

1 **Human transcriptome response to immunization with live-attenuated Venezuelan equine**  
2 **encephalitis virus vaccine (TC-83): Analysis of whole blood**

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5 *Running Title: Human Immune Responses to Vaccination with Live-Attenuated VEEV*

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17 **Keywords:** Venezuelan equine encephalitis virus, gene expression, microarray, vaccination,  
18 **biomarker, transcriptome**

19 **Abstract:**

20

21 Venezuelan equine encephalitis virus (VEEV) is an important human and animal alphavirus  
22 pathogen transmitted by mosquitoes. The virus is endemic in Central and South America, but has  
23 also caused equine outbreaks in southwestern areas of the United States. In an effort to better  
24 understand the molecular mechanisms of the development of immunity to this important  
25 pathogen, we performed whole genome transcriptional analysis from whole, unfractionated  
26 human blood of patients who had been immunized with the live-attenuated vaccine strain of  
27 VEEV, TC-83. We compared changes to the transcriptome between naïve individuals who were  
28 sham vaccinated with saline to responses of individuals who received TC-83. Significant  
29 transcriptional changes were noted at days 2, 7, and 14 post vaccination. The top canonical  
30 pathways revealed at early and intermediate time points (days 2 and 7) included the involvement  
31 of the classic interferon response, interferon-response factors, activation of pattern recognition  
32 receptors, and engagement of the inflammasome. By day 14, the top canonical pathways  
33 included oxidative phosphorylation, the protein ubiquitination pathway, natural killer cell  
34 signaling, and B-cell development. Biomarkers were identified that differentiate between  
35 vaccinees and control subjects, at early, intermediate, and late stages of the development of  
36 immunity as well as markers which were common to all three stages but distinct from the sham-  
37 vaccinated control subjects. The study represents a novel examination of molecular processes  
38 that lead to the development of immunity against VEEV in humans and which may be of value  
39 as diagnostic targets, to enhance modern vaccine design, or molecular correlates of protection.

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41

**42 Introduction:**

43 Venezuelan equine encephalitis virus (VEEV) is a single-stranded, positive sense RNA  
44 virus and a member of the *Alphavirus* genus of the family *Togaviridae*. Among the New World  
45 alphaviruses, VEEV is considered to be one of the most pathogenic for humans.<sup>1</sup> The Trinidad  
46 strain of VEEV was originally isolated from the brain of an infected donkey in 1938 and is  
47 classified as subtype I A/B.<sup>2,3</sup> In total, there are six antigenic varieties or subtypes of VEEV;  
48 however only subtypes I A/B and IC have been associated with epizootic outbreaks.<sup>3</sup>  
49 Vertebrates, especially equids, can serve as both recipients of viral infection and amplification  
50 reservoir hosts. Transmission of the virus typically occurs from the bite of an infected mosquito.  
51 The infectious course of epizootic strains in equines is characterized by high-titer viremia, which  
52 contributes to the disease state as well as the typical transmission cycle, as the infected animal or  
53 human is again fed upon by mosquitoes.<sup>4</sup>

54 VEEV is classified as a Category B biological threat agent by the Centers for Disease  
55 Control (CDC) and has reportedly been developed as a biological weapon in the past.<sup>4,5</sup> The  
56 virus is highly infectious by the aerosol or inhalational route, and incidental infection has been  
57 problematic to laboratory personnel due to accidental exposures.<sup>6,7</sup> Typical disease cases present  
58 with flu-like symptoms, including fever, chills, headache, and malaise.<sup>6</sup> Encephalitis occurs in a  
59 small percentage of cases, and most often in children; additional symptoms of severe disease  
60 include severe headache, photophobia, ataxia, disorientation, and convulsions.<sup>3</sup> Diagnosis of  
61 disease is achieved primarily through direct detection, either by virus isolation from a biological  
62 sample, detection of antigen by enzyme-linked immunosorbent assay (ELISA) or nucleic acid by  
63 polymerase chain reaction, or indirectly by detection of IgM by ELISA. For isolates or IgM

64 positive samples, the plaque reduction neutralization test (PRNT) is particularly useful for  
65 distinguishing VEEV infection from other related alphavirus infections.<sup>3, 8</sup>

66 Currently, there is no FDA-approved vaccine or therapeutic available for the prevention  
67 or treatment of Venezuelan equine encephalitis (VEE). However, there are two investigational  
68 new drug (IND) vaccines that are available for at-risk laboratory personnel.<sup>8</sup> The first vaccine,  
69 TC-83, is a live-attenuated virus developed in 1961 by serial passage of the virulent Trinidad  
70 strain of VEEV through tissue culture in fetal guinea pig heart cells.<sup>9</sup> The second, C-84, is a  
71 formalin-inactivated version of the TC-83 strain.<sup>10</sup>

72 Live-attenuated TC-83 has been used extensively in humans and has demonstrated high  
73 protective data as measured by the production of neutralizing antibodies; however, the rate of  
74 nonresponders is approximately 20-25%, as measured by the failure to produce neutralizing  
75 antibodies against VEEV following immunization.<sup>8</sup> In addition to naturally-occurring  
76 nonresponse, there have been demonstrations of immune interference contributing to the lack of  
77 neutralizing antibody production for individuals receiving sequential alphavirus immunizations,  
78 including circumstances when individuals received eastern equine encephalitis virus (EEEV),  
79 western equine encephalitis virus (WEEV), or Chikungunya virus (CHIK) prior to immunization  
80 with VEEV.<sup>8, 11</sup>

81 In recent years, the reemergence of VEEV has prompted public health concern and  
82 highlighted the persistent need to develop modern vaccines which can achieve FDA-approval or  
83 to develop effective therapeutics which can be licensed. Additionally, high rates of primary  
84 vaccine failure as well as evidence of sexually dimorphic responses to vaccination are  
85 compelling reasons that there is a current need to develop modern, rational vaccines against  
86 VEEV.

87           There are few studies to date which have been conducted to assess the molecular  
88 responses to VEEV. Host transcriptional responses to VEEV have been reported in a small  
89 number of animal model systems (mice and cynomolgus macaques) and in one *in vitro* study of  
90 human PBMC cells.<sup>12-18</sup>

91           VEEV infection has been noted to stimulate the involvement of genes relating to  
92 inflammation and immune processes in mice, nonhuman primates, and in human PBMC cells.<sup>13,</sup>  
93 <sup>14, 18</sup> Transcripts demonstrating increased expression in mouse brain were predominantly  
94 chemokine genes (CXCL9, CXCL10, CXCL11, CXCL13, CCL3, CCL5, and CCL12) which  
95 presented at an intermediate timeframe of viral infection (days 3 and 4 post infection).<sup>14</sup> The  
96 timing of expression of chemokine genes coincided with the first biphasic peak in permeability  
97 of the blood-brain barrier (BBB) at day 3 post infection with virulent VEEV, with inflammation  
98 in the brain denoted by vessel thickening, endothelial cuffing, and infiltration of neutrophils into  
99 the brain.<sup>14, 19</sup> Similar induction of chemokine transcripts was noted in nonhuman primates by  
100 Koterski et al.<sup>13</sup> However, Koterski et al. noted increased expression of inflammatory response  
101 genes (including CXCL11, CCL3, as well as IL1RN, IRF7, and TNFAIP6) more notably in the  
102 spleen than in the brain, as it has been observed in mice.<sup>13</sup> Hammamieh et al.<sup>12</sup> described  
103 transcriptional profiles of PBMCs using the same nonhuman primates reported by Koterski and  
104 colleagues<sup>13</sup> and noted increased transcription of both CCL13 and CCL18 chemokine genes.  
105 Induction of chemokine transcripts in human PBMCs infected with the live-attenuated strain of  
106 VEEV (TC-83) was noted *in vitro* by the increased expression of CXCL11, CCL3, CCL5, CCL7,  
107 and CCRL2 in both naïve and responder PBMC samples from human volunteers who were either  
108 VEEV vaccine-naïve or had previously presented titers in response to VEEV vaccination.<sup>18</sup>

109 Both type I and type II interferon responses to viral infection were observed across  
110 multiple tissue types and species, with increased transcript expression noted for IFNB1, IFNG,  
111 IRF7, several forms of OAS transcripts, MX1, MX2, and STAT1.<sup>13-18</sup> Other notable patterns of  
112 transcript expression that have been previously reported include widespread engagement of  
113 signaling moieties that are key players in pattern recognition receptor (PRR) detection of bacteria  
114 and viruses, including such transcripts as IL6, DDX58, TLR3, TLR7, and CASP1.<sup>18</sup>

115 The purpose of the present study is to examine the molecular changes that occur in  
116 humans in response to VEEV immunization with the overarching goal to provide an in-depth  
117 analysis of the molecular events which contribute to the development of immunity, and hence  
118 may inform any attempts to design a more effective vaccine or therapeutic. Furthermore, there  
119 are significant gaps in the foundation of knowledge surrounding the host cell signaling pathways  
120 required to combat viral infection and propagation.<sup>7</sup> To that end, we have conducted a whole  
121 transcriptome analysis of human genes which are modulated in response to VEEV immunization;  
122 samples were derived from whole, unfractionated blood at various time points, both before and  
123 after immunization, and were compared with sex- and age-matched control samples at each time  
124 point.

