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Proteomic Discovery of Potential Biomarkers in Zika Virus Infected Monkeys

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Zika virus (ZIKV) was first discovered in *Macaca mulatta* (rhesus monkeys) in 1947 and was found in humans in 1952. This virus can be transmitted via the bite of an infected mosquito, through sexual transmission, or from mother to child. Although most infected individuals are asymptomatic, some clinical symptoms of the virus include fever, joint pain, and rash, as well as microcephaly in infants of infected mothers. The World Health Organization (Geneva, Switzerland) announced a public health emergency in 2016 in response to outbreaks of ZIKV and its association with microcephaly; however, there has been limited progress in discerning how this virus impacts the host. Here, we report on the use of a proteomic pipeline to analyze plasma samples of ZIKV-infected macaques to identify biomarkers for the virus. The discovery of ZIKV biomarkers will assist in defining virus behavior during infection and will illustrate how the host is affected.

15. SUBJECT TERMS

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PREFACE

The work described in this report was started in July 2016 and completed in July 2018. At the time this work was performed, the U.S. Army Combat Capabilities Development Command Chemical Biological Center (Aberdeen Proving Ground, MD) was known as the U.S. Army Edgewood Chemical Biological Center.

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This report has been approved for public release.

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PROTEOMIC DISCOVERY OF POTENTIAL BIOMARKERS IN ZIKA VIRUS INFECTED MONKEYS

1. INTRODUCTION

Zika virus (ZIKV) belongs to the *Flaviviridae* virus family and is a positive-sense, single-stranded RNA virus. This family includes other arboviruses that are of clinical importance, such as dengue virus, West Nile virus, and yellow fever virus.[1] All of these are transmitted by a mosquito bite, from one vertebrate to another. Monkeys and apes are the primary hosts, whereas humans serve as incidental hosts. However, anti-Zika antibodies have also been found in sheep, goats, and elephants.[2] Traditionally, symptomatic infections were confined to sporadic cases or small clusters of patients.[3] The first major ZIKV outbreak was identified in the Federated States of Micronesia during 2007. On the Island of Yap, ~13% of the population presented with symptoms, but a total of ~73% were found to be infected.[4] After that initial occurrence, outbreaks occurred in French Polynesia,[5] Cook Islands, Easter Island,[6] and New Caledonia.[7] In 2015, Brazil became the first country in the Americas to have a confirmed outbreak, and by February 2016, the suspected cases numbered as many as 1,300,000.[3]

ZIKV can exist in three states: immature (non-infectious), mature (infectious), and fusogenic (host-membrane binding). Aedes mosquitos have been identified as the carrier for the virus. The infectious cycle normally begins after a mosquito has ingested infected blood from a vertebrate. In the midgut of the insect, the virus replicates in the epithelial cells before moving to the salivary glands. At this point, it takes 10 days for the insect saliva to become infectious.[8] After a mosquito bites a human, the virus attaches to the human's dermal fibroblasts. The infection is enabled by several adhesion and entry factors such as the AXL receptor tyrosine kinase. Cellular autophagy is then used to bring the virion into the dermal fibroblasts. Although replication of other flaviviruses occurs within the endoplasmic reticulum-derived vesicle, antigens for the ZIKV are only found in the nuclei of infected cells.[9] Release from the cell is through vesical fusing with the cell wall.[10] The virus then spreads through the blood to lymph nodes and subsequently infects organs and the nervous system. Of most concern in humans, the virus is able to pass the placental blood barrier to infect the fetus.[11–14] Severe complications in fetal development, including microcephaly and other brain malformations, are reported.[18,19]

The severity of symptomatic infection varies drastically. In ~80% of cases, the ZIKV infection is asymptomatic. Symptomatic infections are usually mild with nonspecific indicators.[4,15] Because of this, misdiagnosis is very common; ZIKV signs and symptoms resemble those of chikungunya and dengue infections. Reported symptoms include fever, joint pain, red eyes, headache, and a maculopapular rash.[16] These symptoms usually last less than a week, and few mortalities are reported from just the initial infection phase. However, complications such as Guillain–Barré syndrome (GBS) and other neurological disorders are triggered in healthy adults post-infection.[17]

To this day, a paucity of knowledge exists regarding how the ZIKV operates to impact its host. Understanding how infections progress may be key to identifying what triggers symptoms in some patients while most display no or mild symptoms. To that end, a proteomic

workflow was employed to identify biomarkers in plasma samples of two species of macaque monkeys (rhesus and cynomolgus) that were infected intravaginally with ZIKV to uncover key host-response biomarkers to ZIKV infections.

2. METHODS

The overall proteomics pipeline is illustrated in Figure 1.

