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Development of Assays on the Meso Scale Diagnostics (MSD) Platform to Assess VEEV Infection in a Non-human Primate (NHP) Vaccination Model

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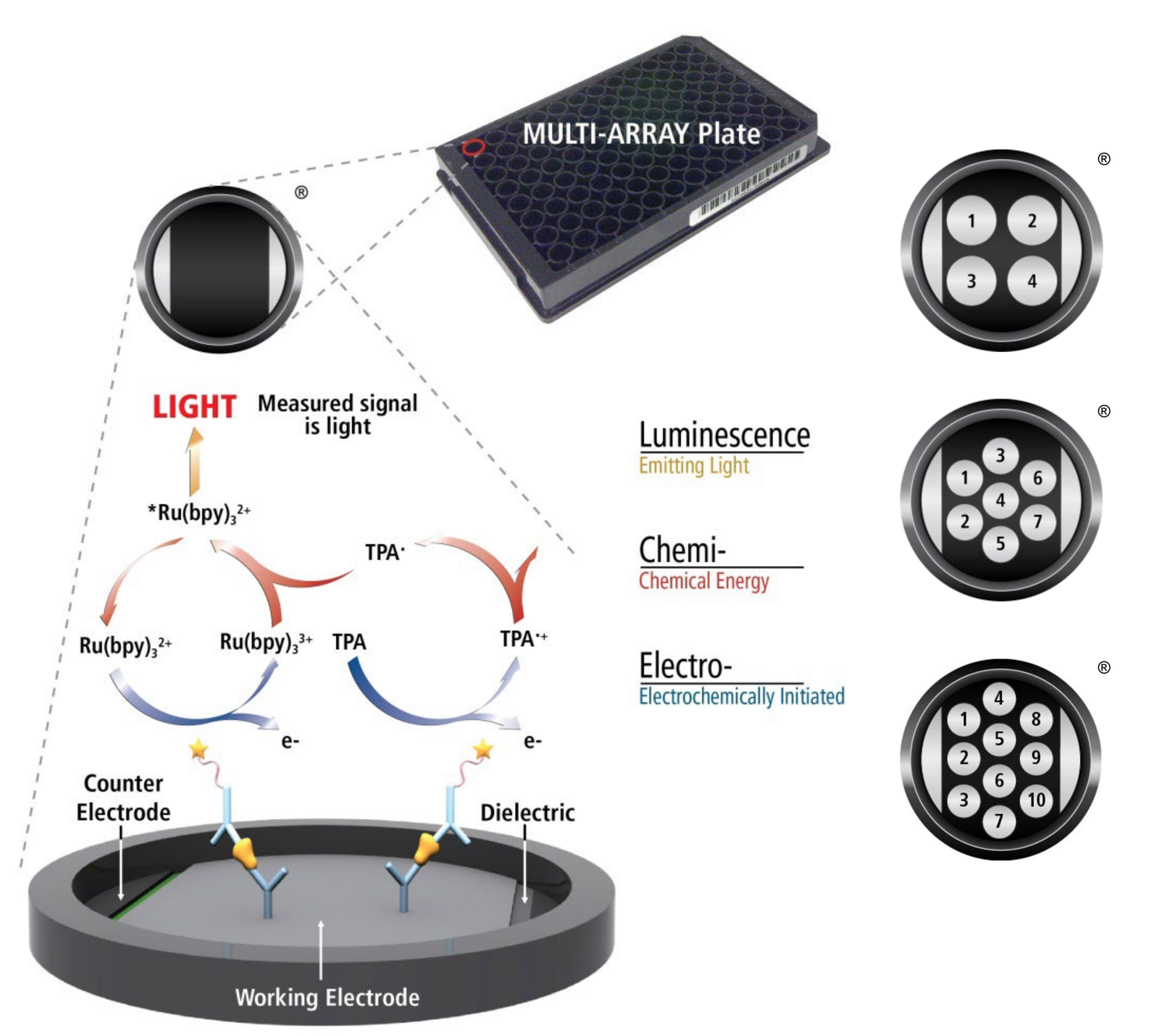
1 Abstract