125

126 **Results:**

127

128 *Overall effects of immunization with TC-83 over time*

129 Comparison of global gene expression values across time (i.e., at 1, 4, 8 hrs and at days 1,  
130 2, 7, 14, 21 and 28 post vaccination), in response to treatment, and as a function of both time and  
131 treatment concomitantly, yielded results that met statistical significance criteria (cut-off p-value)  
132 at days 2, 7, and 14 post vaccination when measured against time-matched control samples  
133 (Table 1). The false discovery rate was set to the limit of 10% using the Step-up multiple test  
134 correction method. At day 2 post vaccination, data analysis revealed 3,511 differentially  
135 expressed transcripts. There were 424 differentially expressed transcripts detected at day 7 and  
136 21,343 transcripts at day 14. In comparison with samples from mock-vaccinated individuals,  
137 there were no statistically significant changes in gene expression levels for TC-83 vaccinated  
138 individuals at any other time point. Data were then further constrained by examining the fold  
139 change of gene expression of each transcript; only transcripts with a fold change of  $\geq 2$  were  
140 included in further analyses (Figure 1). Applying these criteria reduced the total number of  
141 significantly expressed transcripts to 1,142 covering days 2, 7, and 14 post vaccination.

142 Alteration of transcript expression at specific times included thousands of different genes,  
143 with surprisingly little overlap. The first time point where a difference in gene expression in the  
144 TC-83 vaccinated individuals could be detected, relative to sham-vaccinated control subjects,  
145 was at day 2 (Figure 1). Of the 225 transcripts that were differentially expressed on day 2 with at  
146 least a 2-fold change in expression, only 32 overlapped with transcripts at both days 7 and 14.  
147 On day 7, we detected 14 differentially expressed transcripts unique to day 7; while, on day 14  
148 post vaccination, 756 transcripts were detected and unique to this time point. The graphical  
149 interactions displayed by Venn diagramming show a clear distinction in gene expression across

150 time; from these results, the patterns of gene expression were stratified based on early (day 2),  
151 intermediate (day 7), and late (day 14) response to vaccination (Figure 1).

152         The variation induced in transcript expression between treatment groups and samples was  
153 assessed by Principal Component Analysis (PCA) (Figure 2). The primary dimension of the PCA  
154 analysis, reflecting the greatest variation in gene expression, was attributed to the effect  
155 generated by vaccination with TC-83 (treatment, depicted as grouping by color), accounting for  
156 23.8 % of transcript expression variation. The second dimension of variation in transcript  
157 expression was due to changes over time following vaccination (depicted as increasing size of  
158 spheres) and accounted for 7.5 % of the variation observed. Finally, the third dimension of  
159 variation can be explained by the changes that occurred as a factor of the interaction of time and  
160 treatment together, which accounted for 3.5% of the total variation.

161

162 *Cellular pathway analysis for changes in the transcriptome induced by immunization with TC-83*  
163 *in humans*

164         We conducted pathway analysis using Ingenuity Pathway Analysis software (Ingenuity,  
165 Redwood City, CA) to better understand the scope and function of the molecular responses  
166 generated in humans in response to TC-83 vaccination. The observed host responses covered a  
167 variety of pathways involved in disease processes, molecular and cellular functions, and  
168 physiology system development and function (Table 2). On day 2 post vaccination, representing  
169 the early transcriptional response, there was noted involvement of specific transcripts which  
170 were indicative of a strong antimicrobial and inflammatory response, as well as transcripts that  
171 were characteristic of infectious disease, infection mechanisms, and organismal injury (Table 2).  
172 The molecular functions related to transcripts which were differentially expressed on day 2

173 represented cellular movement and development, cellular signaling, post-translational  
174 modification, and protein folding. These molecular functions routinely participate in the systemic  
175 organization of hematological function, immune cell trafficking, tissue development, muscular-  
176 skeletal development, and hematopoiesis. The most prominent signaling pathways induced upon  
177 VEEV vaccination included the interferon signaling pathway, activation of interferon-response  
178 factors by cytosolic pattern recognition receptors, involvement of pattern recognition receptors in  
179 the recognition of viruses and bacteria, the RIG1-like receptors as part of a classical innate  
180 antiviral immune response (i.e., the inflammasome), and the IL-6 signaling pathway. The  
181 responses observed at day 7 post vaccination were similar to those seen at day 2 with regard to a  
182 clear induction of infectious, inflammatory, and antimicrobial responses. In addition to induction  
183 of molecular and cellular functions (e.g., post-translational modification), cellular development  
184 and protein folding functions were also observed at day 2. In contrast, by day 7 post vaccination,  
185 molecular functions expanded to transcripts related to lipid metabolism and molecular transport.  
186 Similarly, overlapping physiological system functions relating to hematological development,  
187 hematopoiesis, immune cell trafficking, and muscular-skeletal development continued to be top  
188 factors through day 7 post vaccination. However, an evolving response was evident by the  
189 induction of transcripts involved with endocrine system development and function. Likewise, the  
190 top canonical pathways that were observed on day 7 post vaccination were predominantly similar  
191 to those seen at day 2 (i.e., interferon signaling, pattern recognition receptor activation of  
192 interferon-response factors, inflammasome-related transcripts) but also included transcripts  
193 which were involved in the pathogenesis of multiple sclerosis. Two parameters characterize a  
194 dramatic shift in the results from day 14 post vaccination: First, the molecular and cellular  
195 processes observed primarily involved those of nucleic acid metabolism, cell to cell signaling,

196 cellular compromise, gene expression, and molecular transport; and secondly, the top canonical  
197 pathways shifted from a strong interferon-driven response to one characterized by oxidative  
198 phosphorylation transcripts, protein ubiquitination, RAN signaling, T cell receptor signaling, and  
199 regulation of eIF4 and p70S6K signaling (Table 2).

200         The involvement of specific canonical pathways in this temporal study of transcriptional  
201 expression allowed us to compare and describe three distinct phases of human VEEV infection *in*  
202 *vivo*. We employed Ingenuity Pathway Analysis (IPA) to describe the involvement of individual  
203 transcripts and canonical pathways in the development of immunity following TC-83  
204 immunization. During the earliest phase (day 2 post vaccination) there was a strong induction of  
205 interferon signaling genes and subsequent interferon-related factors (Table 3). Some of the most  
206 notable transcripts representing interferon signaling included IFIT1, IFIT3, MX1, OAS1, and  
207 IFI34, and many of these transcripts continued to display increased expression through day 7 as  
208 well. However, by day 14 most interferon signaling transcripts had returned to baseline levels  
209 (Table 3). Similarly, activation of interferon related factors was evident by day 2, including  
210 genes comprising the inflammasome (RIG1, also known as DDX58; MDA5, also known as  
211 IFIH1; LGP2, also known as DHX58; and a novel DEXD/H box helicase, DDX60), (Table 3).  
212 Increased transcription of genes involved in IL-6 signal transduction was highest at day 2 (e.g.,  
213 IL1RN, SOCS1, and TNFAIP6) with noted decrease in IL-8 transcription (Table 3). Key  
214 signaling components of the JAK/STAT pathway were also noted to have increased transcription  
215 at day 2 following immunization which was sustained through day 7, but returned to baseline  
216 levels by day 14 (e.g., SOCS1, STAT1, and STAT2) (Table 3).

