Title: Age-specific dynamics of neutralizing antibodies, cytokines, and chemokines in response to La Crosse virus infection in mice

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Running Title: Age-specific cytokines and chemokines following LACV infection

Abstract

La Crosse virus (LACV) is a primary cause of pediatric arboviral encephalitis in the United States, with a notable impact on children aged 16 years or younger. This age-related susceptibility extends to murine models, where weanling mice (≤3 weeks old) succumb to LACV-induced disease, while adults (≥6 weeks old) demonstrate resistance. Despite its clinical significance, our understanding of host responses to LACV remains relatively unexplored. This study aims to elucidate the dynamics of neutralizing antibodies (nAbs), cytokines, and chemokines following LACV infection in both weanling and adult mice. Utilizing a highly infectious dose of LACV, our study reveals age-specific variations in viral titers, neutralizing antibody titers, and survivability. Weanling mice exhibit an early disease onset coupled with heightened peripheral viremia and nAbs compared to adults. Serum cytokine and chemokine profiling showed distinct kinetics and age-specific responses. Notably, adult mice exhibit significantly elevated levels of Th1 cytokines (IFN-γ, IL-18, GM-CSF, IL-1β and IL-12), juxtaposed with elevated levels of Th2 cytokines (IL-6 and IL-4) observed in weanling mice, often coinciding with the onset of symptoms. Furthermore, the heightened levels of Th9/Th17/Th22/Treg cytokines (IL-9, Il-10, IL-17A, IL-22, IL-23, and IL-27) and chemokines (CCL11, CCL3, and CCL5) in infected adult mice underscores their role in mounting protective immune responses against LACV. In conclusion, our study associates cytokines and chemokines with protective immunity in adult mice, contrasting with the pathogenesis observed in weanlings. This work emphasizes the need for further studies into mechanisms of innate and adaptive immune responses to LACV infection.

43

44 **Introduction**

45 La Crosse Virus (LACV) is a single-stranded, negative-sense RNA virus
46 belonging to the *Peribunyaviridae* family in the order *Bunyavirales* (1). Its transmission
47 to humans primarily occurs through the bite of infected Eastern Tree Hole mosquitoes
48 (*Aedes triseriatus*), although other mosquito species may harbor LACV, potentially
49 contributing to the emergence of disease (2). Upon entry into the host, LACV initially
50 replicates in skin muscle cells, facilitating its spread into various tissues and organs (3,
51 4). While the majority of LACV-infected individuals manifest only mild, febrile symptoms,
52 a subset experiences severe neurological complications, including altered behavior,
53 seizures, coma, or fatality, as the virus invades the central nervous system (CNS) (5).
54 Notably, severe disease and neurological manifestations of LACV are predominantly
55 observed in children under the age of 16 years, indicating a higher susceptibility among
56 this age group (6).

57 The clinical significance of LACV, coupled with its ability to also induce disease in
58 murine models, has designated it as a prototype for studying viruses within the
59 *Peribunyaviridae* family. Weanling mice (≤3 weeks old) exhibit susceptibility to LACV
60 infection, whereas adult mice (≥6 weeks old) display resistance, mirroring the age-
61 related susceptibility observed in humans (3, 7, 8). Unraveling the intricacies of host
62 responses to LACV infection is important, particularly in delineating factors contributing
63 to disease resistance or susceptibility. It is believed that the peripheral immune system's
64 control of LACV access to the CNS plays a pivotal role in disease resistance in adult
65 mice (9).

66 Prior investigations have shed light on the significance of both innate and
67 adaptive immune responses following LACV infection (9, 10). Type I interferon
68 responses have garnered considerable attention, demonstrating their indispensable role
69 in curbing LACV replication and mitigating disease severity (11). Early cellular innate
70 immune responses, particularly those imparted by myeloid dendritic cells, proved critical
71 for protection in wild type adult mice infected with LACV (10). The same study also
72 showed that treatment of young wild type mice with type I interferon, particularly IFN-β
73 resulted in protection mediated by myeloid dendritic cells (10). Moreover, the depletion
74 of adaptive immune responses, including both B and T cells, resulted in neurological
75 disease in adult mice infected with LACV, suggesting that these cell types are essential
76 for protection against neuropathology and death (9). However, the broader immune
77 response including cytokines and chemokines remains relatively unexplored. These
78 signaling molecules, produced by various cell types in response to viral infections,
79 orchestrate immune responses and exert influences on disease outcomes (12-15).
80 Thus, deciphering the dynamics of cytokines and chemokines during LACV infection is
81 important for unraveling their contributions to age-specific protection or pathogenesis.

82 Cytokines and chemokines undergo substantial modulation upon LACV infection
83 across diverse cell types. Notably, human neuron-astrocyte co-cultures respond to
84 LACV infection in vitro within 48 hours post infection by eliciting robust proinflammatory
85 cytokines and chemokines including: IFN-γ, IL-6, TNF-α, IL-8, CCL4, CCL5, CXCL10
86 (16). In a separate study, glial cells derived from the brains of young LACV-infected
87 mice produced significant levels of IFN-β (17). Furthermore, preliminary observations
88 hint at a proinflammatory cytokine response in both the periphery and the brain

89 following LACV infection, with elevated levels of IL-6 and IL-12p40, which were reported
90 to be in comparable levels in young and adult mice (10, 18). Interestingly, administration
91 of IL-12 or GM-CSF encoded by a plasmid prior to LACV infection in Ifnar1$^{-/-}$ mice was
92 shown to increase survival rates, suggesting a potential protective role of these
93 cytokines (19). A similar proinflammatory cytokine profile has also been reported with
94 Oropouche virus, a distantly related bunyavirus (20). Cytokines and chemokines seem
95 to act as important contributing factors in the immune response to LACV and other
96 bunyavirus infections.

97 Given the important implications of cytokines and chemokines during LACV
98 infection, this study aims to address a knowledge gap by investigating the involvement
99 of these molecules in age-related susceptibility to LACV infection and their correlation
100 with other components of peripheral immune responses such as neutralizing antibodies
101 (nAbs). To address this knowledge gap, we present an in-depth analysis of cytokine and
102 chemokine levels in the sera of adult and weanling mice throughout the course of a
103 highly infectious dose of LACV infection. We identify age-specific differences in cytokine
104 and chemokine profiles between adult and weanling mice. Moreover, we establish
105 correlations between cytokine and chemokine levels and indicators of viral clearance
106 and neutralizing antibody responses.

