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, particularly affecting children aged 16 years or younger. This age-related susceptibility extends to murine models, where weanling mice (3 weeks old) succumb to LACV infection, while adults (≥6 weeks old) demonstrate resistance. Despite its clinical relevance, the host immune response to LACV is not fully understood. In this study, we investigated the roles of neutralizing antibodies (nAbs), cytokines, and chemokines in weanling and adult mice following infection with $5x10^5$ plaque forming units (PFU) of LACV. We observed significant age-related differences in viral titers and survival. Weanling mice demonstrated early disease onset with elevated peripheral viremia, but passive transfer of adult serum, confirmed to have nAbs, to naïve weanlings prior to infection completely rescued them from death. Cytokine and chemokine profiling revealed distinct kinetics and age-specific immune responses. Adult mice had increased Th1 cytokines, Th9/Th17/Th22/Treg cytokines, and many chemokines. In contrast, weanlings had higher Th2 cytokines, correlating with symptoms onset. Flow cytometry and intracellular cytokine staining further demonstrated that weanling mice produced higher levels of IL-4 by $CD4^+$ and $CD8^+$ T cells compared to adults, regardless of infection status. Conversely, LACV-infected adult mice had increased IFN-γ production by $CD8^+$ T cells compared to uninfected adults. Finally, adoptive transfer of splenocytes from immune adult mice to naïve weanlings delayed neurological symptoms and improved survival, highlighting the protective role of immune adult cells against LACV. In conclusion, this study links nAbs and cytokine and chemokine responses to protective immunity in adult mice, contrasting with the pathogenesis seen in weanlings. These findings underscore the importance of further research into innate and adaptive immune mechanisms in LACV infection.

45 **1. Introduction**

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

57 Vahey et al., recently reported 35-130 LACV disease cases per year using data
58 collected in the United States between 2003 and 2019 (7). The clinical significance of LACV,
59 coupled with its ability to also induce disease in murine models, has designated it as a
60 prototype for studying viruses within the *Peribunyaviridae* family (8). Weanling mice (3 weeks
61 old) exhibit susceptibility to LACV infection, whereas adult mice (≥6 weeks old) display
62 resistance, mirroring the age-related susceptibility observed in humans (3, 8, 9). Unraveling
63 the intricacies of host responses to LACV infection is important, particularly in delineating
64 factors contributing to disease resistance or susceptibility. Peripheral immune control of
65 LACV access to the CNS is believed to be crucial for disease resistance in adult mice. (10).
66 Notably, adult mice exhibit resistance to peripheral infection, even when the virus is
67 introduced via intraperitoneal (IP) and intradermal (ID) routes. In contrast, they are

68 vulnerable to intracranial (IC) infection, further emphasizing the role of peripheral immunity
69 in restricting CNS access by LACV(4, 11).

70 Prior investigations have shed light on the significance of both innate and adaptive
71 immune responses following LACV infection (10, 11). Type I interferon responses have
72 garnered considerable attention, demonstrating their indispensable role in curbing LACV
73 replication and mitigating disease severity (12). Early cellular innate immune responses,
74 particularly those imparted by myeloid dendritic cells, proved critical for protection in wild
75 type adult mice infected with LACV (11). The same study also showed that treatment of
76 young wild type mice with type I interferon, particularly IFN-β, resulted in protection mediated
77 by myeloid dendritic cells (11). Moreover, depletion of adaptive immune cells, including both
78 B and T cells, resulted in neurological disease in adult mice infected with LACV, suggesting
79 that these cell types are essential for protection against neuropathology and death (10).
80 However, the broader immune response including cytokines and chemokines remains
81 underexplored. These signaling molecules, produced by various cell types in response to
82 viral infections, orchestrate immune responses and exert influences on disease outcomes
83 (13-16). Thus, deciphering the dynamics of cytokines and chemokines during LACV infection
84 is important for unraveling their contributions to age-specific protection or pathogenesis.

85 Cytokines and chemokines undergo substantial modulation upon LACV infection
86 across diverse cell types. Notably, human neuron-astrocyte co-cultures respond to LACV
87 infection in vitro within 48 hours post infection by eliciting robust proinflammatory cytokines
88 and chemokines, including IFN-γ, IL-6, TNF-α, IL-8, CCL4, CCL5, CXCL10 (17). In a
89 separate study, glial cells derived from the brains of young LACV-infected mice produced
90 significant levels of IFN-β (18). Furthermore, preliminary observations have hinted at a
91 proinflammatory cytokine response in both the periphery and the brain following LACV

92 infection, with elevated levels of IL-6 and IL-12p40 in infected mice, which were reported to
93 be in comparable levels in young and adult mice (11, 19). Interestingly, administration of IL-
94 12 or GM-CSF encoded by a plasmid prior to LACV infection in *Ifnar1*$^{-/-}$ mice was shown to
95 increase survival rates, suggesting a potential protective role of these cytokines (20).
96 Similarly, proinflammatory cytokines have also been reported during an Oropouche virus
97 infection, a distantly related bunyavirus (21). Cytokines and chemokines therefore seem to
98 act as important contributing factors in the immune response to LACV and other bunyavirus
99 infections.

100 Given the contributions of cytokines and chemokines during LACV infection, this study
101 aims to address a knowledge gap by investigating the involvement of these molecules in
102 age-related susceptibility to LACV infection and their correlation with other components of
103 peripheral adaptive immunity, including neutralizing antibodies (nAbs) and T cells. To
104 address this knowledge gap, we present an in-depth analysis of nAbs, cytokine, and
105 chemokine levels of adult and weanling mice throughout the course of infection with $5x10^5$
106 plaque forming units (PFU) of LACV.

107

108 **2. Results**

109 **2.1 Age-related susceptibility is maintained despite infection with $5x10^5$ PFU of LACV.**

110 An unpublished observation reported no significant difference in cytokine levels
111 between adult and weanling mice infected with 1000 PFU of LACV (11). To investigate the
112 potential impact of infectious dose on immune system responsiveness to LACV infection, we
113 hypothesized that a higher dose might better reveal differences in cytokine and chemokine
114 levels if they exist. Previous studies have shown that infection with 10^5 PFU of LACV

115 maintains age-related susceptibility in mice (11). Peripheral inoculation routes, such as IP
116 and ID, yield consistent age-related susceptibility and survival outcomes, with similar LACV
117 dissemination to muscle cells near the injection site (4, 11). Based on these findings, we
118 used IP injection for LACV infection studies. We aimed to assess the effects of an infectious
119 dose five-fold higher than the previously reported dose by monitoring the development of
120 neurological signs in infected mice and measuring viral titers in peripheral blood. The five-
121 fold increase in infectious dose was chosen to reflect the higher end of the natural infectious
122 dose transmitted by mosquito bites, while maintaining age-specific susceptibility (22).
123 Following infection, serum from adult (n=16) and weanling (n=14) mice were serially
124 collected for downstream processing (Fig. 1).

125 Injection of $5x10^5$ PFU of LACV demonstrated a similar difference in survivability between
126 adult and weanling mice, reinforcing the age-related susceptibility to LACV infection. Adult
127 mice exhibited markedly higher survival rates compared to weanling mice ($p<0.001$), with
128 approximately 50% of adults surviving up to 12 days post-infection (dpi) (Fig. 2A). Weanling
129 mice began exhibiting signs of neurological disease starting at 5 dpi. Our findings show that
130 higher doses of LACV infection results in disease onset similar to that of lower infectious
131 doses that have historically been used (Fig. 2B). Consistent with published reports, we
132 detected LACV viremia between 1-3 dpi in both adult and weanling mice, with levels
133 significantly higher in weanling mice at 2 dpi (Fig. 2C) (3). This observation underscores the
134 retention of age-related susceptibility to LACV even at higher infectious doses, reaffirming
135 the correlation between viremia and survivability.