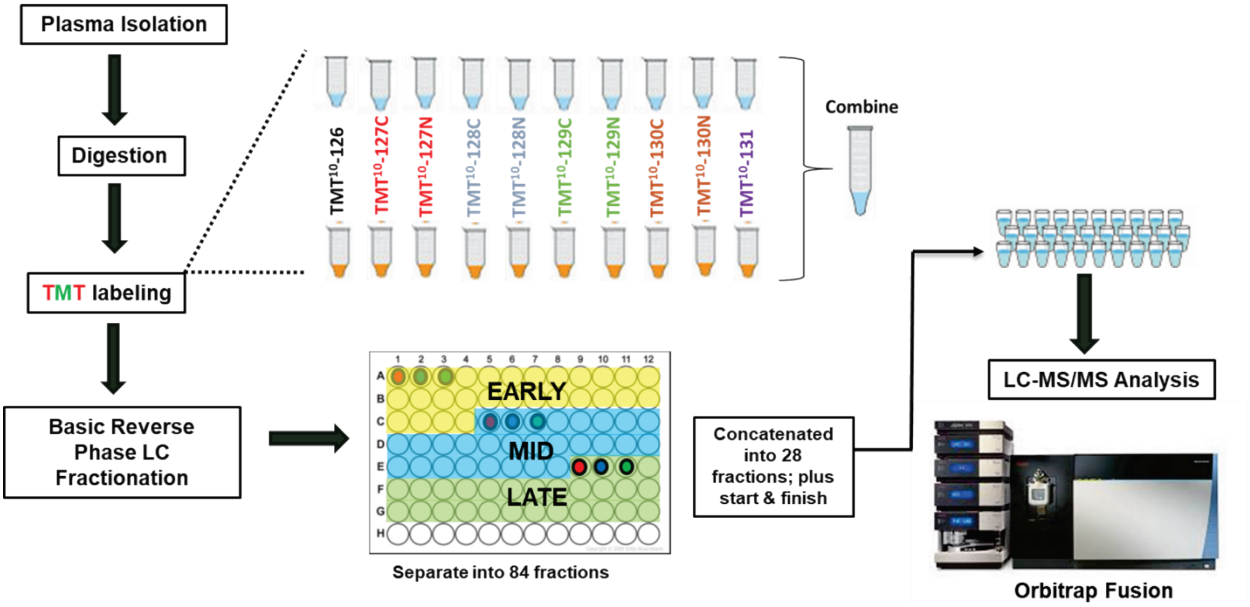

Figure 1. Sample processing pipeline. Plasma was isolated by centrifugation. Equal amounts of protein were digested with trypsin/Lys-C. Samples were labeled with tandem mass tags and then pooled (by animal) for basic reverse-phase fractionation. Concatenated samples were analyzed via liquid chromatography–tandem mass spectrometry (LC–MS/MS) using an Orbitrap Fusion MS system (Thermo Scientific; Waltham, MA).

2.1 Sample Collection

Four female cynomolgus macaques and four female rhesus macaques were intravaginally inoculated with ZIKV. Plasma samples were obtained 7 days pre-infection (control samples) from both species. In addition, the rhesus macaques had plasma taken at 3, 4, 5, 7, 9, 12, 15, and 21 days post-infection, and the cynomolgus macaques had plasma taken at 1, 2, 3, 4, 5, 6, 7, and 9 days post-infection. The infection and collection processes were carried out as described by Haddow and colleagues.[2]

2.2 Sample Preparation

A Pierce BCA protein assay kit (product no. 23225; Thermo Fisher; Waltham, MA) was used to determine the protein concentration of each sample. Aliquots (175 µg) of each sample were removed and placed in 200 µL of 50 mM triethylammonium bicarbonate (TEAB; product no. T-7408; Sigma-Aldrich; St. Louis, MO) for digestion.

Each sample was denatured by the addition of 300 µL of 10 M urea, reduced by the addition of 10 µL of 1 M dithiothreitol (product no. D0632-25G; Sigma-Aldrich), and then incubated on a shaker at 56 °C for 30 min at 400 rpm. Samples underwent alkylation via the addition of 40 µL of 0.5 M iodoacetamide (product no. I1149-25G; Sigma-Aldrich) and were incubated in the dark at room temperature for 30 min. The samples were then diluted with 1 mL of 50 mM TEAB to reduce the urea concentration to 2 M. Trypsin/Lys-C mix (product no. V5071; Promega; Madison, WI) was added at a 50:1 protein–enzyme ratio (4 µg of trypsin added per sample), and the samples were incubated overnight on a shaker at 37 °C and 400 rpm.

After digestion, the samples were acidified with 15 µL of 100% formic acid, and a pH of 2 to 3 was confirmed. The samples were desalted on 1 cc Oasis HLB SPE cartridges (product no. WAT094225; Waters Corp.; Milford, MA) in accordance with the manufacturer's protocol. Samples were then dried in a SpeedVac concentrator (Thermo Fisher Scientific; Waltham, MA). The lyophilized samples were resuspended in 0.1 M TEAB with 10% acetonitrile to a concentration of 30 µg/50 µL. A master mix sample was made for each animal by removing a 10 µL sample at each time point and pooling samples by animal.