107

108 **Results**

109 **Age-related susceptibility is maintained despite a highly infectious dose of LACV**
110 **infection**

111 An unpublished observation reported no significant difference in cytokine levels
112 between adult and weanling mice infected with a 1000 PFU dose of LACV (10). To
113 investigate the potential impact of infectious dose on immune system responsiveness to
114 LACV infection, we hypothesized that a higher dose might better reveal differences in
115 cytokine levels if they exist. Previous studies have shown that infection with 10^5 PFU of
116 LACV maintains age-related susceptibility in mice (10). Here, we aimed to assess the
117 effects of an infectious dose five-fold higher than the previously reported dose by
118 monitoring the development of neurological signs in infected mice and measuring viral
119 titers in peripheral blood.

120 Administration of a $5x10^5$ PFU dose of LACV demonstrated a similar difference in
121 survivability between adult and weanling mice, reinforcing the age-related susceptibility
122 to LACV. Adult mice exhibited markedly higher survival rates compared to weanling
123 mice ($p < 0.001$), with approximately 50% of adults surviving up to 12 days post-
124 infection (dpi) (Fig. 1A). Weanling mice began exhibiting signs of neurological disease
125 starting at 4-5 dpi (Fig. 1B). Our findings show that the higher dose of LACV infection in
126 adult and weanling mice results in a timeline of symptom/disease onset like that of lower
127 infectious doses that have historically been used (Fig. 1B). Consistent with previous
128 reports, viral titers in sera were detectable between 1-3 dpi in both adult and weanling
129 mice, with levels significantly higher in weanling mice at 2 dpi (Fig. 1C) (3). This
130 observation underscores the retention of age-related susceptibility to LACV even at
131 higher infectious doses, reaffirming the correlation between viremia and survivability.

132

133

134 **Neutralizing antibody titers do not correlate with viral clearance in weanling mice**

135 nAb responses are pivotal for viral clearance in many infections, yet our findings
136 reveal an intriguing disparity between nAb titers and viral clearance in weanling mice.
137 Following infection with a highly infectious dose of LACV, nAbs peak in adult mice
138 between 8 and 12 dpi (Fig. 2A, 2C), whereas they peak earlier in weanling mice
139 between 4 and 6 dpi (Fig. 2B, 2C), coinciding with the onset of neurological symptoms.
140 Despite the robust early nAbs responses in weanling mice, 100% succumbed to the
141 infection by 7 dpi (Fig. 1A). These results suggest that viral clearance and the onset of
142 LACV-induced neurological signs may not directly correlate with the nAb responses.

143

144 **Cytokines and chemokines are differentially regulated in adult and weanling mice**

145 To elucidate how serum cytokine and chemokine levels are modulated during
146 LACV infection in adult and weanling mice, we conducted a Luminex panel capable of
147 simultaneously detecting and quantifying Th1/Th2/Th9/Th17/Th22/Treg cytokines and
148 chemokines. Serum samples from adult mice and weanling mice were assessed,
149 revealing significant shifts in 21 cytokines in LACV-infected mice compared to the un-
150 infected phase (0 dpi) (Fig. 3). Notably, elevated levels of all cytokines and chemokines
151 were observed in response to LACV infection in adult and weanling animals, albeit with
152 varying levels, highlighting the importance of a chronological analysis to fully grasp the
153 dynamics of LACV-induced cytokine and chemokine production (Fig. 3).

154 To delineate the potential roles of serum cytokines and chemokines in protection
155 or pathogenesis at different infection stages, we classified the course of infection into

156 three phases: (1) peripheral viremia (1-3 dpi), (2) symptoms onset (4-6 dpi), and (3)
157 survival (>7 dpi). Additionally, we categorized the analyzed cytokines and chemokines
158 into three groups: Th1/Th2 cytokines, Th9/Th17/Th22/Treg cytokines, and chemokines.
159 Among the 21 modulated cytokines and chemokines, 9 belonged to the Th1/Th2
160 cytokine group (GM-CSF, IFN-γ, IL-1β, IL-4, IL-6, IL-12p70, IL-13, IL-18, TNF-α, 6 were
161 classified under the Th9/Th17/Th22/Treg cytokine group (IL-9, IL-10, IL-17A/CTLA-8, IL-
162 22, IL-23, IL-27), and the remaining 6 were identified as elevated chemokines in LACV-
163 infected mice (Eotaxin/CCL11, GROα/CXCL1, IP-10/CXCL10, MCP-1/CCL2, MIP-
164 1α/CCL3, RANTES/CCL5.

165

166 **Dynamics of Th1/Th2 Cytokines during LACV Infection**

167 IFN-γ, IL-18, TNF-α, and IL-13 emerged as the predominant Th1/Th2 cytokines
168 detected in serum samples from both adult and weanling mice (Fig. 3-4). During the
169 peripheral viremia phase, a significant elevation in IL-18 levels was observed as early
170 as 1 dpi, followed by subsequent significant peaks in IL-12p70 and GM-CSF in adult
171 mice (Fig. 4A, 4F, 4H). Notably, levels of IL-18 and IL-12p70 peak during this early
172 phase before gradually declining as the infection progresses. Conversely, weanling
173 mice display a notable significant increase in levels of IL-6 towards the latter part of the
174 peripheral viremia phase, one day prior to symptoms onset (Fig. 4E).

175 At the symptoms onset phase, occurring between 4-6 dpi, several cytokines
176 including IFN-γ, IL-1β, TNF-α, GM-CSF, IL-13, and IL-4 were significantly elevated in
177 both adult and weanling mice (Fig. 4A-D, 4G, 4I). Most cytokines reach their peak levels

178 during this phase, with adult mice exhibiting higher levels of IL-1β, TNF-α, GM-CSF, IL-
179 13, and IFN-γ compared to weanlings. Conversely, IL-4 and IL-6 were significantly
180 elevated in weanlings compared to adult mice (Fig. 4D-E). Interestingly, IL-6 levels in
181 adult mice remained comparable to those during the uninfected phase (Fig. 4E). Finally,
182 during the survival phase (≤>7 dpi) IFN-γ and IL-13 emerged as the primary cytokines
183 specifically elevated during this late phase of infection in adult mice.