136

137

138 **2.2 Neutralizing antibody titers differ between LACV-infected adult and weanling mice,**
139 **with the ability of adult immune serum to protect weanlings from death.**

140 Neutralizing antibody (nAb) responses are pivotal for viral clearance in many
141 infections, yet our findings reveal an intriguing disparity between nAb titers and viral
142 clearance in weanling mice. Following infection with $5x10^5$ PFU of LACV, nAbs peaked in
143 adult mice between 8 and 12 dpi, whereas they peaked earlier in weanling mice between 4
144 and 6 dpi, coinciding with the onset of neurological signs (Fig. 3A-C). Despite the early nAb
145 responses in weanling mice, 100% succumbed to the infection by 7 dpi (Fig. 2A). These
146 results suggest that viral clearance and the onset of LACV-induced neurological signs may
147 not directly correlate with the nAb responses. To assess the protective efficacy of nAbs
148 derived from adult mice, we conducted an adoptive transfer of serum containing nAbs from
149 previously infected adult mice into naïve weanlings. The serum transfer was performed at
150 three distinct time points relative to LACV infection: one day prior to infection, one day post-
151 infection, and at symptom onset (4 dpi). We observed that 100% of weanling mice that
152 received serum one day before LACV infection survived the viral challenge (Fig. 3D). When
153 serum was administered one day post-infection, 50% of the weanling mice survived. Notably,
154 all weanling mice that received the serum at symptom onset succumbed to the infection,
155 similar to the control group that received saline. These findings suggest that the early transfer
156 of serum containing nAbs from previously infected adult mice confers a significant protective
157 effect against LACV in weanling mice.

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159

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161 **2.3 Cytokines and chemokines are differentially regulated in adult and weanling mice.**

162 To elucidate how serum cytokine and chemokine levels are modulated during LACV
163 infection in adult and weanling mice, we conducted a Luminex panel capable of
164 simultaneously detecting and quantifying Th1/Th2/Th9/Th17/Th22/Treg cytokines and
165 chemokines. Aliquots from the same serum samples used to assess viremia and nAbs were
166 assessed in this Luminex assay.

167 To delineate the potential roles of serum cytokines and chemokines in protection or
168 pathogenesis at different infection stages, we classified the course of infection into three
169 phases: (1) peripheral viremia (1-3 dpi), (2) symptoms onset (4-6 dpi), and (3) survival (>7
170 dpi). Additionally, we categorized the analyzed cytokines and chemokines into three groups:
171 Th1/Th2 cytokines, Th9/Th17/Th22/Treg cytokines, and chemokines Among the 21
172 modulated cytokines and chemokines, 9 belonged to the Th1/Th2 cytokine group (GM-CSF,
173 IFN-γ, IL-1β, IL-4, IL-6, IL-12p70, IL-13, IL-18, TNF-α, 6 were classified under the
174 Th9/Th17/Th22/Treg cytokine group (IL-9, IL-10, IL-17A/CTLA-8, IL-22, IL-23, IL-27), and
175 the remaining 6 were identified as chemokines in LACV-infected mice (Eotaxin/CCL11,
176 GROα/CXCL1, IP-10/CXCL10, MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5. Our data
177 reveal significant fluctuations in cytokine and chemokine levels between adult and weanling
178 mice, as shown by the heatmap illustrating p-values that compare these levels during the
179 first 6 days of LACV infection, prior to weanling succumbing to infection (Fig. 4).

180

181 **2.3.1 Dynamics of Th1/Th2 Cytokines during LACV Infection.**

182 IFN-γ, IL-18, TNF-α, and IL-13 emerged as the predominant Th1/Th2 cytokines
183 detected in serum samples from both adult and weanling mice (Fig. 5). During the peripheral

184 viremia phase, a significant elevation in IL-18 levels was observed as early as 1 dpi, followed
185 by subsequent significant peaks in IL-12p70 and GM-CSF in adult mice (Fig. 5A, 5F, 5H).
186 Notably, levels of IL-18 and IL-12p70 peaked during this early phase before gradually
187 declining as the infection progressed. Conversely, weanling mice displayed a notable
188 significant increase in levels of IL-6 towards the latter part of the peripheral viremia phase,
189 one day prior to symptoms onset (Fig. 5E).

190 At the symptoms onset phase, occurring between 4-6 dpi, several cytokines including
191 IFN-γ, IL-1β, TNF-α, GM-CSF, IL-13, and IL-4 were significantly elevated in both adult and
192 weanling mice (Fig. 5A-D, 5G, 5I). Most cytokines reached their peak levels during this
193 phase, with adult mice exhibiting higher levels of IL-1β, TNF-α, GM-CSF, IL-13, and IFN-γ
194 compared to weanlings. Conversely, IL-4 and IL-6 were significantly elevated in weanlings
195 compared to adult mice (Fig. 5D-E). Finally, during the survival phase (>7 dpi) IFN-γ and IL-
196 13 emerged as the primary cytokines specifically elevated during this late phage of infection
197 in adult mice.

198

199 **2.3.2 Dynamics of Th9/Th17/Th22/Treg Cytokines during LACV Infection.**

200 Infection with $5x10^5$ PFU of LACV revealed elevated levels across all
201 Th9/Th17/Th22/Treg cytokines analyzed in both adult and weanling (Fig. 6). Notably, IL-9,
202 IL-10, and IL-17A emerged as the most abundant cytokines within this category throughout
203 the infection course in both age groups. During the early peripheral viremia phase, peaks in
204 IL-9 and IL-17A levels underscored their early involvement in the immune response to LACV
205 infection in adult and weanling mice (Fig. 6A, 6C). Additionally, at 2 dpi, IL-23 levels were

206 significantly higher in adult mice compared to weanlings, mirroring the kinetics of IL-17A,
207 which exhibited significantly elevated levels in adult mice by 3 dpi (Fig. 6C, 6E).

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

214

215 **2.3.3 Dynamics of Chemokines During LACV Infection.**

216 Infection with $5x10^5$ PFU of LACV also triggered a significant increase in CXCL1 and
217 CCL11, followed by CXCL10, in both adult and weanling mice compared to uninfected
218 controls (Fig. 7A-C). This increase manifests as early as 1 dpi for CCL11 and CXCL1.
219 Particularly, CCL11, CCL3, and CCL5 consistently exhibited higher levels in adult mice
220 compared to weanlings throughout the infection course (Figure 7A, 7E, 7F). Intriguingly,
221 CCL3 and CCL5 levels peaked during the survival phase in adult mice, suggesting sustained
222 chemokine responses during later phases of infection.

223 Conversely, distinct chemokine patterns were observed in weanling mice, with certain
224 chemokines showing pronounced elevation whiles others exhibited minimal levels. For
225 example, CCL2 and CXCL10 levels were consistently higher in weanling mice across all
226 infection phases, whereas CXCL1 levels peaked exclusively during the symptom onset
227 phase (Fig. 7B, 7D). CCL5 and CCL3 levels in weanlings remained comparable to baseline
228 levels observed prior to infection (Figure 7E- F).

229

230 **2.3.4 Th1/Th2 cytokines production by $CD4^+$ and $CD8^+$ T cells during LACV infection.**

231 Previous studies have demonstrated the critical role of T cells in protecting adult mice
232 from LACV-induced neurological disease, with *Rag1*$^{-/-}$ adult mice succumbing by 15 dpi (10).
233 Given that $CD4^+$ and $CD8^+$ T cells are key mediators of cytokine production in adaptive
234 immune responses, we aimed to determine whether LACV infection induces specific cytokine
235 expression in these T cell subsets.

236 We analyzed splenic $CD4^+$ and $CD8^+$ T cells from both adult and weanling mice at 5
237 dpi by flow cytometry and intracellular cytokine staining. Our results showed that weanling
238 mice produced higher levels of IL-4 in both $CD4^+$ and $CD8^+$ T cells compared to adults,
239 regardless of LACV infection status (Fig. 8A, 8D). Notably, LACV-infected weanling mice
240 exhibited significantly higher IL-4 production in CD8+ T cells compared to uninfected controls
241 (Fig. 8D). However, IFN-γ production showed a different pattern. While $CD4^+$ T cells
242 produced comparable levels of IFN-γ across age groups and infection statuses, $CD8^+$ T cells
243 from infected adult mice displayed increased IFN-γ production compared to uninfected adults
244 (Fig. 8B, 8E).

