses in severe dengue, a complication of a dengue infection postulated to be due to over-exaggerated host immune responses.[42,45] In addition, a variant of IFN-λ1 (rs7247086) was found to offer protection against dengue haemorrhagic fever, pointing to an important role of IFN-λ1 in dengue pathogenesis.[46] Nevertheless, an increased expression of the more potent type I IFN (α/β) response during the acute phase of dengue infection was linked with less severe outcomes and with increased platelet counts—low platelet counts being a hallmark of severe dengue—pointing to the beneficial effects of a type I IFN response (α/β).[19] Higher IFN-α and IFN-γ responses were also observed upon a primary and secondary DENV infection, respectively.[47] In addition, IFN-γ responses correlated positively with dengue disease severity.[32,33,48] The exact role of the IFN balance in dengue immunopathogenesis remains unclear. Further research is needed to fully elucidate its impact on the outcome of DENV infection, especially the role this IFN balance plays in the development of severe disease. It will therefore be interesting to assess these protein dynamics in patients with dengue with different disease severity. In addition, as accumulating evidence suggests that anti-IFN antibodies modulate various flaviviral diseases, examining the presence of anti-IFN autoantibodies upon a DENV infection would also be highly interesting.[49–52]

Similar to a DENV infection, also SARS-CoV-2 infection might lead to severe disease in a small subset of patients where the immune response gets out of control.[53] It was observed that IFNs can also play a protective role upon a SARS-CoV-2 infection. In critical patients with COVID-19, early IFN-α2b treatment was shown to be associated with reduced mortality, whereas late administration of IFN-α2b was associated with increased mortality and delayed recovery,[54] pointing to a critical role in timing of IFN administration. In addition, recent studies suggested that IFN-β1a may not benefit patients with COVID-19 and could potentially harm those with severe disease requiring advanced respiratory support.[55,56] In contrast, participants who received a single dose of pegylated IFN-λ1 had a significantly lower incidence of hospitalization.[57] Also here the exact role of the IFN balance remains unclear.

In this study, the IFN-inducible proteins GBP1 and GBP2 were significantly increased on day 14 after viral inoculation. Notably, GBP1 was one of the 9 proteins that exhibited a high increase in their concentration on day 14 (Supplementary Figure S2d). Similarly, when comparing acute to convalescent samples from patients with dengue, Garishah and colleagues observed an upregulation of GBP2 using Olink®'s Inflammation panel.[58] As GBP1 is not included in Olink®'s Inflammation panel, it was not studied by Garishah and colleagues. Recently, a decreased expression of both GBP1 and GBP2 was observed during the critical phase in patients that progressed to severe dengue, and both GPB1 and GBP2's expression was shown to be inversely correlated with plasma leakage in severe dengue, pointing to a potential role for GBPs in dengue disease progression.[59] Both guanylate-binding proteins are also upregulated during a ZIKA virus infection.[60]

Furthermore, IFIT3, IL10, TNF-α, and granzyme B showed a significant increase in protein concentration on day 14 after viral inoculation. The IFN-inducible protein IFIT3 is known to play a protective role following the IFN-α response in a DENV infection in human lung epithelial cells.[61] In patients with severe dengue, IL10 was previously reported to be significantly increased,[62] although insufficient to discriminate between severe and non-severe dengue.[63] In addition, the presence IL10, IFN-γ, and TNF producing T cells was associated with mild dengue.[64] Elevated TNF-α levels were associated with dengue disease severity.[65,66] In addition, the TNF-α-308A allele was identified to play a potential role in disease progression.[67] Upon DENV infection, the caspase-like serine protease, granzyme B, is released by cytotoxic lymphocytes to kill virus-infected cells.[68,69]

The immune checkpoint receptor LAG3 was found to have a kinetic protein profile that coincided with the IFN-γ response when evaluating the clustering of proteins based on their profile in participant 202. LAG3 is present on the cell surface of cytotoxic and regulatory T cells.[70,71] As it is highly upregulated on exhausted T cells, it is considered a marker for T cell exhaustion.[72,73] Upon a dengue infection, a subset of CD8+ T cells expressed granzyme B and LAG3.[74] A LAG3 response was also observed upon infection with the rDEN2Δ30 challenge virus after viraemia,[35] pointing to a potential role for LAG3 in the immunopathogenesis of a DENV infection.

In the study presented here, the DENV-elicited proteome response upon a primary dengue infection was evaluated. Symptomatic and severe dengue has been observed to be significantly associated with secondary heterotypic infections spaced by more than 18 months between first and second infection.[75] In contrast, a recent study demonstrated that disease severity was not linked with secondary infections, as primary dengue infections comprised over half of all clinical cases, severe dengue cases, and deaths,[26] pointing to the relevance of studying primary dengue infections.