184

185 **Dynamics of Th9/Th17/Th22/Treg Cytokines during LACV Infection**

186 A highly infectious dose of LACV revealed elevated levels across all
187 Th9/Th17/Th22/Treg cytokines analyzed in both adult and weanling (Fig. 5). Notably, IL-
188 9, IL-10, and IL-17A emerged as the most abundant cytokines within this category
189 throughout the infection course in both age groups (Fig. 3). During the early peripheral
190 viremia phase, peaks in IL-9 and IL-17A levels underscored their early involvement in
191 the immune response to LACV infection in adult and weanling mice (Fig. 5A, 5C).
192 Additionally, at 2 dpi, IL-23 levels were significantly higher in adult mice compared to
193 weanlings, mirroring the kinetics of IL-17A, which exhibited significantly elevated levels
194 in adult mice by 3 dpi (Fig. 5C, 5E).

195 During the symptoms onset phase, peaks in IL-22, IL-23, and IL-27 levels were
196 observed, with a notable increase observed in adult mice (Fig. 5D-F). In contrast, IL-10
197 levels exhibited a significant increase in weanling mice at 4 dpi, followed by a rapid
198 decline one day later, reaching levels significantly lower than those in adult mice. By 6

199 dpi, Th9/Th17/Th22/Treg cytokine levels gradually decreased in adult mice, ultimately
200 returning to baseline levels.

201

202 **Dynamics of Chemokines during LACV Infection**

203 A highly infectious dose of LACV also triggers a significant increase in CXCL1
204 and CCL11, followed by CXCL10, in both adult and weanling mice compared to
205 uninfected controls (Fig. 3, 6A-C). This increase manifests as early as 1 dpi for CCL11
206 and CXCL1. Particularly, CCL11, CCL3, and CCL5 consistently exhibit higher levels in
207 adult mice compared to weanlings throughout the infection course (Figure 6A, 6E, 6F).
208 Intriguingly, CCL3 and CCL5 levels peak during the survival phase in adult mice,
209 suggesting sustained chemokine responses during later phases of infection.

210 Conversely, distinct chemokine patterns were observed in weanling mice, with
211 certain chemokines showing pronounced elevation whiles others exhibit minimal levels.
212 For example, CCL2 and CXCL10 levels were consistently higher in weanling mice
213 across all infection phases, whereas CXCL1 levels peak exclusively during the
214 symptoms onset phase (Fig. 6B, 6D). CCL5 and CCL3 levels in weanlings remain
215 comparable to baseline levels observed prior to infection (Figure 6E- F).

216

217 **Discussion**

218 Our study presents a comprehensive analysis of neutralizing antibody titers and
219 cytokine and chemokine dynamics during a highly infectious dose of LACV infection,

220 shedding light on their roles in protection and pathogenesis. Our findings demonstrate
221 distinct patterns of cytokines and chemokines specific to age and infection phase.
222 Notably, we observed significant increases in Th1/Th2/Th9/Th17/Th22/Treg cytokines
223 and chemokines in response to LACV infection across both adult and weanling mice.
224 Interestingly, we observed age-specific differences in many cytokines and chemokines
225 throughout the course of infection (Fig. 7).

226 Our data emphasize the consistent influence of age-related susceptibility,
227 regardless of the infecting dose. Despite prior indications of age-related vulnerability
228 using lower infectious doses of LACV, our findings expand on this concept by
229 demonstrating that age remains a pivotal determinant of susceptibility, even when using
230 highly infectious doses for infections (3). Specifically, we observed that weanling mice
231 infected with a highly infectious dose exhibited elevated peripheral viral load along with
232 neutralizing antibodies (nAbs) with an early onset of neurological disease. This
233 underscores the correlation between peripheral viremia and survivability, highlighting
234 the relevance of our model for investigating both immune response dynamics and
235 disease pathology.

236 Our investigation into viral neutralization yielded unexpected findings. Despite the
237 early and robust production of nAbs in weanling mice, these titers failed to correlate with
238 viral clearance, prompting speculation regarding the role of nAbs in early disease
239 progression, Notably, different neutralization capabilities have been associated with
240 isotype switching, a phenomenon documented in other viral infections such as HIV and
241 SARS-CoV-2 (21, 22). Interestingly, adult mice exhibited significantly lower nAb titers
242 during the symptoms onset phase, suggesting either rapid nAbs consumption in vivo or

243 the involvement of non-neutralizing antibodies in protection. However, given that
244 weanling nAbs were incapable of effectively eliminating the virus in vivo, the former
245 explanation seems less likely.

246 A possible explanation for these unexpected findings may stem from an
247 imbalance in the Th1/Th2 response among weanling mice, impacting viral clearance
248 and disease progression. Upon encountering pathogens, naive $CD4^+$ T cells
249 differentiate into effector Th1 and Th2 cells, with Th1 cells producing inflammatory
250 cytokines crucial for cell-mediated immune responses, while Th2 cells facilitate B cell
251 activation and antibody production (23). An imbalanced response can lead to ineffective
252 immunity and contribute to pathogenesis. Thus, a Th2-skewed response may account
253 for disparities in the role of nAbs. Further exploration of immune response dynamics,
254 particularly the balance between Th1 and Th2 responses, holds promise for elucidating
255 the mechanisms underlying our findings.

256 To advance this research direction, it is essential to conduct a thorough
257 characterization of not only Th1/Th2 cytokines but also Th9/Th17/Th22/Treg cytokines
258 and chemokines during LACV infection. The investigation of cytokine profiles becomes
259 particularly crucial in the context of age-dependent viral infections infiltrating the central
260 nervous system (CNS) (24). This study demonstrated that cytokine and chemokine
261 profiles in children and adolescents with tick-borne encephalitis vary depending on age
262 (24). Such variability in cytokine and chemokine responses may significantly influence
263 the outcome of viral infections and the subsequent risk of severe disease outcomes.

264 In the case of LACV infection, whether increased cytokines and chemokines are
265 a correlate of protection or pathogenesis remains to be fully investigated. However, in
266 most cases, an increase in specific cytokines at particular phases of infection in
267 susceptible hosts, such as weanling mice, may suggest a potential contribution of these
268 pathways to susceptibility and disease pathogenesis. Conversely, the increase in
269 cytokines and chemokines in disease-resistant hosts, such as adult mice, may indicate
270 a protective role facilitated by these molecules.