The mosquito-borne alphavirus Venezuelan equine encephalitis virus (VEEV) is endemic to South, Central, and North America. VEEV outbreaks in both human and equine populations have been recorded since the 1920s. Due to its high replicative capacity in vitro, its ability to be transmitted through aerosols, and the lack of effective treatments or vaccines, VEEV is considered a biological threat agent. Work to address this threat through the development of new vaccines requires new assay tools to support VEEV vaccine research and animal studies. We identified and developed reliable, non-invasive biomarker assays relevant to different stages (lymphatic, viremic, and encephalitic) of VEEV infection. Sixteen host biomarker assays and several VEEV protein assays were used to screen serum and plasma samples collected from non-vaccinated and vaccinated cynomolgus macaques exposed to aerosolized VEEV. The circulating levels of IFN-γ, IL-6, MCP-1, and IP-10 in serum and plasma increased up to 40-fold during the lymphatic stage of infection compared to the pre-vaccination and pre-challenge baseline levels. Viremia was associated with detectable levels of the VEEV E2 envelope glycoprotein only in non-vaccinated NHPs. Neurofilament L is the strongest indicator of the encephalitic stage of infection, with up to a 30-fold increase in circulating levels compared to baseline. Selected assays are currently being assessed in real-time, point-of-care testing of vaccinated and unvaccinated NHPs following viral challenge. Additional screening of archived NHP samples with new or improved biomarker assays will be carried out including a higher sensitivity assay for VEEV E2. Similar approaches could be used for developing biomarker panels for other viral infections including, but not limited to, filoviruses, flaviviruses and other alphaviruses.

2 Methods

MSD's electrochemiluminescent detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.



Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analytes yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- The carbon electrode surface has a 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

3 Biomarkers Assay Development & Typical Performance

MSD developed 18 assays against host biomarkers and an assay against the VEEV E2-ectodomain protein. Inflammatory cytokine assays such as for IFNγ, IL-6, IP-10, MCP-1, TNFα, RANTES, MIP-1α, IP-10, IL-1α, IL-1β, MMP-9, and PDGFR-β were developed to follow the lymphatic stage of infection. Brain injury marker (S100B, GFAP, UCH-L1, NfL, ICAM-1, and Tau) assays may be used to assess the encephalitic stage of VEEV infection. Assays against the VEEV E2-ectodomain protein are designed to detect the viremia stage of VEEV infection.

The assays were combined into five multiplexed panels (Table 1) and formulated as kits containing 96-well MULTI-SPOT 10-spot plates, calibrators, controls, detection antibody solutions, diluents, and read buffer. The assays were run following the 2-step protocol shown below and used to test against NHP serum, plasma, and CSF samples collected during vaccination and infection studies.

