

1 **Axl deficiency promotes the neuroinvasion of Japanese encephalitis virus by**
2 **enhancing IL-1 α production from pyroptotic macrophages**

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16 **Running title:** Axl deficiency promotes neuroinvasion of Japanese encephalitis virus.

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23 **Abstract**

24 Japanese encephalitis virus (JEV) is a flavivirus that causes Japanese encephalitis (JE),
25 which has an unclear pathogenesis. Despite vaccination, thousands of deaths
26 attributed to JE are reported annually. In this study, we report that mice deficient for
27 Axl, a receptor tyrosine kinase that plays multiple roles in flaviviral infection,
28 displayed greater mortality upon JEV infection. The effect of Axl deficiency on JEV
29 infection was mediated by markedly elevated serum interleukin-1 α (IL-1 α) levels,
30 which devastated the blood-brain-barrier and promoted viral neuroinvasion within 24
31 h post infection. Using an in situ infection model, we showed that dead macrophages
32 were the primary source of observed increased serum IL-1 α levels. Axl deficiency
33 enhanced cell death and caused pyroptosis in 80% of JEV-infected macrophages by
34 disrupting PI3K-Akt signaling. Intriguingly, the primary effector released by
35 pyroptotic macrophages in our model was IL-1 α rather than IL-1 β . Finally, we
36 assessed the effect of an IL-1 α antagonist and demonstrated that it effectively
37 prevented the incidence of JE. Our results indicate that Axl plays a protective role in
38 JEV infection, identify IL-1 α released by pyroptotic macrophages as a crucial factor
39 promoting JEV neuroinvasion, and suggest that an IL-1 α antagonist may be a
40 candidate for JE therapy.

41 **Importance**

42 Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus that causes Japanese
43 encephalitis (JE), the most commonly diagnosed viral encephalitis worldwide. The
44 fatality rate of JE is 20%, and nearly half of the surviving patients develop

45 neuropsychiatric sequelae. Axl is a receptor tyrosine kinase that plays multiple roles
46 in flaviviral infections. Currently, the involvement of Axl in JEV infection remains
47 enigmatic. In this study, we demonstrate that Axl impedes the pathogenesis of severe
48 JE in mice by maintaining blood-brain-barrier (BBB) integrity and restricting viral
49 neuroinvasion. Furthermore, serum IL-1 α is a key mediator of this process and is
50 primarily released by JEV-infected pyroptotic macrophages to elicit BBB breakdown,
51 while an IL-1 α antagonist can effectively reduce the incidence of severe JE. Our work
52 uncovers the protective role of Axl in antagonizing severe JE and shows that the use
53 of IL-1 α antagonist may be a promising tactic to prevent severe JE.

54

55 **Introduction**

56 Japanese encephalitis virus (JEV), a member of the same genus (*Flavivirus*) as
57 dengue virus (DENV), Zika virus (ZIKV), and West Nile virus (WNV), is transmitted
58 by *Culex* mosquitos and causes Japanese encephalitis (JE), which is endemic in 24
59 countries and is the most important viral encephalitis worldwide(1). Although
60 vaccination has dramatically reduced the incidence of JE, approximately 67,900 cases
61 of JE still occur annually(2). Unfortunately, even after surviving the encephalitis,
62 nearly half of patients bear permanent neuropsychiatric sequelae(1). Moreover, due to
63 a number of unfavorable factors, such as insufficient vaccination coverage and global
64 warming, the incidence of JE is still increasing in some developing countries(3).
65 Nevertheless, to date, we have neither enough knowledge regarding the mechanism of
66 JEV infection nor a specific therapy for JE.

67

68 As a member of the receptor tyrosine kinase family TAM (Tyro3, Axl, and Mertk),
69 which recognizes phosphatidylserines (PtdSers) exposed on surface of apoptotic cells
70 and enveloped viruses(4, 5), Axl has been proposed to be an entry receptor for various
71 enveloped viruses, such as Lassa virus(6), vaccinia virus(7), and ebolavirus(8).
72 Regarding flaviviruses, a group to which JEV belongs, Axl has been thought to serve
73 as an entry receptor for ZIKV, DENV, and WNV(9-12). However, accumulating
74 evidence has shown that its roles in flaviviral infection are diverse and vary based on
75 the virus and host type. For example, Axl mediates DENV entry via Gas6
76 bridging(13), while Axl knockout has no impact on WNV replication in target organs
77 but increases its neuroinvasion(14). The involvement of Axl in ZIKV infection is
78 more complicated and controversial. Although Axl promotes ZIKV infection in many
79 cell types in vitro, its knockout has no impact on ZIKV replication or pathogenesis in
80 mouse models, human neural progenitor cells or cerebral organoids(15-17). Recently,
81 Axl knockout mice were shown to be resistant to ZIKV pathogenesis in an
82 age-dependent manner, corresponding to lower pro-interleukin-1 β (pro-IL-1 β)
83 production and decreased apoptosis in microglia(18). The complexity of the
84 contributions of Axl to flaviviral infection is potentially associated with its versatile
85 functions. Axl not only mediates the removal of dead cells by phagocytes and
86 maintains immune and inflammatory homeostasis, but also regulates all forms of
87 programmed cell death(19-21). Therefore, Axl has additional roles beyond that of a

88 receptor in flaviviral infection, and its roles in infection need to be investigated on an
89 individual basis for each flavivirus.

90

91 In this study, using Axl deficient mice, we discovered that Axl suppresses JE
92 pathogenesis by inhibiting IL-1 α production from pyroptotic macrophages induced by
93 JEV. IL-1 α mediates the early neuroinvasion of JEV by disrupting BBB integrity, and
94 an IL-1 α antagonist was shown to potently reduce the incidence of severe JE. The
95 results of our study revealed the crucial roles of Axl and IL-1 α in JEV infection and
96 suggest that IL-1 α antagonists may candidate drugs for JE therapy.

97

98 **Results**

99 **1. Axl deficiency promotes higher mortality after JEV infection**

100 To study the role of Axl in JEV infection, four-week-old Axl deficient mice (Axl^{-/-})
101 and their littermate control mice (Axl^{+/+}) received an intraperitoneal (i.p.) injection of
102 different doses of JEV (10⁴, 10⁵, and 10⁶ PFU per mouse). Axl^{+/+} mouse littermates
103 were used as controls because they are more comparable with respect to genetic
104 background, age, and nutrition status, all of which have important impacts on
105 mortality upon JEV infection according to our experience. At 6-8 days post infection
106 (dpi), 17-91% of mice, which depended on the dose, began to lose weight and became
107 sluggish. As the disease progressed, these mice gradually manifested ruffled fur,
108 hunched back, limb paralysis, and finally died from encephalitis. At a dose of 10⁴

109 PFU of JEV, the mortality of the $Axl^{-/-}$ mice was 50% (11/22), which was significantly
110 higher than the 17% (2/12) observed in the $Axl^{+/-}$ mice ($p<0.05$) (**Fig. 1A**),
111 demonstrating that the $Axl^{-/-}$ mice were more susceptible to JEV infection. As the
112 infection dose increased, the mortality gap between the $Axl^{-/-}$ and $Axl^{+/-}$ mice
113 decreased and vanished when 10^6 PFU of JEV was injected, which led to death in
114 more than 90% of mice (**Fig. 1B-C**). Compared to the $Axl^{+/-}$ mice, the $Axl^{-/-}$ mice
115 displayed faster and more severe body weight loss after disease onset at all doses of
116 JEV ($p<0.01$ for 10^4 and 10^5 PFU, $p=0.06$ for 10^6 PFU) (**Fig. 1D-F**). These results
117 indicated that Axl may be a protective factor against JE pathogenesis that improves
118 the survival of JEV infection.

119
120 One possible explanation for these results is that Axl inhibited JEV replication. To test
121 this hypothesis, we measured viral RNA loads in serum and peripheral organs and
122 analyzed JEV infection in primary macrophages, which are reported to be the primary
123 target cells during JEV infection. After i.p. infection with JEV, viral RNA was
124 detectable in serum at 6 h post infection (hpi) and lasted for at least 7 days (**Fig. 2A**).
125 The $Axl^{-/-}$ and control mice demonstrated similar levels of viral RNA in serum (**Fig.**
126 **2A**) and all assayed peripheral organs at different time points (**Fig. 2B-D**). Peritoneal
127 macrophages isolated from the $Axl^{-/-}$ and control mice were infected with an
128 equivalent dose of JEV in vitro but showed no difference regarding viral infection rate
129 and replication dynamics (**Fig. 2E-G**). To further test if Axl is only a restrictive factor
130 in the brain, we injected JEV directly into mouse brains. All mice died within 7 days

131 (Fig. 2H), which was accompanied by similar body weight loss (Fig. 2I) and viral
132 RNA loads in the brain (Fig. 2J). Thus, Axl is not likely to be a restrictive factor that
133 directly inhibits JEV replication. Considering that the Axl^{-/-} mice had similar viral
134 loads in peripheral organs but were at greater risk of developing encephalitis, Axl
135 possibly plays a protective role by regulating the BBB permeability for JEV.

136

137 2. Axl deficiency increases the probability of JEV brain infection

138 To investigate if Axl protects brains from JEV infection, we measured the brain viral
139 loads of the Axl^{-/-} and control mice from 1 to 7 dpi after i.p. infection with JEV. Viral
140 RNA was barely detectable in mouse brains until 5 dpi (Fig. 3A). The Axl^{-/-} mice
141 displayed a higher positive rate of JEV and greater viral loads in the brain than control
142 mice (Fig. 3A), indicating that Axl^{-/-} mouse brains were more accessible to JEV. We
143 further observed the target cell usage of JEV in mouse brains (cerebral cortex)
144 through immunofluorescence (IF) staining of NeuN (a specific marker for neurons),
145 GFAP (a specific marker for astrocytes), Iba1 (a specific marker for microglia), and
146 JEV antigens, but no differences between the Axl^{-/-} and control mice were observed
147 (data not shown), suggesting the protective role of Axl is achieved by inhibiting JEV
148 entry into the brain rather than altering its cell tropism in the brain.

149

150 To confirm the CNS entry-inhibition role of Axl, mice received an i.p. injection of $5 \times$
151 10^4 PFU of Renilla luciferase-harboring JEV (Rluc-JEV), and the luciferase substrate
152 was injected at different time points to allow viral replication and spread to be

153 continuously monitored via bioluminescent imaging (BLI). Rluc-JEV was first
154 detected in the abdomens (**Fig. 3B**), the site of infection, in all mice by 2 dpi and later
155 in the brains of a few mice (**Fig. 3B**). The $Axl^{-/-}$ and control mice exhibited no
156 difference in signal intensity in the abdomens (**Fig. 3C**). However, more $Axl^{-/-}$ mice
157 showed luciferase-positive brains (7/15) than the control mice (1/15) by 10 dpi (**Fig.**
158 **3D**), in agreement with the results obtained with the wild-type JEV. Taken together,
159 Axl deficiency remarkably increased the probability of JEV entry into brain.