217         In sharp contrast, the canonical pathways that are highly represented by transcripts with  
218 increased expression by day 14 post immunization include that of oxidative phosphorylation

219 (e.g., COX7A2, COX16, UQCRB, UQCRH, PPA1), the protein ubiquitination pathway (e.g.,  
220 UBR1, USP1, PSMA3, PSMC6, BIRC2, BIRC3, HSP90AA1), the ERK5 Signaling pathway  
221 (e.g., IL6ST, NRAS, RRAS2, ATF2), the Natural Killer Cell Signaling pathway (e.g., KLRC2,  
222 FYN, PRKC1, KLRK1, KLRC3, RRAS2, NRAS), and the B-Cell Development pathway (e.g.,  
223 IL7R, IGKC, IGL@, IGHM) (Table 3).

224

#### 225 *Biomarker analysis and identification using Ingenuity Pathway Analysis*

226 The temporal transcriptional responses from TC-83 vaccinated subjects and unvaccinated  
227 control subjects were evaluated with IPA to establish biomarkers following immunization; we  
228 employed analysis filters to enrich for biomarkers previously identified in biological fluids (e.g.,  
229 blood, sera, plasma, and urine). The results were clustered into groups representing early,  
230 intermediate, and late biomarkers (Table 4) categories which distinguished biomarkers that were  
231 either unique to each stage of immune development or were common across all days following  
232 immunization. The biomarkers displayed in Table 4 were selected by restricting the analysis to  
233 the top 10 transcripts showing the greatest levels of change in expression, as well as showing  
234 consistent expression profiles over time, and for all potential probe sets which correspond to  
235 each transcript.

236

#### 237 *HLA phenotype and post vaccination titer*

238 All study subjects were assessed for the development of neutralizing antibodies against  
239 live attenuated TC-83 at 28 days post vaccination; production of neutralizing antibody in  
240 response to vaccination is currently the gold standard measure of an immunity correlate of  
241 protection and denotes successful primary vaccination. Results of neutralizing antibody

242 production were compared with HLA phenotype to describe the potential contribution of MHC  
243 haplotype to the immunological response induced by the vaccine (Table 5). Control study  
244 subjects receiving only a saline injection were also included in this portion of the study to  
245 demonstrate the lack of antibody response as a result of mock vaccination. Table 5 displays a  
246 subset of MHC Class II haplotypes (i.e., DRB1 and DQB1). A single volunteer (Vaccinee 1)  
247 who displayed the HLA DQB1\*0301 allele is included in the table; however the gene expression  
248 data from that individual was removed from the microarray data analysis due to primary vaccine  
249 failure. We noted that two of three “low” responders (Day 28 post vaccination titer < 100)  
250 displayed a shared HLA haplotype (DQB1 \*0302). The DQB1 \*0302 phenotype was also  
251 present in one of the “high” vaccine responders (Day 28 post vaccination titer >100). Complete  
252 HLA phenotype data for study subjects, including all MHC Class I and Class II haplotypes, may  
253 be requested from the corresponding author.

254 To address the potential role or contribution of certain HLA DQB1 alleles to vaccine  
255 outcome, a second ANOVA was performed to include neutralizing titer as a variable (i.e., low  
256 titer <100, high titer >100) (Supplemental Data Table 1). Temporal gene expression values in  
257 low and high titer immune response groups were compared to describe changes which could be  
258 observed between these two groups (Supplemental Data Table 2). While the expression of many  
259 genes met the criteria of statistical significance for differential expression, none of the significant  
260 genes met the cut off of 2-fold or higher change in expression level used in the primary analysis,  
261 suggesting that the pathways and processes that are critical for vaccine success or failure in  
262 humans are tightly regulated and may be influenced even by small changes in transcriptional  
263 expression.

264

265 **Discussion:**

266

267 VEEV is a reemerging pathogen with potential risk as both a public health and  
268 biological threat.<sup>25</sup> VEEV is classified as a category B biological threat agent by the US Centers  
269 for Disease Control and Prevention (CDC). The human disease caused by VEEV infection is  
270 difficult to assess as symptoms are clinically similar to other, more commonly occurring  
271 diseases, such as Dengue Fever.<sup>20</sup> Confirmatory diagnosis of VEEV requires specialized  
272 laboratory tests that are frequently unavailable in countries with limited medical and public  
273 health resources.<sup>26</sup> Small outbreaks of epizootic VEEV have been detected in endemic countries  
274 for decades; however, recent surveillance data covering Latin American nations (particularly  
275 Mexico, Panama, and Peru) have suggested that the annual number of VEEV cases have been  
276 seriously underrepresented, in large part because of misdiagnoses of infection with VEEV as  
277 Dengue Fever. Such misdiagnoses may comprise between 0.1 to 7% of all Dengue Fever  
278 infections.<sup>26</sup> As such, VEEV infection remains an important public health threat.

279 The advent and widespread application of vaccines has been hailed as one of the most  
280 profound achievements for public health in the 20<sup>th</sup> century. Successful vaccination is ideally  
281 mediated through both B and T cell mediated responses. Vaccine responses, as correlates of  
282 protection, are often measured by the ability of the vaccine to generate measurable levels of  
283 neutralizing antibodies and are usually the only correlate of protection data available in  
284 vaccination studies.<sup>27</sup>

285 Currently, there is no FDA-approved vaccine for human immunization against VEEV,  
286 although there is an Investigational New Drug (IND) vaccine, live-attenuated VEEV TC-83,  
287 which has been used for decades by military and at-risk laboratory personnel.<sup>8, 21</sup> The mechanism  
288 of protection induced by vaccination with TC-83 is believed to be through the production of

289 neutralizing antibody, but other molecular mechanisms of protection are not well understood or  
290 defined.<sup>8, 21</sup> The present study explored the sequential molecular events, *in vivo*, which occur  
291 following human immunization with TC-83, and which lead to the development of immunity.

292 The early and sustained engagement of interferon signals and interferon response factors  
293 beginning on day 2 and extending to day 7 observed post vaccination are indicative of a  
294 traditional innate antiviral immune response. There is an extensive overlap between the  
295 molecules that exhibit changes in transcript expression and the canonical pathways in which they  
296 participate, particularly between genes of the interferon response, interferon-response factors,  
297 activation of pattern recognition receptors, and engagement of the inflammasome. Potent  
298 induction of expression in IFIT1 (ISG54), IFIT3, IRF7, TLR7, and OAS 1-3 represent induction  
299 of a classic type I interferon signaling mediated in response to single-stranded RNA viruses.<sup>22-25</sup>  
300 IFIT1 has been shown to act as a molecular receptor for 5' tri-phosphorylated RNA and  
301 consequently inhibit viral replication.<sup>24</sup> We also observed increased transcription of IFIT3 which  
302 contributes to antiviral signaling by bridging mitochondrial antiviral signaling and TBK1.<sup>26</sup>

303 Early induction of the broad-spectrum innate inflammasome response was noted as a  
304 consequence of immunization, spanning days 2 through 7. Engagement of the inflammasome  
305 has been shown to be classically mediated through TLR7, DDX58 (also known as RIG-1), IFIH1  
306 (also known as MDA5), and DHX58 (also known as LGP2).<sup>27</sup> We found that TC-83  
307 immunization caused transcriptional induction of DDX60, an RNA helicase related to DDX58  
308 which has also been demonstrated in functional genomics studies to be required for RIG-1 or  
309 MDA5-dependent signaling in response to viral infection.<sup>28-29</sup> Satoh et al.<sup>30</sup> describe the  
310 importance of DHX58 (LGP2), an ATP-dependent RNA helicase, as a key modulator of both  
311 RIG-1 and MDA5-mediated responses ostensibly through activity which makes viral RNA more

312 accessible to either RIG-1 or MDA5 directly or by altering the cellular location of viral  
313 ribonucleoprotein complexes for greater access. Other proteins that can initiate anti-viral  
314 responses include IFIT2 (ISG56), RSAD2 (viperin), and ISG15; our results demonstrate strongly  
315 increased transcription for each of these transcripts on both days 2 and 7 following vaccination  
316 suggesting that the type I interferon response is primarily regulated through IRF3 activation.<sup>31</sup>  
317 Over expression of RSAD2 has been linked to expression and regulation by histone deacetylase  
318 1 (HDAC1) which results in transcriptional repression; during VEEV-induced early engagement  
319 of the inflammasome, HDAC1 expression was not altered. Indeed, HDAC1 expression was not  
320 altered significantly until day 14 post vaccination.<sup>32</sup> Regulation of HDAC1 has been shown to be  
321 dependent both on the cell type and influenced by the physiological environment.<sup>32</sup>