271 Our study reveals distinct patterns of Th1/Th2 cytokines around symptoms onset
272 during LACV infection in weanling mice. Particularly intriguing is the role of IL-6, a key
273 factor in determining the balance between Th1 and Th2 responses. Our data suggest
274 that IL-6, along with IL-4, promotes Th2-related responses during symptoms onset,
275 correlating with higher nAb levels in weanling mice, supporting the Th2 skey hypothesis
276 (25). Additionally, the substantial and sustained increase in Th1-related cytokines in
277 adult mice underscores their protective role against disease, consistent with previous
278 observations (10, 19).

279 Moreover, our findings in the context of Th1/Th2 cytokines are consistent with
280 previous studies on CNS viral infections. For instance, elevated IL-6 levels in the CNS
281 in infections with dengue and Japanese encephalitis have been associated with various
282 neurological manifestations and poor outcomes (26, 27). On the contrary, our
283 observation of the substantial and sustained upregulation of Th1-related cytokines,
284 including IL-18, IFN-γ, IL-12, and GM-CSF, throughout LACV infection supports
285 previously reported data of protective roles of GM-CSF and IL-12 in Ifnar1$^{-/-}$ mice and

286 underscores their role in eliciting protective responses in mice resistant to the disease
287 (19).

288 Among these cytokines, the pronounced elevation of IL-18 in adult mice stands
289 out as the most significant modulation induced by LACV. IL-18 has been demonstrated
290 to stimulate the proliferation and differentiation of T cells toward a Th1 response and
291 acts as a crucial cofactor in IL-12-induced IFN-γ production by natural killer (NK) and T
292 cells (28). Moreover, IL-18 exerts its proinflammatory effects by upregulating cytokines
293 such as IL-13, IL-1β, and TNF-α, consistent with our findings (29). The protective role of
294 IL-18 during viral infections is well-documented, as evidenced by studies showing that
295 administration of IL-18 induces antiviral activities in mice challenged with a lethal dose
296 of encephalomyocarditis virus (EMCV) (30). These findings highlight the importance of
297 IL-18 in mounting effective immune responses against LACV infections and suggest its
298 potential as a therapeutic target for enhancing antiviral immunity. Furthermore, IFN-γ
299 emerged as one of the most robust responses, particularly increased in adult mice,
300 especially during the survival phase. Our findings align with previous observations
301 indicating increased IFN-γ production from neuron/astrocyte co-cultures infected with
302 LACV in a dose-dependent manner (16). IFN-γ serves not only as a marker of Th1
303 $CD4^+$ and $CD8^+$ T cells and NK cells but also as a crucial antiviral mediator central to
304 eliminating viruses from the CNS (31).

305 In our analysis of Th9/Th17/Th22/Treg cytokines, all exhibited increased levels in
306 adult mice in response to LACV infection. Among these, IL-23 and IL-27 were among
307 the most significantly increased. Although these cytokines have been predominantly
308 studied in the context of autoimmune diseases, emerging evidence suggests their

309 crucial roles during viral infections (32, 33). Specifically, IL-23 has been implicated in the
310 control of viral infections within the brain, indicating its potential importance in host
311 defense mechanisms against neurotropic viruses such as LACV (32). Similarly, IL-27
312 has been found to mediate NK cell effector functions during viral infections, highlighting
313 its involvement in orchestrating innate immune responses against viral pathogens (33).

314 Chemokines, as a distinct group of immune mediators, demonstrated elevated
315 levels in response to LACV infection, with increases observed in CCL5, followed by
316 CCL3, in adult mice. In contrast, CCL2 increased in weanling mice throughout the
317 course of infection. The expression and release of CCL2 and CCL3 on cerebral
318 endothelium suggest an essential role for these chemokines in regulating the trafficking
319 of inflammatory cells across the blood-brain barrier (BBB) during CNS inflammation
320 (34). Furthermore, CCL3 has been shown to contribute to the differentiation and
321 migration of effector T cells in response to viral infection within the CNS (35).
322 Additionally, CCL5 plays a crucial role in the homing and migration of effector and
323 memory T cells during acute CNS infections (36). CCL2 emerges as a major player in
324 mediating monocyte infiltration and the trafficking of inflammatory monocytes to the
325 brain during viral encephalitis (37). Our findings, indicating increased levels of CCL2 in
326 weanling mice, which by logic, potentially contributes to pathogenesis possibly mediated
327 through the recruitment of an overwhelming inflammatory response in the infected brain.
328 However, LACV-induced lymphocyte infiltration of CNS itself is less likely to contribute
329 to pathogenesis according to a recent study (9). With this study being one of the few to
330 focus specifically on LACV infections, significant gaps persist in our understanding of
331 immunity correlates and the mechanisms underlying immune-mediated viral clearance.

332 More often than not, successful clearance of virus infection from the CNS requires the
333 elimination of virus from the cells without causing damage to neurons. Ideally, immune-
334 mediated virus clearance from neurons occurs mainly through early cytolytic processes,
335 followed by non-cytolytic processes that are mediated by antibody specific for the virus
336 and IFN-γ, which enable neurons to survive (38). Building upon this observation, a
337 better understanding of adaptive immune responses is needed to address LACV-
338 induced neurological disease.

339 It is important to acknowledge the limitations of our study. Characterization of
340 cytokines and chemokines secreted into the serum may not fully capture the immune
341 activity within the infected host, as the brain-resident immune response may differ from
342 the systemic immune response. Additionally, we cannot definitively conclude whether a
343 specific skew in Th1/Th2 responses occurred, as the cellular source of the cytokines
344 detected in the serum remains unknown. In conclusion, our study contributes to a
345 deeper understanding of immune responses to neurotropic bunyavirus infections,
346 offering insights into potential therapeutic targets and biomarkers for disease
347 management. Future research should focus on elucidating the underlying mechanisms
348 driving age-related susceptibility, further characterizing the immune response dynamics,
349 and exploring therapeutic interventions to mitigate the neurological complications
350 associated with LACV infection. By advancing our understanding of host-virus
351 interactions, we can develop more effective strategies for the prevention, diagnosis, and
352 treatment of LACV-induced disease, ultimately enhancing public health preparedness
353 against emerging viral threats.