245 To further explore the impact of LACV infection on cytokine balance, we calculated
246 the IFN-γ/IL-4 ratio in both $CD4^+$ and $CD8^+$ T cells. In uninfected controls, these ratios were
247 similar across age groups. However, in LACV-infected weanling mice, the IFN-γ/IL-4 ratio
248 was significantly lower in $CD4^+$ T cells, indicating a shift toward IL-4 production (Fig. 8C). A
249 similar trend was observed in $CD8^+$ T cells, although the difference was not statistically
250 significant (Fig. 8F).

251 Given these differences in cytokine profiles between weanling and adult mice, we
252 hypothesized that the more balanced cytokine profiles in adult mice may contribute to their
253 greater resistance to LACV-induced disease. To test this, we adoptively transferred bulk
254 splenocytes from immune adult mice into naïve weanlings prior to LACV infection. This
255 transfer conferred partial protection, evidenced by improved survival rates and delayed onset
256 of neurological disease compared to weanling controls that received naïve adult splenocytes
257 (Fig. 8G). These findings suggest that adult T cells and the cytokine environment they
258 establish may play a protective role in controlling LACV infection in weanling mice.

259 **3. Discussion**

260 This study presents an analysis of nAb titers and cytokine and chemokine dynamics
261 during LACV infection, shedding light on their roles in protection and pathogenesis. Our
262 findings demonstrate distinct patterns of cytokines and chemokines specific to age and
263 infection phase. We observed significant increases in Th1/Th2/Th9/Th17/Th22/Treg
264 cytokines and chemokines in response to LACV infection across both adult and weanling
265 mice. Interestingly, we observed age-specific differences in many cytokines and chemokines
266 throughout the course of infection (Fig. 9).

267 Our data emphasize the consistent influence of age-related susceptibility, regardless
268 of the infecting dose. Despite prior indications of age-related vulnerability using lower
269 infectious doses of LACV, our findings expand on this concept by demonstrating that age
270 remains a pivotal determinant of susceptibility, even when animals are infected with $5x10^5$
271 PFU of LACV (3). Specifically, we observed that weanling mice infected with $5x10^5$ PFU of
272 LACV exhibited elevated peripheral viremia along with nAbs with an early onset of
273 neurological disease. This underscores the correlation between viremia and survivability,

274 highlighting the relevance of our model for investigating both immune response dynamics
275 and disease pathology.

276 Our investigation into viral neutralization yielded unexpected findings. Despite the
277 early production of nAbs in weanling mice, these titers failed to correlate with viral clearance,
278 prompting speculation regarding the role of nAbs in early disease progression in weanling
279 mice. A plausible explanation is that the virus infects the brain in weanlings by 3 dpi,
280 rendering peripheral antibodies ineffective, which could explain the high titers observed.
281 However, the detection of nAbs in the CNS of weanling mice by 6 dpi suggests that these
282 antibodies can infiltrate to the CNS, where the virus is actively replicating (10). Nevertheless,
283 these findings indicate that nAbs are ineffective in controlling the infection within the CNS as
284 well. Notably, different neutralization capabilities have been associated with isotype
285 switching, a phenomenon documented in other viral infections such as HIV and SARS-CoV-
286 2 (23, 24). Interestingly, adult mice exhibited significantly lower nAb titers during the
287 symptoms onset phase, suggesting either rapid nAbs consumption in vivo or the involvement
288 of non-neutralizing antibodies in protection. However, given that weanling nAbs were
289 incapable of effectively eliminating the virus in vivo, the former explanation seems less likely.

290 The complete protection observed in weanling mice following the early adoptive
291 transfer of immune adult serum containing nAbs to weanlings emphasizes the importance of
292 the humoral response in mediating protection. This finding raises several important
293 questions, particularly regarding how adults achieve this rapid level of nAbs. One key area
294 that remains unclear is whether the difference in protective potential between weanlings and
295 adults is due to functional differences in the antibodies produced, the timing of their release,
296 or a combination of both factors. Understanding these nuances is crucial, as it could shed
297 light on the mechanisms that confer immunity and guide the development of targeted

antibody-based therapies. To clarify these mechanisms, further studies focusing on antibody isotypes, function, and dynamics are necessary. While antibodies are likely key players, the protection might also be mediated by other components in the serum, such as complement or cytokines. The exact contribution of these factors remains to be clarified.

Our data demonstrate that cytokines and chemokines play a key role in age-specific susceptibility to LACV infection. We observed significant shifts in these immune mediators between adult and weanling mice during LACV infection. Given that cytokine- and chemokine-driven responses are essential for both innate and adaptive immunity, understanding their dynamics is especially important in neurotropic viral infections like LACV, which can infiltrate the CNS (25). Variability in cytokine and chemokine responses has been shown to significantly influence viral infection outcomes and the risk of severe disease(25). Additionally, previous studies have reported differences in cytokine and chemokine levels between adults and young individuals, even under healthy conditions (26). These findings suggest that age-related variations in these immune mediators could contribute to differing susceptibilities to viral infections, such as LACV. Therefore, further investigation into cytokine and chemokine profiles is warranted to better understand their role in the increased vulnerability of younger hosts to LACV infection.

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

322 Our study reveals distinct patterns of Th1/Th2 cytokines around symptom onset
323 during LACV infection in weanling mice. Particularly intriguing is the role of IL-6, a key factor
324 in determining the balance between Th1 and Th2 responses. Our data suggest that IL-6,
325 along with IL-4, promotes Th2-related responses during symptom onset, correlating with
326 higher nAb levels in weanling mice, supporting the Th2 skew hypothesis (27). Additionally,
327 the substantial and sustained increase in Th1-related cytokines in adult mice underscores
328 their protective role against disease, consistent with previous observations (11, 20).

329 Moreover, our findings in the context of Th1/Th2 cytokines are consistent with
330 previous studies on CNS viral infections. For instance, elevated IL-6 levels in the CNS in
331 infections with dengue or Japanese encephalitis viruses have been associated with various
332 neurological manifestations and poor outcomes (28, 29). In contrast, our observation of the
333 substantial and sustained upregulation of Th1-related cytokines, including IL-18, IFN-γ, IL-
334 12, and GM-CSF, throughout LACV infection supports previously reported data of protective
335 roles of GM-CSF and IL-12 in *Ifnar1*$^{-/-}$ mice and underscores their role in eliciting protective
336 responses in mice resistant to the disease (20).

337 Among these cytokines, the pronounced elevation of IL-18 in adult mice stands out
338 as the most significant shift induced by LACV. IL-18 has been demonstrated to stimulate the
339 proliferation and differentiation of T cells toward a Th1 response and acts as a crucial cofactor
340 in IL-12-induced IFN-γ production by natural killer (NK) and T cells (30). Moreover, IL-18
341 exerts its proinflammatory effects by upregulating cytokines such as IL-13, IL-1β, and TNF-
342 α, consistent with our findings (31). Furthermore, IFN-γ emerged as one of the most robust
343 responses, particularly increased in adult mice, especially during the survival phase. Our
344 findings align with previous observations indicating increased IFN-γ production from
345 neuron/astrocyte co-cultures infected with LACV in a dose-dependent manner (17). IFN-γ

346 serves not only as a marker of Th1 $CD4^+$ and $CD8^+$ T cells and NK cells but also as a crucial
347 antiviral mediator central to eliminating viruses from the CNS (32).

348 In our analysis of Th9/Th17/Th22/Treg cytokines, all exhibited increased levels in
349 adult mice in response to LACV infection. Among these, IL-23 and IL-27 were among the
350 most significantly increased. Although these cytokines have been predominantly studied in
351 the context of autoimmune diseases, emerging evidence suggests their crucial roles during
352 viral infections (33, 34). Specifically, IL-23 has been implicated in the control of viral infections
353 within the brain, indicating its potential importance in host defense mechanisms against
354 neurotropic viruses such as LACV (33). Similarly, IL-27 has been found to mediate NK cell
355 effector functions during viral infections, highlighting its involvement in orchestrating innate
356 immune responses against viral pathogens (34).