160

161 **3. Axl deficiency increases BBB permeability at an early stage of JEV infection**

162 To understand the mechanism underlying the protective role of Axl against JE
163 pathogenesis, we utilized an Evans blue (EB) leakage test to gauge the permeability of
164 the BBB after JEV infection, which is the key structure preventing viral entry into the
165 brain. In the uninfected groups, both the $Axl^{-/-}$ and control mice showed very little and
166 comparable EB leakage into the brain parenchyma, indicating that Axl deficiency has
167 no effect on BBB permeability under physiological conditions (**Fig. 3E-F**, 0 dpi).
168 After JEV infection, EB leakage into the brain parenchyma significantly increased
169 and reached the first peak at 2 dpi, when the $Axl^{-/-}$ mice showed significantly
170 enhanced EB leakage compared to that observed in control mice, indicating that Axl
171 deficiency promoted the BBB permeability at early phase of infection (**Fig. 3E-F**, 2
172 dpi). After 2 dpi, both the $Axl^{-/-}$ and control mice showed gradually decreased EB
173 leakage into the brain parenchyma until 6 dpi, when EB leakage in the $Axl^{-/-}$ mice
174 increased again, while the control mice showed a continual decrease in EB leakage

175 (Fig. 3E-F, 2-7 dpi). The $Axl^{-/-}$ mice showed significantly enhanced EB leakage
176 compared to the control mice at 6 and 7 dpi, indicating that Axl deficiency also
177 promoted BBB permeability at late phase of infection (Fig. 3E-F, 6 and 7 dpi).
178 Moreover, in both the $Axl^{-/-}$ and $Axl^{+/-}$ mice, there was a strong positive linear
179 correlation between BBB permeability and brain viral load (slope=0.6917, $R^2=0.37$,
180 $p<0.05$ for $Axl^{-/-}$ mice; slope=0.7484, $R^2=0.50$, $p<0.05$ for $Axl^{+/-}$ mice; and
181 slope=0.7375, $R^2=0.55$, $p<0.001$ for all mice) (Fig. 3G-I), suggesting that greater
182 BBB permeability facilitates JEV entry into brains. We also used a fluorescein
183 leakage test to confirm the results obtained in the EB leakage test. In the uninfected
184 groups, all mice showed slight but comparable fluorescein leakage into the brain
185 parenchyma. In contrast, the $Axl^{-/-}$ mice showed significantly greater fluorescein
186 leakage than the $Axl^{+/-}$ mice after JEV infection (7 dpi) (Fig. 3J-K). These data
187 indicate that Axl deficiency significantly increases BBB permeability and
188 consequently promotes JEV entry into brain.

189

190 In the results described above, we noted that the $Axl^{-/-}$ mice displayed greater BBB
191 permeability at an early stage of JEV infection, much earlier than the appearance of
192 encephalitis. To confirm this phenomenon and investigate the underlying mechanisms,
193 we elucidated the dynamic changes in the expression and distribution of three
194 important tight junction (TJ) proteins in the brain, namely, claudin-5, occludin, and
195 ZO-1 using IF staining. In the uninfected mice (0 dpi), claudin-5 displayed the
196 strongest signal and was located at the interfaces between adjacent endothelial cells,

197 as revealed using an antibody against CD31 (a specific marker of vascular endothelial
198 cells) (**Fig. 4A**). Occludin and ZO-1 displayed similar distribution as claudin-5 (**Fig.**
199 **4A**). No difference between the $Axl^{-/-}$ and control mice was observed with respect to
200 the IF staining intensity of all the three TJ proteins (**Fig. 4B**). After JEV infection, in
201 both the $Axl^{-/-}$ and control mice, the IF staining intensity of the three TJ proteins was
202 apparently reduced (**Fig. 4C**). Remarkably, the $Axl^{-/-}$ mice showed lower IF staining
203 intensity of claudin-5 than the control mice at 1 dpi (**Fig. 4D**). At 2 dpi, the $Axl^{-/-}$ mice
204 displayed even lower IF staining intensity of claudin-5, occludin, and ZO-1 than the
205 control mice (**Fig. 4E-F**). We also detected the levels of claudin-5, occludin, and
206 ZO-1 mRNA in the brains of the $Axl^{-/-}$ and control mice at 0, 1, and 2 dpi using
207 qRT-PCR, and observed that the RNA abundance of the three TJ proteins were
208 consistent with their protein levels detected by IF staining (**Fig. 4G-I**). Taken together,
209 our results suggest that prior to the occurrence of encephalitis, the expression of TJ
210 proteins in the brains of $Axl^{-/-}$ mice have already been disrupted, making it easier for
211 JEV to penetrate the BBB.

212

213 **4. IL-1 α mediates early JEV neuroinvasion in Axl deficient mice**

214 As changes in BBB permeability and TJ proteins levels rapidly occurred after JEV
215 infection, we wondered whether $Axl^{-/-}$ mice triggered the release of some humoral
216 factors to disrupt BBB integrity soon after JEV infection. To address the question, we
217 measured the serum levels of a panel of cytokines (IL-1 α , IL-1 β , IL-2, IL-4, IL-6,
218 IL-10, TNF- α , IFN- γ , CCL-2, and CCL-5) that may affect BBB permeability at 1 and

219 7 dpi. Without JEV infection, no obvious difference was observed in the serum levels
220 for any of the assayed cytokines between the $Axl^{-/-}$ and $Axl^{+/-}$ mice (**Fig. 5**, mock). In
221 contrast, upon JEV infection, the serum levels of all measured cytokines increased at
222 1 dpi and returned to a lower level at 7 dpi that was comparable to those observed in
223 the mock treated group (**Fig. 5**, mock, 1 and 7 dpi). Compared to the $Axl^{+/-}$ mice, the
224 $Axl^{-/-}$ mice showed higher levels of IL-1 α , IL-1 β , IL-2, IL-4, IL-6, and IFN- γ but
225 lower levels of CCL2 and CCL5 in serum at 1 dpi (**Fig. 5**, 1 dpi). Of the six cytokines
226 with increased levels, IL-1 α (1 dpi) and IL-6 (1 dpi) were greatly increased in $Axl^{-/-}$
227 mice (**Fig. 5**, 1 dpi), suggesting that they may be the mediators responsible for the
228 observed BBB breakdown.

229
230 To further investigate the effect of IL-1 α and IL-6 on JE pathogenesis, we treated
231 JEV-infected $Axl^{-/-}$ mice with IL-1 α , IL-6, or vehicle (sterile normal saline) daily.
232 Compared to the vehicle-treated mice, the IL-6-treated mice showed slightly higher
233 body weights (**Fig. 6A**) but no differences in mortality (**Fig. 6B**) or BBB permeability
234 (**Fig. 6C-D**), which excluded it as a crucial contributor to JE pathogenesis in $Axl^{-/-}$
235 mice.

236
237 In contrast, mice treated with IL-1 α showed more severe body weight loss and JE
238 signs than vehicle-treated mice (**Fig. 7A-B**), and the mortality rate in the
239 IL-1 α -treated group (11/14) was significantly greater than that observed for the
240 vehicle-treated group (5/13) (**Fig. 7C**). In microscopic observations, JEV infection

241 induced obvious neuroinflammation, including neuron cell death, angiectasis,
242 perivascular cuffing, and inflammatory cell infiltration (**Fig. 7D-E**). Moreover,
243 IL-1 α -treated mice showed significantly larger areas of neuron death (**Fig. 7D and F**)
244 and inflammatory cell infiltration than the vehicle-treated mice (**Fig. 7E and G**) at 7
245 dpi, suggesting that IL-1 α treatment enhanced JEV-induced brain lesions. We also
246 detected the viral load in mouse brains by qRT-PCR, and observed that the
247 IL-1 α -treated mice showed greater viral loads in the brain than the vehicle-treated
248 mice (**Fig. 7H**). Taken together, these results show that IL-1 α promotes the
249 pathogenesis of severe JE by promoting the invasion of JEV into the brain.

250

251 To elucidate the impact of IL-1 α on BBB permeability in JEV-infected mice, we
252 evaluated BBB permeability by quantifying endogenous IgG leakage and assessing
253 the expression and distribution of claudin-5, occludin, and ZO-1 in mouse brains by
254 IF staining. In contrast to the vehicle-treated mice, the IL-1 α -treated mice showed
255 significantly increased endogenous IgG leakage into the brain parenchyma (**Fig.**
256 **8A-B**). In agreement with this result, the expression of claudin-5, occludin, and ZO-1
257 in the IL-1 α -treated mice was conspicuously lower than that observed in the
258 vehicle-treated mice, and the distribution of the three TJ proteins was more severely
259 disrupted in the IL-1 α -treated mice than in the vehicle-treated mice (**Fig. 8C-D**).
260 Taken together, these results indicate that IL-1 α but not IL-6 disrupted the BBB
261 integrity and promoted JEV invasion into brains.

262

263 To ensure IL-1 α was the factor inducing BBB and TJ breakdown at the early stage of
264 JEV infection, an i.p. injection of a mouse recombinant IL-1 α was administered to
265 Axl^{-/-} mice and its direct effect on BBB permeability and brain TJs was assessed at 6 h
266 post treatment, when viral RNA was first detected in serum. BBB permeability was
267 measured by EB leakage assays and by quantifying the levels of claudin-5, occludin,
268 and ZO-1 mRNA were by qRT-PCR and assessing their distribution by IF staining.
269 Compared to the vehicle-treated mice, the IL-1 α -treated mice showed greater EB
270 leakage into the brain parenchyma (2.6-fold), indicating that IL-1 α alone could
271 immediately increase BBB permeability (**Fig. 9A-B**). Furthermore, the IL-1 α -treated
272 mice showed significantly lower mRNA levels of claudin-5, occludin, and ZO-1 (**Fig.**
273 **9C**) and much weaker IF staining intensity for these TJ proteins than was observed in
274 the vehicle-treated mice (**Fig. 9D-E**). Moreover, unlike in the vehicle-treated mice,
275 where the TJ proteins clearly located at the interface between two neighboring
276 endothelial cells, the TJ proteins in the IL-1 α -treated mice were dispersed and lost
277 their typical distribution patterns (**Fig. 9D**), indicating that IL-1 α alone could rapidly
278 disrupt the distribution of TJ proteins and increase the BBB permeability.

279

280 **5. IL-1 α is released by dead JEV-infected in situ peritoneal macrophages**

281 To identify the source of IL-1 α in the JEV-infected mice, we measured the IL-1 α
282 content in the brain, serum and peritoneal wash at 24 hpi. No significant alteration in
283 the brain IL-1 α content was observed (**Fig. 10A**). Consistent with previous
284 measurements, serum IL-1 α levels sharply increased (**Fig. 10B**). Remarkably,

285 peritoneal wash levels of IL-1 α showed a decreasing tendency after JEV infection
286 (**Fig. 10C**), and there was a negative correlation with respect to the IL-1 α content
287 between the serum and peritoneal wash (**Fig. 10D**). The above data suggested that
288 serum IL-1 α was primarily derived from the peritoneal cavity rather than the brain,
289 and it was probably achieved by releasing the pre-existing IL-1 α inside cells rather
290 than increasing IL-1 α expression. The serum IL-1 α content in Axl^{-/-} mice was much
291 greater than that observed in the control mice (**Fig. 10B**), whereas the IL-1 α content
292 in the peritoneal wash samples from Axl^{-/-} mice was lower than that detected in the
293 control mice (**Fig. 10C**), suggesting that Axl deficiency accelerated the release of
294 IL-1 α by peritoneal cells.

