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.

Introduction

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

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

Prior investigations have shed light on the significance of both innate and adaptive immune responses following LACV infection (9, 10). Type I interferon responses have

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

80 Cytokines and chemokines undergo substantial modulation upon LACV infection
81 across diverse cell types. Notably, human neuron-astrocyte co-cultures respond to LACV
82 infection in vitro within 48 hours post infection by eliciting robust proinflammatory
83 cytokines and chemokines including: IFN-γ, IL-6, TNF-α, IL-8, CCL4, CCL5, CXCL10
84 (16). In a separate study, glial cells derived from the brains of young LACV-infected mice
85 produced significant levels of IFN-β (17). Furthermore, preliminary observations hint at a
86 proinflammatory cytokine response in both the periphery and the brain following LACV
87 infection, with elevated levels of IL-6 and IL-12p40, which were reported to be in
88 comparable levels in young and adult mice (10, 18). Interestingly, administration of IL-12

89 or GM-CSF encoded by a plasmid prior to LACV infection in Ifnar1$^{-/-}$ mice was shown to
90 increase survival rates, suggesting a potential protective role of these cytokines (19). A
91 similar proinflammatory cytokine profile has also been reported with Oropouche virus, a
92 distantly related bunyavirus (20). Cytokines and chemokines seem to act as important
93 contributing factors in the immune response to LACV and other bunyavirus infections.

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

104

105 **Results**

106 **Age-related susceptibility is maintained despite a highly infectious dose of LACV**
107 **infection**

108 An unpublished observation reported no significant difference in cytokine levels
109 between adult and weanling mice infected with a 1000 PFU dose of LACV (10). To
110 investigate the potential impact of infectious dose on immune system responsiveness to

111 LACV infection, we hypothesized that a higher dose might better reveal differences in
112 cytokine levels if they exist. Previous studies have shown that infection with 10^5 PFU of
113 LACV maintains age-related susceptibility in mice (10). Here, we aimed to assess the
114 effects of an infectious dose five-fold higher than the previously reported dose by
115 monitoring the development of neurological signs in infected mice and measuring viral
116 titers in peripheral blood.

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

129

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

131 nAb responses are pivotal for viral clearance in many infections, yet our findings
132 reveal an intriguing disparity between nAb titers and viral clearance in weanling mice.

133 Following infection with a highly infectious dose of LACV, nAbs peak in adult mice
134 between 8 and 12 dpi (Fig. 2A, 2C), whereas they peak earlier in weanling mice between
135 4 and 6 dpi (Fig. 2B, 2C), coinciding with the onset of neurological symptoms. Despite the
136 robust early nAbs responses in weanling mice, 100% succumbed to the infection by 7 dpi
137 (Fig. 1A). These results suggest that viral clearance and the onset of LACV-induced
138 neurological signs may not directly correlate with the nAb responses.

139

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

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

150 To delineate the potential roles of serum cytokines and chemokines in protection
151 or pathogenesis at different infection stages, we classified the course of infection into
152 three phases: (1) peripheral viremia (1-3 dpi), (2) symptoms onset (4-6 dpi), and (3)
153 survival (>7 dpi). Additionally, we categorized the analyzed cytokines and chemokines
154 into three groups: Th1/Th2 cytokines, Th9/Th17/Th22/Treg cytokines, and chemokines.

155 Among the 21 modulated cytokines and chemokines, 9 belonged to the Th1/Th2 cytokine
156 group (GM-CSF, IFN-γ, IL-1β, IL-4, IL-6, IL-12p70, IL-13, IL-18, TNF-α, 6 were classified
157 under the Th9/Th17/Th22/Treg cytokine group (IL-9, IL-10, IL-17A/CTLA-8, IL-22, IL-23,
158 IL-27), and the remaining 6 were identified as elevated chemokines in LACV-infected
159 mice (Eotaxin/CCL11, GROα/CXCL1, IP-10/CXCL10, MCP-1/CCL2, MIP-
160 1α/CCL3, RANTES/CCL5.

161

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

163 IFN-γ, IL-18, TNF-α, and IL-13 emerged as the predominant Th1/Th2 cytokines
164 detected in serum samples from both adult and weanling mice (Fig. 3-4). During the
165 peripheral viremia phase, a significant elevation in IL-18 levels was observed as early as
166 1 dpi, followed by subsequent significant peaks in IL-12p70 and GM-CSF in adult mice
167 (Fig. 4A, 4F, 4H). Notably, levels of IL-18 and IL-12p70 peak during this early phase before
168 gradually declining as the infection progresses. Conversely, weanling mice display a
169 notable significant increase in levels of IL-6 towards the latter part of the peripheral
170 viremia phase, one day prior to symptoms onset (Fig. 4E).