357 Chemokines, as a distinct group of immune mediators, demonstrated elevated levels
358 in response to LACV infection, with increases observed in CCL5, followed by CCL3, in adult
359 mice. In contrast, CCL2 peaked in weanling mice. The expression and release of CCL2 and
360 CCL3 on cerebral endothelium suggest an essential role for these chemokines in regulating
361 the trafficking of inflammatory cells across the blood-brain barrier (BBB) during CNS
362 inflammation (35). Furthermore, CCL3 has been shown to contribute to the differentiation
363 and migration of effector T cells in response to viral infection within the CNS (36). Additionally,
364 CCL5 plays a crucial role in the homing and migration of effector and memory T cells during
365 acute CNS infections (37). CCL2 emerges as a major player in mediating monocyte
366 infiltration and the trafficking of inflammatory monocytes to the brain during viral encephalitis
367 (38). Our findings, indicating increased levels of CCL2 in weanling mice, which by logic,
368 potentially contributes to pathogenesis, potentially mediated through the recruitment of an
369 overwhelming inflammatory response in the infected brain.

370 However, LACV-induced lymphocyte infiltration of CNS itself is less likely to contribute
371 to pathogenesis according to a recent study (10). With this study being one of the few to
372 focus specifically on LACV infections, significant gaps persist in our understanding of
373 immune correlates and the mechanisms underlying immune-mediated viral clearance. More
374 often than not, successful clearance of virus infection from the CNS requires the elimination
375 of virus from the cells without causing damage to neurons. Ideally, immune-mediated virus
376 clearance from neurons occurs mainly through early cytolytic processes, followed by non-
377 cytolytic processes that are mediated by antibody specific for the virus and IFN-γ, which
378 enable neurons to survive (39). Building upon this observation, a better understanding of
379 adaptive immune responses is needed to address LACV-induced neurological disease.

380 Our data indicate that adaptive immune responses mediated by T cells, specifically
381 $CD4^+$ and $CD8^+$ T cells, differ significantly between adult and weanling mice during LACV
382 infection. The analysis of IL-4 and IFN-γ production by $CD4^+$ and $CD8^+$ T cells during LACV
383 infection provides insights into the underlying mechanisms that contribute to differential
384 immune responses in weanling versus adult mice. IL-4 and IFN-γ are key cytokines
385 representing opposing arms of the immune response, with IL-4 linked to Th2 responses that
386 promote humoral immunity and tissue repair, and IFN-γ associated with Th1 responses that
387 drive cell-mediated immunity and viral clearance (40-43). In our study, the elevated IL-4
388 production in LACV infected weanling mice, particularly in $CD8^+$ T cells, suggests a skewing
389 toward a Th2-type response, which may be less effective in controlling LACV replication and
390 mitigating disease severity. Published work have identified a subset of IL-4 producing-$CD8^+$
391 T cells that have been described in humans in several diseases including HIV infection and
392 *Mycobacterium tuberculosis* infection (44, 45). In contrast, the increased IFN-γ production
393 observed in adult $CD8^+$ T cells indicate a more robust Th1 response, which likely contributes

394 to better viral control and protection from severe neurological disease. This is consistent with
395 the observation that adult mice are generally more resistant to LACV-induced disease
396 compared to weanling mice. Moreover, the shift in the IFN-γ/IL-4 ratio produced by $CD4^+$ and
397 $CD8^+$ T cells in weanling mice toward a Th2 profile supports the notion that a balanced
398 Th1/Th2 response is necessary for effective viral clearance and protection from severe
399 disease.

400 Furthermore, the functional significance of a balanced Th1/Th2 response mediated
401 by immune cells in LACV infection was demonstrated by the delayed disease onset and
402 improved survival following total splenocyte transfer from previously infected adults to
403 weanling mice. The fact that adult splenocytes were able to confer partial protection to
404 weanling mice suggests that the enhanced IFN-γ production from adult CD8 T cells may play
405 a critical role in mediating this protective effect. This emphasizes the need to investigate the
406 functionality and specificity of these T cells, particularly whether they target viral antigens.
407 However, it remains unclear whether the protective effect is primarily mediated by $CD4^+$ or
408 $CD8^+$ T cells, and further detailed characterization is required to define their specific
409 contributions.

410 It is important to acknowledge the limitations of our study. Characterization of
411 cytokines and chemokines secreted into the serum may not fully capture the immune activity
412 within the infected host, as the brain-resident immune response may differ from the systemic
413 immune response. Additionally, we cannot definitively conclude whether a specific skew in
414 Th1/Th2 responses occurred, as the cellular source of the cytokines detected in the serum
415 remains unknown This study is primarily descriptive and based on observational data. While
416 it provides valuable insights into the LACV-induced peripheral cytokine and chemokine

417 response, experimental investigations are required to further elucidate the cell types and
418 underlying mechanisms driving these dynamics in nAbs, cytokines, and chemokines.

419 In conclusion, our study contributes to a deeper understanding of immune responses
420 to neurotropic bunyavirus infections, offering insights into potential therapeutic targets and
421 biomarkers for disease management. Future research should focus on elucidating the
422 underlying mechanisms driving age-related susceptibility, further characterizing the immune
423 response dynamics, and exploring therapeutic interventions to mitigate the neurological
424 complications associated with LACV infection. By advancing our understanding of host-virus
425 interactions, we can develop more effective strategies for the prevention, diagnosis, and
426 treatment of LACV-induced disease, ultimately enhancing public health preparedness
427 against emerging viral threats.

428

429 **Methods**

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

431 All animal studies were approved by the Rutgers IACUC (protocol number
432 202300007). Wildtype (C57Bl/6) mice were purchased from Jackson Laboratories and
433 maintained in a breeding colony at the Child Health Institute of New Jersey. LACV (La Crosse
434 Virus, NR-540), a human isolate was purchased obtained from BEI Resources. Mice at 3
435 (weanling) or 6-8 (adult) weeks of age were inoculated with $5x10^5$ plaque forming units (PFU)
436 of LACV in phosphate-buffered saline (PBS) intraperitoneally in a volume of 200 µl/mouse.
437 Mice were observed daily for signs of neurological disease that included hunched posture,
438 seizures, reluctance, or inability to move normally or paralysis. Animals with clear clinical
439 signs of neurological disease were scored as clinical and euthanized immediately.

440 **Serum sample collection**

441 Submandibular blood was collected into heparin coated tubes. Blood samples were
442 centrifuged at 2000 xg for 10 minutes. After centrifugation, sera was aliquoted and stored in
443 -80°C for subsequent analysis. Serum samples were collected from a total of 14-16 mice in
444 each age group (Fig. 1). Those mice were divided into two subgroups, with each group being
445 bled every other day. Accordingly, serum samples were collected on 0, 1, 2, 3, 4, 5, 6, 7, 8
446 and 12 dpi. This study design was implemented to reduce the number of mice used for ethical
447 purposes and to ensure that the serum sample volume from each mouse remained within
448 ethical guidelines. Serum samples collected on odd days were pooled by gender. The
449 sample size decreased over time as some mice did not survive the infection.

450 To assess the correlation between cytokine and chemokine levels and indicators of
451 viral clearance and neutralizing antibody responses, each serum sample was used in three
452 assays: viremia, neutralization, and cytokine/chemokine quantification.

453

454 **Neutralizing antibody detection**

455 For quantification of neutralizing antibodies, sera samples were serially diluted in
456 quadruplicates and mixed with an equal volume of LACV corresponding to 100 PFU per in a
457 final volume of 100 µl in DMEM/10% FBS/1% Pen Strep. The mixture was preincubated for
458 2 h at 37°C. The 100 µl mixtures were then added to confluent Vero cells in a 96-well plate
459 and incubated for 3 days. After incubation, plasma/virus mixture was removed, and cells
460 were stained with 0.1% crystal violet for 2 h. After staining, cells were rinsed with deionized
461 water. Neutralizing antibody titers were determined by the highest plasma dilution protecting
462 50% of the infected wells.