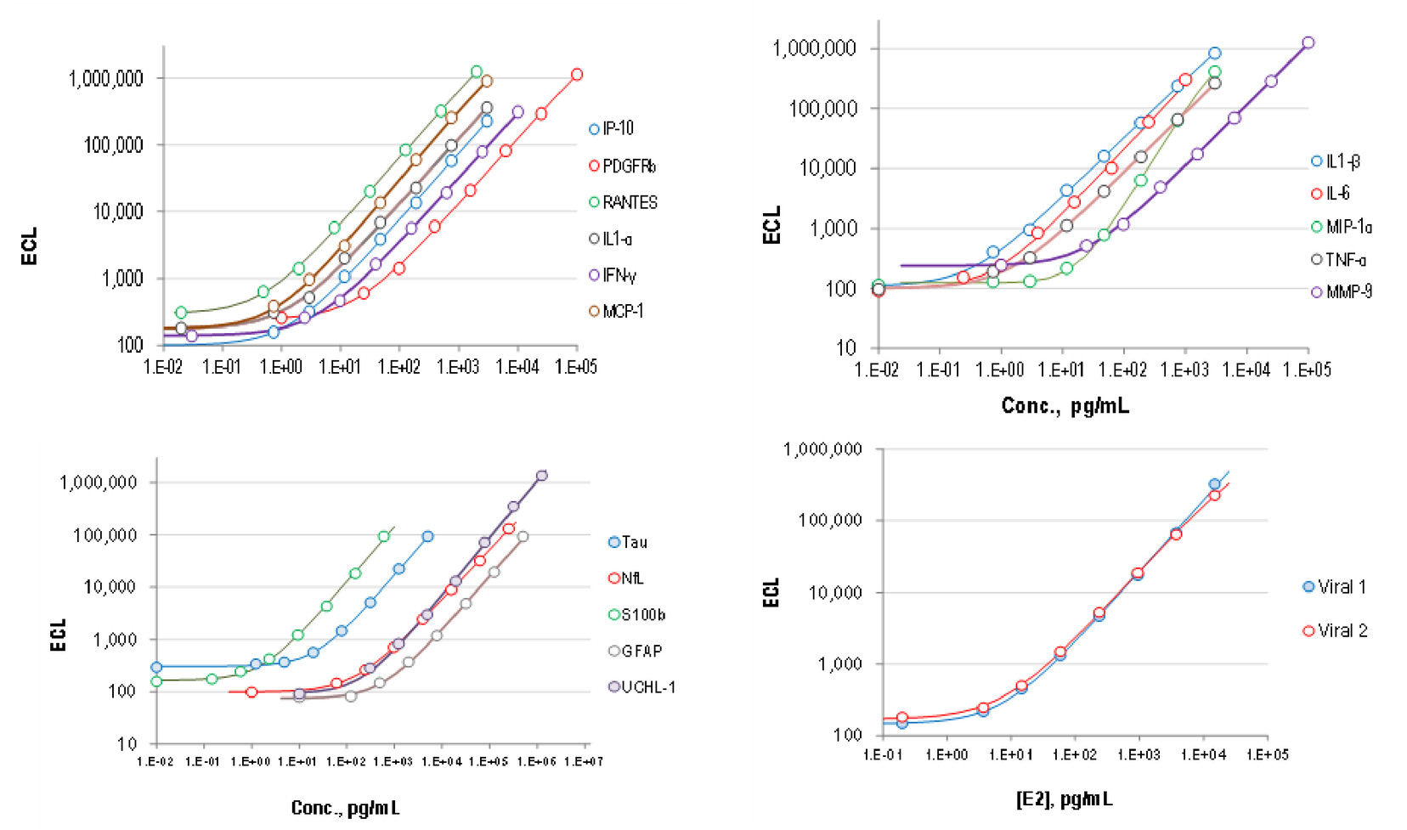


Figure 1. Typical calibration curves for MSD® host biomarker and viral assays tested in this study.

Protocol at Glance

1. Add calibrator, control, or sample (50 µL/per well). Incubate 1 hour at room temperature (RT).
2. Wash and add detection antibody solution (50 µL per well). Incubate 1 hour at RT.
3. Wash and add read buffer (150 µL per well). Analyze with MSD® instrument

#	Assay Panel	Biomarker Name	Typical LLOD, pg/mL	Normal Range Serum (n=29)	Normal Range Plasma (n=30)	Normal Range CSF (n=12)
1	Panel 1	IFN-γ	1.8	ND - 6.2 pg/mL	ND - 6.4 pg/mL	ND - 41 pg/mL
2		IL-1α	0.48	ND - 0.6 pg/mL	ND - 0.6 pg/mL	ND - 1.3 pg/mL
3		IL-1β	0.21	ND - 0.5 pg/mL	ND - 0.5 pg/mL	ND - 2.1 pg/mL
4		IL-6	0.42	0.3 - 2.6 pg/mL	ND - 5.1 pg/mL	ND - 5.7 pg/mL
5		IP-10	0.88	82 - 826 pg/mL	177 - 733 pg/mL	156 - 11,000 pg/mL
6		MCP-1	0.32	59 - 359 pg/mL	44 - 248 pg/mL	51 - 1075 pg/mL
7		MIP-1α	9.1	7 - 38 pg/mL	5.3 - 24 pg/mL	ND - 222 pg/mL
8		TNF-α	0.76	ND - 1 pg/mL	ND - 1 pg/mL	ND - 17 pg/mL
9		Tau	7.5	ND - 26 pg/mL	ND - 72 pg/mL	5.4 - 97 pg/mL
10	Panel 2	RANTES	0.11	1 - 98 ng/mL	0.2 - 21 ng/mL	ND - 63 ng/mL
11		PDFGRβ	5.8	4 - 58 ng/mL	ND - 70 ng/mL	ND - 11 ng/mL
12		MMP-9	8.6	33 - 547 ng/mL	ND - 135 ng/mL	ND - 265 ng/mL
13	Panel 3	NFL	107	0.4 - 100 ng/mL	ND - 80 ng/mL	3 - 576 ng/mL
14		ICAM-1	3563	7 - 24 ng/mL	ND - 27 ng/mL	ND - 1.2 ng/mL
15		S100b	0.55	0.01 - 2 ng/mL	ND - 0.13 ng/mL	0.4 - 104 ng/mL
16	Panel 4	GFAP	517	ND - 1 ng/mL	ND - 1 ng/mL	0.6 - 944 ng/mL
17		UCHL-1	137	ND - 2 ng/mL	ND - 0.6 ng/mL	0.8 - 163 ng/mL
18	Panel 5	Viral Assay 1	3.2	ND	ND	ND
19		Viral Assay 2	2.7	ND	ND	ND

Table 1. Concentration ranges of biomarkers measured in non-vaccinated and non-infected animals. Normal ranges are based on samples collected from 3 vaccinated animals prior to vaccinations and from 3 non-vaccinated animals prior to viral challenge. Non-detectable values are marked as ND.