171 At the symptoms onset phase, occurring between 4-6 dpi, several cytokines
172 including IFN-γ, IL-1β, TNF-α, GM-CSF, IL-13, and IL-4 were significantly elevated in both
173 adult and weanling mice (Fig. 4A-D, 4G, 4I). Most cytokines reach their peak levels during
174 this phase, with adult mice exhibiting higher levels of IL-1β, TNF-α, GM-CSF, IL-13, and
175 IFN-γ compared to weanlings. Conversely, IL-4 and IL-6 were significantly elevated in
176 weanlings compared to adult mice (Fig. 4D-E). Interestingly, IL-6 levels in adult mice

177 remained comparable to those during the uninfected phase (Fig. 4E). Finally, during the
178 survival phase (≤>7 dpi) IFN-γ and IL-13 emerged as the primary cytokines specifically
179 elevated during this late phase of infection in adult mice.

180

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

182 A highly infectious dose of LACV revealed elevated levels across all
183 Th9/Th17/Th22/Treg cytokines analyzed in both adult and weanling (Fig. 5). Notably, IL-
184 9, IL-10, and IL-17A emerged as the most abundant cytokines within this category
185 throughout the infection course in both age groups (Fig. 3). During the early peripheral
186 viremia phase, peaks in IL-9 and IL-17A levels underscored their early involvement in the
187 immune response to LACV infection in adult and weanling mice (Fig. 5A, 5C). Additionally,
188 at 2 dpi, IL-23 levels were significantly higher in adult mice compared to weanlings,
189 mirroring the kinetics of IL-17A, which exhibited significantly elevated levels in adult mice
190 by 3 dpi (Fig. 5C, 5E).

191 During the symptoms onset phase, peaks in IL-22, IL-23, and IL-27 levels were
192 observed, with a notable increase observed in adult mice (Fig. 5D-F). In contrast, IL-10
193 levels exhibited a significant increase in weanling mice at 4 dpi, followed by a rapid decline
194 one day later, reaching levels significantly lower than those in adult mice. By 6 dpi,
195 Th9/Th17/Th22/Treg cytokine levels gradually decreased in adult mice, ultimately
196 returning to baseline levels.

197

198

199 **Dynamics of Chemokines during LACV Infection**

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

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

213

214 **Discussion**

215 Our study presents a comprehensive analysis of neutralizing antibody titers and
216 cytokine and chemokine dynamics during a highly infectious dose of LACV infection,
217 shedding light on their roles in protection and pathogenesis. Our findings demonstrate
218 distinct patterns of cytokines and chemokines specific to age and infection phase.
219 Notably, we observed significant increases in Th1/Th2/Th9/Th17/Th22/Treg cytokines
220 and chemokines in response to LACV infection across both adult and weanling mice.

221 Interestingly, we observed age-specific differences in many cytokines and chemokines
222 throughout the course of infection (Fig. 7).

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

233 Our investigation into viral neutralization yielded unexpected findings. Despite the
234 early and robust production of nAbs in weanling mice, these titers failed to correlate with
235 viral clearance, prompting speculation regarding the role of nAbs in early disease
236 progression, Notably, different neutralization capabilities have been associated with
237 isotype switching, a phenomenon documented in other viral infections such as HIV and
238 SARS-CoV-2 (21, 22). Interestingly, adult mice exhibited significantly lower nAb titers
239 during the symptoms onset phase, suggesting either rapid nAbs consumption in vivo or
240 the involvement of non-neutralizing antibodies in protection. However, given that weanling
241 nAbs were incapable of effectively eliminating the virus in vivo, the former explanation
242 seems less likely.

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

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

261 In the case of LACV infection, whether increased cytokines and chemokines are a
262 correlate of protection or pathogenesis remains to be fully investigated. However, in most
263 cases, an increase in specific cytokines at particular phases of infection in susceptible
264 hosts, such as weanling mice, may suggest a potential contribution of these pathways to

265 susceptibility and disease pathogenesis. Conversely, the increase in cytokines and
266 chemokines in disease-resistant hosts, such as adult mice, may indicate a protective role
267 facilitated by these molecules.

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

276 Moreover, our findings in the context of Th1/Th2 cytokines are consistent with
277 previous studies on CNS viral infections. For instance, elevated IL-6 levels in the CNS in
278 infections with dengue and Japanese encephalitis have been associated with various
279 neurological manifestations and poor outcomes (26, 27). On the contrary, our observation
280 of the substantial and sustained upregulation of Th1-related cytokines, including IL-18,
281 IFN-γ, IL-12, and GM-CSF, throughout LACV infection supports previously reported data
282 of protective roles of GM-CSF and IL-12 in Ifnar1$^{-/-}$ mice and underscores their role in
283 eliciting protective responses in mice resistant to the disease (19).