463

464 **qPCR**

465 Viral titers were quantified by qPCR. Total viral RNA was extracted from serum using
466 Pure link viral RNA mini kit (Invitrogen) following manufacturer's protocol. cDNA was
467 prepared from RNA samples using the SuperScript IV VILO reverse transcription kit
468 (Invitrogen) following the manufacturer's instructions. cDNA samples were diluted fivefold in
469 Rnase-free water and those diluted samples were analyzed by qPCR using PowerTrack
470 SYBR Green kit (Appliedbiosystems) to detect LACV RNA. The primer sequences used in
471 this study are: forward (ATTCTACCCGCTGACCATTG), and reverse
472 (GTGAGAGTGCCATAGCGTTG). CT values were measured using a QuantStudio 3 Flex
473 (Applied Biosysterms) machine. QuantStudio Real-Time PCR software was used to extract
474 the data.

475 **Adoptive serum transfer**

476 Adult mice previously infected with LACV were sacrificed at 14 days dpi, and serum
477 was collected via cardiac puncture. For the adoptive transfer experiments, weanling mice
478 received 200 μL of serum intraperitoneally at three distinct time points: one day before LACV
479 challenge, one day after LACV challenge, or on the day of symptom onset (4 dpi). As a
480 control, a separate group of weanling mice received 200 μL of sterile saline intraperitoneally
481 at each of the corresponding time points.

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

483 To quantify the levels of cytokines in LACV infected serum samples, a magnetic bead-
484 based multiplex immunoassay was performed using a MAGPIX® (Luminex Corporation,
485 USA) instrument system. A mouse cytokine magnetic 26-plex panel (Invitrogen) was utilized

486 to determine immune responses mediated through cytokine and chemokine such as (1)
487 Th1/Th2: GM-CSF, IFN-γ, IL-1 beta , IL-2 , IL-4 , IL-5, IL-6, IL-12p70, IL-13, IL-18, TNF alpha,
488 and (2) Th9/Th17/Th22/Treg: IL-9, IL-10, IL-17A (CTLA-8), IL-22, IL-23, IL-27, (3)
489 Chemokines: Eotaxin (CCL11), GRO alpha (CXCL1), IP-10 (CXCL10), MCP-1 (CCL2),
490 MCP-3 (CCL7), MIP-1 alpha (CCL3), MIP-1 beta (CCL4), MIP-2, RANTES (CCL5) following
491 the manufacturer's instructions.

492 **Tissues processing for flow cytometry and intracellular staining (ICS)**

493 Spleens from LACV-infected and uninfected mice were harvested at 5 dpi, as
494 described previously (10). Spleens were processed into single-cell suspensions by passing
495 them through a 70 μM mesh filter, followed by red blood cell lysis using RBC Lysis Buffer
496 (Thermo Fisher Scientific). Isolated cells were then seeded at a density of 2 x 10^6 cells per
497 well in 96-well U-bottom plates and cultured. Cells stimulated with PMA served as a positive
498 control, and Brefeldin A was added during the 4-hour incubation. After incubation, cells were
499 stained for viability and Fc receptors were blocked. Cells were stained for extracellular
500 markers (CD8a, CD4, and CD3), fixed and permeabilized, and stained for intracellular
501 cytokines (IL-4 and IFN-γ). Flow cytometry was performed using the Aurora system (Cytek®
502 Biosciences), and data were analyzed with FlowJo software, gating on live cells and
503 excluding doublets.

504 **Adoptive transfer of total splenocytes**

505 Donor mice are euthanized at 14 dpi, spleens are harvested, and splenocytes are
506 generated as described above. Naïve recipient mice receive transfusions of 10^6 total cells in
507 100 ul PBS via tail vein injection and are subsequently challenged 1 day post transfusion
508 with 10^3 PFU LACV via intraperitoneal injection.

Statistical analysis

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

Data Availability

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

Acknowledgements

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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

			Blood Collection (days post-infection)												
	Group	n	0	1	2	3	4	5*	6*	7	8	9*	10	11	12*
Weanling	A	7	●	-	●	-	●	-	●	-	-	-	-	-	-
	B	7	●	●	-	●	-	●	-	-	-	-	-	-	-
Adult	A	7	●	-	●	-	●	-	●	-	●	-	-	-	●
	B	9	●	●	-	●	-	●	-	●	-	-	-	-	●

Figure 1. Sample collection schema. Adult and weanling mice were divided into two groups: Group A was bled on even days and Group B on odd days. Serum samples from each mouse were aliquoted for subsequent analysis of viremia, antibody neutralization, and cytokine and chemokine levels. Samples collected on day 0 served as controls. *, days when mice were removed from the study upon reaching the euthanasia endpoint. Red shaded squares, all weanling mice succumbed to infection. -, indicates no blood collection.

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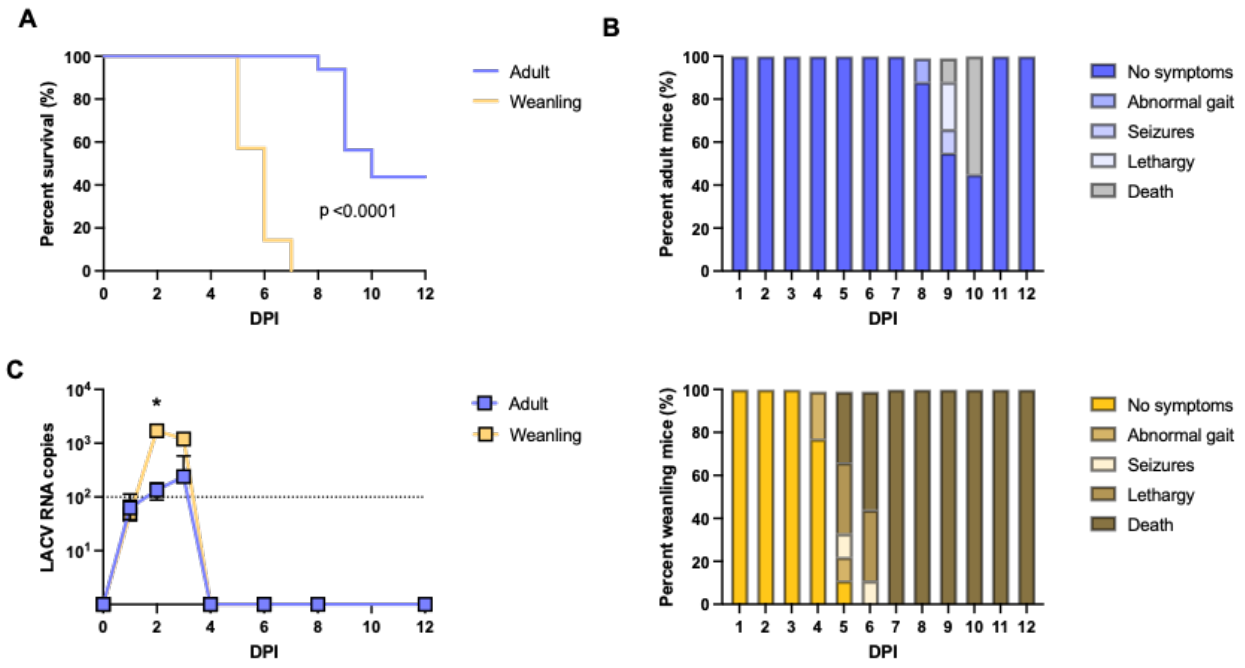

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725 **Figure 2. Impact of 5x10^5 PFU of LACV infection on viremia and symptoms. (A)** Percent survival

726 of adult and weanling LACV-infected mice. Survival analysis was performed using the log-rank test

727 ($p<0.001$) (n=7 weanling mice, n=9 adult mice). **(B)** Percent mice showing LACV-induced outcomes

728 in adult (top) and weanling (bottom) mice. **(C)** Viral titers in sera of LACV-infected adult and weanling

729 mice, represented by LACV RNA copies quantified by qPCR. Statistical analysis was performed using