From each 30 µg/50 µL resuspension, 50 µL (including the master mix samples) was removed for tandem mass tag (TMT) labeling. A TMT10plex isobaric label reagent set (product no. 90110; Thermo Scientific) was used for TMT labeling. One kit was used per set of four animals, in accordance with the manufacturer's protocol. After TMT labeling was complete, all samples for each time point were pooled according to animal, for a total of eight TMT-labeled pools. These pools were dried in a SpeedVac concentrator before basic reverse-phase (BRP) peptide fractionation was performed.

Each pool was run separately on the BRP system. The pool was resuspended in 100 µL of acetonitrile and shaken at 400 rpm for 5 min at 37 °C. BRP mobile phase A (100 µL), consisting of 90% H_2O, 10% acetonitrile, and 20 mM ammonium formate at pH 10, was added to the pool, and it was shaken for another 5 min at 37 °C and 400 rpm. Mobile phase A (800 µL) was then added to the pool, and the entire volume was loaded onto an XBridge C18 5 µm 4.6 × 250 mm column at a rate of 0.1 mL/min. Samples were fractionated into 3 early-phase fractions, 5 late-phase fractions, and 84 mid-phase fractions that were concatenated into 28 fractions. The 36 fractions were then acidified with 100 µL of 10% formic acid, dried in a SpeedVac concentrator, and resuspended in 20 µL of 5% acetonitrile and 0.1% formic acid. The 3 early fractions and 5 late fractions were combined into single tubes, respectfully, for a total of 30 fractions.

2.3 LC–MS/MS Analysis

The 30 fractions were analyzed on an Orbitrap Fusion MS/MS system. Each sample (2 µL) was injected onto a 2 µm, 100 A, 75 µm × 75 cm Easy-Spray LC column (Thermo Scientific) and run using a 190 min gradient.

2.4 Data Analysis

Perseus 2.1 software (Max Planck Institute of Biochemistry; Martinsried, Germany) was used for analyzing LC–MS/MS data. UniprotKB databases (European Bioinformatics Institute; Cambridgeshire, U.K.) were used to analyze the rhesus and cynomolgus data. Proteins were filtered by high-protein false discovery rate (FDR) confidence, and had to have at least two identified peptides and one peptide unique to that protein group in order to be considered for protein quantitation. An additional parameter for being qualified for quantification was that the protein had to be detected in all four individuals of a species at least at one time point. Statistically significant proteins were determined by two methods: an analysis of variance (ANOVA) test using a 1% FDR, or identification of proteins with a ±2-fold change without regard to ANOVA significance. A normalization strategy was optimized to show the best reproducibility between animals and was accomplished by using animal-grouped Z-scores (Figure 2).

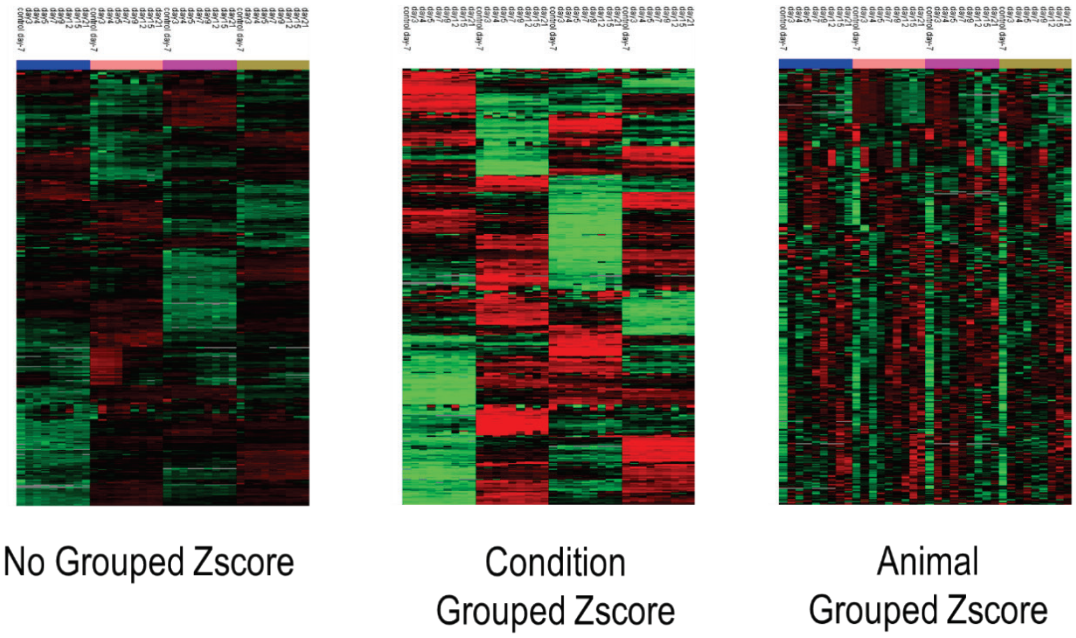

Figure 2. The normalization strategy was optimized using rhesus data and was then applied to both the rhesus and cynomolgus data sets. Reporter ion intensities were transformed to $\log_2$ across the entire data set (not grouped), grouped for each condition, or grouped by animal. Only the grouped Z-score by animal showed the best data reproducibility between each animal.