284 Among these cytokines, the pronounced elevation of IL-18 in adult mice stands out
285 as the most significant modulation induced by LACV. IL-18 has been demonstrated to
286 stimulate the proliferation and differentiation of T cells toward a Th1 response and acts
287 as a crucial cofactor in IL-12-induced IFN-γ production by natural killer (NK) and T cells

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

301 In our analysis of Th9/Th17/Th22/Treg cytokines, all exhibited increased levels in
302 adult mice in response to LACV infection. Among these, IL-23 and IL-27 were among the
303 most significantly increased. Although these cytokines have been predominantly studied
304 in the context of autoimmune diseases, emerging evidence suggests their crucial roles
305 during viral infections (32, 33). Specifically, IL-23 has been implicated in the control of
306 viral infections within the brain, indicating its potential importance in host defense
307 mechanisms against neurotropic viruses such as LACV (32). Similarly, IL-27 has been
308 found to mediate NK cell effector functions during viral infections, highlighting its
309 involvement in orchestrating innate immune responses against viral pathogens (33).

310 Chemokines, as a distinct group of immune mediators, demonstrated elevated
311 levels in response to LACV infection, with increases observed in CCL5, followed by CCL3,
312 in adult mice. In contrast, CCL2 increased in weanling mice throughout the course of
313 infection. The expression and release of CCL2 and CCL3 on cerebral endothelium
314 suggest an essential role for these chemokines in regulating the trafficking of
315 inflammatory cells across the blood-brain barrier (BBB) during CNS inflammation (34).
316 Furthermore, CCL3 has been shown to contribute to the differentiation and migration of
317 effector T cells in response to viral infection within the CNS (35). Additionally, CCL5 plays
318 a crucial role in the homing and migration of effector and memory T cells during acute
319 CNS infections (36). CCL2 emerges as a major player in mediating monocyte infiltration
320 and the trafficking of inflammatory monocytes to the brain during viral encephalitis (37).
321 Our findings, indicating increased levels of CCL2 in weanling mice, which by logic,
322 potentially contributes to pathogenesis possibly mediated through the recruitment of an
323 overwhelming inflammatory response in the infected brain. However, LACV-induced
324 lymphocyte infiltration of CNS itself is less likely to contribute to pathogenesis according
325 to a recent study (9). With this study being one of the few to focus specifically on LACV
326 infections, significant gaps persist in our understanding of immunity correlates and the
327 mechanisms underlying immune-mediated viral clearance. More often than not,
328 successful clearance of virus infection from the CNS requires the elimination of virus from
329 the cells without causing damage to neurons. Ideally, immune-mediated virus clearance
330 from neurons occurs mainly through early cytolytic processes, followed by non-cytolytic
331 processes that are mediated by antibody specific for the virus and IFN-γ, which enable

332 neurons to survive (38). Building upon this observation, a better understanding of
333 adaptive immune responses is needed to address LACV-induced neurological disease.

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

349

350 **Materials and Methods**

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

352 All animal studies were approved by the Rutgers University IACUC (protocol
353 number 202300007). Wildtype (C57BL/6) mice were purchased from Jackson

354 Laboratories and maintained in a breeding colony at the Child Health Institute of New
355 Jersey. LACV (La Crosse Virus, NR-540), a human isolate was purchased obtained from
356 BEI Resources. Mice at 3 (weanling, n=13) or 6-8 (adult, n=13) weeks of age were
357 inoculated with $5x10^5$ plaque forming units (PFU) of LACV in phosphate-buffered saline
358 (PBS) intraperitoneally in a volume of 200 µl/mouse. Mice were observed daily for signs
359 of neurological disease that included abnormal gait, seizures, and lethargy. Animals with
360 clear clinical signs of neurological disease were scored as clinical and euthanized
361 immediately and humanely.

362

363 **Serum sample collection**

364 Serum samples were collected from a total of 13 adult mice and 13 weanling mice
365 throughout the course of infection, including 0 (baseline uninfected phase) and 1, 2, 3, 4,
366 5, 6, 7, 8 and 12 dpi. Submandibular blood or cardiac blood (on days in which animals
367 were euthanized) were collected into heparin coated tubes. Blood samples were
368 centrifuged at 2000 x g for 10 minutes. After centrifugation, sera were aliquoted and
369 stored in -80°C until subsequent analysis.