354

355 **Materials and Methods**

356 **Infection of mice with LACV and neurological disease progression criteria**

357 All animal studies were approved by the Rutgers University IACUC (protocol
358 number 202300007). Wildtype (C57BL/6) mice were purchased from Jackson
359 Laboratories and maintained in a breeding colony at the Child Health Institute of New
360 Jersey. LACV (La Crosse Virus, NR-540), a human isolate was purchased obtained
361 from BEI Resources. Mice at 3 (weanling) or 6-8 (adult) weeks of age were inoculated
362 with $5x10^5$ plaque forming units (PFU) of LACV in phosphate-buffered saline (PBS)
363 intraperitoneally in a volume of 200 µl/mouse. Mice were observed daily for signs of
364 neurological disease that included abnormal gait, seizures, and lethargy. Animals with
365 clear clinical signs of neurological disease were scored as clinical and euthanized
366 immediately and humanely.

367

368 **Serum sample collection**

369 Serum samples were collected from individual mice on 0, 1, 2, 3, 4, 5, 6, 7, 8 and 12
370 dpi. Submandibular blood was collected into heparin coated tubes. Blood samples were
371 centrifuged at 2000 x g for 10 minutes. After centrifugation, sera were aliquoted and
372 stored in -80°C for subsequent analysis.

373

374 **Neutralizing antibody assay**

375 A microneutralization assay was performed to detect neutralizing antibodies. 100
376 µl of the sera were diluted in phosphate buffer saline (PBS), and serial dilutions were
377 prepared in a 96-well plate. For each dilution, contact was performed with a 100 PFU
378 LACV dose for two hours at 37°C in a 5% CO_2 incubator. The complexes were then
379 added to Vero cells (ATCC) CCL-81, $4x10^4$ cells/well. After 3-4 days of incubation, the
380 cytopathogenic effects were investigated with 0.1% crystal violet staining (SigmaAldrich,
381 St. Louis, MO) for 2-4 hours at room temperature prior to washing with water. The
382 neutralizing titer was defined as the inverse of the highest dilution resulting in a
383 infectious reduction of 50%. A titer lower than 1:20 was considered as negative if
384 cytopathic effects were not observed. Samples were considered as positive if the titer
385 was >1:20.

386

387 **qPCR**

388 Viral titers were quantified by qPCR. Total viral RNA was extracted from serum
389 using Pure link viral RNA mini kit (ThermoFisher, Waltham, MA) following
390 manufacturer's protocol. cDNA was prepared from RNA samples using the SuperScript
391 IV VILO reverse transcription kit (ThermoFisher, Waltham, MA) following the
392 manufacturer's instructions. cDNA samples were diluted fivefold in Rnase-free water
393 and those diluted samples were analyzed by qPCR using PowerTrack SYBR Green kit
394 (ThermoFisher, Waltham, MA) to detect LACV RNA. The following primers were used:
395 forward (ATTCTACCCGCTGACCATTG) and reverse (GTGAGAGTGCCATAGCGTTG).
396 CT values were measured using a QuantStudio 3Flex instrument (Applied Biosysterms,
397 Waltham, MA). QuantStudio Real-Time PCR software was used to extract the data.

398

399 **Bead-based multiplex immunoassay (Luminex)**

400

401 To quantify the levels of cytokines in LACV infected serum samples, a magnetic
402 bead-based multiplex immunoassay was performed using a MAGPIX® (ThermoFisher,
403 Waltham, MA instrument system. A mouse cytokine magnetic 26-plex panel (Invitrogen)
404 was utilized to determine immune responses mediated through cytokine and chemokine
405 such as (1) Th1/Th2: GM-CSF, IFN-γ, IL-1β , IL-2 , IL-4 , IL-5, IL-6, IL-12p70, IL-13, IL-
406 18, TNF-α, and (2) Th9/Th17/Th22/Treg: IL-9, IL-10, IL-17A/CTLA-8, IL-22, IL-23, IL-27,
407 (3) Chemokines: Eotaxiv/CCL11, GROα/CXCL1, IP-10/CXCL10, MCP-1/CCL2, MCP-
408 3/CCL7, MIP-1α/CCL3, MIP-1β/CCL4, MIP-2α/CXCL2, RANTES/CCL5, MCP-3/CCL7
409 following the manufacturer's instructions. 5 (IL-2, IL-5, MIP-1β/CCL4, MIP-2α/CXCL2,
410 and MCP-3/CCL7) of the analyzed cytokines were out of range and therefore were not
411 included in the analysis for both adult and weanling mice.

412

413 **Statistical analysis**

414 All statistical analyses were performed using Prism software Version 7.01
415 (GraphPad) and are described in the figure legends.

416

417 **Data Availability**

418 The data that support the findings of this study are available within the
419 manuscript or upon request from the corresponding author.

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Conflicts of Interest

BBH is a co-founder of Mir Biosciences, Inc., a biotechnology company focused on T cell-based diagnostics and vaccines for infectious diseases, cancer, and autoimmunity.

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549 **Figures**

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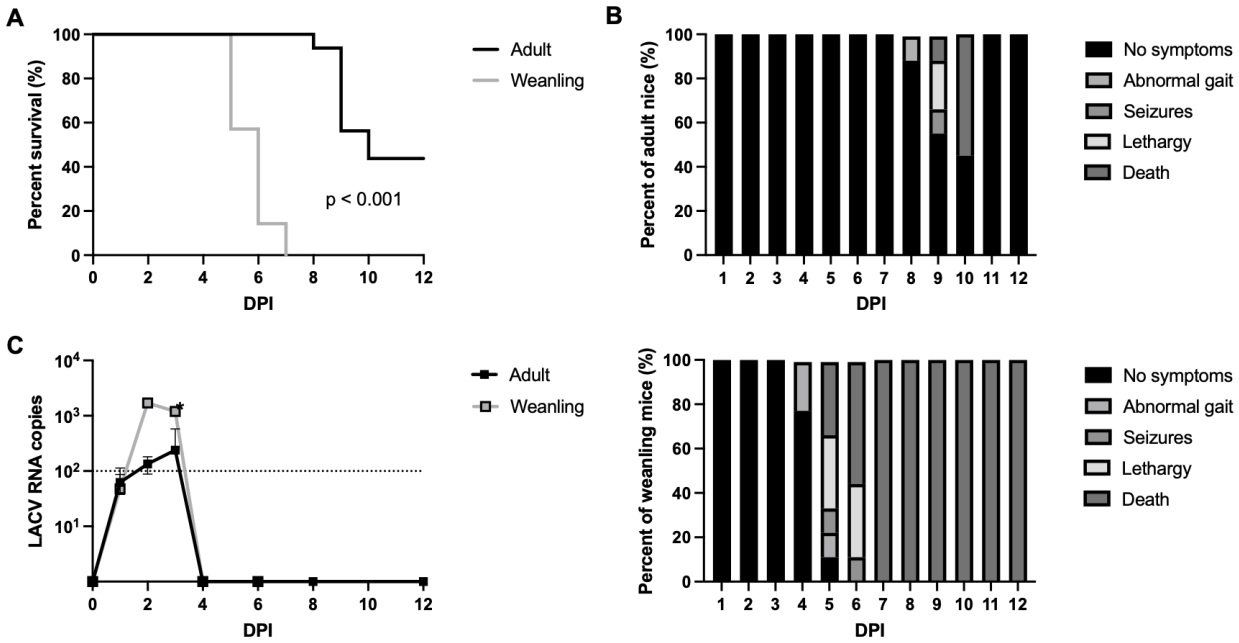