322 The HLA-DQB1 phenotype has previously been associated with autoimmune disorders  
323 and suggested to be involved with hyporesponsiveness to vaccination.<sup>18, 33-35</sup> A number of studies  
324 also suggest that certain combinations of the DQB1 allele play an important role in linkage  
325 disequilibrium patterns.<sup>36-38</sup> From the current study, nine of ten vaccinated volunteers produced  
326 an effective immune response, as measured by the production of neutralizing antibody against  
327 VEEV. However, no trend in either HLA-DRB1 or HLA-DQB1 phenotype could be definitively  
328 determined with respect to linking the phenotype allele to an immunization outcome. We  
329 previously reported results of an *in vitro* assessment of changes in transcription in PBMCs from  
330 volunteers previously vaccinated with VEEV TC-83 in which it was suggested that there may be  
331 an inverse association between HLA DQB1 alleles and production of neutralizing titer.<sup>18</sup> In that  
332 instance, either the HLA DQB1 \*0301 or \*0302 allele was present in the samples of volunteers  
333 with the lowest neutralizing antibody titer. Interestingly, specific alleles of the HLA DQB1  
334 haplotypes, including DQB1\*0201 and DQB1\*0302, have been reported to confer up to 50% of

335 the risk of heritable Type I diabetes.<sup>38</sup> We noted decreased transcription of several genes related  
336 to insulin signaling, IRS2, SGK, and IGF1R, during the course of vaccination and immune  
337 development, suggesting that the insulin signaling pathway may be involved in early responses  
338 to vaccination. Additionally, within the DRB1 haplotype, the DRB1 \*1501 allele has been  
339 associated with Multiple Sclerosis.<sup>39</sup> The data suggest that the association between vaccine  
340 failure (i.e., vaccine nonresponders) and responders with low neutralizing titer may not  
341 necessarily be due to a random association with DQB1 \*0301 or \*0302 alleles, but rather these  
342 results prompt further study to test the hypothesis that primary vaccine failure and weak vaccine  
343 take can be explained, at least in part, by association with specific HLA haplotypes. Indeed, the  
344 answer to such questions may not ultimately rest on only one haplotype (e.g., DQB1) but may be  
345 influenced by the combination of specific DRB1 and DQB1 alleles. While the results are  
346 intriguing, it is clear that there are additional factors that affect both disease outcome and  
347 vaccination success; further work will need to be conducted to address the questions that such  
348 results inspire and with greater numbers of subjects to achieve statistical significance.

349 We queried the IPA analysis to evaluate the effects of vaccination on the microRNA  
350 population; changes in the expression of certain microRNA may represent an avenue of future  
351 investigation to suggest regulatory mechanisms for differentially expressed genes. Several  
352 microRNA factors were identified as having been effected by VEEV infection, including let-7,  
353 miR-21, miR30, miR-101, and miR-214; the presence of these microRNA suggest that the  
354 regulation of transcription of certain genes may also be influenced by microRNA. Further studies  
355 are needed to pinpoint the hypothesized involvement of specific roles these microRNA and what  
356 role each factor may play in the transcriptional regulation of genes and the timing of interaction

357 (transcription, translation, or post-translational modification of genes) (Supplemental Data Table  
358 3).

359 The study is not without limitations. We were able to detect statistically significant  
360 changes in gene expression at days 2, 7, and 14, but at no other time points in the study. This  
361 could have been in part due to the restrictive statistical parameters used (i.e., 95% power, 0.001  
362 two-sided t-test, 2-fold change filter, 0.5 CV). Future studies should include a time point between  
363 day 7 and day 14 to bridge the changes associated with a largely interferon-driven response and  
364 the beginning of development of immunity. Follow on studies may benefit from using a larger  
365 sample size to detect more discreet changes of gene expression, and potentially determine  
366 whether a correlation between HLA DRB1 or DQB1 alleles and neutralizing antibody production  
367 could be established. This would provide further support of previously published *in vitro* data.<sup>18</sup>  
368 The present study also utilized only male volunteers between the ages of 23-48 as a strategy to  
369 control confounding factors such as age and female sex hormone signaling; future studies should  
370 address potential differences in immune response due to age and gender, as well.

371 The changes observed from whole blood sampling of the transcriptome of subjects  
372 vaccinated with live-attenuated VEEV TC-83 provide the first glimpse of the molecular  
373 epidemiology events that contribute to the specific development of alphaviral immunity in a  
374 human host. The most profound changes were noted at days 2, 7, and 14 post vaccination and  
375 represent early, intermediate, and late transcriptional events. By day 14, it is not surprising that  
376 many of the top molecules which are differentially expressed are related to immunoglobulin  
377 genes (Table 2, Table 3, and Table 4). While the early and intermediate phases are dominated by  
378 interferon responses, driving innate anti-viral host responses, the events that occur at day 14 are  
379 among the most interesting and are represented by changes relating to oxidative phosphorylation,

380 protein ubiquitination, MAPK-related cell signaling pathways, and both natural killer signaling  
381 and B-cell development. These changes are similar to reports of involvement of ubiquitination  
382 in other alphaviruses. Indeed, nsP2 proteins of Sindbis, Semliki Forest, and Chikungunya viruses  
383 have been shown to inhibit cellular transcription by ubiquitination of Rpb1, a catalytic subunit of  
384 the RNAPII complex, suggesting a possible mechanism utilized by Old World alphaviruses to  
385 subvert the cellular antiviral response.<sup>40</sup> Differentially expressed transcripts for the MAPK  
386 pathway and for the pore-forming protein perforin and the family of granzymes have been  
387 suggested as a potential antiviral role in cytotoxic T lymphocyte (CTL) and natural killer (NK)  
388 cells in another positive sense RNA virus, the Japanese encephalitis virus infection.<sup>41</sup> The  
389 exploitation of similar mechanisms by VEEV, as suggested by our results, may represent highly  
390 conserved responses.

391 Biomarkers which are unique to each phase or common across all stages of infection have  
392 been identified with the potential to serve as a molecular signature of infection or as molecular  
393 correlates of protection. The HLA phenotype data combined with analysis of the immunity  
394 process in humans to VEEV vaccination establish new frontiers for further evaluation of  
395 identified HLA phenotypes and induced host genes for their contribution to genomic instability  
396 of certain phenotypes and production of neutralizing antibody titers, which are currently the  
397 gold-standard in terms of correlates of immunity.<sup>18, 42</sup> Additionally, the suggested host  
398 mechanisms affected by vaccination with live-attenuated VEEV TC-83 in humans revealed  
399 potential viral subversion strategies to achieve productive infection, which could be manipulated  
400 therapeutically or in immunization intervention protocols to achieve full protection against  
401 VEEV and related alphaviruses.

402

403

404 **Patients and Methods:**

405

406 *Selection of volunteers:*

407           The research protocol was conducted under Good Clinical Practice (GCP) quality  
408 standards, approved by the USAMRIID Institutional Review Board (IRB), and volunteers signed  
409 a written informed consent document (ICD) prior to enrollment in the study which described the  
410 purpose of the study, as well as the manner in which samples would be collected, used, and  
411 disposed. The study consisted of twenty male volunteers between the ages of 23 and 48 years.  
412 Male volunteers were selected for the study to reduce the confounding impact of hormonal  
413 variation on global gene expression. Additionally, each vaccinee was age-matched to a control  
414 volunteer. Study participants were individuals who had not previously received any alphavirus  
415 IND vaccines (i.e., against WEEV, EEEV, or VEEV). Prior to enrollment and participation in  
416 the study, all study participants were screened for antibodies by ELISA and PRNT<sup>18</sup> for prior  
417 exposure to new world Alphaviruses (VEEV, EEEV, and WEEV) and demonstrated to be  
418 negative for previous exposure. Participants were also genotyped for Human Leukocyte Antigen  
419 (HLA) allele expression, as previously described.<sup>18</sup> The *in vivo* study, conducted under Good  
420 Clinical Practice quality standards and approved human use protocol FY06-17, included ten  
421 vaccinees who received 0.5 ml of live-attenuated TC-83 VEEV (NDBR-102 vaccine) [roughly  
422 equivalent to  $1.7 \times 10^5$  plaque forming units (PFU) of the virus] administered subcutaneously  
423 (SC) in the upper outer aspect of the arm, as well as ten control subjects who were administered  
424 0.5 ml saline via the same procedure. Whole, unfractionated blood was collected at specific time  
425 points immediately prior to (0 h) and following vaccination (1, 4, 8 h and days 1, 2, 7, 14, 21,  
426 and 28). On day 56 post vaccination, serum was drawn from volunteers to assess development of  
427 neutralizing antibody titer against VEEV. The dataset is comprised of expressed transcripts from

428 9 responder vaccinees and 10 control subjects; one vaccinated subject was removed due to  
429 primary vaccine immunization failure. Total blood RNA samples from these individuals were  
430 subjected to microarray analysis.