4 Vaccination and Infection Study: Animal Handling & Sample Collection

All animal handling procedures were conducted in accordance with the UTMB Institutional Animal Care and Use Committee-approved protocols. Six cynomolgus macaques (Macaca fascicularis) (NHPs) weighing between 2.9-3.4 kg were screened by hemagglutination testing to be free of prior infection with alphaviruses (i.e., Venezuelan and eastern equine encephalitis, Sindbis, Semliki Forest, and chikungunya viruses) as well as simian immunodeficiency virus, simian type D retrovirus and simian T-lymphotropic virus. The NHPs were housed individually in open metal caging, allowing visual contact with other NHP in the room. A standard primate chow and fresh fruits and vegetables were provided daily. Following restraint via the cage-squeeze mechanism, an intramuscular injection of ketamine hydrochloride for anesthetization was given prior to collecting blood samples, vaccination, or challenge.

Vaccination: On Day 0, three out of six animals were vaccinated subcutaneously in the right leg with approximately 0.5 mL of VEEV-IRES vaccine at a titer of approximately 4.0x10^4 plaque-forming units (PFU) per mL. No signs of disease or distress were noted following vaccination.

Infection: On Day 42 after vaccination, the animals were challenged by the aerosol administration of approximately 10^5 PFU, or 100 infectious dose 50% (ID50) units of cDNA clone-derived VEEV epizootic/epidemic subtype IAB strain Trinidad donkey (TrD). A head-only, 16-liter dynamic inhalation aerosol exposure using the Biaera Aero3G delivery system was used to challenge each NHP. Collison nebulizer and all-glass impinger samples were used to determine actual infectious doses received by each NHP. For unknown reasons, animals received infectious doses of 3.5 x 10^4 to 9.1 x 10^4 PFU, which was smaller than the expected 10^5 to 10^6 PFU dose.

Sample Collection: Blood samples were collected on Day 0 prior to vaccination and on Days 1, 2, 3, 4, 7, and 28 post-vaccination. On the day of viral challenge (Day 42) blood was collected prior to the challenge and also on Days 43, 45, 46, 49, and 62. Blood samples were taken by venipuncture of the inguinal vein. Serum and plasma were separated, transferred to cryovials, and maintained at -80 °C for later analyses. CSF was also collected via lumbar puncture from six animals (3 vaccinated, 3 sham-vaccinated) on Days 7 and 28 after vaccination as well as Days 42, 49, and 62 (Days 1, 7, and 20 after challenge).

5 Biomarker Responses to Vaccination

Vaccinated NHP — Assay Name / Animal ID

DAY	IFN-γ B04077	IFN-γ B04302	IFN-γ B04350	IL-6 B04077	IL-6 B04302	IL-6 B04350	IP-10 B04077	IP-10 B04302	IP-10 B04350	MCP-1 B04077	MCP-1 B04302	MCP-1 B04350	MIP-1α B04077	MIP-1α B04302	MIP-1α B04350	Tau B04077	Tau B04302	Tau B04350	TNF-α B04077	TNF-α B04302	TNF-α B04350	IL1-α B04077	IL1-α B04302	IL1-α B04350	IL1-β B04077	IL1-β B04302	IL1-β B04350	MMP-9 B04077	MMP-9 B04302	MMP-9 B04350	PDGFRb B04077	PDGFRb B04302	PDGFRb B04350	RANTES B04077	RANTES B04302	RANTES B04350	ICAM-1 B04077	ICAM-1 B04302	ICAM-1 B04350	NFL B04077	NFL B04302	NFL B04350	S100b B04077	S100b B04302	S100b B04350	UCHL1 B04077	UCHL1 B04302	UCHL1 B04350
0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	13	12	4	5	5	13	27	23	44	9	11	10	3	3	3	6	2	2	3	4	7	3	5	9	5	8	8	0	1	0	0	1	0	0	22	0	1	1	1	1	1	1	0	1	1	0	0	1
2	4	3	1	2	1	3	6	8	7	2	2	3	1	1	2	1	0	1	1	2	3	2	2	3	1	2	3	0	0	0	1	1	1	2	2	0	1	1	1	1	1	1	0	5	1	0	0	0
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14	0	0	0	0	0	1	0	0	0	1	1	1	2	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	3	3	1	5	32	6	1	1	1	1	0	0	0	2	0	1	2	1