730 Mann-Whitney U test. *$p<0.05$ indicates significant differences. DPI, days post-infection.

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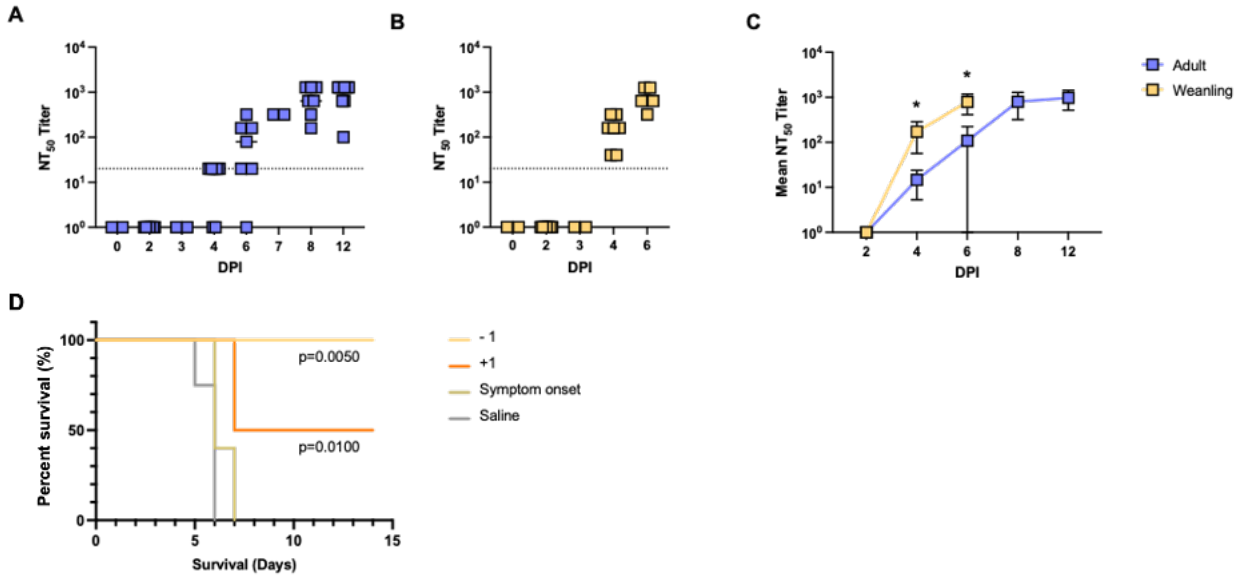

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744 **Figure 3. Neutralizing antibody titers differ between LACV-infected adult and weanling mice,**

745 **with the ability of immune adult serum to protect weanlings from death. (A, B)** NT_{50} titers

746 from LACV-infected adult and weanling mice throughout the course of infection. **(C)** Mean NT_{50}

747 titers between adult and weanling mice. Statistical analysis was performed using the Mann-Whitney

748 U test; *$p<0.05$. **(D)** Percent survival of weanling mice following adoptive transfer of immune adult

749 serum or saline prior or post-LACV challenge. -1, immune adult serum transferred to naïve

750 weanling mice one day prior to LACV challenge (n=6; p=0.005). +1, immune adult serum

751 transferred to naïve weanling mice one day post-LACV challenge (n=4; p=0.01). Symptoms onset,

752 immune adult serum transferred to naïve weanling mice at 4 days post-infection (n=4; not

753 significant). Saline, saline transferred to naïve weanling mice one day prior to LACV challenge as

754 control (n=4; not significant). Survival analysis was performed using the log-rank test.

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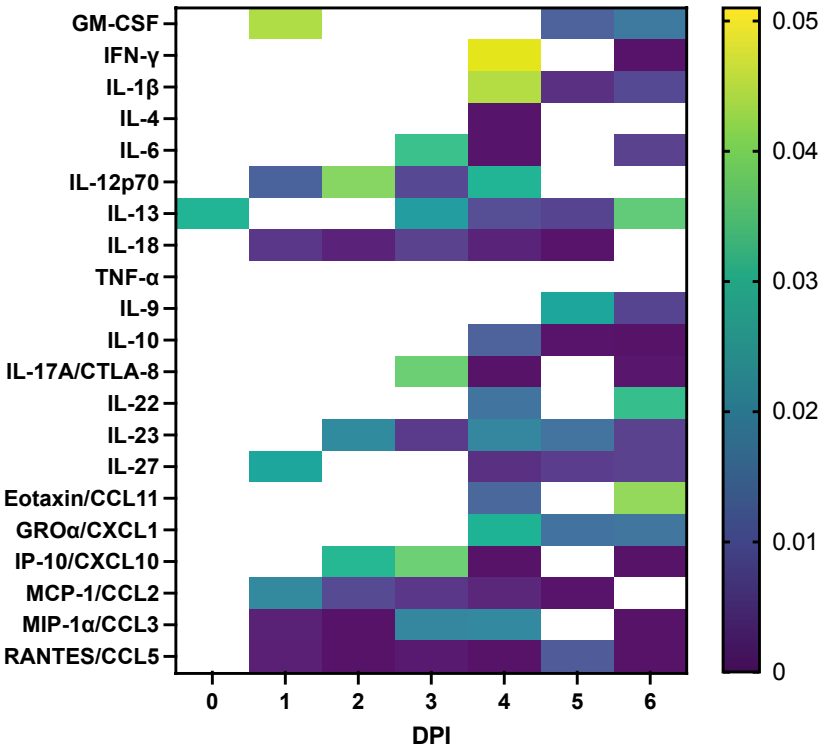


Figure 4. Serum cytokines and chemokines levels differ in adult and weanling mice. Heatmap of p-values derived from Mann-Whitney U test comparing cytokine and chemokine levels in adult and weanling mice during the course of LACV infection (1-6 days post-infection [DPI]). The rows represent cytokines and chemokines and the columns represent the time at which the sample was collected post-infection. Darker colors represent higher p-value.