295
296 To elucidate the mechanism underlying the Axl-mediated regulation of IL-1 α release
297 by peritoneal cells, we isolated mouse peritoneal cells and infected them with JEV in
298 vitro. Most of the peritoneal cells were F4/80 positive (**Fig. 10E**), suggesting they
299 were macrophages. In the uninfected groups, Axl^{-/-} and control macrophages showed
300 similar IL-1 α content in the supernatant. However, the IL-1 α content in the
301 supernatant of Axl^{-/-} macrophages immediately increased after infection, which was
302 maintained at higher levels than that of the control macrophages at all tested time
303 points, suggesting that Axl deficiency promotes the release of IL-1 α (**Fig. 10F**).
304 However, the magnitude of the increase of IL-1 α in the supernatant of Axl^{-/-}
305 macrophages was not enough to explain the drastic rise in serum IL-1 α levels.
306 Moreover, at 24 hpi, the expression of IL-1 α inside peritoneal macrophages did not

307 decrease significantly (**Fig. 10G**), and the IL-1 α content in the supernatant was
308 slightly elevated (**Fig. 10H**). We also measured IL-1 α production by mouse brain
309 microvascular endothelial cells (bEND.3) and primary splenocytes in vitro in the
310 absence of Axl signaling, which was achieved by Axl deficiency or inhibition with
311 R428, but these cells produced even less IL-1 α than the peritoneal macrophages (**Fig.**
312 **10I-J**). Compared to the JEV-infected cells, LPS-stimulated peritoneal macrophages
313 showed a conspicuous decrease in intracellular IL-1 α expression (**Fig. 10G**) and a
314 sharp increase in the IL-1 α content in the supernatant (**Fig. 10H**). IL-1 α can be
315 released by either LPS induction or cell death(22, 23). Since IL-1 α release by
316 peritoneal macrophages after JEV infection occurred through a different mechanism
317 than LPS stimulation, it was speculated that it may have been released by dead cells
318 as an alarm molecule.

319

320 We noticed that the peritoneal macrophages cultured in vitro were not highly
321 susceptible to JEV infection (**Fig. 2E-F**), and no detectable death was observed after
322 infection. Thereafter, we avoided the in vitro infection model and instead directly
323 isolated the peritoneal macrophages from JEV-infected mice and made smears, which
324 closely reflected the in situ infection characteristics of peritoneal macrophages. We
325 observed that in situ macrophages were highly susceptible to JEV infection, with an
326 infection rate of up to 80% (**Fig. 10K-L**). There was no significant difference in the
327 infection rate between the Axl^{-/-} and control macrophages (**Fig. 10L**), which was in
328 accordance with the similar viral titers observed in the peritoneal wash samples (**Fig.**

329 **10M)** and with the previous results (**Fig. 2F-G**). In both the $Axl^{-/-}$ and control
330 macrophages, IL-1 α was largely localized to the nucleus, which was consistent with
331 previous reports(24). At 24 hpi, the expression of intracellular IL-1 α significantly
332 increased, and the $Axl^{-/-}$ macrophages showed slightly greater expression of IL-1 α
333 than that observed in the control macrophages (**Fig. 10K and N**). These results
334 suggest that IL-1 α is released not only by induction but more importantly by
335 immediately liberating preexisting intracellular IL-1 α .

336

337 To assess whether serum IL-1 α was released by dead macrophages after JEV infection,
338 we first used the Trypan blue (TB) staining method to discriminate live cells
339 (unstained, TB $^{-}$) and dead cells (blue stained, TB $^{+}$) (**Fig. 10O**), At 24 hpi, the
340 proportion of TB $^{+}$ cells in the peritoneal wash samples of the $Axl^{-/-}$ mice was
341 significantly increased, much more than that observed for the control mice (**Fig.**
342 **10O-P**). To determine whether additional IL-1 α can be released after cell death, we
343 used a hypotonic treatment to induce the death of $Axl^{-/-}$ peritoneal macrophages,
344 observing that IL-1 α levels in the supernatant increased by more than two orders of
345 magnitude (**Fig. 10Q**). Taken together, these results suggest that macrophages lacking
346 Axl die more readily after JEV infection, which triggers a substantial release of IL-1 α
347 from cells.

348

349 **6. Axl deficiency promotes the pyroptosis of JEV-infected peritoneal**
350 **macrophages.**

351 To further elucidate the mode of death of JEV-infected macrophages, we isolated
352 peritoneal macrophages from JEV-infected mice at 24 hpi and analyzed
353 transcriptomic changes through RNA-Seq analysis. Hundreds of differentially
354 expressed genes were identified between the $Axl^{-/-}$ and control macrophages after
355 infection (**Fig. 11A**). Many differentially expressed genes were enriched in cell
356 death-related biological processes, such as necrosis, apoptosis, and pyroptosis (**Fig.**
357 **11B**). Remarkably, compared to the control macrophages, most of these cell
358 death-related genes in the $Axl^{-/-}$ macrophages were upregulated after infection,
359 suggesting that Axl deficiency enhanced the transcription of cell death-related genes
360 (**Fig. 11B**). We also assessed the transcription of IL-1 α , IL-1 β , and IL-1 receptor
361 antagonist (IL-1Ra), observing that only IL-1 β was upregulated in JEV-infected $Axl^{-/-}$
362 macrophages (**Table 1**). After infection, the transcription of caspase-1 and GSDMD
363 (key mediators of pyroptosis) was notably enhanced but showed no difference
364 between the $Axl^{-/-}$ and control macrophages (**Table 1**). Although the transcription of
365 RIPK1 (a key mediator of necroptosis) was unchanged, its suppressor BIRC3 showed
366 greater transcription in $Axl^{-/-}$ macrophages than in control macrophages after infection
367 (**Table 1**). The transcription of caspase-8, caspase-9, and caspase-3 (all are crucial
368 regulators of apoptosis and pyroptosis) was enhanced after infection, but no
369 differences were observed between the $Axl^{-/-}$ and control macrophages (**Table 1**). The
370 expression of the pro-apoptotic gene BCL-10 was upregulated in the $Axl^{-/-}$
371 macrophages after infection (**Table 1**). In addition, Nox1, which promotes the
372 generation of ROS and cell death, was more expressed in the $Axl^{-/-}$ macrophages than

373 that observed in the control macrophages after infection (**Table 1**). PI3K-Akt
374 signaling is an important pathway that promotes cell survival. After infection, the
375 transcription levels of PI3K and Akt were similar in the $Axl^{-/-}$ and control
376 macrophages (data not shown), but the PI3K inhibitors PI3KIP1 and WDR91 as well
377 as the Akt inhibitor TRIB3 showed increased expression in the $Axl^{-/-}$ macrophages
378 compared to that observed in the control macrophages (**Table 1**), suggesting that
379 during infection, PI3K-Akt signaling may be inhibited in $Axl^{-/-}$ macrophages.

380

381 Subsequently, we verified the RNA-Seq revealed cell death pathways (apoptosis,
382 pyroptosis, and necroptosis) using IF staining. At 24 hpi, the proportion of
383 GSDMD-N terminal⁺ (a marker of pyroptosis) cells and TUNEL⁺ (a marker of
384 apoptosis) cells increased significantly (**Fig. 12A-C**), but that of RIPK1⁺ (a marker of
385 necroptosis) cells did not change significantly (**Fig. 12D-E**), indicating that the
386 JEV-infected peritoneal macrophages primarily underwent pyroptosis and apoptosis
387 rather than necroptosis, which was consistent with the RNA-Seq results. The
388 pyroptosis and apoptosis rates in the $Axl^{-/-}$ macrophages were much greater than those
389 observed in the control mice (**Fig. 12B-C**), suggesting that *Axl* deficiency promotes
390 the pyroptosis and apoptosis of JEV-infected macrophages. Remarkably, pyroptosis
391 was the predominant death mode, with approximately 80% of JEV-infected $Axl^{-/-}$
392 macrophages undergoing pyroptosis (**Fig. 12F**). We also assessed the expression of
393 cleaved caspase-1 (another maker of pyroptosis) and GSDMD-N in the in situ
394 peritoneal macrophages by immunoblotting, and observed that consistent with the IF

395 staining results, *Axl*^{-/-} macrophages showed greater expression of the cleaved
396 caspase-1 and GSDMD-N than the control macrophages after JEV infection (**Fig.**
397 **12G-I**). In subsequent experiments, caspase-8, a key molecule regulating cell death
398 mode, was observed to be significantly upregulated in JEV-infected macrophages, but
399 no significant difference in caspase-8 expression was observed between the *Axl*^{-/-} and
400 control macrophages (**Fig. 13A**). The changes in caspase-9 and caspase-3 levels were
401 similar to that observed for caspase-8 (**Fig. 13B-C**).

402

403 *Axl* has been shown to promote cell survival and inhibit cell apoptosis and pyroptosis
404 by activating PI3K-Akt signaling(25, 26), and the RNA-Seq results also indicated that
405 PI3K-Akt signaling was inhibited in *Axl*^{-/-} macrophages. To confirm this result, we
406 examined the expression of PI3K-p110 and phosphorylated Akt (pAkt) in
407 JEV-infected in situ macrophages using IF staining (**Fig. 13D-E**), observing that the
408 expression of PI3K-p110 (**Fig. 13D and F**) and pAkt (**Fig. 13E and G**) were
409 significantly lower in *Axl*^{-/-} macrophages than in control macrophages. Moreover, the
410 PI3K inhibitor LY294002 could effectively reduce the differences in the pyroptosis
411 (**Fig. 12A-B**) and apoptosis rates (**Fig. 12A and C**) between the *Axl*^{-/-} macrophages
412 and control macrophages. We also verified the expression of PI3K-p110, cleaved
413 caspase-1, and GSDMD-N after JEV infection and LY294002 treatment by
414 immunoblotting, the results of which were consistent to those of IF staining (**Fig.**
415 **12G-I and Fig. 13H-I**). Taken together, these results suggest that *Axl* impedes the
416 pyroptosis and apoptosis of JEV-infected peritoneal macrophages via activating

417 PI3K-Akt signaling.

418

419 7. IL-1 α antagonist prevents JE pathogenesis

420 Since IL-1 α promoted JE pathogenesis, we wondered if its antagonist could hinder the
421 incidence of JE. To test this hypothesis, Axl^{-/-} mice were challenged with 10⁴ PFU of
422 JEV and then treated with IL-1Ra (a natural IL-1 α antagonist) or vehicle (sterile
423 normal saline) every two days. The IL-1Ra-treated mice showed no occurrence of JE
424 or mortality (0/13), whereas the vehicle-treated mice showed severe signs (**Fig.**
425 **14A-B**) and increased mortality of JE (7/14) (**Fig. 14C**), suggesting the IL-1 α
426 antagonist potently reduced the incidence of JE. Next, we assessed the pathological
427 changes in the brain, observing that JEV infection in the mouse brain caused neuron
428 death, angiectasis, and inflammatory cell infiltration. Compared to the vehicle-treated
429 mice, the IL-1Ra-treated mice showed significantly fewer area of neuron death (**Fig.**
430 **14D-E**) and inflammatory cell infiltration (**Fig. 14D and F**), suggesting that the IL-1 α
431 antagonist potently relieved brain lesions during JEV infection. To elucidate the
432 mechanism by which IL-1Ra protects mice from JE, we assessed the viral load and
433 BBB permeability in mouse brains, observing that the IL-1Ra-treated mice exhibited
434 reduced viral loads in the brain (**Fig. 14G**) and significantly decreased endogenous
435 IgG leakage into the brain parenchyma (**Fig. 14H-I**). These data indicate that IL-1Ra
436 may prevent JE pathogenesis by inhibiting increased BBB permeability and inhibiting
437 JEV invasion of the brain during infection.