Non Vaccinated NHP — Assay Name / Animal ID

DAY	IFN-γ B04140	IFN-γ B04165	IFN-γ B04352	IL-6 B04140	IL-6 B04165	IL-6 B04352	IP-10 B04140	IP-10 B04165	IP-10 B04352	MCP-1 B04140	MCP-1 B04165	MCP-1 B04352	MIP-1α B04140	MIP-1α B04165	MIP-1α B04352	Tau B04140	Tau B04165	Tau B04352	TNF-α B04140	TNF-α B04165	TNF-α B04352	IL1-α B04140	IL1-α B04165	IL1-α B04352	IL1-β B04140	IL1-β B04165	IL1-β B04352	MMP-9 B04140	MMP-9 B04165	MMP-9 B04352	PDGFRb B04140	PDGFRb B04165	PDGFRb B04352	RANTES B04140	RANTES B04165	RANTES B04352	ICAM-1 B04140	ICAM-1 B04165	ICAM-1 B04352	NFL B04140	NFL B04165	NFL B04352	S100b B04140	S100b B04165	S100b B04352	UCHL1 B04140	UCHL1 B04165	UCHL1 B04352
0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
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14	1	0	2	1	0	1	1	0	1	1	0	1	1	0	1	0	0	0	0	0	1	1	0	1	1	0	1	0	0	0	1	0	0	2	0	0	1	1	1	2	2	0	1	0	0	0	1	0

Scale
<2
2 - 5
5 - 10
>10

Table 2. Changes in circulating levels of biomarkers in serum during the post-vaccination period in vaccinated (top table) and non-vaccinated (bottom table) animals. Concentration values for each animal were normalized to pre-vaccination measurements taken on the day of vaccination (Day 0). Data from matching plasma samples (not shown) exhibited similar trends. Significant increases in circulating levels of IFNγ, IL-6, MCP-1, and IP-10 were observed in all animals after vaccination

6 Biomarker Response to Infection

Vaccinated NHP — Assay Name / Animal ID

Day	IFN-γ B04077	IFN-γ B04302	IFN-γ B04350	IL-6 B04077	IL-6 B04302	IL-6 B04350	IP-10 B04077	IP-10 B04302	IP-10 B04350	MCP-1 B04077	MCP-1 B04302	MCP-1 B04350	MIP-1α B04077	MIP-1α B04302	MIP-1α B04350	Tau B04077	Tau B04302	Tau B04350	TNF-α B04077	TNF-α B04302	TNF-α B04350	IL1-α B04077	IL1-α B04302	IL1-α B04350	IL1-β B04077	IL1-β B04302	IL1-β B04350	MMP-9 B04077	MMP-9 B04302	MMP-9 B04350	PDGFRb B04077	PDGFRb B04302	PDGFRb B04350	RANTES B04077	RANTES B04302	RANTES B04350	ICAM-1 B04077	ICAM-1 B04302	ICAM-1 B04350	NFL B04077	NFL B04302	NFL B04350	S100b B04077	S100b B04302	S100b B04350	GFAP B04077	GFAP B04302	GFAP B04350	UCHL1 B04077	UCHL1 B04302	UCHL1 B04350	Viral 1 B04077	Viral 1 B04302	Viral 1 B04350	Viral 2 B04077	Viral 2 B04302	Viral 2 B04350
42	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
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Non-Vaccinated NHP — Assay Name / Animal ID