3. RESULTS

A selection of ANOVA-significant and twofold-changed proteins were identified in all four animals in the rhesus and cynomolgus species, which demonstrated that all animals in the study experienced an immune response to the virus.

3.1 Viremia and Serological Response

None of the subjects exhibited obvious clinical signs of infection. Only two of the four rhesus monkeys and two of the four cynomolgus monkeys showed a viremia response and a serological response following inoculation (Table 1).[20]

Table 1. Viremia and Serological Response

Macaque	Sex	Viremia* (log_{10} PFU/mL) DPI							Serological response† ($PRNT_{80}$) DPI			
		≤ 2	3	4	5	6	7	≥ 9	7	15	21	28
Rhesus 1	F	–	–	2.2	3.4	2.2	–	–	–	>1:640	>1:640	>1:640
Rhesus 2	F	–	–	–	–	–	–	–	–	–	–	–
Rhesus 3	F	–	–	–	–	–	–	–	–	–	–	–
Rhesus 4	F	–	–	2.2	4.0	2.2	–	–	–	>1:640	>1:640	>1:640
Cynomolgus 1	F	–	–	–	–	–	–	–	–	–	–	–
Cynomolgus 2	F	–	–	–	–	–	–	–	–	–	–	–
Cynomolgus 3	F	–	–	1.0	1.9	3.4	3.5	–	–	>1:640	>1:640	>1:640
Cynomolgus 4	F	–	1.0	1.9	3.4	2.8	–	–	–	>1:640	>1:640	>1:640

Notes:
Serological responses of four female rhesus macaques after intravaginal inoculation of ZIKV. Values are titers.
PFU, plaque-forming unit; DPI, days post-inoculation; –, absence of detectable viremia or serological response; PRNT80, 80% plaque reduction neutralization test.
*Limit of detection was 1.0 log_{10} PFU/mL. Adapted from Andrew D. Haddow and colleagues.[20]
† Limit of detection was a titer of 1:20.

3.2 Rhesus Protein Expression

In the rhesus macaques, 417 proteins were identified for quantitation. Of those, 12 proteins were ANOVA-significant, and 206 proteins exhibited a twofold or greater expression change from the control sample. Of the 206 twofold-changed proteins, 9 were ANOVA-significant.

Cluster analysis of the twofold-changed proteins revealed several distinct clusters. Enrichment analysis of those clusters showed that one cluster (Figure 3, cluster 195) was involved in antioxidant activity and inflammation mediated by chemokine and cytokine signaling. Another cluster (Figure 3, cluster 198) was enriched in proteins involved in glycolysis, cellular organization, and response to interleukin (IL)-7.