The nine participants in this study are representative for the antiviral immune responses upon a primary DENV-1 infections. Nevertheless, our population is limited in size, as the primary objective of this clinical trial was to assess the safety of the DENV-1-LVHC viral strain intended for use in a DHIM, and an attenuated DENV-1 was used. Characterizing the clinical, immunologic and virologic responses following exposure to the DENV-1-LVHC viral strain as a DHIM was the secondary objective of this clinical trial and the focus of this manuscript. Neither age, sex, nor fever, could be identified to clearly affect the proteome dynamics upon a primary DENV-1 infection. Only baseline MICB_-MICA levels were observed to be associated to dengue induced leukopenia. Interestingly, leukopenia is described as a potential predictive marker for progression to severe dengue during emergency department admission[76] and genome-wide association studies identified the MICB variant rs3132468 to be associated with dengue shock syndrome,[77–79] potentially pointing to insights in dengue pathogenesis. In addition, increased expression of MICB_MICA on cells is known to result in increased susceptibility to cytotoxicity from NK and CD8+ T cell activities.[80] Nevertheless, the small sample size might have limited the possibility to identify correlation effects, and multicollinearity must be considered when interpreting these results. Likewise, the diversity of this cohort is limited (Table 1) and little difference in pathology was observed. Including earlier time points between day 0 and day 7 would be of interest to evaluate the dynamics of the early innate immune response, although immune responses would be more localized in this case. Thus, it is important to emphasize

	All participants (n = 9)
Age (years)	
Mean (SD)	34.6 (8.8)
Median	33.0
Min, Max	20, 45
Sex, n (%)	
Male	3 (33.3)
Female	6 (66.7)
Ethnicity, n (%)	
Hispanic or Latino	3 (33.3)
Non-Hispanic or Latino	6 (66.7)
Race, n (%)	
White	7 (77.8)
Black or African American	1 (11.1)
American Indian or Alaska Native	0 (0.0)
Asian	0 (0.0)
Native Hawaiian or Other Pacific Islander	0 (0.0)
Other or Multiple	1 (11.1)

Table 1: **Study participants demographics and characteristics (Adapted from Waickman et al., 2022, with permission from AAAS).**

that the findings of this study provide starting points for further investigation to explore the observed protein dynamics in larger populations, during mild and severe natural infections and in secondary infections.

Moreover, as observed in this study, the importance of sample collection timing must be noted. In a controlled DHIM, differences in timing of protein responses were observed upon a primary dengue infection, as one participant showed an IFN-γ response that was delayed as compared to the other participants, concurrent with a delayed viral load. Based on sample collection timing certain responses might be missed in field or clinical studies, which is critically important when interpreting dengue studies.

In conclusion, our analyses provide detailed insights into the temporal dynamics of protein responses and the complex immunomechanisms and -pathways upon a primary DENV-1 infection. In the future, it will be of interest to utilize these insights as starting points to further explore protein dynamics in mild and severe natural dengue infections with different DENV serotypes. These proteins could give insights into the complex immunopathogenesis of dengue. Identifying biomarkers that can predict disease progression may enable healthcare providers to prioritize treatment for patients having a higher risk of developing disease complications, potentially reducing morbidity and mortality associated with dengue.

Contributors

LVW, ATW, FR, GHT, SJT, and MVL designed the clinical study. CS, KVDH, and OL designed, analysed, and interpreted the proteomics study. CS prepared the draft manuscript and coordinated its finalization. OL is responsible for the decision to submit the manuscript. CS, KVDH, LVW, ATW, FR, GHT, SJT, MVL, and OL read and approved the final version of the manuscript and had full access to and verified the underlying study data.

Data sharing statement

Clinical participant data is available in the manuscript of Waickman and colleagues.[28] Raw data to perform the proteome analyses are available in supplementary information to all readers.

Declaration of interests

CS, LVW, FR, GHT, MVL, and OL are current employees of Johnson and Johnson. LVW, FR, GHT, MVL, and OL own stock or stock options in that company. KVDH provided consultancy to Johnson and Johnson. In the context of dengue research, the State University of New York Upstate Global Health Institute received grants and/or contracts from Merck, the National Institutes of Health, Island Pharmaceuticals, Johnson and Johnson, and the US Department of Defence. ATW and SJT provided consultancy to Takeda Pharmaceuticals and Merck. ATW is inventor of the patent "IgA monoclonal antibodies for treating flavivirus infections" and co-founder and CEO of Azimuth Biologics. SJT participated on advisory boards for Takeda Pharmaceuticals, Merck, and Island Pharmaceuticals. SJT owns stock or stock options from Island Pharmaceuticals.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2025.105728.

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