Day	IFN-γ B04077	IFN-γ B04302	IFN-γ B04350	IL-6 B04077	IL-6 B04302	IL-6 B04350	IP-10 B04077	IP-10 B04302	IP-10 B04350	MCP-1 B04077	MCP-1 B04302	MCP-1 B04350	MIP-1α B04077	MIP-1α B04302	MIP-1α B04350	Tau B04077	Tau B04302	Tau B04350	TNF-α B04077	TNF-α B04302	TNF-α B04350	IL1-α B04077	IL1-α B04302	IL1-α B04350	IL1-β B04077	IL1-β B04302	IL1-β B04350	MMP-9 B04077	MMP-9 B04302	MMP-9 B04350	PDGFRb B04077	PDGFRb B04302	PDGFRb B04350	RANTES B04077	RANTES B04302	RANTES B04350	ICAM-1 B04077	ICAM-1 B04302	ICAM-1 B04350	NFL B04077	NFL B04302	NFL B04350	S100b B04077	S100b B04302	S100b B04350	GFAP B04077	GFAP B04302	GFAP B04350	UCHL1 B04077	UCHL1 B04302	UCHL1 B04350	Viral 1 B04077	Viral 1 B04302	Viral 1 B04350	Viral 2 B04077	Viral 2 B04302	Viral 2 B04350
42	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
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Scale
<2
2 - 5
5 - 10
>10

Table 3. Changes in circulating levels of biomarkers in serum during the post-challenge period in vaccinated (top table) and non-vaccinated (bottom table) animals. Concentration values for each animal were normalized to pre-challenge measurements taken on the day of the aerosol viral challenge (Day 42). Data from matching plasma samples (not shown) exhibited similar trends. Vaccinated animals did not show significant increases in the concentration of any biomarkers after the aerosol viral challenge. The majority of non-vaccinated animals showed significant increases in IFNγ, IL-6, MCP-1, IP-10, and NfL levels in response to a viral challenge event.

7 Selected Biomarker Concentration Profiles

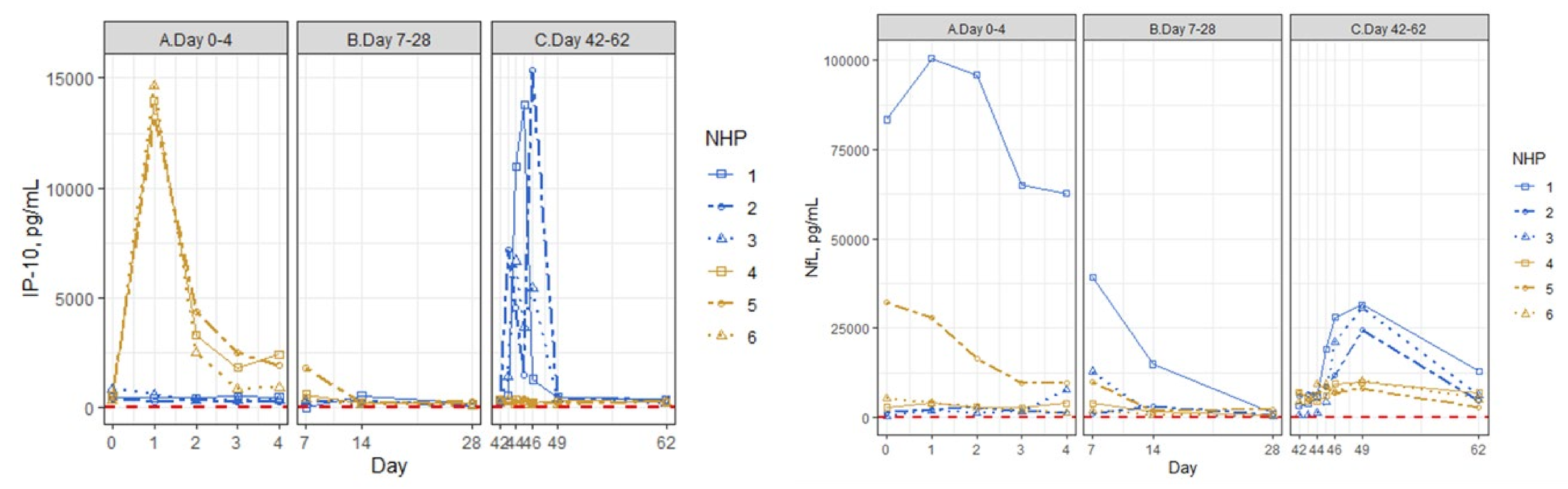

Figure 2. Circulating levels of IP-10 (left) and NfL (right) in serum samples from 3 vaccinated (yellow series) and 3 non-vaccinated (blue series) animals. The red dotted line represents the limit of detection of the assay. Graphs are divided into 3 panels. The first panel (A) shows data for the first 4 days post-vaccination; the second panel (B) Day 7 to Day 28 period between vaccination and viral challenge; the third panel (C) post-challenge period from Day 42 to Day 62. A similar pattern was observed in plasma and CSF samples (data not shown).