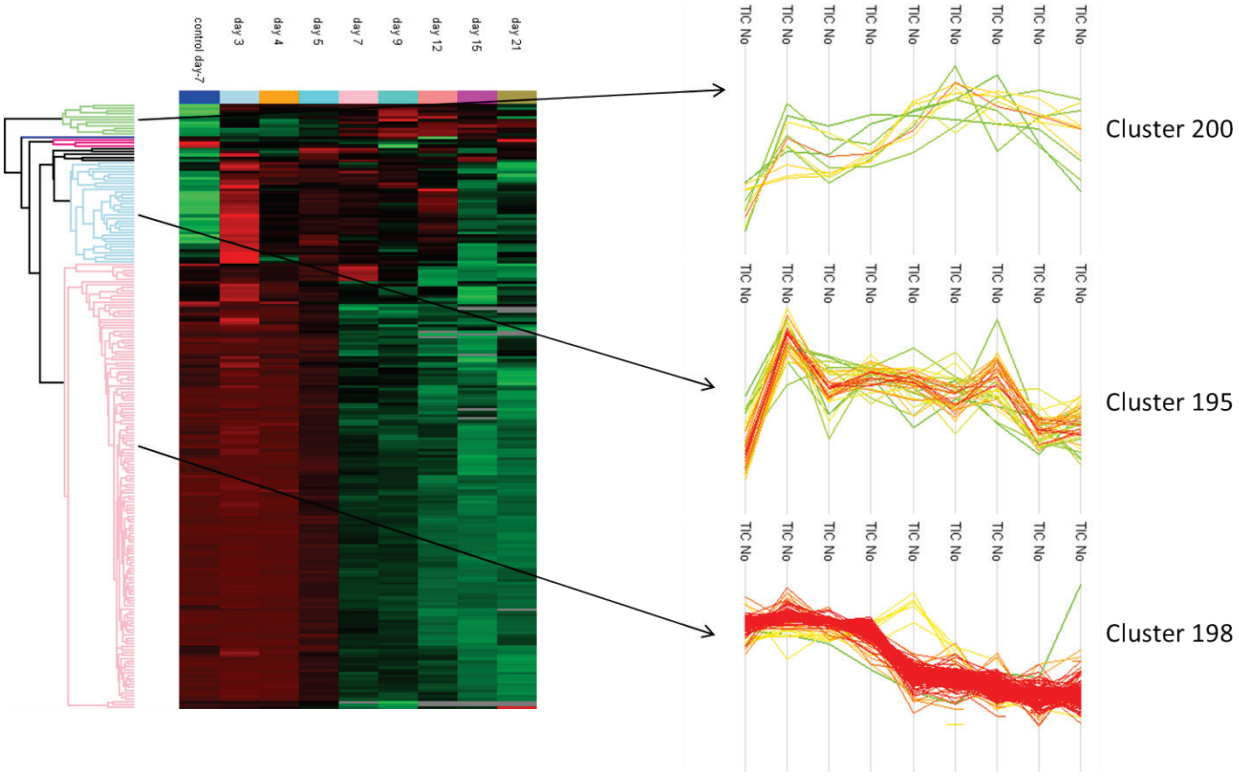

Figure 3. Heat map of all 206 rhesus proteins that changed ±2-fold, separated into three main clusters (>10 proteins). Gene annotation enrichment analysis was performed using Perseus software.

3.3 Cynomolgus Protein Expression

In the cynomolgus macaques, 451 proteins were identified for quantitation. Of those, 35 exhibited an expression change of twofold or greater from the control sample.

Cluster analysis of the twofold-changed proteins revealed several distinct clusters. Enrichment analysis of those clusters showed that one cluster (Figure 4, cluster 29) was enriched in proteins involved in threonine-type endopeptidase activity, whereas another (Figure 4, cluster 26) was enriched in proteins involved in cellular detoxification, catabolic processes, catalytic activity, and oxidoreductase activity.

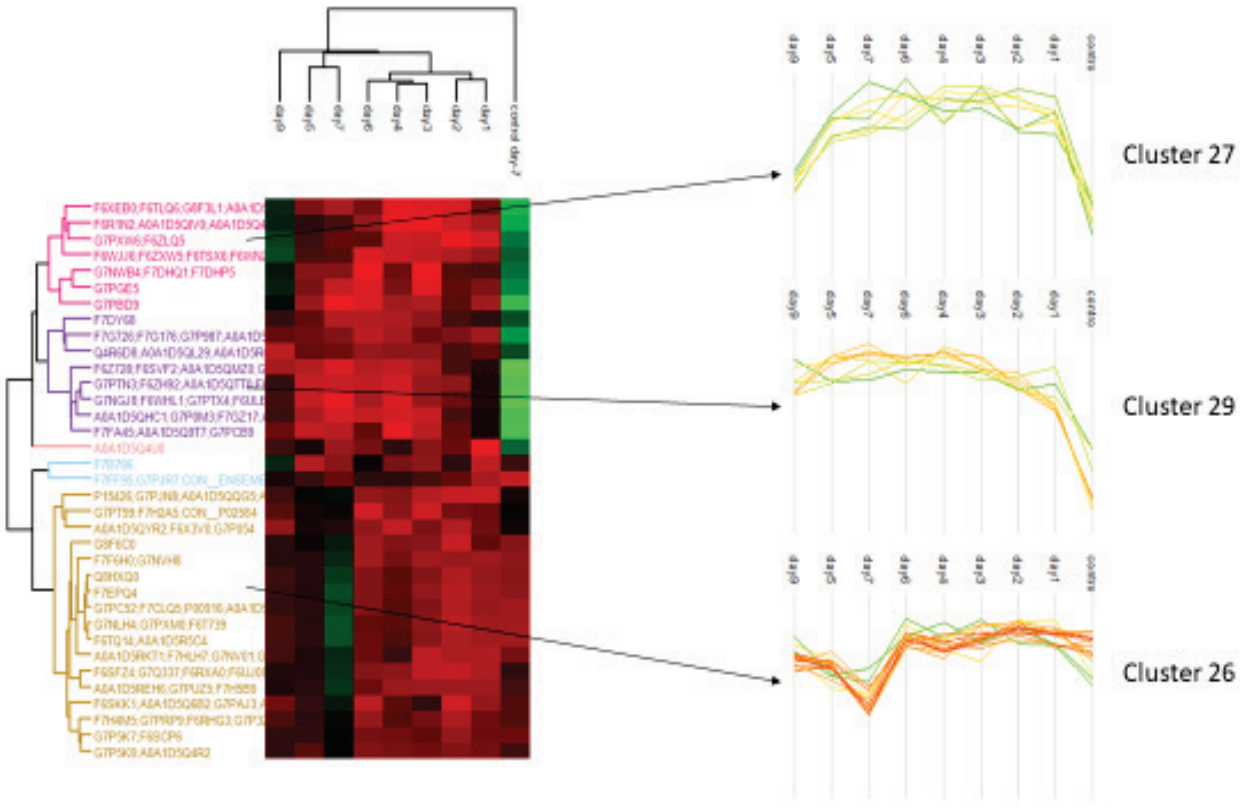

Figure 4. Heat map of all 35 cynomolgus proteins that changed ±2-fold, separated into three main clusters (≥7 proteins). Gene annotation enrichment analysis was performed using Perseus software.