431

432 *RNA Isolation and sample preparation for microarray analysis:*

433 RNA was isolated from whole, unfractionated blood using the PAXgene Blood RNA kit  
434 according to manufacturer's instructions (Qiagen, Valencia, CA). Briefly, RNA from whole  
435 blood was collected in PAXgene Blood RNA tubes from each volunteer at each time point.  
436 Samples were subjected to quality and concentration analysis using the Agilent RNA 6000 Nano  
437 BioAnalyzer kit, according to manufacturer's instructions (Agilent, Santa Clara, CA). Total  
438 RNA samples were then prepared for hybridization to the Affymetrix Human Genome U133 plus  
439 2.0 Gene chip arrays according to manufacturer's specifications (Affymetrix, Inc., Santa Clara,  
440 CA). The microarray hybridizations were performed at the Core Laboratory Facility at the  
441 Virginia Bioinformatics Institute (Blacksburg, VA).

442

443 *Microarray Data Analysis:*

444 The gene expression data (Affymetrix .CEL files) were imported into Partek Genomics  
445 Suite v6.0 software (Partek Inc., St. Louis, MO). Using the Robust Multi-array Average (RMA)  
446 algorithm,<sup>43</sup> the gene expression data (Affymetrix gene probe sets) were normalized and log<sub>2</sub>  
447 transformed. To detect differential expression, a 4-way ANOVA was constructed by using the  
448 restricted maximum likelihood (REML) approach to produce an unbiased estimate of variance.<sup>44</sup>  
449 The following equation describes the partitioning of time, vaccine type, and subject variability  
450 from variability due to biological and experimental noise:

451

452 Equation 1: 
$$Y_{ijklm} = \mu + \text{Scan Date}_i + T_j + V_k + S(V)_{kl} + T * V_{jk} + \varepsilon_{ijklm}$$

453 Where  $Y_{ijklm}$  represents the  $m^{\text{th}}$  observation on the  $i^{\text{th}}$  Scan Date,  $j^{\text{th}}$  Time Point,  $k^{\text{th}}$  Treatment,  $l^{\text{th}}$   
 454 Subject. The common effect for the whole experiment is represented by  $\mu$ , and  $\varepsilon_{ijklm}$  represents  
 455 the random error present in the  $m^{\text{th}}$  observation on the  $i^{\text{th}}$  Scan Date,  $j^{\text{th}}$  Time Point,  $k^{\text{th}}$  Treatment,  
 456  $l^{\text{th}}$  Subject. The errors  $\varepsilon_{ijklm}$  are assumed to be normally and independently distributed with mean  
 457 0 and standard deviation  $\delta$  for all measurements. The symbols T, V, VT, and S(V) represent  
 458 effects due to time, vaccination type, treatment-by-time interaction, and subject-nested-within-  
 459 treatment, respectively. Vaccine type and time are fixed effects; scan date and subject are  
 460 random effects. Using this ANOVA model, gene expression data from 9 individuals from the  
 461 VEE vaccine group were contrasted against those from 10 individuals of the placebo vaccination  
 462 group (control group). The p-value for each condition was then corrected using the step-up false  
 463 discovery rate (FDR) multiple test correction with a cut-off value of 0.1 to produce the list of  
 464 significantly modulated genes (Table 1).<sup>45</sup> Contrasts between vaccinated and control subjects at  
 465 each time point were achieved using Fisher's Least Significant Difference (LSD) of  $\text{Log}_2$   
 466 transformed data and applying a further restriction of at least 2-fold change in gene expression  
 467 (either up or down).<sup>46</sup> Requests for the complete microarray data should be directed to the  
 468 corresponding author.

469 *Ingenuity Pathway Analysis:*

470 For the cellular pathway analysis, gene expression values for the significantly modulated  
 471 genes were imported into the Ingenuity Pathway Analysis (IPA) software to identify canonical  
 472 pathways associated with genes from the Ingenuity Pathways Analysis library.<sup>24</sup> The genes  
 473 associated with a canonical pathway were measured in two ways: 1) Ratio of the number of

474 genes from the data set that map to the pathway is displayed. The ratio provides the percentage  
475 of genes in the dataset that were part of a defined list of genes associated with a particular  
476 pathway. 2) Fisher's exact test was used to calculate a p-value, which expresses the probability  
477 that the association between the genes in the dataset and the canonical pathway can be explained  
478 by chance alone; highly significant p-values support an alternate hypothesis that suggests that the  
479 interaction is not due to random chance.

480

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482  
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492

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622       **Figure Legends**

623

624       Figure 1: Venn diagram depicting the number of transcripts that were differentially expressed at  
625       day 2, day 7, and day 14 post-immunization. The common and unique transcripts shown are  
626       indicative of those which were statistically significant (FDR-corrected Step-up p-value  $\leq 0.1$ ) as  
627       well meeting a minimum criteria of a twofold change in gene expression (either up or down)  
628       over baseline levels of expression.

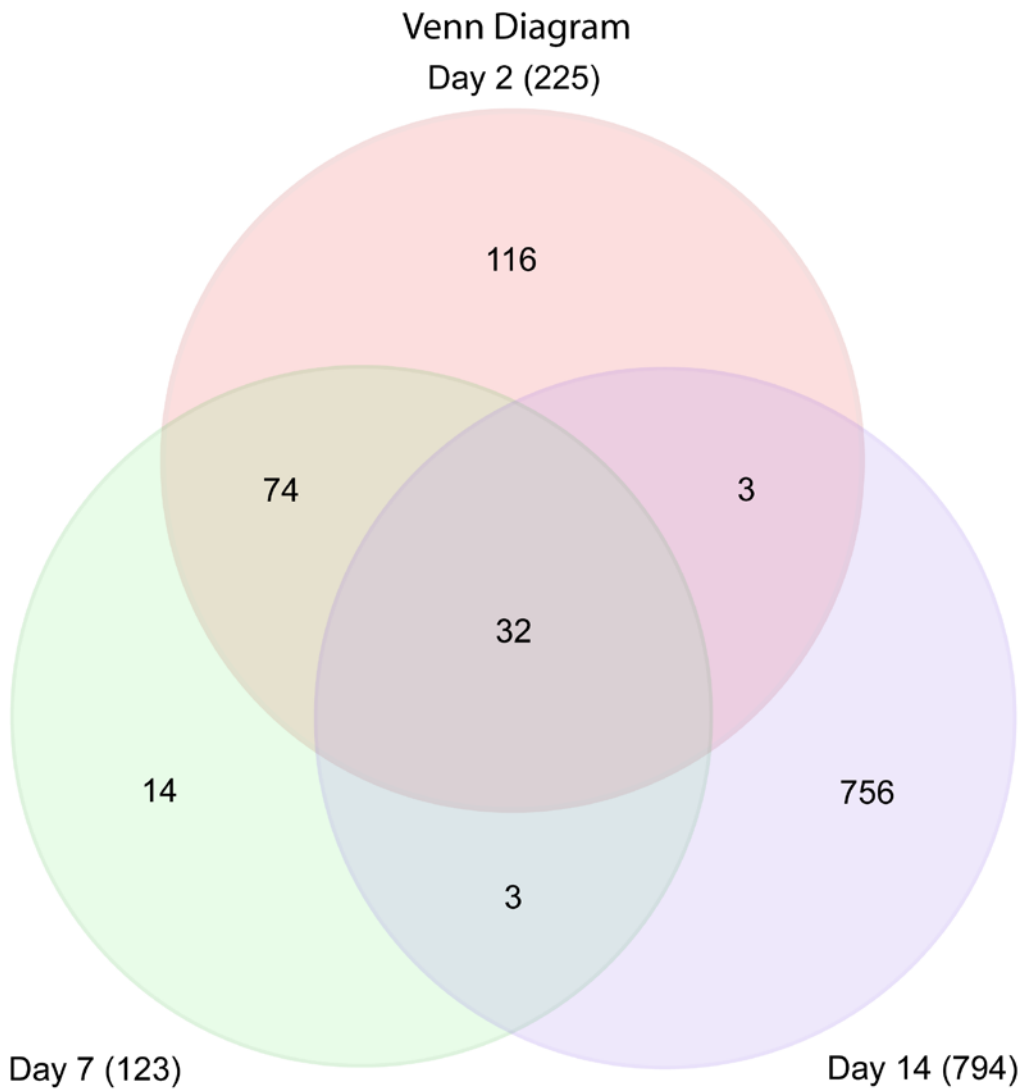
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630