Significant increases in NfL levels in two animals during the vaccination and post-vaccination periods were linked to an altercation between one of the vaccinated and one non-vaccinated animals. Based on veterinary notes, the non-vaccinated animal suffered more damage than the vaccinated animal, which could be traced by significantly higher levels of NfL in the former animal's samples. NfL levels dropped to the normal level in both animals before viral challenge (Day 42).

8 Ultra-Sensitive E2-ectodomain protein Assay

The standard MSD viral assays that we developed were capable of detecting the VEEV E2-ectodomain protein during the viremia stage (see assays Viral 1 & 2 in Table 4). However, in some tested animals, viral assay responses were relatively low. Significant improvement in E2-ectoprotein assay sensitivity was observed with an optimized antibody pair and using the ultra-sensitive S-PLEX® MSD platform.

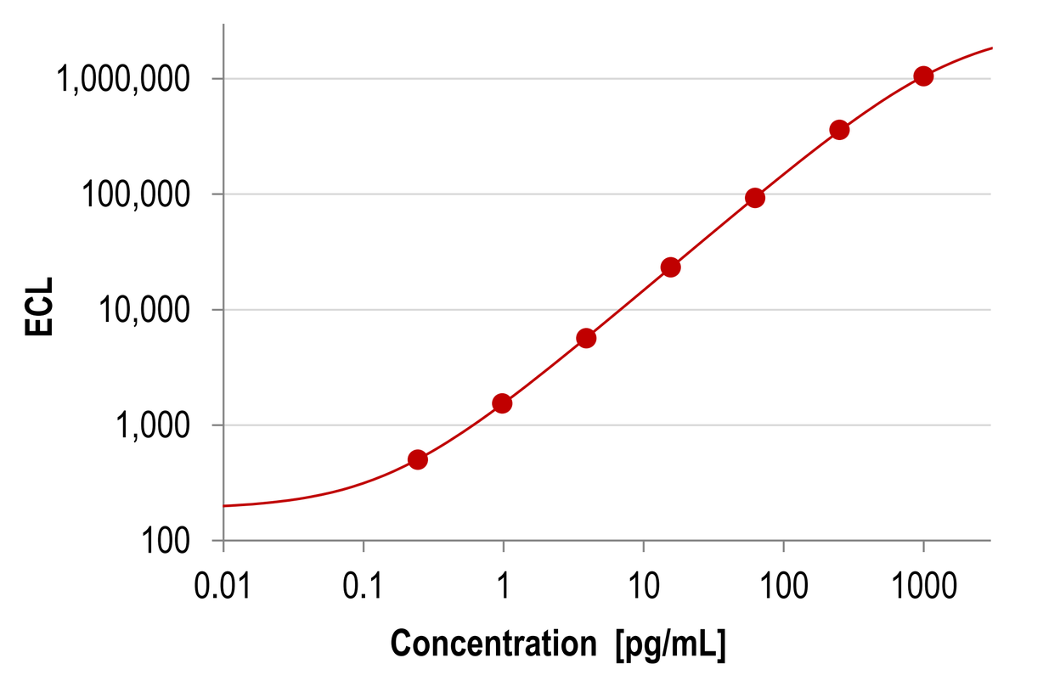

Figure 3. Typical calibration curve of VEEV E2-ectodomain protein on MSD S-PLEX platform.

Analyte	Est LOD S-PLEX	Est LOD Std. Assay	Units	S-PLEX vs Std. Assay improvement in LOD
rec E2-ectodomain	0.05	3.0	pg/mL	60
TC-83	94	785	PFU/mL	8
Trinidad Donkey	1,298	36,190	PFU/mL	28
IRES	6.0	105	PFU/mL	17
IRES-vaccine	122	2,513	PFU/mL	21
rec VLP	7	109	pg/mL	16

Table 4. Typical limits of detection for standard and S-PLEX MSD assays for VEEV E2-ectoprotein and different strains of VEEV.