3.4 Rhesus and Cynomolgus Comparison

Data for the rhesus and cynomolgus species were compared by running a combined search for both species in the FASTA databases (European Bioinformatics Institute). This comparison revealed an overlap of nearly 20% of proteins that exhibited a twofold or greater change in expression (Figure 5 and Table 2). Of the 13 twofold-changed proteins that were present in both species, only a few showed similar expression patterns in both species (Table 3).

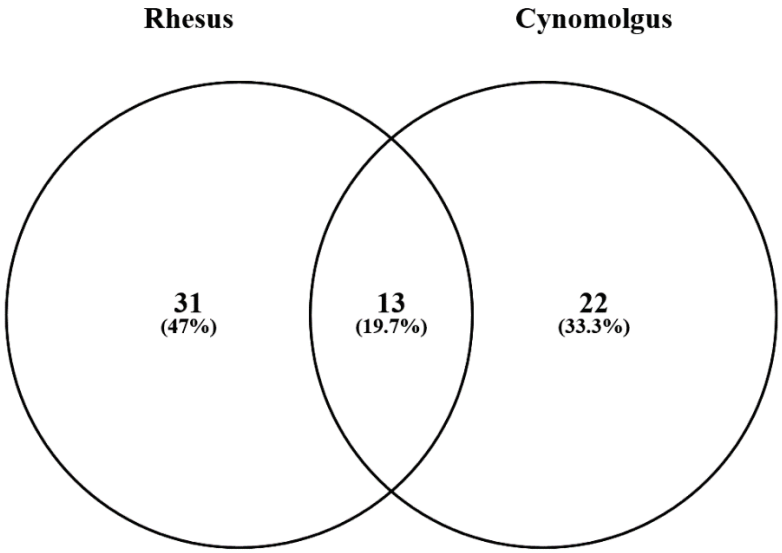

Figure 5. Venn diagram showing twofold-changed proteins in common between rhesus and cynomolgus macaque species.

Table 2. Twofold-Changed Proteins in Common

			Rhesus								Cynomolgus							
Majority protein IDs	**Protein names**	**Gene names**	**Day 3/Day-7**	**Day 4/Day-7**	**Day 5/Day-7**	**Day 7/Day-7**	**Day 9/Day-7**	Day 12/Day-7	Day 15/Day-7	Day 21/Day-7	Day 1/Day-7	Day 2/Day-7	**Day 3/Day-7**	**Day 4/Day-7**	**Day 5/Day-7**	Day 6/Day-7	**Day 7/Day-7**	**Day 9/Day-7**
G7P5K8;A0A1D5Q4R2			25.50	3.82	7.89	3.88	5.03	5.35	2.15	3.08	1.81	2.22	2.40	2.50	1.46	2.14	0.68	0.98
F6R1N2;A0A1D5QIV0;A0A1D5Q4A7;G7PQL6;F7E6F6	L-lactate dehydrogenase	LDHA	1.04	1.13	0.88	0.48	0.38	0.40	0.32	0.22	2.02	2.20	2.45	2.65	1.72	2.21	1.79	1.34
G7P5K7;F6SCP6	**C-X-C motif chemokine**	**PPBP**	**11.27**	**3.02**	**4.45**	**2.76**	**3.10**	3.26	1.50	2.48	1.93	**2.21**	**3.66**	**4.46**	0.99	2.00	**0.58**	**0.82**
F6TQ14;A0A1D5R5C4		CA2	2.67	2.02	2.76	2.49	2.10	2.92	1.49	1.78	1.26	1.33	1.28	1.21	0.79	2.32	0.34	0.85
F6Z728;F6SVF2;A0A1D5QMZ0;G7PPR0	**Alpha-1,4 glucan phosphorylase**	**PYGM**	**6.13**	**5.43**	**6.66**	**6.17**	**5.52**	5.22	5.18	2.94	4.34	5.22	**8.29**	**9.35**	**8.72**	8.20	**9.66**	**7.95**
G7PXW6;F6ZLQ5		CKM	2.70	2.02	2.34	1.75	1.16	1.31	0.87	0.91	4.05	4.79	4.36	6.90	3.78	2.67	2.54	1.57
G7PC52;F7CLQ5;P00916	Carbonic anhydrase 1	CA1	2.80	1.97	2.42	2.38	1.96	2.63	1.40	1.67	1.24	1.34	1.21	1.21	0.78	2.23	0.37	0.83
G7NWB4;F7DHQ1		CRP	3.88	4.78	2.14	1.67	2.87	3.54	1.24	1.79	1.90	1.92	2.66	2.02	1.87	3.36	2.10	1.27
F7DY68	**Serum amyloid A protein**	**LOC694944**	**3.36**	**6.28**	**5.04**	**7.26**	**4.03**	2.74	0.89	0.94	1.55	1.57	**1.93**	**2.16**	**2.33**	4.34	**4.09**	**2.03**
F7EPQ4		ALOX15	2.59	2.08	2.43	2.22	2.05	2.90	1.46	1.71	1.32	1.46	1.40	1.35	0.80	2.63	0.40	0.93
F7FF95			0.60	0.53	0.47	0.39	0.34	0.40	0.43	0.61	1.28	1.11	1.25	1.48	3.13	4.30	4.17	2.37
G7NLH4;G7PXM0		BLVRB	2.84	2.04	2.62	2.47	2.00	2.82	1.49	1.85	1.26	1.39	1.31	1.30	0.79	2.44	0.35	0.92
G7PTN3;F6ZH92;A0A1D5QTT0;F6ZH99;F6Y1M7;G7PTN4;A0A1D5Q9D7;F6ZIC3;G7PTN2		**MYH1;MYH2;MYH8**	**30.42**	**23.48**	**24.74**	**25.56**	**19.73**	8.09	7.62	2.61	3.20	5.06	**6.02**	**6.90**	**6.84**	6.12	**7.05**	**3.44**