438

439 Finally, we assessed the expression and distribution of brain claudin-5, occludin, and
440 ZO-1, observing that in the vehicle-treated mice, the expression of claudin-5 and
441 occludin were significantly reduced and exhibited a sparse distribution along the
442 microvascular endothelial cells (**Fig. 14J**). The expression of ZO-1 was also reduced,
443 and its distribution became dispersed (**Fig. 14J**). Notwithstanding, in the
444 IL-1Ra-treated mice, the expression and distribution of the three TJ proteins were
445 comparable to that observed in the uninfected mice, where they were regularly spaced
446 at the interfaces of microvascular endothelial cells (**Fig. 14J**). The above results
447 suggest that the IL-1 α antagonist can efficiently safeguard the expression and
448 distribution of TJ proteins. In summary, the IL-1 α antagonist protected mice from
449 developing JE by reducing BBB permeability and viral invasion into brain during JEV
450 infection.

451

452 We also verified the effect of the IL-1 α antagonist on JE pathogenesis in wild-type
453 BALB/c mice. BALB/c mice were administered IL-1Ra (n=10) or vehicle (n=10) in
454 the same manner as described for the Ax1^{-/-} mice and then challenged with an even
455 higher dose of JEV (10⁵ PFU per mouse). Compared to the vehicle-treated mice, the
456 IL-1Ra-treated mice showed significant reductions in mortality, body weight loss, and
457 sign scores after JEV infection (**Fig. 14K-M**), suggesting that the IL-1 α antagonist
458 also impedes JE pathogenesis in wild-type BALB/c mice.

459

460 Discussion

461 In this study, we discovered that Axl deficiency causes increased mortality in mice
462 infected with JEV. Axl deficiency did not directly affect JEV replication, rather it
463 significantly increased the neuroinvasion of JEV, which was correlated with an
464 enhanced BBB permeability. IL-1 α , which was primarily produced by the
465 JEV-infected peritoneal macrophages, was shown to be the key mediator that induced
466 BBB breakdown and promoted JEV neuroinvasion at the early phase of infection.
467 Furthermore, an IL-1 α antagonist effectively reduced the incidence of severe JE by
468 antagonizing JEV-induced brain TJ impairment and increased BBB permeability. The
469 results of our study suggest that Axl is a protective factor that reduces the incidence of
470 JE and that IL-1 α promotes early JEV neuroinvasion.

471

472 Axl is a crucial and complicated host factor in flaviviral infection. Although many
473 studies have proposed that Axl serves as an entry receptor for several flaviviruses,
474 accumulating evidence suggests that Axl plays unique roles in a virus type-dependent
475 manner. For example, Axl mediates the entry of DENV(13), inhibits WNV
476 neuroinvasion without affecting viral replication in target organs(14), and also
477 enhances ZIKV pathogenesis in an age dependent manner by exacerbating IL-1 β
478 production and apoptosis in microglia(18). Our results show that Axl impedes JEV
479 neuroinvasion by antagonizing viral-induced pyroptosis in peritoneal macrophages
480 and subsequent IL-1 α release. These results further suggest that Axl plays unique
481 roles in the infection processes of different flaviviruses.

482

483 In our study, we identified IL-1 α as a pivotal mediator of JEV infection and
484 demonstrated that it functions at an early rather than late stage of JEV infection.
485 Cytokine screening results showed that IL-1 α was the most increased
486 proinflammatory cytokine in serum from Axl deficient mice at 1 dpi, and it potently
487 inhibited brain TJ protein expression and distribution, resulting in increased BBB
488 permeability. Importantly, an IL-1 α antagonist effectively restored these TJ
489 impairments and BBB disruptions and subsequently protected mice from developing
490 severe JE. Currently, there are few reports regarding the specific role of IL-1 α in
491 modulating BBB integrity and they primarily focus on nonviral diseases. In an in vitro
492 human BBB model, IL-1 α immediately increased BBB permeability within 6 h by
493 disrupting the distribution and expression of TJ proteins, which was accompanied by
494 cytoskeleton alteration, and its blockage by IL-1Ra could prevent such changes(27).
495 Gloor et al reported that IL-1 α increased BBB permeability in an in vitro porcine
496 BBB model by downregulating plasma membrane-associated tyrosine phosphatase
497 activity(28). Indeed, we also observed that IL-1 α immediately downregulated the
498 expression of claudin-5, occludin, and ZO-1 in an in vitro mouse BBB model, which
499 is related to actin filament and MyD88 alterations (data not shown). In addition to
500 acting on brain microvascular endothelial cells, IL-1 α also accounted for increasing
501 pulmonary vascular permeability by downregulating a the TJ protein cadherin during
502 acute lung injury(23). IL-1 β is a homolog of IL-1 α and uses the same receptor as
503 IL-1 α . Pan et al reported that DENV infection induces IL-1 β secretion from
504 macrophages in patients and mice, and IL-1 β induces vascular leakage in both in vitro

505 vascular endothelial monolayers and in mice, while IL-1Ra alleviates these
506 IL-1 β -mediated effects(29). In agreement with the above studies, the results of our
507 work suggest that IL-1 α is a crucial mediator that promotes the incidence of JE by
508 disrupting BBB integrity at a very early stage of infection. Moreover, IL-1 α is a
509 promising target for JE therapy and IL-1Ra is an effective antagonist.

510

511 In our results, pyroptosis, rather than apoptosis or necroptosis, was identified as the
512 predominant cell death mode in JEV-infected in situ macrophages, suggesting its
513 pivotal role in JEV infection. Interestingly, pyroptosis is also observed in the infection
514 of other flaviviruses. DENV infection in macrophages induces pyroptosis together
515 with the release of IL-1 β (30, 31). ZIKV infection elicits pyroptosis in the brains of
516 fatal microcephaly cases and increases IL-1 β expression(32). In addition, WNV
517 infection in the brain also provokes the expression of pyroptosis markers (caspase-1,
518 IL-1 β , and IL-18)(33). Elucidation of the significance of viral infection-induced
519 pyroptosis will deepen our understanding of the nature of flavivirus infections.

520

521 Intriguingly, our investigations showed that JEV-induced pyroptosis was accompanied
522 by the release of large amounts of IL-1 α rather than IL-1 β . The cleavage of pro-IL-1 β
523 into mature IL-1 β is a key step in pyroptosis that promotes inflammation and is also a
524 feature that distinguishes pyroptosis from other types of cell death(34). In contrast to
525 IL-1 β , the release of IL-1 α has been largely less studied in pyroptosis. Indeed, IL-1 α
526 and IL-1 β share the same receptor and have similar biological functions, but the

527 distinct differences in the manner they are released(35). IL-1 α is typically
528 preexpressed in cells and immediately released to serve as an alarm factor by dead
529 cells(36). In our study, IL-1 α levels increased more significantly than IL-1 β in Axl
530 deficient mice. We isolated in situ peritoneal macrophages from JEV-infected mice
531 and observed that both Axl deficient macrophages and control macrophages expressed
532 large amounts of IL-1 α in the cytosol and nucleus. However, Axl deficient
533 macrophages died more readily (pyroptosis and apoptosis) than control cells after JEV
534 infection, and the dead macrophages readily released the pre-expressed intracellular
535 IL-1 α and boosted serum IL-1 α levels. The results of mechanistic studies indicated
536 that Axl could activate PI3K-Akt signaling, which downregulated the expression of
537 cleaved caspase-1 and GSDMD-N and thus inhibited the pyroptotic death of
538 macrophages, averting large amount of IL-1 α being released by dead cells. Since
539 IL-1 α is pre-expressed in macrophages and IL-1 β is merely expressed upon
540 stimulation, IL-1 α is likely to act earlier and faster than IL-1 β , which may explain
541 why IL-1 α is more crucial in the early phase of JEV infection. Previous studies on
542 pyroptosis in flaviviral infection typically focused on IL-1 β and not IL-1 α . Therefore,
543 whether the release of IL-1 α prior to IL-1 β is a universal phenomenon during
544 pyroptosis is an interesting issue to be addressed in future studies.

545

546 In summary, in this study, we discovered that Axl is a protective factor that impedes
547 the occurrence of JE by suppressing IL-1 α production from pyroptotic macrophages
548 and IL-1 α -induced BBB breakdown. Neuroinvasive pathogens penetrate BBB in

549 unique ways, and our results reveal a novel mechanism in which, BBB penetration is
550 driven by IL-1 α . In view of the apparent protection of IL-1 α blockage in JEV
551 infection, IL-1 α can thus be a potential therapeutic target to prevent severe JE.
552 Although the above findings are interesting, there are still some issues needing to be
553 further solved. JEV is often transmitted by mosquito bites, but our experimental
554 system used an i.p. injection, and while macrophages are one of the primary target
555 cells for both infection routes, whether Axl and IL-1 α play the same roles in JEV
556 infection resulting from mosquito bites or a subcutaneous route remains to be
557 elucidated.

558

559 **Materials and Methods**

560 **Cells and Viruses**

561 C6/36 cells (*Aedes albopictus* lava cells) were grown in RPMI 1640 medium (Gibco,
562 USA) supplemented with 10% fetal bovine serum (FBS, PAN, Germany) and
563 maintained at 28°C. Vero cells (African green monkey kidney cells) were raised in
564 minimum essential medium (MEM, Gibco, USA) supplemented with 5% FBS.
565 bEND.3 cells (murine brain microvascular endothelia cells) were maintained in
566 Dulbecco's modified eagle medium (DMEM, Gibco, USA) supplemented with 10%
567 FBS.

568

569 JEV Beijing strain-1 (JEV)(37) and *Renilla reniformis* luciferase reporter JEV
570 (Rluc-JEV)(38) were propagated in C6/36 cells and titrated on Vero cells by plaque

571 assay as previously reported(39).