370

371 **Neutralizing antibody assay**

372 A microneutralization assay was performed to detect neutralizing antibodies. 100
373 µl of the sera were diluted in phosphate buffer saline (PBS), and serial dilutions were
374 prepared in a 96-well plate. For each dilution, contact was performed with a 100 PFU
375 LACV dose for two hours at 37°C in a 5% CO_2 incubator. The complexes were then added

376 to Vero cells (ATCC) CCL-81, $4x10^4$ cells/well. After 3-4 days of incubation, the
377 cytopathogenic effects were investigated with 0.1% crystal violet staining (SigmaAldrich,
378 St. Louis, MO) for 2-4 hours at room temperature prior to washing with water. The
379 neutralizing titer was defined as the inverse of the highest dilution resulting in a infectious
380 reduction of 50%. A titer lower than 1:20 was considered as negative if cytopathic effects
381 were not observed. Samples were considered as positive if the titer was >1:20.

382

383 **qPCR**

384 Viral titers were quantified by qPCR. Total viral RNA was extracted from pooled
385 serum samples from LACV-infected adult and weanling mice using Pure link viral RNA
386 mini kit (ThermoFisher, Waltham, MA) following manufacturer's protocol. Given that
387 submandibular blood collection results in small quantities of serum isolation necessitated
388 pooling of sera samples prior to viral RNA extraction. cDNA was prepared from RNA
389 samples using the SuperScript IV VILO reverse transcription kit (ThermoFisher, Waltham,
390 MA) following the manufacturer's instructions. cDNA samples were diluted fivefold in
391 Rnase-free water and those diluted samples were analyzed by qPCR using PowerTrack
392 SYBR Green kit (ThermoFisher, Waltham, MA) to detect LACV RNA. The following
393 primers were used: forward (ATTCTACCCGCTGACCATTG) and reverse
394 (GTGAGAGTGCCATAGCGTTG). CT values were measured using a QuantStudio 3Flex
395 instrument (Applied Biosysterms, Waltham, MA). QuantStudio Real-Time PCR software
396 was used to extract the data.

397

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

399

400 Sera samples from adult male (n=3) and female (n=3) and weanling male (n=3)
401 and female(n=3) mice at 0, 1, 3, 5, and 7 dpi were pooled for analysis. Additionally, sera
402 samples from adult (n=7; 4 male, 3 female) and weanling (n=7; 4 male, 3 female) mice at
403 2, 4, 6, 8, and 12 dpi were individually analyzed. To quantify the levels of serum cytokines
404 and chemokines in LACV-infected adult and weanling mice, a magnetic bead-based
405 multiplex immunoassay was performed using a MAGPIX® (ThermoFisher, Waltham, MA)
406 instrument. A mouse cytokine magnetic 26-plex panel (Invitrogen) was utilized to
407 determine immune responses mediated through cytokine and chemokine such as (1)
408 Th1/Th2: GM-CSF, IFN-γ, IL-1β , IL-2 , IL-4 , IL-5, IL-6, IL-12p70, IL-13, IL-18, TNF-α,
409 and (2) Th9/Th17/Th22/Treg: IL-9, IL-10, IL-17A/CTLA-8, IL-22, IL-23, IL-27, (3)
410 Chemokines: Eotaxiv/CCL11, GROα/CXCL1, IP-10/CXCL10, MCP-1/CCL2, MCP-
411 3/CCL7, MIP-1α/CCL3, MIP-1β/CCL4, MIP-2α/CXCL2, RANTES/CCL5, MCP-3/CCL7
412 following the manufacturer's instructions. 5 (IL-2, IL-5, MIP-1β/CCL4, MIP-2α/CXCL2,
413 and MCP-3/CCL7) of the analyzed cytokines were out of range and therefore were not
414 included in the analysis for both adult and weanling mice.

415

416 **Statistical analysis**

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

419

Data Availability

The data that support the findings of this study are available within the 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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Figures