551

552 **Figure 1. Impact of infectious dose on viremia and susceptibility to LACV infection. (A)**
553 Percent survival of adult (black) and weanling (gray) LACV-infected mice with highly infectious
554 dose (P<0.001). **(B)** Percent mice showing LACV-induced outcomes in adult (top) and weanling

555 (bottom) mice. **(C)** Viral titer in sera of LACV-infected adult and weanling mice represented by
556 LACV RNA copies quantified by qPCR. *, p<0.05.

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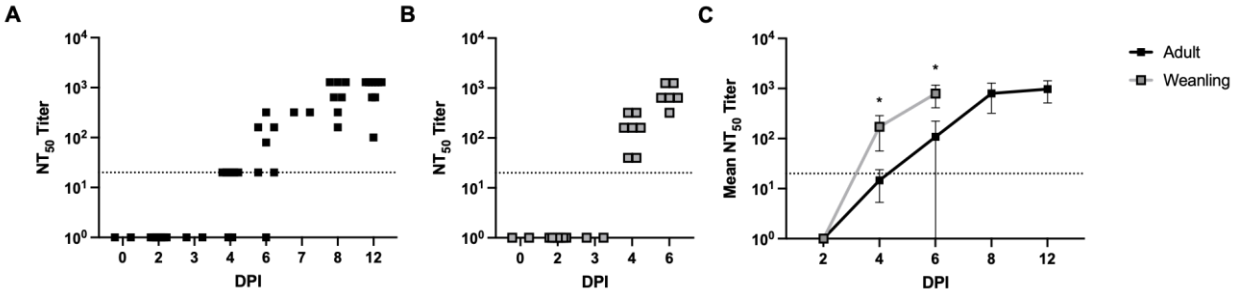

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563 **Figure 2. Neutralizing antibody titers do not correlate with viral clearance in weanling**
564 **mice. (A, B)** NT50 titers in sera from LACV-infected adult (black) and weanling (gray) mice
565 throughout the course of infection. **(C)** Mean NT50 titers between adult and weanling mice. *,
566 p<0.05.

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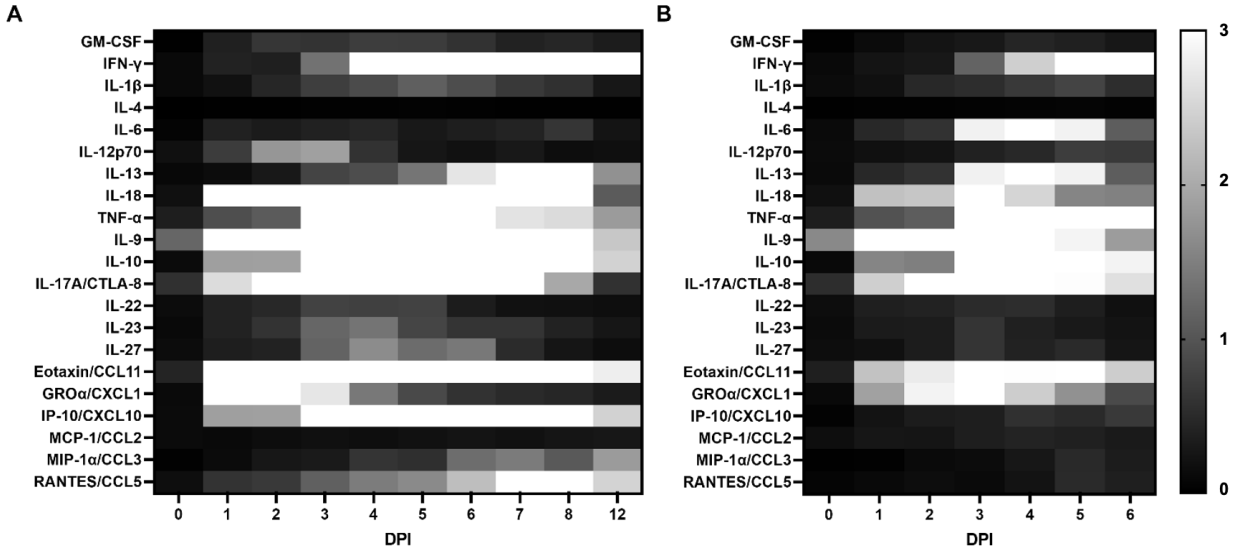

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579 **Figure 3. Cytokines and chemokines are differentially regulated in adult and weanling**
580 **mice.** A heatmap for cytokine and chemokine levels analyzed in **(A)** adult and **(B)** weanling
581 mice during the course of LACV infection (1-12 dpi) compared to uninfected phase (0 dpi). The
582 rows represent cytokines and chemokines and the columns represent 1-12 dpi. The scale is
583 represented in an arbitrary format, depicting low (0) to high (3) cytokines and/or chemokines
584 detected in sera.