572

573 Mice

574 Axl deficient ($Axl^{-/-}$) mice were kind courtesy of Dr. Greg Lemke (Salk Institute for
575 Biological Studies, La Jolla, CA). The F_0 $Axl^{-/-}$ mice were generated from C57BL/6J
576 mice and were mated with F_0 wild type ($Axl^{+/+}$) C57BL/6J mice to generate F_1
577 heterozygous ($Axl^{+/-}$) mice. The F_0 $Axl^{-/-}$ mice were then mated with F_1 $Axl^{+/-}$ mice to
578 generate F_2 $Axl^{-/-}$ mice (experimental group) and littermate F_2 $Axl^{+/-}$ mice (control
579 group). The littermate $Axl^{+/-}$ mice are ideal controls for $Axl^{-/-}$ mice due to same age,
580 similar nutritional and living status, and genetic background. The mice were bred and
581 maintained under a specific pathogen free animal facility at Capital Medical
582 University, Beijing. The genotypes were identified using PCR, and three primers were
583 used: Wt: 5'-GCCGAGGTATAGTCTGTCACAG-3', Mut:
584 5'-TTTGCCAAGTTCTAATTCCATC-3', WtMut:
585 5'-AGAAGGGGTTAGATGAGGAC-3'. The sizes of PCR products are 350 bp for
586 $Axl^{+/+}$ mice, 350 and 200 bp for $Axl^{+/-}$ mice, and 200 bp for $Axl^{-/-}$ mice.

587

588 Four-week-old female BALB/c mice were purchased from Vital River Laboratories
589 (Beijing, China) and used for validating the protection of IL-1Ra during JEV
590 infection.

591

592 Animal infection

593 $Axl^{+/-}$ and $Axl^{-/-}$ mice were i.p. injected with 10^4 , 10^5 , or 10^6 PFU of JEV in 200 μ L of
594 phosphate buffered saline (PBS), or just 200 μ L of PBS in mock-treated mice. For
595 intracerebral infection, mice were intracerebrally injected with 10^3 PFU of JEV in 20
596 μ L of PBS. For bioluminescent imaging, mice were i.p. injected with 5×10^4 PFU of
597 Rluc-JEV in 200 μ L of PBS.

598

599 **Scoring the signs of JEV infection**

600 To quantitatively analyze the severity of JEV infection, signs were scored according
601 to the following standard: well=0; sluggish=1; partially ruffled fur=2; wholly ruffled
602 fur=3; hunched back=4; one limb paralysis=5; two limb paralysis=6; more than two
603 limb paralysis=7; death=8.

604

605 **Hematoxylin and Eosin (HE) staining**

606 Brains of JEV-infected or mock-treated mice were collected at 7 dpi. The brains were
607 immersed in 4% paraformaldehyde (PFA in PBS) for paraffin embedding and section.
608 Paraffin embedded brains were sagittally sectioned into 5 μ m slides, which were then
609 performed with HE staining according to a standard protocol. The analyses of HE
610 staining results were completed by using Image-Pro Plus 6.0 software.

611

612 **Immunofluorescent (IF) staining**

613 For IF staining, tissue sections or cell monolayers were subjected to membrane
614 permeability with 0.5% Triton (in PBS) for 5 min and blockage with 1% bovine

615 serum albumin (BSA) for 1 h, then incubated with specific primary antibodies
616 (1:250-1:300) at 4°C overnight and followed by incubation with Alexa Fluor 488
617 labeled donkey anti-mouse IgG antibody (1:500, A21202, Thermo) and/or Alexa
618 Fluor 594 labeled donkey anti-rabbit IgG antibody (1:500, A21207, Thermo) at room
619 temperature for 50 min. The primary antibodies used in IF staining include anti-JEV
620 serum (self-made), anti-NeuN (ab104224, Abcam), anti-GFAP (ab7260, Abcam),
621 anti-Iba1 (10904-1-AP, Proteintech), anti-CD31 (ab9498, Abcam), anti-claudin-5
622 (AF0130, Affinity Biosciences), anti-Occludin (71-1500, Thermo), anti-ZO-1
623 (61-7300, Thermo), anti-F4/80 (ab100790, Abcam), anti-IL-1 α (DF6893, Affinity
624 Biosciences), anti-GSDMD-N (AF4013, Affinity Biosciences), anti-RIPK1 (AF7377,
625 Affinity Biosciences), anti-caspase-8 (ab227430, Abcam), anti-caspase-9 (ab202068,
626 Abcam), anti-caspase-3 (9664S, CST), anti-PI3K-p110 (AF5112, Affinity
627 Biosciences), anti-pAkt (AF0016, Affinity Biosciences) antibodies. The analyses of IF
628 staining results were accomplished using Image-Pro Plus 6.0 software. Specifically,
629 the IF staining images were collected under fixed excitation light intensity and
630 exposure time. For each slide from a mouse, at least eight random scopes were
631 collected under appropriate magnification. Then the original images were subjected to
632 fluorescent intensity gauge for specific staining signals by using the Count/Size
633 function of Image-Pro Plus 6.0 software. The selected measurements were Area and
634 Density (red). The mean density of all software-identified specific staining signals
635 was extracted as the fluorescent intensity for an image (scope). The average
636 fluorescent intensity of at least eight images (scopes) for each slide was defined as the

637 fluorescent intensity for an independent mouse.

638

639 **RNA extraction in serum and major target organs**

640 Serum, brain, spleen, liver, and kidney were collected at 1, 4, and 7 dpi. Total RNA in

641 serum was extracted using EasyPure Viral DNA/RNA Kit (ER201-01, TransGen).

642 Total RNA in organs was extracted using TransZol (ET101-01, TransGen).

643

644 **Quantitative real time PCR (qRT-PCR)**

645 For viral load detection, a previously reported(40) FAM-TAMRA probe based

646 qRT-PCR method was used to quantify JEV genome RNA copies in cell supernatant,

647 serum, and brain. We also developed an SYBR based qRT-PCR method to reflect viral

648 loads in spleen, liver, and kidney (in these organs, viral loads were quite low), and the

649 results were expressed as JEV copies/GAPDH copies. Primer set for JEV is sense:

650 5'-CGTTTCGTGCTGGCTCTTAT-3'; antisense:

651 5'-CCAAGTTCTCGTTTGAAACT-3'. Primer sets for GAPDH is sense:

652 5'-CCTGGAGAAACCTGCCAAGT-3'; antisense:

653 5'-GGAGTTGCTGTTGAAGTCGC-3'. For brain tight junction RNA quantification,

654 a previously reported(41) qRT-PCR method was modified and used. Specifically, the

655 primer sets for brain tight junctions (claudin-5, occludin, and ZO-1) are kept the same

656 as the reported method, while the normalization control is GAPDH in our system

657 rather than HRPT used by the reported method.

658

659 **Isolation, culture, and infection of primary cells**

660 To isolate peritoneal macrophages, four-week-old mice were euthanized and
661 disinfected, followed by i.p. injection with 5 mL of DMEM and abdominal massage
662 for 3 min and stand for 7 min. Then, the injected DMEM in abdominal cavity was
663 collected and centrifuged at 2,000 rpm for 10 min. The resulting cell pellet was
664 washed and recovered in DMEM supplemented with 10% FBS. The cells were
665 cultured for 4 h, then washed with PBS to remove non-adherent cells, and finally
666 added fresh DMEM supplemented with 10% FBS. Isolated peritoneal macrophages
667 were stained with anti-F4/80 antibody to monitor the cell purity. And using this
668 method, the macrophage purity can be no less than 98%. For infection, peritoneal
669 macrophages were infected with JEV at MOI=1 for 1.5 h.

670

671 To isolate splenocytes, the spleen was collected from mice and grinded on a 200-mesh
672 filter. The filtrate was collected and subjected to density gradient centrifugation with
673 Mouse 1x Lymphocyte Separation Medium (DKW33R0100, Dakewe). The
674 splenocytes were cultured with 10% FBS RPMI 1640 medium. For infection,
675 splenocytes were infected with JEV at MOI=1 for 1.5 h.

676

677 **Bioluminescence imaging (BLI) of Rluc-JEV infection in mice**

678 $Axl^{+/-}$ (n=15) and $Axl^{-/-}$ (n=15) mice were i.p. injected with 5×10^4 PFU of Rluc-JEV.
679 The mice were subjected to bioluminescence detection by a bioluminescence
680 detecting system (PerkinElmer, US) at 2, 4, 5, 8 and 10 dpi. Each mouse was i.p.

681 injected with 0.148 mM $\times$ 200 μ L of vivi Ren substrate (P1232, Promega) 10 min
682 prior to immediate imaging for 90 s at 8 bins.

683

684 **BBB permeability evaluation**

685 Three independent methods were used to measure BBB permeability, namely, Evans
686 blue (EB) leakage test, sodium fluorescein (NaF) leakage test, and endogenous IgG
687 leakage detection.

688

689 For EB brain leakage test, mice were intravenously injected with 100 μ L of 2% EB
690 (Macklin, E808783) solution (in normal saline). After 45 min to allow the circulation
691 of EB, the mice were anesthetized with 200 μ L of 3.5% chloral hydrate solution. The
692 anesthetized mice were then transcardially perfused with 30 mL of normal saline to
693 completely remove EB in circulation. The mouse brains were then collected, weighed,
694 and homogenized in dimethylformamide (DMFA) (200 mg brain tissues/500 μ L
695 DMFA). The homogenates were stand at 60 $^{\circ}$ C for 24 h, followed by centrifugation at
696 12, 000 g for 5 min. 100 μ L of resulting supernatant was transferred into a 96-well
697 plate to measure absorbance at 620 nm. The content of EB in brain was calculated
698 according to the standard curve, and expressed as EB content μ g/g brain tissues. To
699 facilitate the linear correlation analysis between brain viral load ($\log_{10}$) and BBB
700 permeability, the EB content was normalized to the average of Axl^{+/-} mice at 0 dpi.

701

702 NaF leakage test was performed according to a method previously reported(42).

703 Briefly, mice were i.p. injected with 100 μ L of 100 mg/mL NaF. Then circulating
704 blood was gleaned to separate serum. The serum (60 μ L) was mixed with 60 μ L of 15%
705 trichloroacetic acid (TCA) and centrifuged. The supernatant was then mixed with 30
706 μ L of 5 M NaOH and 7.5% TCA and NaF content was measured as below. Euthanized
707 mice were transcardially perfused with 30 mL of normal saline. The brain tissues (200
708 mg) were homogenized in 7.5% TCA (600 μ L) and centrifuged to separate
709 supernatant. The supernatant (120 μ L) was mixed with 30 μ L of 5 M NaOH. NaF in
710 mixture was determined using a spectrophotometer (Multiskan spectrum, Thermo
711 scientific) with excitation at 485 nm and emission at 530 nm. Standard curve (0-25
712 μ g/mL) was used to calculate the NaF content in samples. NaF leakage into brain
713 parenchyma was expressed as brain NaF content/serum NaF content to normalize
714 different blood levels of the dye.