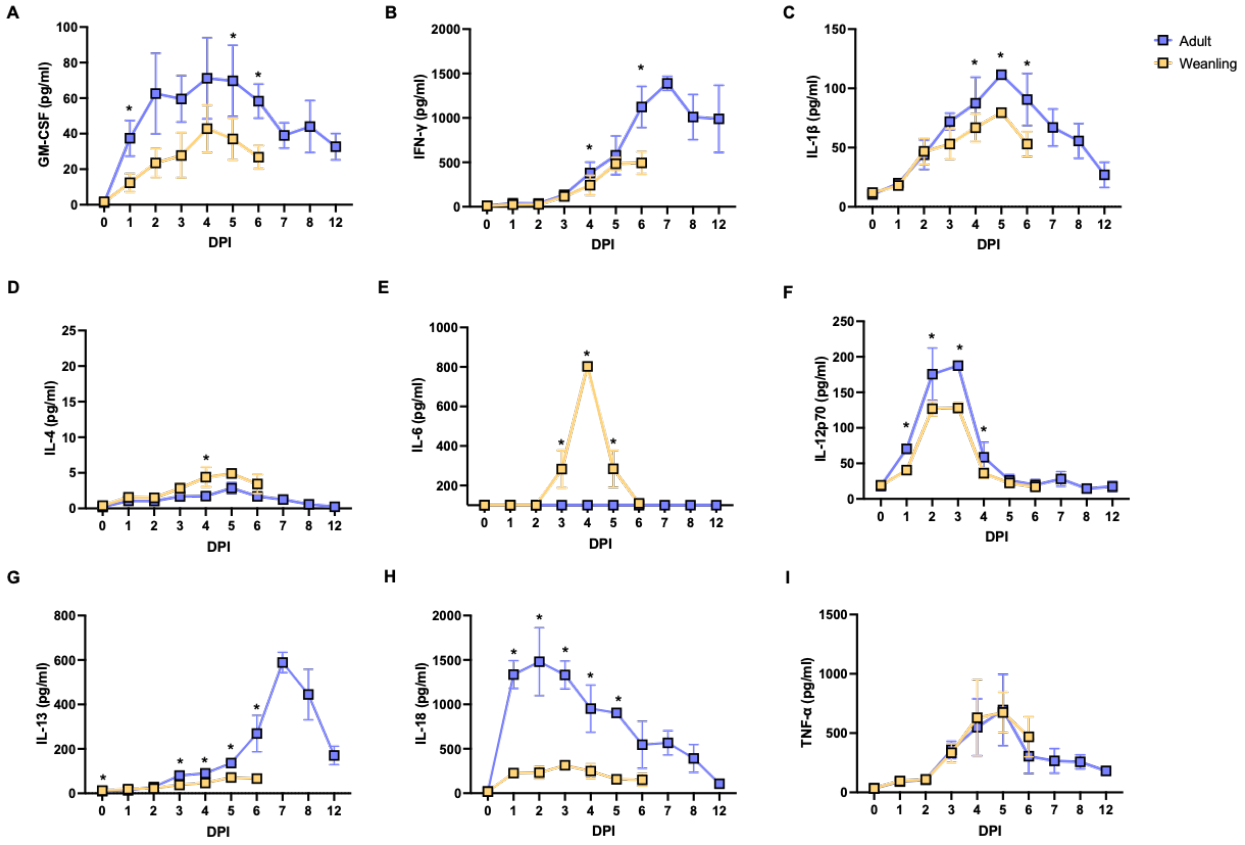
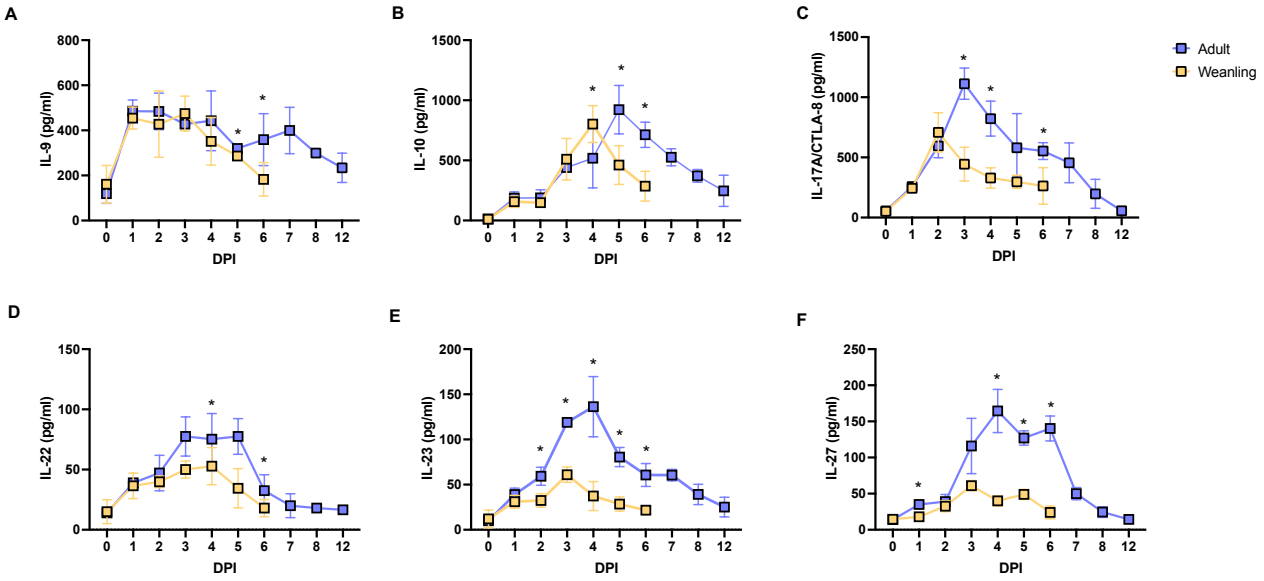


Figure 5. Dynamics of Th1/Th2 cytokines during LACV infection. Line charts representing levels of Th1/Th2 related cytokines (A-I) in sera of adult and weanling mice during LACV infection. Data represent average ± standard deviation. Statistical analysis was performed using the Mann-Whitney U test. *$p<0.05$ indicates significant differences.



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807 **Figure 6. Dynamics of Th9/Th17/Th22/Treg cytokines during LACV infection.** Line charts
808 representing levels of Th9/Th17/Th22/Treg related cytokines (A-F) in sera of adult and weanling mice
809 during LACV infection. Data represent average ± standard deviation. Statistical analysis was
810 performed using the Mann-Whitney U test. *$p<0.05$ indicates significant differences.

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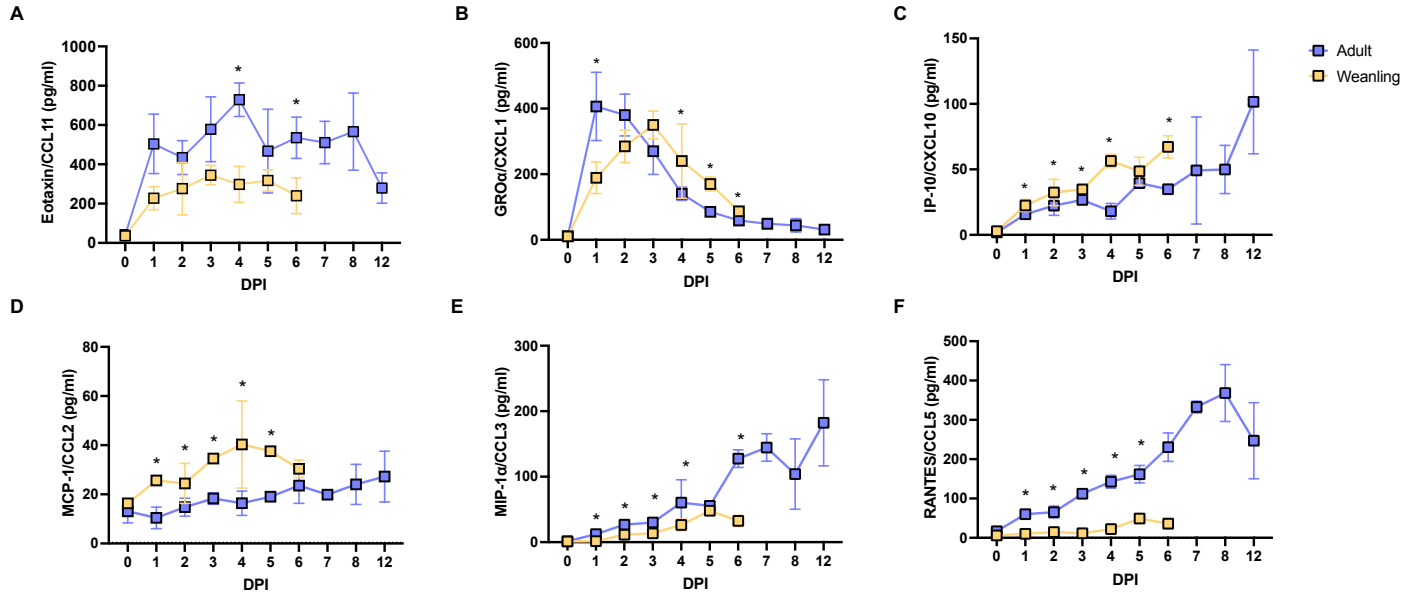

Figure 7. Dynamics of chemokine levels during LACV infection. Line charts representing levels of chemokines (A-F) in sera of adult and weanling mice during LACV infection. Data represent average ± standard deviation. Statistical analysis was performed using the Mann-Whitney U test. *$p<0.05$ indicates significant differences.