Notes:
Twofold-changed proteins in common between rhesus and cynomolgus species.
Red designates upregulated expression; blue designates downregulated expression.

Table 3. Overlapping Time Points of Twofold-Changed Proteins in Common

Majority protein IDs	Protein names	Gene names	Species	Day 3/Day-7	Day 4/Day-7	Day 5/Day-7	Day 7/Day-7	Day 9/Day-7
G7P5K8;A0A1D5Q4R2			**Rhesus**	25.5	3.82	7.89	3.88	5.03
			Cynomolgus	2.4	2.5	1.46	0.68	0.98
F6R1N2;A0A1D5QIV0;A0A1D5Q4A7;G7PQL6;F7E6F6	L-lactate dehydrogenase	LDHA	**Rhesus**	1.04	1.13	0.88	0.48	0.38
			Cynomolgus	2.45	2.65	1.72	1.79	1.34
G7P5K7;F6SCP6	C-X-C motif chemokine	PPBP	**Rhesus**	**11.27**	**3.02**	**4.45**	**2.76**	**3.1**
			Cynomolgus	**3.66**	**4.46**	0.99	**0.58**	**0.82**
F6TQ14;A0A1D5R5C4		CA2	**Rhesus**	2.67	2.02	2.76	2.49	2.1
			Cynomolgus	1.28	1.21	0.79	0.34	0.85
F6Z728;F6SVF2;A0A1D5QMZ0;G7PPR0	Alpha-1,4 glucan phosphorylase	PYGM	**Rhesus**	**6.13**	**5.43**	**6.66**	**6.17**	**5.52**
			Cynomolgus	**8.29**	**9.35**	**8.72**	**9.66**	**7.95**
G7PXW6;F6ZLQ5		CKM	**Rhesus**	2.7	2.02	2.34	1.75	1.16
			Cynomolgus	4.36	6.9	3.78	2.54	1.57
G7PC52;F7CLQ5;P00916	Carbonic anhydrase 1	CA1	**Rhesus**	2.8	1.97	2.42	2.38	1.96
			Cynomolgus	1.21	1.21	0.78	0.37	0.83
G7NWB4;F7DHQ1		CRP	**Rhesus**	3.88	4.78	2.14	1.67	2.87
			Cynomolgus	2.66	2.02	1.87	2.1	1.27
F7DY68	Serum amyloid A protein	LOC694944	**Rhesus**	**3.36**	**6.28**	**5.04**	**7.26**	**4.03**
			Cynomolgus	**1.93**	**2.16**	**2.33**	**4.09**	**2.03**
F7EPQ4		ALOX15	**Rhesus**	2.59	2.08	2.43	2.22	2.05
			Cynomolgus	1.4	1.35	0.8	0.4	0.93
F7FF95			**Rhesus**	0.6	0.53	0.47	0.39	0.34
			Cynomolgus	1.25	1.48	3.13	4.17	2.37
G7NLH4;G7PXM0		BLVRB	**Rhesus**	2.84	2.04	2.62	2.47	2
			Cynomolgus	1.31	1.3	0.79	0.35	0.92
G7PTN3;F6ZH92;A0A1D5QTT0;F6ZH99;F6Y1M7;G7PTN4;A0A1D5Q9D7;F6ZIC3;G7PTN2		MYH1;MYH2;MYH8	**Rhesus**	**30.42**	**23.48**	**24.74**	**25.56**	**19.73**
			Cynomolgus	**6.02**	**6.9**	**6.84**	**7.05**	**3.44**

Notes:
Twofold-changed proteins in common between rhesus and cynomolgus species, highlighting the proteins that showed similar trends during overlapping time points (in yellow).
Upregulated proteins are designated in red text; downregulated proteins are designated in blue text.