715

716 For endogenous IgG detection, brains were embedded in OCT and cryo-sectioned into
717 6 μ m slides, anti-claudin-5 antibody was used to demonstrate BBB, and Alexa Fluor
718 488 labeled donkey anti-mouse IgG was used to visualize endogenous IgG.

719

720 **Multiplex immunoassays and enzyme-linked immunosorbent assay (ELISA)**

721 $Axl^{+/-}$ and $Axl^{-/-}$ mice were i.p. injected with 10^4 PFU of JEV, and sera were collected
722 at 1 dpi and 7 dpi. Supernatants from cultured cells were collected at indicated time
723 points. The sera were subjected to a bead-based immunoassay (Aimplex, Multiplex
724 Immunoassays for Flow, Beijing Quantobio Co., LTD.) to measure a panel of

725 cytokines (IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, TNF- α , IFN- γ , CCL2, and CCL5).
726 Specially, the contents of IL-1 α in peritoneal washes, supernatants of peritoneal
727 macrophages and other cells were gauged by ELISA (ELM-IL1 α , Raybiotech).

728

729 **R428 treatment**

730 bEND.3 cells were pretreated with culture medium containing 1 μ M of R428 (21523,
731 Cayman Chemical) or vehicle (0.1% DMSO) for 30 min, then infected with JEV at
732 MOI=1 for 1.5 h in the present of 1 μ M of R428 or vehicle. After infection, the viral
733 inoculates were completely removed and replaced with fresh culture medium
734 containing 1 μ M of R428 or vehicle. The cell supernatants were collected at 24 hpi
735 and subjected to IL-1 α measurement.

736

737 **Trypan blue (TB) staining**

738 The isolated peritoneal macrophages were stained with 0.04% of TB solution (final
739 concentration) for 3 min and then immediately smeared on a slide for cell death
740 counting and photographing.

741

742 **TUNEL staining**

743 In situ peritoneal macrophages were isolated from JEV-infected mice and
744 immediately made into smears, followed by TUNEL staining with One Step TUNEL
745 Apoptosis Assay Kit (C1086, Beyotime)

746

747 **Immunoblotting**

748 The isolated peritoneal macrophages were lysed with 100 μ L of 1x cell lysis buffer
749 (9803S, CST) and ultrasonicated, mixed with 25 μ L of 5x SDS loading buffer (P0015,
750 Beyotime), and finally boiled for 15 min. The resulting samples were subjected to an
751 immunoblotting protocol(43) for detection of specific proteins.

752

753 **Drug administration**

754 Mice ($Axl^{-/-}$) in IL-1 α or IL-6 treated group were i.p. injected with 200 ng of a
755 recombinant mouse IL-1 α (50114-MNAE, Sino Biological) or 60 ng of a recombinant
756 mouse IL-6 (50136-MNAE, Sino Biological) per mouse 30 min prior to i.p. injection
757 with 10^4 PFU of JEV, then a daily i.p. injection of 200 ng of IL-1 α or 60 ng of IL-6
758 was administrated. Mice (including wild-type BALB/c mice and $Axl^{-/-}$ mice) in
759 IL-1Ra-treated group were i.p. injected with 10 μ g of a recombinant rat IL-1Ra (a
760 natural IL-1 α antagonist, 80073-R01H, Sino Biological) per mouse 1 h prior to i.p.
761 injection with 10^4 PFU of JEV, then an every-two-day i.p. injection of 5 μ g of IL-1Ra
762 was administrated. Mice (including wild-type BALB/c mice and $Axl^{-/-}$ mice) in
763 vehicle-treated group were i.p. injected with 200 μ L of sterile normal saline 30 min
764 prior to i.p. injection with 10^4 PFU of JEV, then a daily i.p. injection of 200 μ L of
765 sterile normal saline was administrated. $Axl^{+/-}$ mice were i.p. injected with 1.25 mg of
766 LY294002 (PI3K inhibitor, HY-10108, MCE) per mouse 1 h prior to i.p. injection
767 with 10^4 PFU of JEV. At 24 hpi, peritoneal macrophages were isolated and made into
768 smears for cell death studies.

769

770 **Statistical analysis**

771 The quantitative data were expressed as Mean $\pm$ SEM and all the statistical analyses
772 were performed on GraphPad Prism 7.00 software. Student's t test or with Welch's
773 correction and Mann-Whitney test were used to compare quantitative data between
774 two groups. Two-way ANOVA and multiple t test were used to compare grouped
775 quantitative data. Specially, Log-rank test was used to compare survival curve.
776 Fisher's exact test was used to compare the probability of JEV entry into brain.
777 Rank-sum test was used to compare sign scores. Linear regression model was used to
778 analyze the correlation between brain viral load and BBB permeability, and the
779 correlation between IL-1 α content in serum and IL-1 α content in peritoneal wash.
780 Comparisons were considered statistically different when $p < 0.05$.

781

782 **Ethical statement**

783 All the animal experiments were reviewed and approved by the Experimental Animal
784 Welfare and Animal Ethics Committee of Capital Medical University, Beijing, China
785 (permission code: EEI-2015-048; permission date: April 20, 2015).

786

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794

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801

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956
957 **Figure legends**

958
959 **Fig. 1. Effects of Axl on Japanese encephalitis virus (JEV) infection.**

960 Four-week-old Axl^{-/-} and Axl^{+/-} mice receive an intraperitoneal (i.p.) injection of
961 different dose of JEV (10⁴, 10⁵, and 10⁶ PFU). (A-C) Survival curves of Axl^{+/-} and
962 Axl^{-/-} mice infected with 10⁴ PFU (A, n=12 for Axl^{+/-}, n=22 for Axl^{-/-}), 10⁵ PFU (B,
963 n=19 for Axl^{+/-}, n=14 for Axl^{-/-}), and 10⁶ PFU of JEV (C, n=11 for Axl^{+/-}, n=19 for
964 Axl^{-/-}). The survival curves are compared by Log-rank test. (D-F) Body weight
965 changes of Axl^{+/-} and Axl^{-/-} mice infected with 10⁴ PFU (D, n=26 for Axl^{+/-}, n=19 for
966 Axl^{-/-}), 10⁵ PFU (E, n=7 for Axl^{+/-}, n=9 for Axl^{-/-}), and 10⁶ PFU of JEV (F, n=11 for

967 $Axl^{+/-}$, n=19 for $Axl^{-/-}$). The body weight changes are compared by two-way ANOVA.
968 Data are expressed as the means $\pm$ SEM, * denotes $p<0.05$, ** denotes $p<0.01$, and ns
969 denotes no significance ($p>0.05$); each result is the representative of three
970 independent experiments.

971

972 **Fig. 2. Effects of Axl on peripheral and brain JEV infection.**

973 **(A-D)** Four-week-old $Axl^{-/-}$ and $Axl^{+/-}$ mice receive an i.p. injection of 10^4 PFU of JEV.
974 Viral loads in serum **(A)**, spleen **(B)**, liver **(C)**, and kidney **(D)** at the indicated time
975 points post infection. LOD (green dashed line) denotes limit of detection. Each dot
976 denotes a mouse, and the viral loads are compared by two-way ANOVA and multiple t
977 tests. **(E-G)** Primary peritoneal macrophages are infected with JEV at MOI=1. **(E)**
978 Immunofluorescent (IF) staining of JEV antigens (green) in peritoneal macrophages at
979 24 hpi. **(F)** Infection rates of peritoneal macrophages at 24 hpi. n=4 for each group,
980 and the infection rates are compared by unpaired t test. **(G)** Viral loads in supernatants
981 of peritoneal macrophages. n=4 for each group, and the data are compared by
982 two-way ANOVA and multiple t tests. **(H-J)** Four-week-old $Axl^{-/-}$ mice (n=8) and
983 $Axl^{+/-}$ mice (n=12) are intracerebrally injected with 10^3 PFU of JEV. **(H)** Survival
984 curves, which are compared by Log rank test. **(I)** Body weight changes, which are
985 compared by two-way ANOVA. **(J)** Viral loads in brain at 6 dpi, each dot denotes a
986 mouse, and the viral loads are compared by unpaired t test. All the data are expressed
987 as the means $\pm$ SEM, ns denotes no significance ($p>0.05$); each result is the
988 representative of three independent experiments.

989

990 **Fig. 3. Effects of Axl on JEV neuroinvasion and blood-brain barrier (BBB)**
991 **permeability.**

992 Four-week-old Axl^{-/-} and Axl^{+/-} mice receive an i.p. injection of 10⁴ PFU of JEV (**A**,
993 **E-K**) or 5 × 10⁴ PFU of Rluc-JEV for bioluminescent imaging (BLI, **B-D**). (**A**) Viral
994 loads in brains, each dot denotes a mouse, and viral loads are compared by two-way
995 ANOVA and multiple t tests. (**B**) Representative BLI image of Rluc-JEV infection. (**C**)
996 Quantification of the BLI signal intensity in peritoneal cavity, n=15 for each group,
997 and the data are compared by two-way ANOVA and multiple t tests. (**D**) Cumulative
998 percentage of the mice with Rluc-JEV entry into brain, n=15 for each group, and the
999 data are compared by Fisher's exact test. (**E**) Representative image of Evans blue (EB)
1000 leakage into brain parenchyma. (**F**) Quantification of EB leakage into brain
1001 parenchyma after JEV infection, n=5-8 for each group, and the data are compared by
1002 two-way ANOVA and multiple t tests. (**G-I**) Correlation analysis of BBB permeability
1003 and brain viral load in Axl^{+/-} mice (**G**), Axl^{-/-} mice (**H**), and mixed mice (**I**), each dot
1004 denotes a mouse, and the data are analyzed by linear regression model. (**J**)
1005 Representative image of sodium fluorescein (NaF) leakage into brain parenchyma. (**K**)
1006 Quantification of NaF leakage into brain parenchyma under uninfected and
1007 JEV-infected (7 dpi) conditions, each dot denotes a mouse, and the data are compared
1008 by multiple t tests. All the data are expressed as the means ± SEM, * denotes p<0.05,
1009 ** denotes p<0.01, and ns denotes no significance (p>0.05); each result is the
1010 representative of three independent experiments.

1011

1012 **Fig. 4. Effects of Axl on the integrity of tight junctions in the brain.**

1013 Four-week-old Axl^{-/-} and Axl^{+/-} mice receive an i.p. injection of 10⁴ PFU of JEV. (A,
1014 C, E) IF staining of claudin-5, occludin, and ZO-1 (tight junction proteins, red), CD31
1015 (microvascular endothelial cell marker, green), and DAPI (nucleus, blue) in brain at 0
1016 dpi (A), 1 dpi (C), and 2 dpi (E), where each image is the representative from 3-5
1017 mice, scale bar=20 μ m. (B, D, F) Quantification of the fluorescent intensity of
1018 claudin-5, occludin, and ZO-1 in brain at 0 dpi (B), 1 dpi (D), and 2 dpi (F), each dot
1019 denotes a mouse, and the data are compared by unpaired t test. (G-I) Relative mRNA
1020 abundance of claudin-5, occludin, and ZO-1 in mouse brains at 0 dpi (G), 1 dpi (H),
1021 and 2 dpi (I), normalized to the average of Axl^{+/-} mice, each dot denotes a mouse, and
1022 the data are compared by unpaired t test. All the data are expressed as the means $\pm$
1023 SEM, * p<0.05, ** p<0.01; each result is the representative of three independent
1024 experiments.