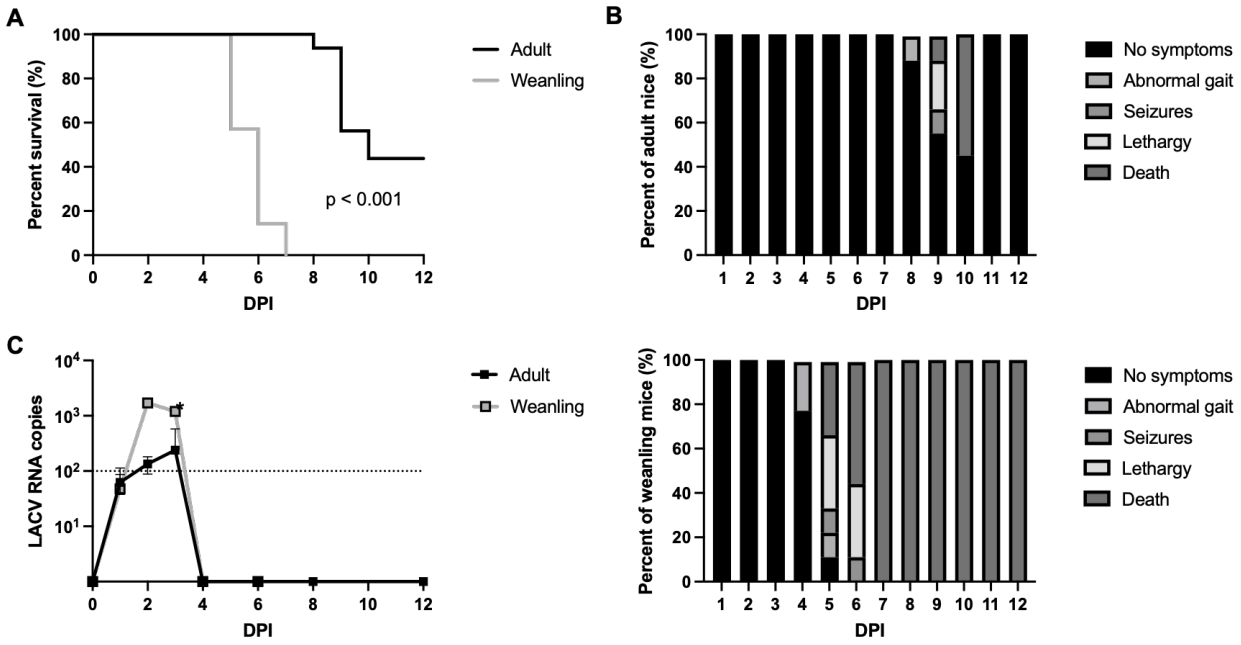

Figure 1. Impact of infectious dose on viremia and susceptibility to LACV infection. (A) Percent survival of adult (black) and weanling (gray) LACV-infected mice with highly infectious dose (P<0.001). **(B)** Percent mice showing LACV-induced outcomes in adult (top) and weanling (bottom) mice. **(C)** Viral titer in sera of LACV-infected adult and weanling mice represented by LACV RNA copies quantified by qPCR. *, p<0.05.

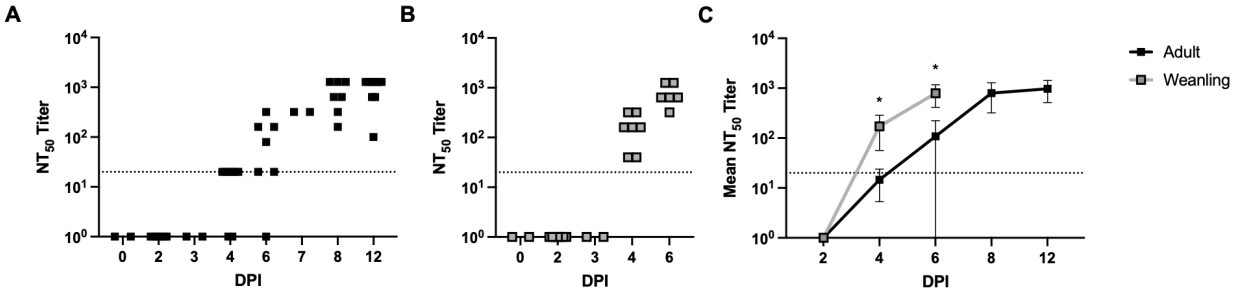

Figure 2. Neutralizing antibody titers do not correlate with viral clearance in weanling mice. **(A, B)** NT50 titers in sera from LACV-infected adult (black) and weanling (gray) mice throughout the course of infection. **(C)** Mean NT50 titers between adult and weanling mice. *, $p<0.05$.