631       Figure 2: Principal Component Analysis (PCA) Mapping of TC-83 *in vivo* vaccination  
632       microarray data. PCA, as a visual representation of the experimental conditions which elicit the  
633       greatest variability in the data, illustrated that the greatest differences in the data were due to the  
634       effect of the treatment, with the samples from vaccinees immunized with TC-83 showing the  
635       greatest diversity of gene expression. The second greatest factor which separates the data are the  
636       changes due to or that occur over time following vaccination. The remaining factor which  
637       describes the further diversification of data are the changes due to differences which are  
638       introduced by the intersection of time and treatment.

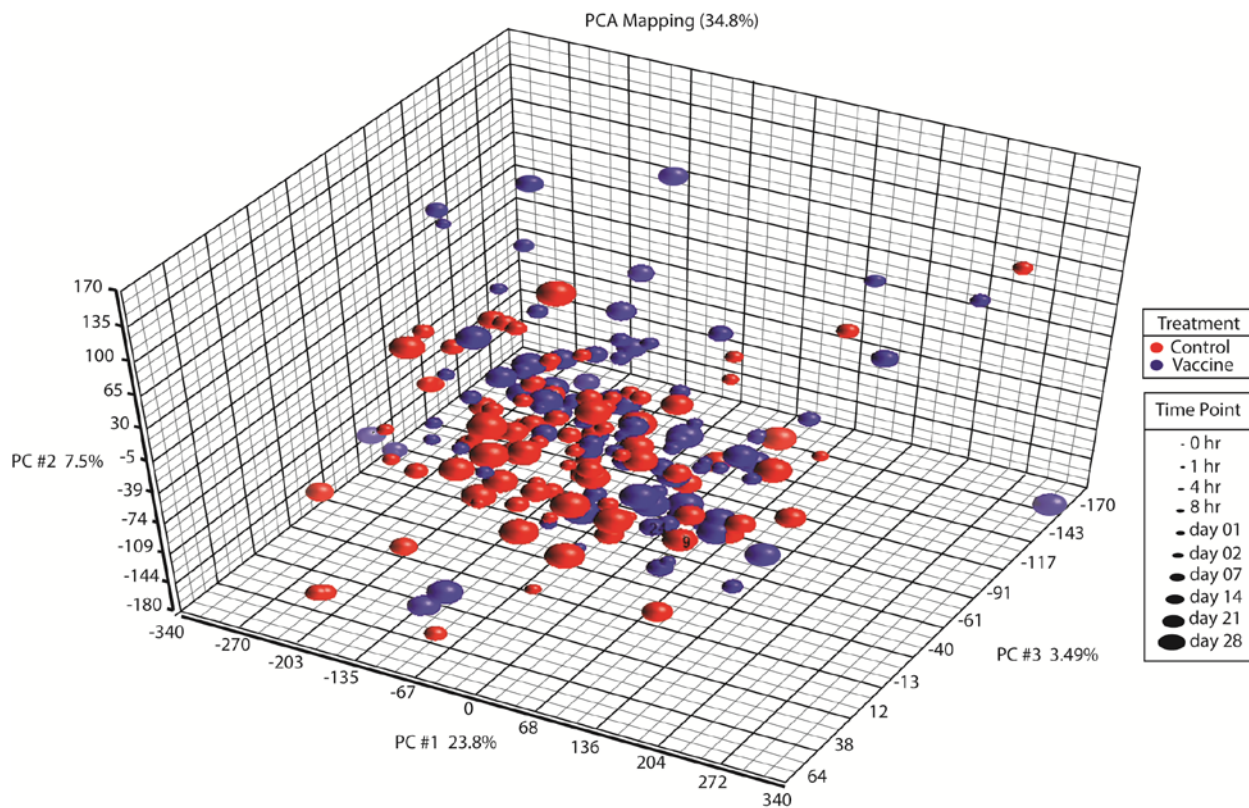
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640 Figure 1:  
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646 Figure 2: PCA mapping for *in vivo* experiments



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650 Table 1: FDR Report

651

<b>FDR Report</b>		
<b>Significance Level: 0.1; Total number of p-values: 54675</b>		
<b>Method: Step Up</b>		
<b>Variable Name</b>	<b>Cutoff Value</b>	<b># of Significant p-values</b>
p-value(Time Point)	1.49E-02	8,128
p-value(Treatment)	1.10E-05	6
p-value(Time Point * Treatment)	1.84E-02	10,055
p-value(0 h * Vaccine vs. 0 h * Control)	1.83E-06	0
p-value(1 h * Vaccine vs. 1 h * Control)	1.83E-06	0
p-value(4 h * Vaccine vs. 4 h * Control)	1.83E-06	0
p-value(8 h * Vaccine vs. 8 h * Control)	1.83E-06	0
p-value(day 1 * Vaccine vs. day 1 * Control)	1.83E-06	0
p-value(day 2 * Vaccine vs. day 2 * Control)	6.42E-03	3,511
p-value(day 7 * Vaccine vs. day 7 * Control)	7.75E-04	424
p-value(day 14 * Vaccine vs. day 14 * Control)	3.90E-02	21,343
p-value(day 21 * Vaccine vs. day 21 * Control)	1.83E-06	0
p-value(day 28 * Vaccine vs. day 28 * Control)	1.83E-06	0

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655 Table 2: Overview of Pathway Analysis Summary.  
656

Summary of IPA Analysis			
TOP BIO FUNCTIONS	VEE Day 2	VEE Day 7	VEE Day 14
<b>Diseases and Disorders</b>	Antimicrobial Response	Organismal Injury and Abnormalities	Immunological Disease
	Inflammatory Response	Antimicrobial Response	Hematological Disease
	Organismal Injury and Abnormalities	Inflammatory Response	Cancer
	Infection Mechanism	Infection Mechanism	Reproductive System Disease
<b>Molecular &amp; Cellular Function</b>	Infectious Disease	Genetic Disorder	Genetic Disorder
	Cellular Movement	Post-Translational Modification	Nucleic Acid Metabolism
	Cellular Development	Protein Folding	Cell-to-Cell Signaling and Interaction
	Cell-to-Cell Signaling and Interaction	Cellular Development	Cellular Compromise
	Post-Translational Modification	Lipid Metabolism	Gene Expression
<b>Physiology System Development &amp; Function</b>	Protein Folding	Molecular Transport	Molecular Transport
	Hematological System Development and Function	Endocrine System Development and Function	Tissue Development
	Immune Cell Trafficking	Hematological System Development and Function	Tumor Morphology
	Tissue Development	Hematopoiesis	Immune Cell Trafficking
	Skeletal and Muscular System Development and Function	Skeletal and Muscular System Development and Function	Nervous System Development and Function
<b>TOP CANONICAL PATHWAYS</b>	Hematopoiesis	Immune Cell Trafficking	Organ Morphology
	Interferon Signaling	Interferon Signaling	Oxidative Phosphorylation
	Activation of IRF by Cytosolic Pattern Recognition Receptors	Activation of IRF by Cytosolic Pattern Recognition Receptors	Protein Ubiquitination Pathway
	Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	Regulation of eIF4 and p70S6K Signaling
	Role of RIG1-like Receptors in Antiviral Innate Immunity	Pathogenesis of Multiple Sclerosis	RAN Signaling
IL-6 Signaling	Role of RIG1-like Receptors in Antiviral Innate Immunity	T Cell Receptor Signaling	
<b>Top Molecules - UP</b>	RSAD2, IFI44L, IFIT1, AMPK2, ISG15, LAMP3, IFI44, HERC5, MX1, OAS3 (includes EG:4940)	IFI27, RSAD2, IFI44L, IFI44, ISG15, CMPK2, IFIT1, OAS3 (includes EG:4940), HERC5, OAS1	IFI27, IGI, IGL@, IFI44, IGHM, IFI44L, RSAD2, TNFRSF17, TXNDC5, IGHA1
<b>Top Molecules - DOWN</b>	FCER1A, IL8, ITM2A, SGK1, GRAMD1C, IRS2, CLC, THBD, IGF1R, FAM101B	PI3, TUBB2A, EPB42, SLC4A1, SNCA, IGF1R, MARCH8, CPA3, FAM101B, CCR3	PI3, EPB42, TNS1, SLC4A1, TUBB2A, SELENBP1, SNCA, GMPR, KRT1, BLVRB,