4. DISCUSSION

In 2016, an outbreak of ZIKV was linked to an increase in cases of microcephaly, GBS, and other neurological disorders, prompting the World Health Organization (Geneva, Switzerland) to declare a public health emergency. Despite this, to this day, a paucity of knowledge exists regarding how the virus operates to impact its host. To that end, we employed a proteomic workflow to identify biomarkers in plasma samples of two species of macaque monkeys, rhesus and cynomolgus (4 animals per species; total $n = 8$), that were infected intravaginally with ZIKV. Only half of the monkeys in both the rhesus and cynomolgus groups showed a viremic and

serological response to the infection, and absolutely none of the subjects showed an obvious clinical response to the infection. The proteomic analysis revealed that all eight monkeys had some measure of immune response to the ZIKV infection. When we ran the combined analysis with both species, we found that the proteomic response was for the most part species-specific; however, four proteins showed a significant change in expression and displayed similar expression trends in both species: C-reactive protein (CRP); serum amyloid A protein (SAA, gene ID no. LOC694944); α-1,4-glucan phosphorylase (PYGM); and creatine kinase M-type (CKM).

The proteomics results revealed that expression of SAA and CRP, both well-documented biomarkers of innate immune system response, were upregulated immediately following ZIKV infection. CRP, a known biomarker of inflammation, binds to ligands containing phosphocholine, which in turn activates the classical complement pathway. SAA is another protein known to be involved in inflammation response. In fact, SAA has been shown to suppress infectivity of hepatitis C, which is another class of flavivirus.[21] SAA and CRP are acute-phase response proteins. Elevated SAA and CRP levels began returning to baseline by day 15 in rhesus macaques. (This experiment was not carried out past day 9 in cynomolgus macaques, so it is unknown when baseline levels would have returned in that species.) The initial upregulation of these proteins demonstrates that an acute-phase response occurred in all eight animals, even though only half of them displayed viremia or a serological response at 0–9 days post-inoculation.

The functions of PYGM and CKM proteins reveal a deeper understanding of the macaques' response to ZIKV infection. PYGM is an enzyme involved in glycogen phosphorylase activity, which catalyzes the reaction $[(1\rightarrow4)\text{-α-D-glucosyl}]_{(n)}$ + phosphate = $[(1\rightarrow4)\text{-α-D-glucosyl}]_{(n-1)}$ + α-D-glucose 1-phosphate. In doing this, the enzyme assists in breaking down the glycogen and producing α-D-glucose-1-phosphate, which in turn can be used in glycolysis or in starch and sucrose metabolism. More interestingly, PYGM has been identified as an effector molecule specific to *Rac*1 in IL-2 stimulated cells, and it has been shown that inhibition of *Rac*1 activation and PYGM activity results in inhibition of T-cell proliferation in response to IL-2 stimulation.[22] CKM is a muscle-type creatine kinase. It catalyzes the reversible reaction in which phosphate is transferred from ATP to creatine, producing phosphocreatine and ADP; this reaction is part of the arginine and proline metabolism pathway. CKM levels were also upregulated during the early time points in both species, and they returned to baseline at 7 or 9 days post-infection. PYGM levels remained upregulated throughout the study, even throughout the 21-day experiment in the rhesus species. PYGM is known to interact with the GTPase *Rac*1 to stimulate T-cell proliferation,[23] which suggests that these animals were experiencing an adaptive immune response to the ZIKV infection.

Taken together, these four proteins were shown to be robust potential biomarkers. They were clearly indicative of an immune response on the protein level, even when initial clinical observations revealed no obvious physical signs of immune response. All four proteins had very similar expression patterns despite being from different macaque species. These expression patterns make sense in the context of how a mammal would respond to viral infection.

Although more research is needed to confirm these results in a larger study, and perhaps in other animal models, these proteins show strong evidence for being reliable biomarkers to track the immune response to ZIKV infection.

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ACRONYMS AND ABBREVIATIONS

ANOVA	analysis of variance
BRP	basic reverse phase
CKM	creatine kinase M-type
CRP	C-reactive protein
FDR	false discovery rate
GBS	Guillain–Barré syndrome
IL	interleukin
LC	liquid chromatography
MS/MS	tandem mass spectrometry
PYGM	α-1,4-glucan phosphorylase
SAA	serum amyloid A
TEAB	triethylammonium bicarbonate
TMT	tandem mass tag
ZIKV	Zika virus

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