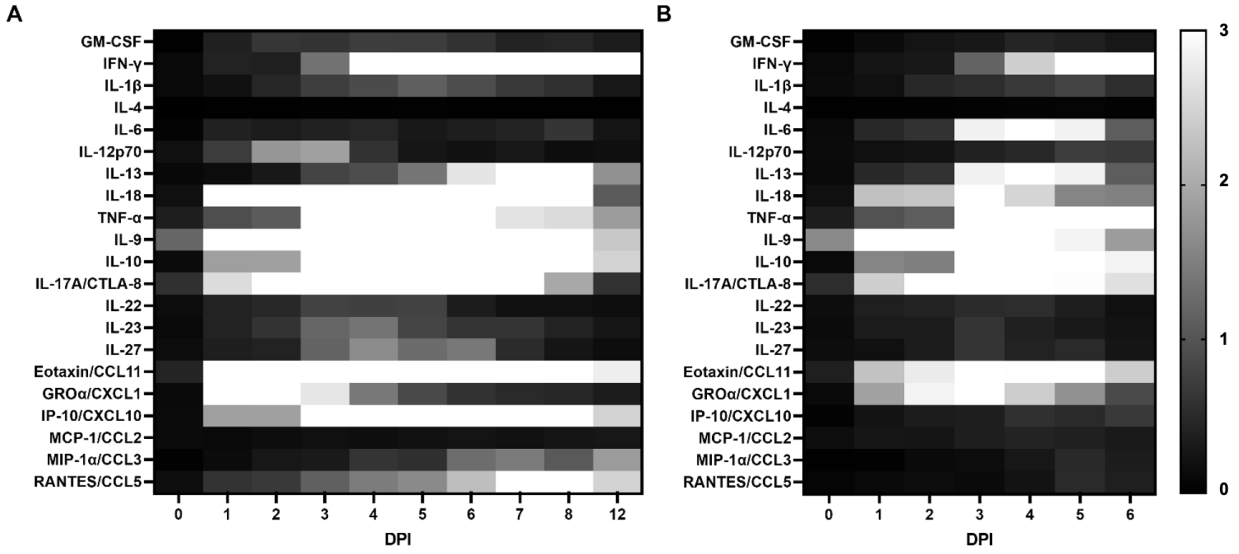

Figure 3. Cytokines and chemokines are differentially regulated in adult and weanling mice. A heatmap for cytokine and chemokine levels analyzed in **(A)** adult and **(B)** weanling mice during the course of LACV infection (1-12 dpi) compared to uninfected phase (0 dpi). The rows represent cytokines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

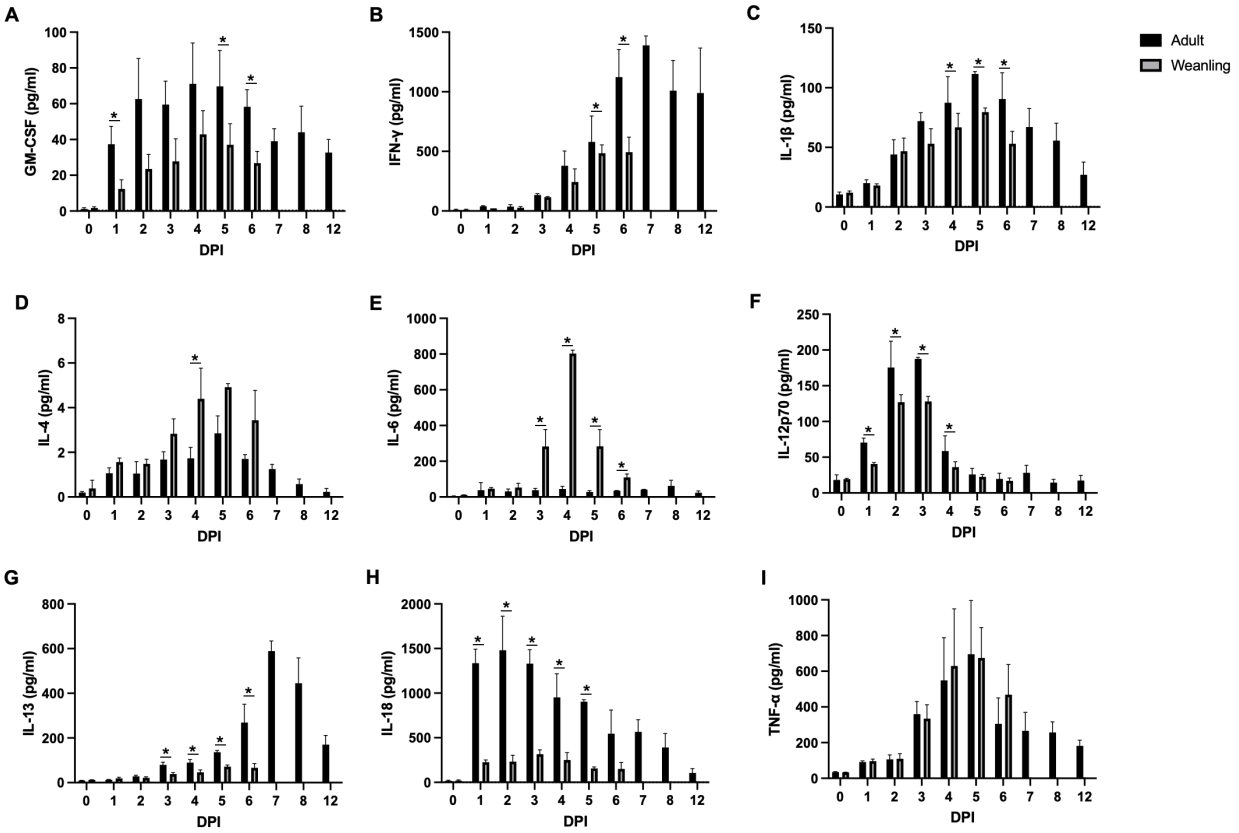

Figure 4. Dynamics of Th1/Th2 Cytokines During LACV Infection. (A-I) Bar charts representing levels of Th1/Th2 related cytokines in sera of adult (black) and weanling (gray) mice during LACV infection. *, $p<0.05$.