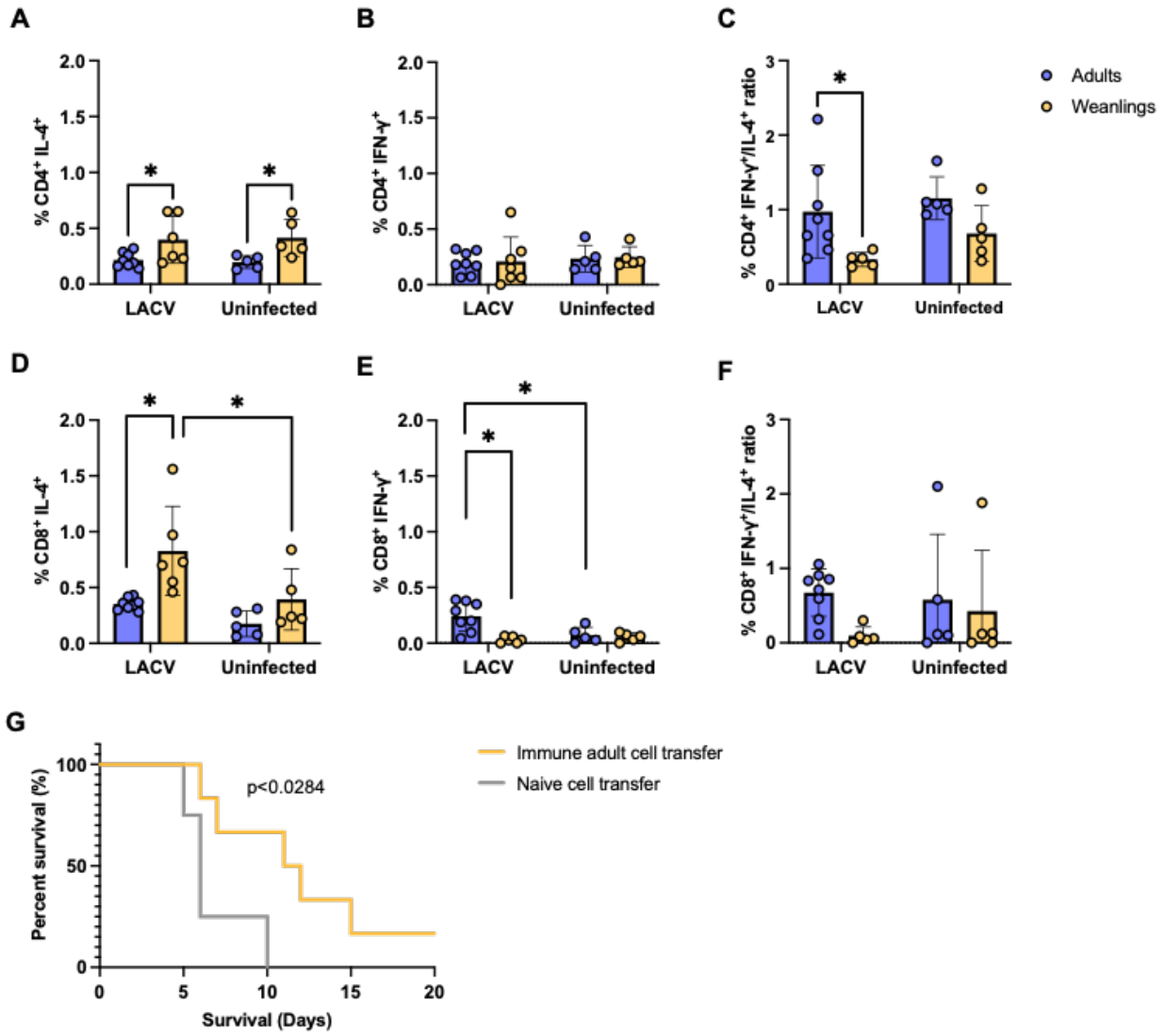

Figure 8. Th1/Th2 cytokine production by CD4⁺ and CD8⁺ T cells during LACV infection. (A-F) Bar charts showing CD4⁺ (A-B) and CD8⁺ (D-E) T cells positive for IL-4 or IFN-γ, measured by intracellular staining (ICS). IFN-γ⁺/IL-4⁺ ratios for CD4⁺ and CD8⁺ T cells are shown in (C) and (F), respectively (n=5-8). Data represent the mean ± standard deviation. Statistical analysis was performed using the Mann-Whitney U test. *p<0.05 indicates significant differences. **(G)** Percent survival of weanling mice following adoptive transfer of total splenocytes from previously infected adult mice (immune adult cell transfer), then challenged with LACV (n=6) compared to weanlings

receiving cells from naïve adult mice (n=6). Survival analysis was performed using the log-rank test ($p<0.0284$).

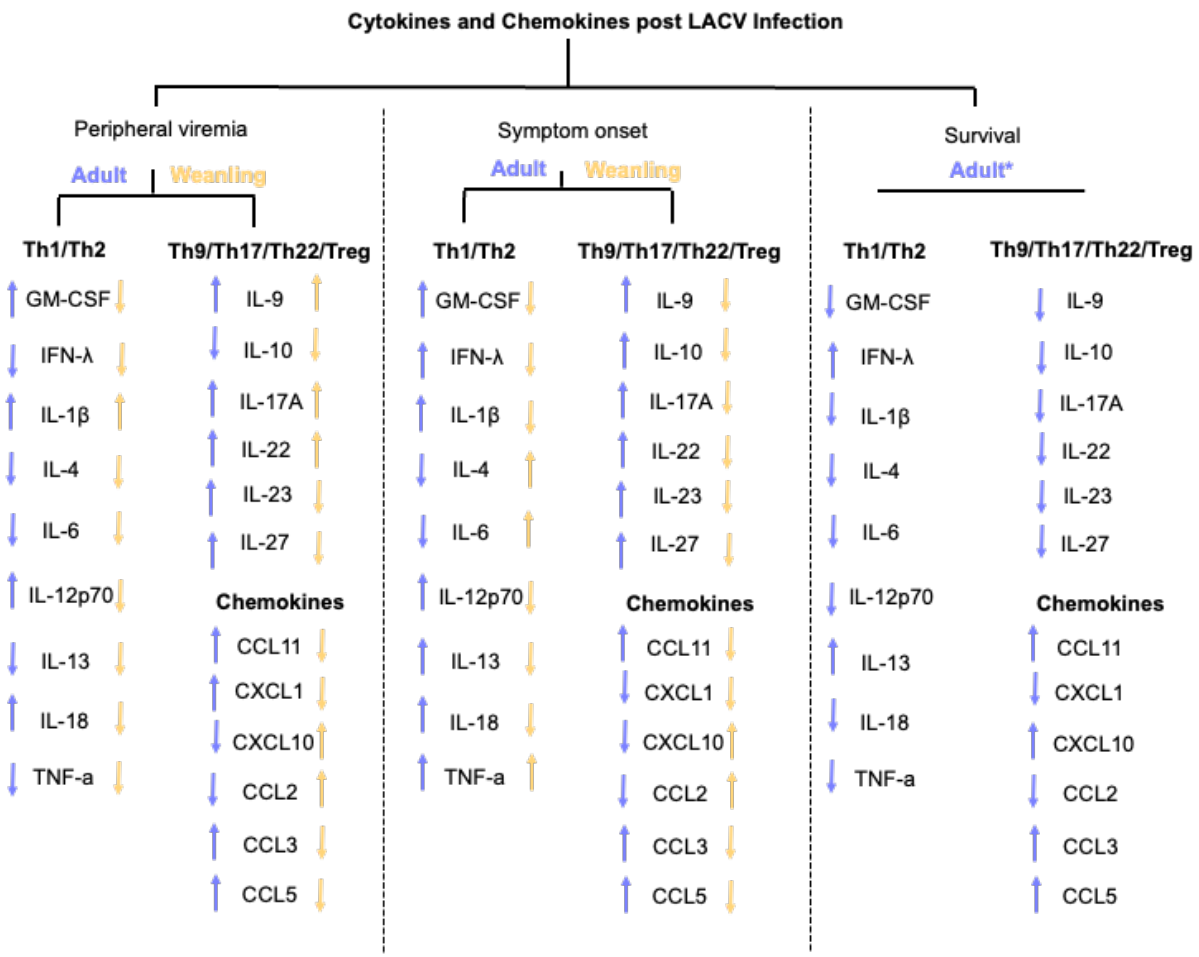

Figure 9. Summary of age-specific dynamic changes of cytokines and chemokines in LACV-infected adult and weanling mice. Relative shift in cytokine or chemokine levels across different stages of LACV infection, categorized as peripheral viremia, symptom onset, and survival stage, in adult and weanling mice. Purple arrows represent trends observed in adult mice, while yellow arrows represent trends observed in weanling mice. *, level compared to symptoms onset stage in adult mice.