1025

1026 **Fig. 5. Cytokine profiling in serum.**

1027 Four-week-old Axl^{-/-} and Axl^{+/-} mice receive an i.p. injection of 10⁴ PFU of JEV or
1028 200 μ L of PBS (mock) per mouse, and serum samples are obtained at 1 and 7 dpi. A
1029 panel of cytokines including IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, TNF- α , IFN- γ ,
1030 CCL2, and CCL5 in sera are detected by a bead-based immunoassay (Aimplex), n=2
1031 for mock, and n=6 for 1 and 7 dpi, and the data are compared by two-way ANOVA
1032 and multiple t tests. All the data are expressed as means $\pm$ SEM, * denotes p<0.05;

1033 this result is the representative of three independent experiments.

1034

1035 **Fig. 6. Effects of IL-6 on JEV infection outcome and BBB permeability.**

1036 (A-B) Four-week-old $Axl^{-/-}$ mice receive an i.p. injection of recombinant mouse IL-6
1037 (60 ng per mouse, n=10) or vehicle (sterile normal saline, n=10) 30 min prior to an i.p.
1038 injection of 10^4 PFU of JEV, and then treated daily with IL-6 (60 ng per mouse) or
1039 vehicle (sterile normal saline). (A) Body weight changes, which are compared by
1040 two-way ANOVA. (B) Survival curves, which are compared by Log-rank test. (C-D)
1041 Four-week-old $Axl^{-/-}$ mice receive a single i.p. injection of IL-6 (60 ng per mouse) or
1042 vehicle (sterile normal saline), and BBB permeability is measured 6 h later by an EB
1043 leakage test. (C) Representative image showing EB leakage into brain parenchyma.
1044 (D) Quantification of EB content in brain, normalized to the average of
1045 vehicle-treated mice, where each dot denotes a mouse, and the data are compared by
1046 unpaired t test. All the data are expressed as means $\pm$ SEM, ** denotes $p < 0.01$, and ns
1047 denotes no significance ($p > 0.05$); each result is the representative of three
1048 independent experiments.

1049

1050 **Fig. 7. Effects of IL-1 α on JEV infection outcome.**

1051 Four-week-old $Axl^{-/-}$ mice receive an i.p. injection of recombinant mouse IL-1 α (200
1052 ng per mouse, n=14) or vehicle (sterile normal saline, n=13) 30 min prior to an i.p.
1053 injection of 10^4 PFU of JEV and then are treated daily with IL-1 α or vehicle. (A)
1054 Body weight changes, which are compared by two-way ANOVA. (B) Scores of the

1055 infection signs, which are compared by Rank sum test. (C) Survival curves, which are
1056 compared by Log-rank test. (D) Pathological lesions in cerebral cortex and
1057 hippocampus at 7 dpi. (E) Perivascular infiltration of inflammatory cells in cerebral
1058 cortex at 7 dpi. (F-G) Quantification of the area of neuron death (F) and the area of
1059 inflammatory cell infiltration (G) in cerebral cortex per high power field (HPF),
1060 normalized to the average of vehicle-treated mice, n=5 for each group, and the data
1061 are compared by unpaired t test. (H) Viral load in mouse brains at 7 dpi, green dashed
1062 line denotes the limit of detection, n=5 for each group, and the data are compared by
1063 unpaired t test. All the data are expressed as the means $\pm$ SEM, * denotes $p<0.05$, and
1064 ** denotes $p<0.01$; each result is the representative of three independent experiments.

1065

1066 **Fig. 8. Effects of IL-1 α on BBB permeability and tight junction integrity during**
1067 **JEV infection.**

1068 (A) Representative image displaying endogenous IgG (green) leakage into brain
1069 parenchyma. Claudin-5 (red) is used to position BBB, DAPI is used to show nucleus,
1070 scale bar=20 μ m. (B) Quantification of endogenous IgG leakage into brain
1071 parenchyma at 7 dpi, n=5, and the data are compared by unpaired t test. (C)
1072 Representative image depicting brain claudin-5, occludin, and ZO-1 (red), CD31
1073 (microvascular endothelial cell marker, green), and DAPI (nucleus, blue), scale
1074 bar=20 μ m. (D) Quantification of the staining intensity of claudin-5, occludin, and
1075 ZO-1 in the cerebral cortex, n=5, and the data are compared by unpaired t test. All the
1076 data are expressed as the means $\pm$ SEM, * denotes $p<0.05$, and ** denotes $p<0.01$;

1077 each result is the representative of three independent experiments.

1078

1079 **Fig. 9. Effects of IL-1 α alone on BBB permeability and tight junction integrity.**

1080 Four-week-old Axl^{-/-} mice receive an i.p. injection of recombinant mouse IL-1 α (200
1081 ng per mouse) or vehicle (sterile normal saline). At 6 h post treatment, BBB
1082 permeability is gauged by an EB leakage test, and brain tight junction proteins are
1083 observed by IF staining. (A) Representative image showing EB leakage into brain
1084 parenchyma. (B) BBB permeability change after IL-1 α treatment, n=5, and the data
1085 are compared by unpaired t test. (C) mRNA abundance of claudin-5, occludin, and
1086 ZO-1 in mouse brains after IL-1 α treatment, normalized to the average of
1087 vehicle-treated mice, n=5, and the data are compared by unpaired t test. (D) IF
1088 staining of claudin-5, occludin, and ZO-1 (red), CD31 (microvascular endothelial cell
1089 marker, green), and DAPI (nucleus, blue), scale bar=20 μ m. (E) Quantification of the
1090 IF staining intensity of claudin-5, occludin, and ZO-1, n=5 for each group, and the
1091 data are compared by unpaired t test. All the data are expressed as the means $\pm$ SEM,
1092 * denotes p<0.05, and ** denotes p<0.01; each result is the representative of three
1093 independent experiments.

1094

1095 **Fig. 10. Effect of Axl on IL-1 α production during JEV infection.**

1096 (A-D) Four-week-old Axl^{-/-} and Axl^{+/-} mice receive an i.p. injection of 10⁴ PFU of JEV.
1097 IL-1 α content in the brain (A), serum (B), and peritoneal wash (C) at 24 hpi, each dot
1098 denotes a mouse, and the data are compared by unpaired t test. (D) Correlation

1099 analysis of IL-1 α content in serum and peritoneal wash at 24 hpi, the data are
1100 analyzed by linear regression model. **(E-H)** Peritoneal macrophages are isolated from
1101 Axl^{-/-} and Axl^{+/-} mice, cultured and infected with JEV (MOI=1) or stimulated with
1102 LPS (1 μ g/mL) in vitro. **(E)** IF staining of F4/80 (marker of macrophages) in
1103 untreated Axl^{-/-} and Axl^{+/-} peritoneal macrophages. Scale bar=20 μ m. **(F)** Dynamics of
1104 IL-1 α contents in the supernatants of peritoneal macrophages, n=4 for each group, and
1105 the data are compared by two-way ANOVA. **(G)** IF staining of intracellular IL-1 α in
1106 peritoneal macrophages at 24 hpi. Scale bar=20 μ m. **(H)** IL-1 α contents in the
1107 supernatants of peritoneal macrophages at 24 hpi, n=3-6 for each group, and the data
1108 are compared by unpaired t test. **(I-J)** Murine cerebral microvascular endothelial cells
1109 (bEND.3) and primary splenocytes are infected with JEV (MOI=1). **(I)** IL-1 α contents
1110 in the supernatants of bEND.3 cells at 24 hpi, n=5, the data are compared by unpaired
1111 t test. **(J)** IL-1 α contents in the supernatants of primary splenocytes at 24 hpi, n=6, the
1112 data are compared by unpaired t test. **(K)** IF staining of JEV antigens and IL-1 α inside
1113 the in situ peritoneal macrophages at 24 hpi. Scale bar=20 μ m. **(L)** Infection rates of
1114 the in situ peritoneal macrophages at 24 hpi, n=3 for Axl^{+/-}, and n=5 for Axl^{-/-}, the data
1115 are compared by unpaired t test. **(M)** Viral loads in peritoneal washes at 24 hpi, n=7
1116 for each group, the data are compared by unpaired t test. **(N)** IF staining intensity of
1117 intracellular IL-1 α in the in situ peritoneal macrophages at 24 hpi, n=4-7 for each
1118 group, the data are compared by unpaired t test. **(O)** Trypan blue staining of the in situ
1119 peritoneal macrophages at 24 hpi. Scale bar=20 μ m. **(P)** Cell death rates of the in situ
1120 peritoneal macrophages at 24 hpi, n=5-7, the data are compared by unpaired t test. **(Q)**

1121 IL-1 α content in the supernatant of live and hypotonically dead Axl^{-/-} peritoneal
1122 macrophages, n=8 for each group, the data are compared by unpaired t test. All the
1123 data are expressed as the means $\pm$ SEM, * denotes p<0.05, ** denotes p<0.01, and ns
1124 denotes no significance (p>0.05); each result is the representative of three
1125 independent experiments.

1126

1127 **Fig. 11. Transcriptomic analysis of the cell death pathways in the in situ**
1128 **peritoneal macrophages.**

1129 Four-week-old Axl^{-/-} and Axl^{+/-} mice receive an i.p. injection of 10⁴ PFU of JEV. At 24
1130 hpi, the in situ peritoneal macrophages are isolated and subjected to transcriptomic
1131 sequencing. (A) Volcano plot showing the upregulated, downregulated, and
1132 unchanged genes. JEV-infected Axl^{-/-} macrophages vs JEV-infected Axl^{+/-}
1133 macrophages, each dot denotes a specific gene, n=5 for each group. (B) GO
1134 enrichment analysis of the cell death-related biological processes, JEV-infected Axl^{-/-}
1135 macrophages vs JEV-infected Axl^{+/-} macrophages, n=5 for each group.