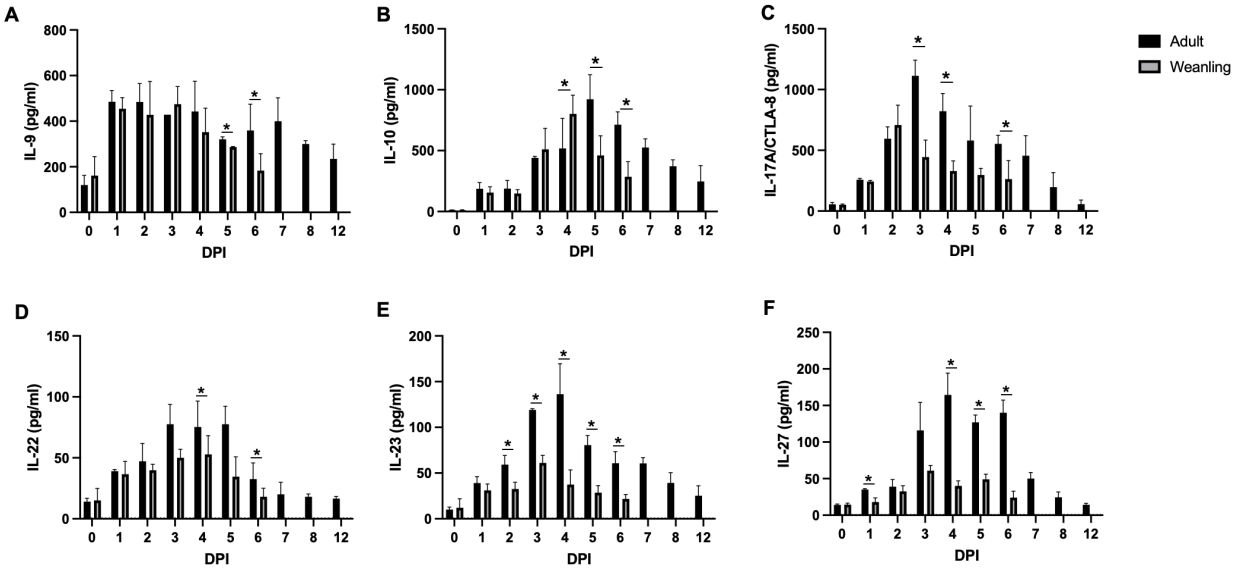

Figure 5. Dynamics of Th9/Th17/Th22/Treg Cytokines During LACV Infection. (A-F) Bar charts representing levels of Th9/Th17/Th22/Treg related cytokines in sera of adult (black) and weanling (gray) mice during LACV infection. Data represent average from 9 adult and 9 weanling mice with SD. *, $p<0.05$.

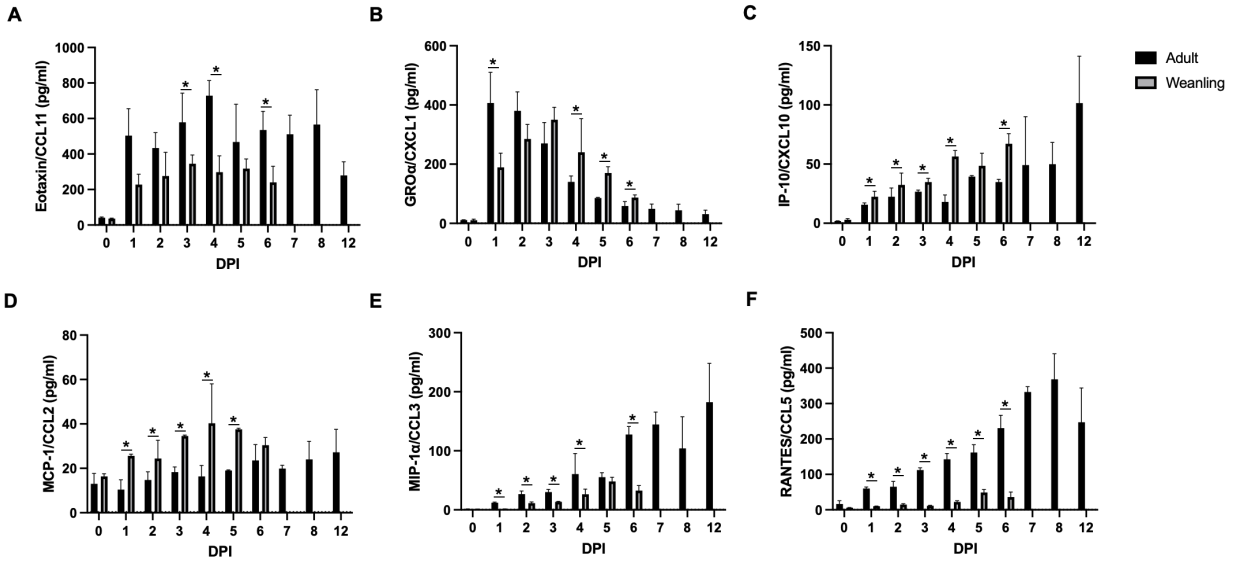

Figure 6. Dynamics of Chemokine Levels During LACV Infection. (A-F) Bar charts representing levels of chemokines in sera of adult (black) and weanling (gray) mice during LACV infection. *, $p<0.05$.