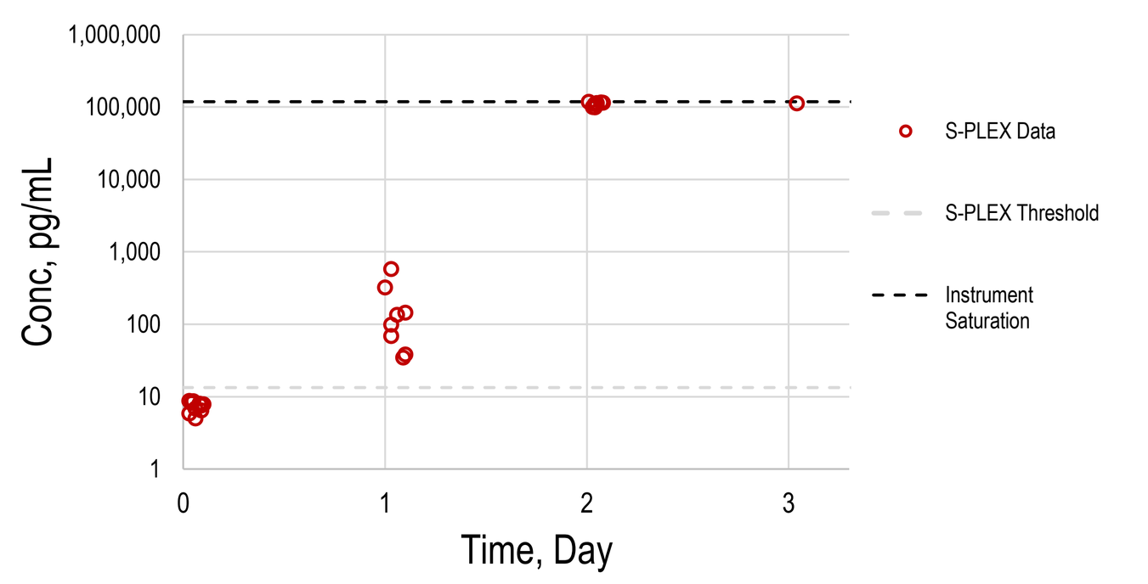

Figure 4. Concentrations of VEEV E2-ectodomain protein in A129 mice during TC-83 infection. Concentrations were estimated based on the calibration curve shown in Figure 3. 50-fold diluted samples were used

A small study of TC-88 VEEV in mice was performed to test the utility of the developed viral assay to measure viral load and the concentration of E2-ectoprotein in infected mice. Briefly, eight Type I interferon receptor knockout mice (strain A129) were used in the study. The A129 mouse, lacking the interferon type I receptor, is more susceptible to viral infection *in vivo*, yielding high titer viremia.

A129 mice were injected subcutaneously with 10^4 PFU of TC-83 virus. Blood was collected on Days 0 (prior to infection), 1, and 2, and from the only one surviving mouse on Day 3. Serum samples were separated and tested on the S-PLEX E2-ectodomain protein assay. Due to limited sample volumes (approximately 40 µL per sample), samples were diluted 50-fold in MSD sample diluent. The S-PLEX assay consistently detected E2-ectoprotein in the mouse blood samples on Day 1 after infection even at 50X (see graph) and 500X (data not shown) dilutions.

9 Conclusions:

- Significant increases in circulating levels of IFNγ, IL-6, MCP-1, and IP-10 were observed in all animals after vaccination.
- Vaccinated animals did not show significant increases in the concentration of these biomarkers after aerosol viral challenge. These findings suggest that the proposed biomarkers could be valuable tools in vaccine efficacy studies.
- The majority of non-vaccinated animals showed significant increases in IFNγ, IL-6, MCP-1, IP-10, and NfL levels in response to a viral challenge event. These biomarkers had different time profiles in response to viral challenge. Concentrations of IFNγ, IL-6 and MCP-1 peaked on Day 1 after challenge and decreased to pre-challenge level after 3–4 days. An elevated IP-10 concentration as a response to viral challenge persisted till Days 4–5, while NfL levels peaked on Days 5–7 and remained slightly elevated even after 20 days post-challenge. Different response times of these biomarkers could be useful for development of a "time-from-exposure" algorithm.
- An ultra-sensitive assay for the VEEV envelope protein (E2-ectodomain protein) was developed and could become a valuable tool for detection of VEEV viremia.
- The approaches described in this poster could be used for developing biomarker panels for other viral infections including, but not limited to, filoviruses, flaviviruses and other alphaviruses.

10 Future Directions:

- The biomarkers of VEEV infection identified in this study will be measured in a near real-time NHP study using assays in MSD's cartridge based platform.

11 Acknowledgements:

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