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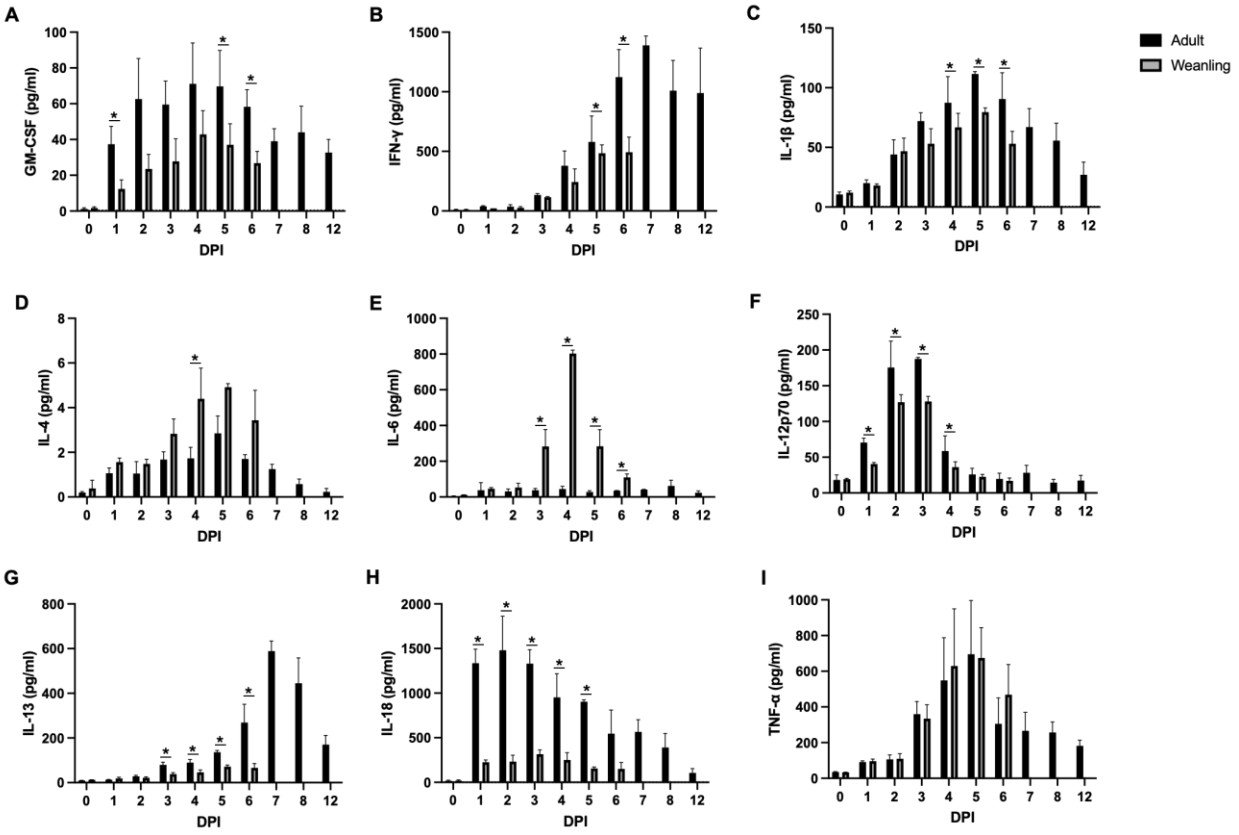

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594 **Figure 4. Dynamics of Th1/Th2 Cytokines During LACV Infection. (A-I)** Bar charts
595 representing levels of Th1/Th2 related cytokines in sera of adult (black) and weanling (gray)
596 mice during LACV infection. *, $p<0.05$.

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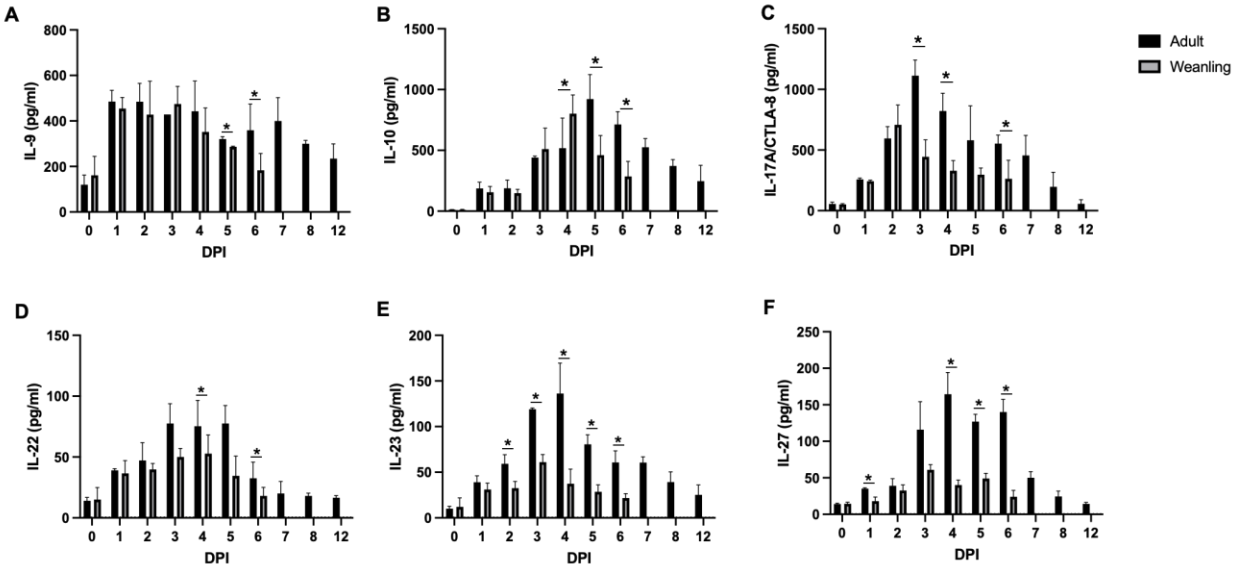

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604 **Figure 5. Dynamics of Th9/Th17/Th22/Treg Cytokines During LACV Infection. (A-F)** Bar
605 charts representing levels of Th9/Th17/Th22/Treg related cytokines in sera of adult (black) and
606 weanling (gray) mice during LACV infection. Data represent average from 9 adult and 9
607 weanling mice with SD. *, $p<0.05$.