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658  
659

660 Table 3: Top Canonical Pathways in response to Live-Attenuated VEEV (TC-83) Vaccination.

Top Canonical Pathways							
Pathway	Molecules	Day 2		Day 7		Day 14	
		Fold Change	p-value*	Fold Change	p-value*	Fold Change	p-value*
Interferon Signaling	IFI35	3.86	2.94E-14	2.84	9.43E-09	1.33	1.01E-01
	IFIT1	13.11	1.02E-06	10.95	2.53E-05	2.95	3.88E-02
	IFIT3	7.03	3.47E-08	7.34	2.57E-10	1.88	7.64E-02
	IFITM1	2.38	1.66E-07	2.35	1.30E-06	1.61	6.35E-03
	MX1	9.93	3.69E-09	6.89	4.17E-06	1.71	1.73E-01
	OAS1	7.18	2.32E-08	7.42	6.46E-08	2.71	7.01E-03
	SOCS1	2.43	5.90E-09	1.58	4.65E-02	-1.20	1.19E-01
Activation of IRF by Cytosolic Factors	DDX58	5.00	2.85E-09	2.79	1.86E-03	1.85	2.69E-02
	DHX58	2.00	9.01E-10	1.50	4.94E-03	1.03	8.26E-01
	IFIH1	4.50	7.41E-08	3.03	8.02E-04	2.36	4.12E-03
	IFIT2	5.54	1.67E-12	4.36	1.81E-04	1.89	6.96E-02
	IRF7	3.70	2.35E-09	3.68	1.85E-08	1.40	1.27E-01
	ISG15	11.39	3.77E-11	11.20	2.76E-10	2.66	1.07E-02
	ZBP1	3.94	1.32E-12	2.85	3.73E-09	1.72	3.11E-03
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	DDX58	5.00	2.85E-09	2.79	1.86E-03	1.85	2.69E-02
	EIF2AK2	3.27	8.67E-10	2.96	1.42E-07	1.35	1.25E-01
	IFIH1	4.50	7.41E-08	3.03	8.02E-04	2.36	4.12E-03
	IRF7	3.70	2.35E-09	3.68	1.85E-08	1.40	1.27E-01
	OAS2	5.35	4.31E-12	4.97	2.57E-10	2.24	1.42E-03
OAS3	9.07	3.35E-12	8.20	1.58E-10	2.27	1.22E-02	
Role of RIG1-like Receptors in Antiviral Innate Immunity	DDX58	5.00	2.85E-09	2.79	1.86E-03	1.85	2.69E-02
	DDX60	3.53	1.44E-04	4.43	1.18E-05	3.49	3.78E-04
	DHX58	2.00	9.01E-10	1.50	4.94E-03	1.03	8.26E-01
	TLR7	1.58	1.39E-06	1.12	7.93E-01	-1.10	3.08E-01
Interleukin-6 Signaling	IL8	-3.11	2.25E-02	1.36	8.85E-01	1.74	1.62E-01
	IL1RN	3.46	4.00E-09	2.28	1.20E-03	-1.19	5.68E-02
	TNFAIP6	3.87	2.39E-06	2.84	2.31E-03	1.53	2.19E-01
Communication between Innate and Adaptive Immune	CXCL10	3.79	1.31E-12	2.10	5.95E-04	1.21	3.10E-01
	IL1RN	3.46	4.00E-09	2.28	1.20E-03	-1.19	5.68E-02
TNFSF13B	2.48	1.70E-05	1.96	1.27E-02	1.35	2.47E-01	
JAK/STAT Signaling	SOCS1	2.43	5.90E-09	1.58	4.65E-02	-1.20	1.19E-01
	STAT1	2.67	1.59E-05	1.99	2.68E-02	1.57	1.89E-02
	STAT2	2.83	5.50E-09	2.19	1.10E-04	1.46	3.75E-02
Oxidative Phosphorylation	ATP5J	-1.30	4.94E-01	1.34	8.34E-01	2.20	9.53E-03
	COX7B	-1.40	5.53E-01	1.60	8.10E-01	2.20	6.43E-02
	COX7A2	-1.27	5.13E-01	1.43	7.64E-01	2.04	1.35E-02
	COX6C	-1.16	7.86E-01	1.80	6.65E-01	2.50	1.59E-02
	UQCRCB	-1.24	7.46E-01	1.67	8.08E-01	2.32	7.13E-02
	UQCQRH	-1.13	7.88E-01	1.46	7.79E-01	2.35	7.71E-03
	PPA1	-1.06	8.71E-01	1.44	6.94E-01	2.23	2.18E-03
	NDUFA6	-1.25	5.26E-01	1.48	7.12E-01	2.11	7.78E-03
	UQCRCQ	-1.18	7.41E-01	1.56	7.68E-01	2.22	2.44E-02
Protein Ubiquitination Pathway	PSMA3	1.17	7.02E-01	1.43	7.86E-01	2.42	5.05E-03
	UBR1	-1.27	5.23E-01	1.16	9.30E-01	2.48	3.00E-03
	USP1	-1.74	7.37E-02	1.06	9.70E-01	2.32	8.32E-03
	UBE3A	-1.54	1.91E-01	1.18	8.48E-01	2.07	7.17E-03
	USP53	-1.08	6.88E-01	1.21	9.10E-01	2.28	9.92E-03
	PSMC6	-1.72	3.37E-01	1.20	9.48E-01	2.71	2.57E-02
	USP47	-1.56	1.99E-01	1.21	8.97E-01	2.11	8.24E-03
	USP16	-1.64	1.67E-01	1.22	8.66E-01	2.22	6.24E-03
	PSMA4	1.10	8.61E-01	1.69	7.09E-01	2.54	1.08E-02
	HSP90AA1	-1.02	9.78E-01	1.51	7.94E-01	2.91	4.74E-03
	BIRC3	-1.50	2.52E-01	1.09	9.63E-01	2.12	1.62E-02
	BIRC2	-1.38	4.13E-01	1.08	9.70E-01	2.22	5.80E-03
ERK5 signaling	YWHAQ	-1.25	4.08E-01	1.20	8.27E-01	2.27	4.00E-04
	IL6ST	-1.43	2.98E-01	1.24	8.51E-01	2.07	8.76E-03
	RP56KB1	-1.58	1.82E-01	1.09	9.15E-01	2.11	7.34E-03
	NRAS	-1.24	4.26E-01	1.21	7.60E-01	2.08	1.65E-03
	RRAS2	-1.39	2.32E-01	1.17	8.92E-01	2.31	4.53E-04
	ATF2	-1.48	2.86E-01	1.04	9.27E-01	2.32	5.48E-03
Natural Killer Cell Signaling	FYN	-1.45	1.65E-01	1.22	7.18E-01	2.09	1.44E-03
	PRKCI	-1.31	3.57E-01	1.26	7.35E-01	2.04	3.45E-03
	NRAS	-1.24	4.26E-01	1.21	7.60E-01	2.08	1.65E-03
	RRAS2	-1.39	2.32E-01	1.17	8.92E-01	2.31	4.53E-04
	PIK3C2A	-1.57	1.79E-01	1.23	6.55E-01	2.03	9.01E-03
	KLRK1	-1.11	7.89E-01	1.21	8.78E-01	2.01	8.80E-03
B Cell Development	IL7R	nd	nd	nd	nd	2.13	4.13E-03
	IGKC	-1.02	9.57E-01	1.04	9.79E-01	2.44	6.13E-04
	IGL@	1.30	8.19E-02	1.29	8.52E-01	6.01	2.87E-12
	IGHM	-1.48	2.48E-01	-1.28	8.48E-01	4.99	7.60E-04
T Cell Receptor Signaling	FYN	-1.45	1.65E-01	1.13	9.13E-01	2.09	1.44E-03
	CD28	-1.57	1.72E-01	1.05	9.79E-01	2.57	9.73E-04
	CAMK4	-1.86	5.56E-02	1.04	9.83E-01	2.10	4.89E-03
	RRAS2	-1.39	2.32E-01	1.17	8.92E-01	2.31	4.53E-04
	PI3KC2A	-1.57	1.79E-01	-1.03	9.88E-01	2.03	9.01E-03
	RASGRP1	-1.48	1.70E-01	1.23	8.50E-01	2.44	3.48E-04
	CD3D	-1.14	7.40E-01	1.50	7.02E-01	2.24	4.84E-03
	ITK	-1.44	1.83E-01	1.15	9.04E-01	2.12	1.44E-03