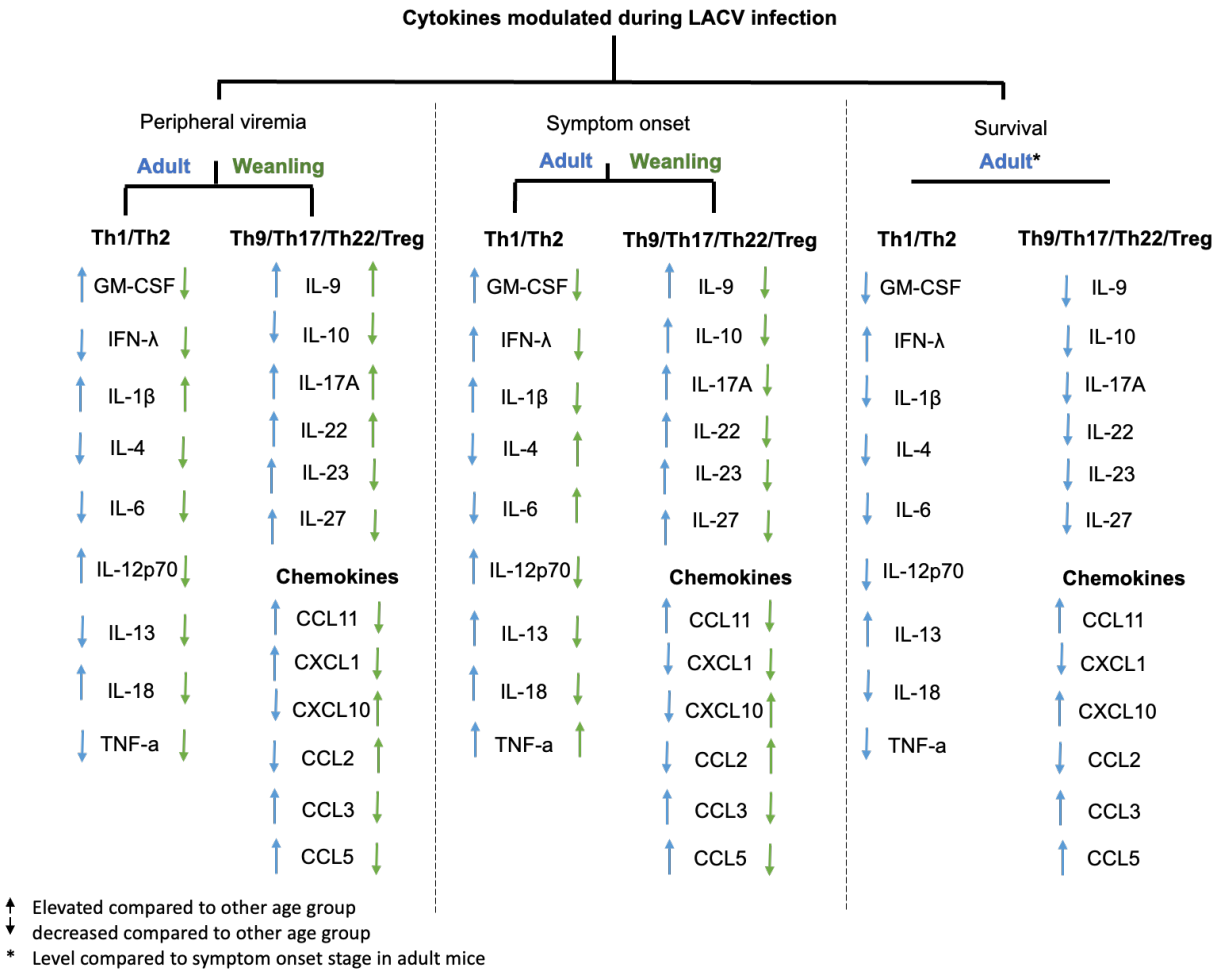

Figure 7. Summary of serum cytokine and chemokine responses during phases of LACV infection. Roadmap illustrating the relative changes in cytokine and chemokine levels across different phases of LACV infection, categorized as peripheral viremia, symptom onset, and survival stage. Blue arrows indicate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