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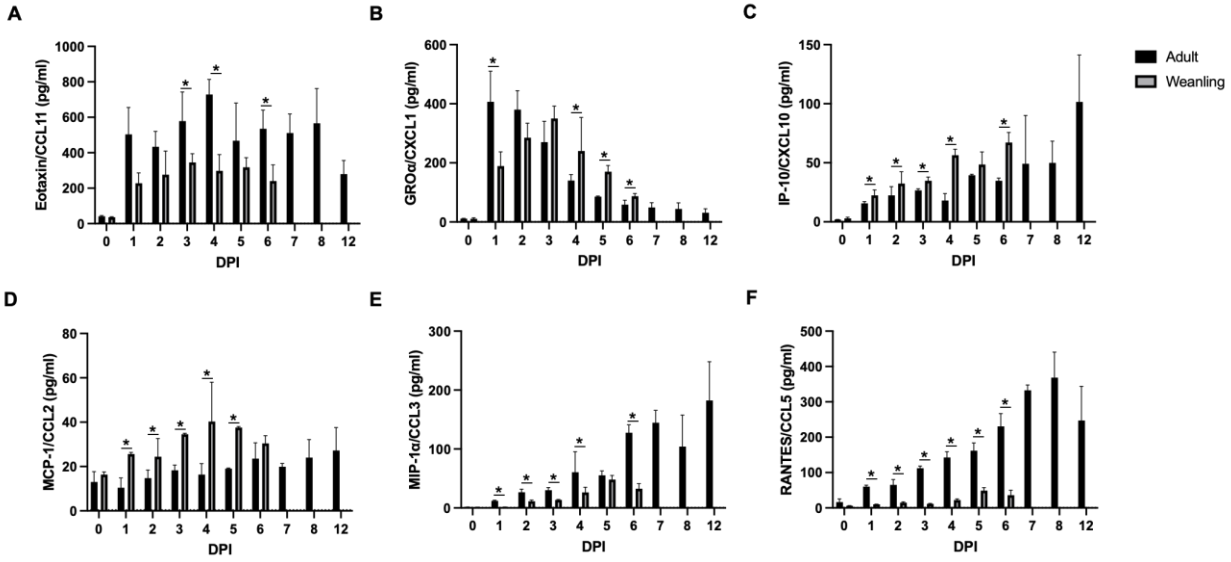

Figure 6. Dynamics of Chemokine Levels During LACV Infection. (A-F) Bar charts representing levels of chemokines in sera of adult (black) and weanling (gray) mice during LACV infection. *, $p<0.05$.

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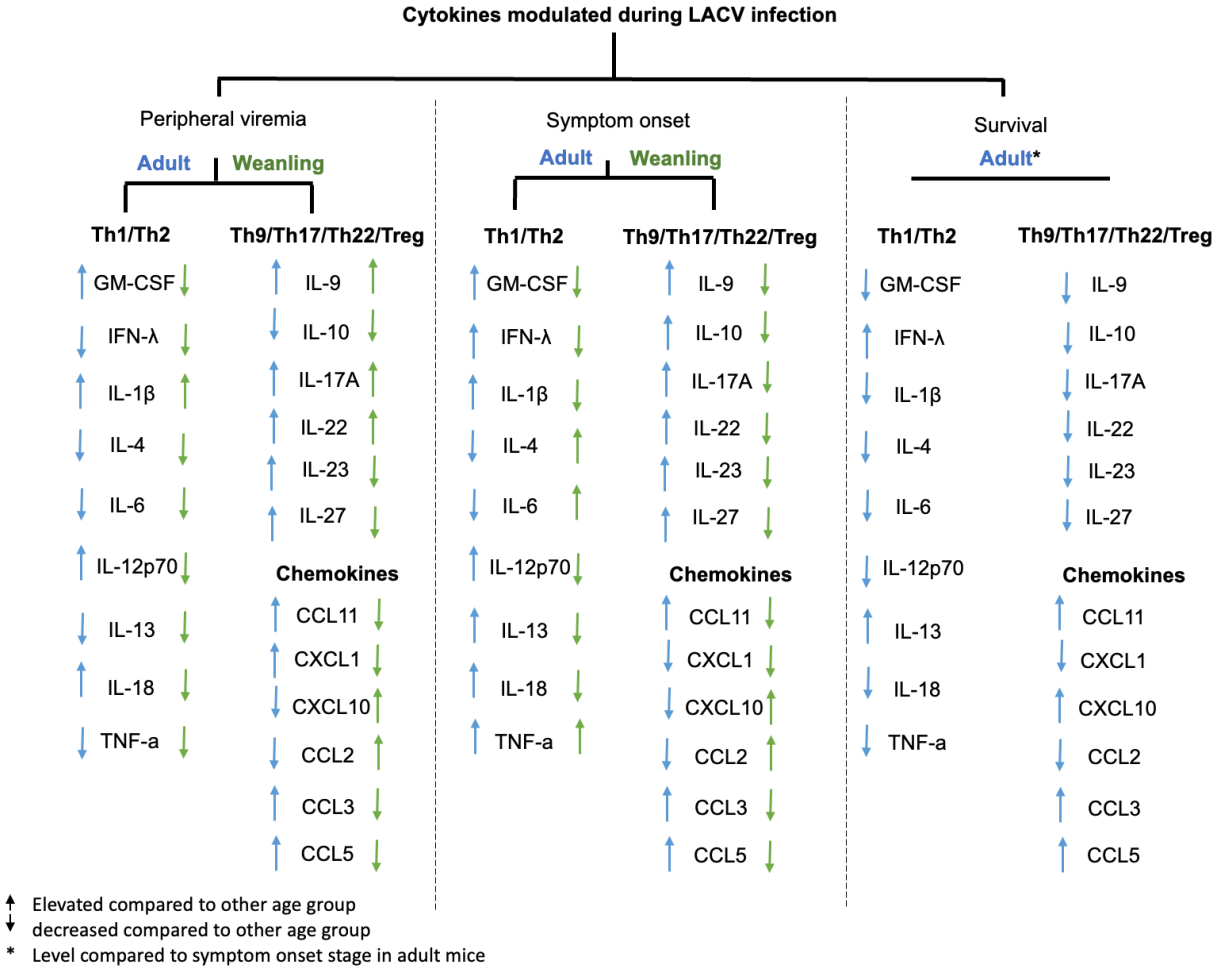

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644 **Figure 7. Summary of serum cytokine and chemokine responses during phases of LACV**
645 **infection.** Roadmap illustrating the relative changes in cytokine and chemokine levels across
646 different phases of LACV infection, categorized as peripheral viremia, symptom onset, and
647 survival stage. Blue arrows indicate trends observed in adult mice, while green arrows represent
648 trends observed in weanling mice.