\*p-values shown are the FDR-corrected Step up values.  
nd = not detected

662 Table 4: Identification of Biomarkers following Vaccination with Live-Attenuated (TC-83)  
 663 VEEV  
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Table 4: Top Biomarkers							
Gene symbol	Gene Name	Cellular Location	Fold Change	Day 2	Day 7	Day 14	Common
GBP4	guanylate binding protein 4	Cytoplasm	3.5	X			
MT1X	metallothionein 1X	unknown	3.2	X			
ANKRD22	ankyrin repeat domain 22	Nucleus	3.2	X			
CCL2	chemokine (C-C motif) ligand 2	Extracellular Space	3.1	X			
BST2	bone marrow stromal cell antigen 2	Plasma Membrane	3.0	X			
LIPA	lipase A, lysosomal acid, cholesterol esterase	Cytoplasm	2.8	X			
TRIM14	tripartite motif containing 14	Cytoplasm	2.3		X		
CCR1	chemokine (C-C motif) receptor 1	Plasma Membrane	2.2		X		
SMA5	glucuronidase, beta pseudogene	unknown	2.2		X		
PPM1K	protein phosphatase, Mg2+/Mn2+ dependent, 1K	Cytoplasm	2.1		X		
SHISA5	shisa homolog 5 (Xenopus laevis)	Nucleus	2.0		X		
C18orf49	chromosome 18 open reading frame 49	unknown	2.0		X		
IGJ	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	Extracellular Space	6.8			X	
IGL@	immunoglobulin lambda locus	Nucleus	6.0			X	
IGHM	immunoglobulin heavy constant mu	Plasma Membrane	5.0			X	
TNFRSF17	tumor necrosis factor receptor superfamily, member 17	Plasma Membrane	4.1			X	
TXNDC5	thioredoxin domain containing 5 (endoplasmic reticulum)	Cytoplasm	4.0			X	
NDUFA5	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5, 13kDa	Cytoplasm	3.4			X	
CMPK2	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	Cytoplasm	3.5 to 11.9				X
RSAD2	radical S-adenosyl methionine domain containing 2	Cytoplasm	4.2 to 29.2				x
DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	unknown	3.5 to 4.4				X
EPSTI1	epithelial stromal interaction 1 (breast)	unknown	4.1 to 8.2				X
HERC5	hect domain and RLD 5	Cytoplasm	2.2 to 10.2				X
LY6E	lymphocyte antigen 6 complex, locus E	Plasma Membrane	2.4 to 6.5				X
RTP4	receptor (chemosensory) transporter protein 4	Plasma Membrane	2.2 to 4.3				X
XAF1	XIAP associated factor 1	Nucleus	2.3 to 6.2				X

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669 Table 5: HLA phenotype and post vaccination titer of Study Volunteers

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Treatment	HLA-DRB1 Phenotype	HLA-DQB1 Phenotype	Day 28 Post-TC-83 Vaccination Titer
Control 1	0401/1501	0302/0602	<10
Control 2	0401/0701	0301/0319 / 0202	<10
Control 3	0301/0701	0201/0202	<10
Control 4	0701/1302	0202/0302	<10
Control 5	0101/0701	0501/0303	<10
Control 6	0301/1602	0201/0502	<10
Control 7	0401/0701	0302/0202	<10
Control 8	0101/0701	0501/0303	<10
Control 9	0401/0701	0301/0319 / 0202	<10
Control 10	0302/1503	0402/0602	<10
Vaccinee 1	1101/1302	0301/0319 / 0604/0634	<10*
Vaccinee 2	0402/0701	0302/0202	20
Vaccinee 3	0301/0401	0202/0602	40
Vaccinee 4	0402/0701	0302/0202	80
Vaccinee 5	0801/1501	0402/0602	160
Vaccinee 6	1101/1302	0301/0319 / 0609	160
Vaccinee 7	0401/0701	0202/0302	320
Vaccinee 8	0701/1501	0202/0602	320
Vaccinee 9	0701/1401	0303/0503	1280
Vaccinee 10	1501	0602	1280

\* Titer repeated at Day 56; Subject confirmed as NonResponder

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672 \*Day 56 titer indicates that Vaccinee 1 was a Nonresponder.

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675 Supplemental Data Table 1: FDR Report for ANOVA analysis with the added variable of  
 676 vaccine response

FDR Report		
Significance Level: 0.1; Total number of p-values: 54675		
Method: Step Up		
Variable Name	Cutoff Value	# of Significant p-values
p-value(Time Point)	1.24E-02	6795
p-value(Treatment)	3.66E-06	2
p-value(Vaccine Response(Treatment))	1.83E-06	0
p-value(Time Point * Treatment)	1.72E-03	939
p-value(Time Point * Vaccine Response(Treatment))	3.66E-06	2
p-value(Vaccine * 0 hr * High and Control * 0 hr * Control vs. Vaccine * 0 hr * Low and Control * 0 hr * Control)	1.83E-06	0
p-value(Vaccine * 1 hr * High and Control * 1 hr * Control vs. Vaccine * 1 hr * Low and Control * 1 hr * Control)	1.83E-06	1
p-value(Vaccine * 4 hr * High and Control * 4 hr * Control vs. Vaccine * 4 hr * Low and Control * 4 hr * Control)	1.83E-06	0
p-value(Vaccine * 8 hr * High and Control * 8 hr * Control vs. Vaccine * 8 hr * Low and Control * 8 hr * Control)	1.28E-05	7
p-value(Vaccine * day 01 * High and Control * day 01 * Control vs. Vaccine * day 01 * Low and Control * day 01 * Control)	1.83E-06	0
p-value(Vaccine * day 02 * High and Control * day 02 * Control vs. Vaccine * day 02 * Low and Control * day 02 * Control)	1.83E-06	1
p-value(Vaccine * day 07 * High and Control * day 07 * Control vs. Vaccine * day 07 * Low and Control * day 07 * Control)	1.04E-04	57
p-value(Vaccine * day 14 * High and Control * day 14 * Control vs. Vaccine * day 14 * Low and Control * day 14 * Control)	2.38E-05	13
p-value(Vaccine * day 21 * High and Control * day 21 * Control vs. Vaccine * day 21 * Low and Control * day 21 * Control)	1.83E-06	0
p-value(Vaccine * day 28 * High and Control * day 28 * Control vs. Vaccine * day 28 * Low and Control * day 28 * Control)	1.83E-06	0

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- 679 Supplemental Data Table 2: 5-Way ANOVA\_Time-Treatment vs Titer (See attached Excel file,  
680 file is too large to embed).
- 681 Supplemental Data Table 3: MicroRNA Target Filter List for VEEV Vax Days 2-7-14  
682 (Attached).