1136

1137 **Fig. 12. Effect of Axl on the cell death mode of in situ peritoneal macrophages.**

1138 Four-week-old Axl^{-/-} and Axl^{+/-} mice receive an i.p. injection of 10⁴ PFU of JEV.
1139 Specifically, for PI3K inhibition in the in situ Axl^{+/-} peritoneal macrophages, an i.p.
1140 injection of LY294002 (1.25 mg per mouse) is administered 1 h prior to i.p. infection.
1141 At 24 hpi, the in situ peritoneal macrophages are isolated. (A) IF staining of
1142 GSDMD-N (marker of pyroptosis) and TUNEL staining (marker of apoptosis) of the

1143 in situ peritoneal macrophages. **(B)** Pyroptosis (GSDMD-N⁺) rates of the in situ
1144 peritoneal macrophages, n=3-4 for each group, the data are compared by unpaired t
1145 test. **(C)** Apoptosis (TUNEL⁺) rates of the in situ peritoneal macrophages, n=3-4 for
1146 each group, the data are compared by unpaired t test. **(D)** IF staining of RIPK1
1147 (marker of necroptosis) in the in situ peritoneal macrophages. **(E)** Quantification of
1148 the fluorescence intensity of RIPK1 in the in situ peritoneal macrophages, n=3 for
1149 each group, the data are compared by unpaired t test. **(F)** Pyroptosis (GSDMD-N⁺)
1150 rate of JEV-infected (JEV⁺) in situ peritoneal macrophages, n=3-4 for each group, the
1151 data are compared by unpaired t test. **(G)** Immunoblotting detection of cleaved
1152 caspase-1 and GSDMD-N in the in situ peritoneal macrophages at 24 hpi. GAPDH
1153 was used as a loading control, n=3. **(H-I)** Quantification of the gray density of
1154 cleaved caspase-1 **(H)** and GSDMD-N **(I)**, normalized to the average of mock-treated
1155 Axl^{+/-} macrophages, n=3 for each group, the data are compared by unpaired t test. All
1156 the data are expressed as the means $\pm$ SEM, * denotes p<0.05, and ** denotes p<0.01;
1157 each result is the representative of three independent experiments.

1158

1159 **Fig. 13. Effects of Axl on the expression of cell death regulatory proteins in the in**
1160 **situ peritoneal macrophages.**

1161 Four-week-old Axl^{-/-} and Axl^{+/-} mice receive an i.p. injection of 10⁴ PFU of JEV. At 24
1162 hpi, the in situ peritoneal macrophages are isolated. **(A-C)** IF staining and
1163 quantification of caspase-8 (extrinsic apoptosis initiator, **A**), caspase-9 (intrinsic
1164 apoptosis initiator, **B**), and caspase-3 (apoptosis executor, **C**), where these genes are

1165 also crucial regulators of pyroptosis, $n=3$ for each group, the data are compared by
1166 unpaired t test. Scale bar=20 μm . **(D-E)** IF staining of PI3K-p110 **(D)** and
1167 phosphorylated Akt (pAkt, **E**) of the in situ peritoneal macrophages. Scale bar=20 μm .
1168 **(F-G)** Quantification of the fluorescent intensity of PI3K-p110 **(F)** and pAkt **(G)** of
1169 the in situ peritoneal macrophages, $n=3-4$ for each group, the data are compared by
1170 unpaired t test. **(H)** Immunoblotting detection of PI3K-p110 in the in situ peritoneal
1171 macrophages at 24 hpi. GAPDH is used as a loading control. **(I)** Quantification of the
1172 gray density of PI3K-p110, normalized to the average of mock-treated $\text{Axl}^{+/-}$
1173 macrophages, $n=3$ for each group, the data are compared by unpaired t test. All the
1174 data are expressed as the means $\pm$ SEM, * denotes $p<0.05$, and ** denotes $p<0.01$;
1175 each result is the representative of three independent experiments.

1176

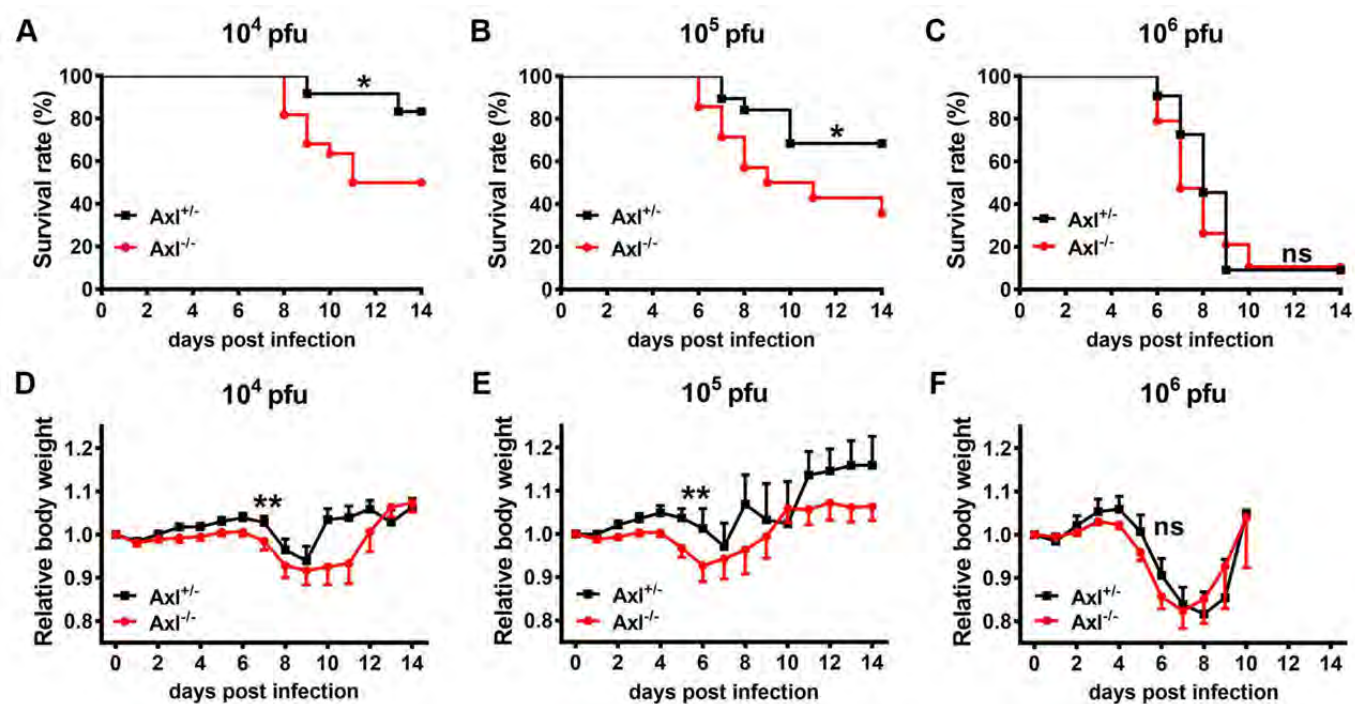
1177 **Fig. 14. Effects of an IL-1 α antagonist on JEV infection.**

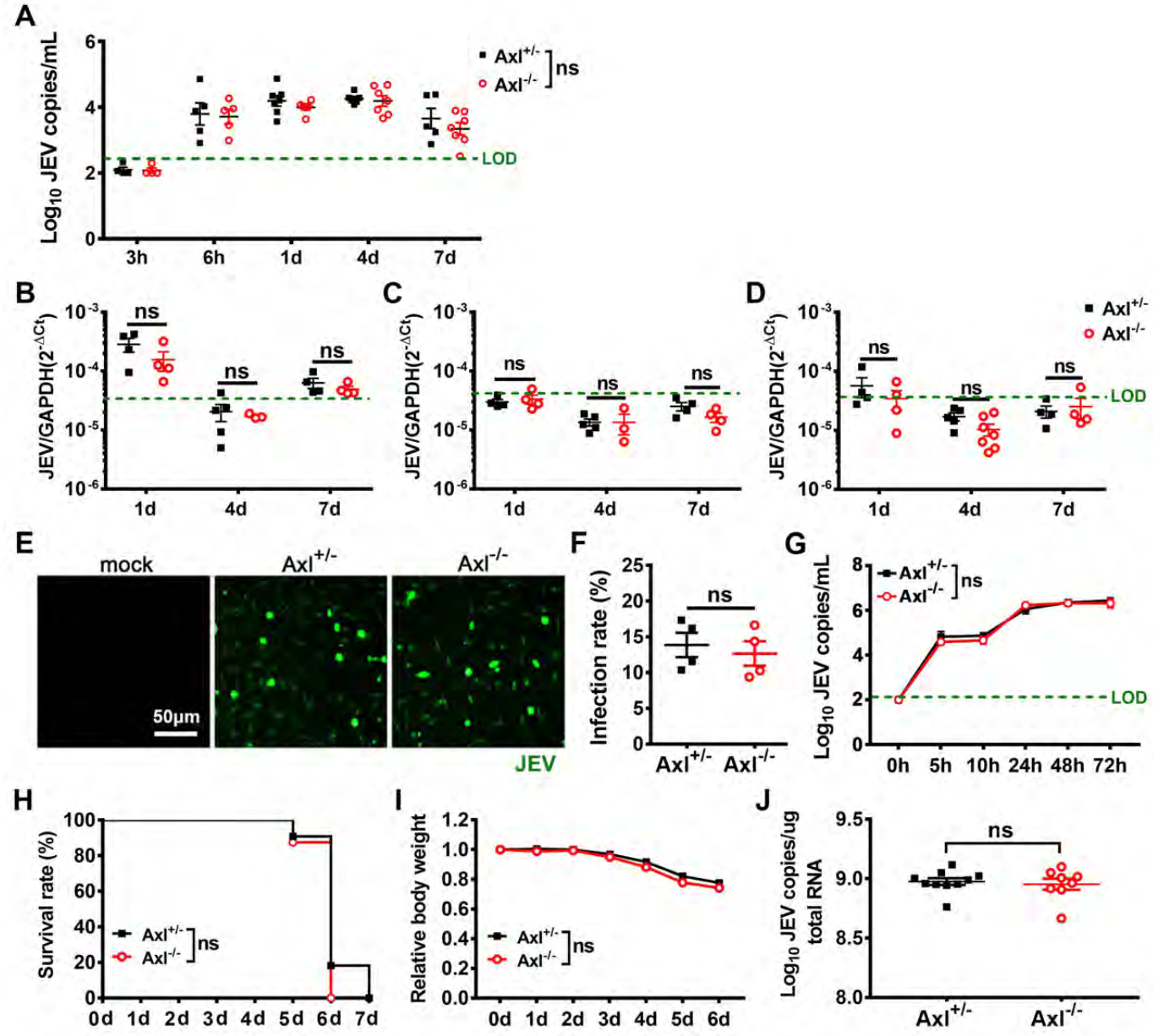
1178 Four-week-old $\text{Axl}^{+/-}$ mice receive an i.p. injection of recombinant rat IL-1Ra (10 μg
1179 per mouse, $n=13$) or vehicle (sterile normal saline, $n=14$) 1 h prior to receiving an i.p.
1180 injection of 10^4 PFU of JEV and then are treated with IL-1Ra (5 μg per mouse) or
1181 vehicle every two days. **(A)** Representative image showing clinical manifestations in
1182 the IL-1Ra- and vehicle-treated mice with JEV infection. The IL-1Ra-treated mice
1183 showed no apparent signs of JE, while vehicle-treated mice displayed typical signs of
1184 JE, such as ruffled fur and limbs paralysis. **(B)** Sign scores of IL-1Ra- and
1185 vehicle-treated mice with JEV infection, which are compared by Rank sum test. **(C)**
1186 Survival curves, which are compared by Log-rank test. **(D)** Pathological changes in

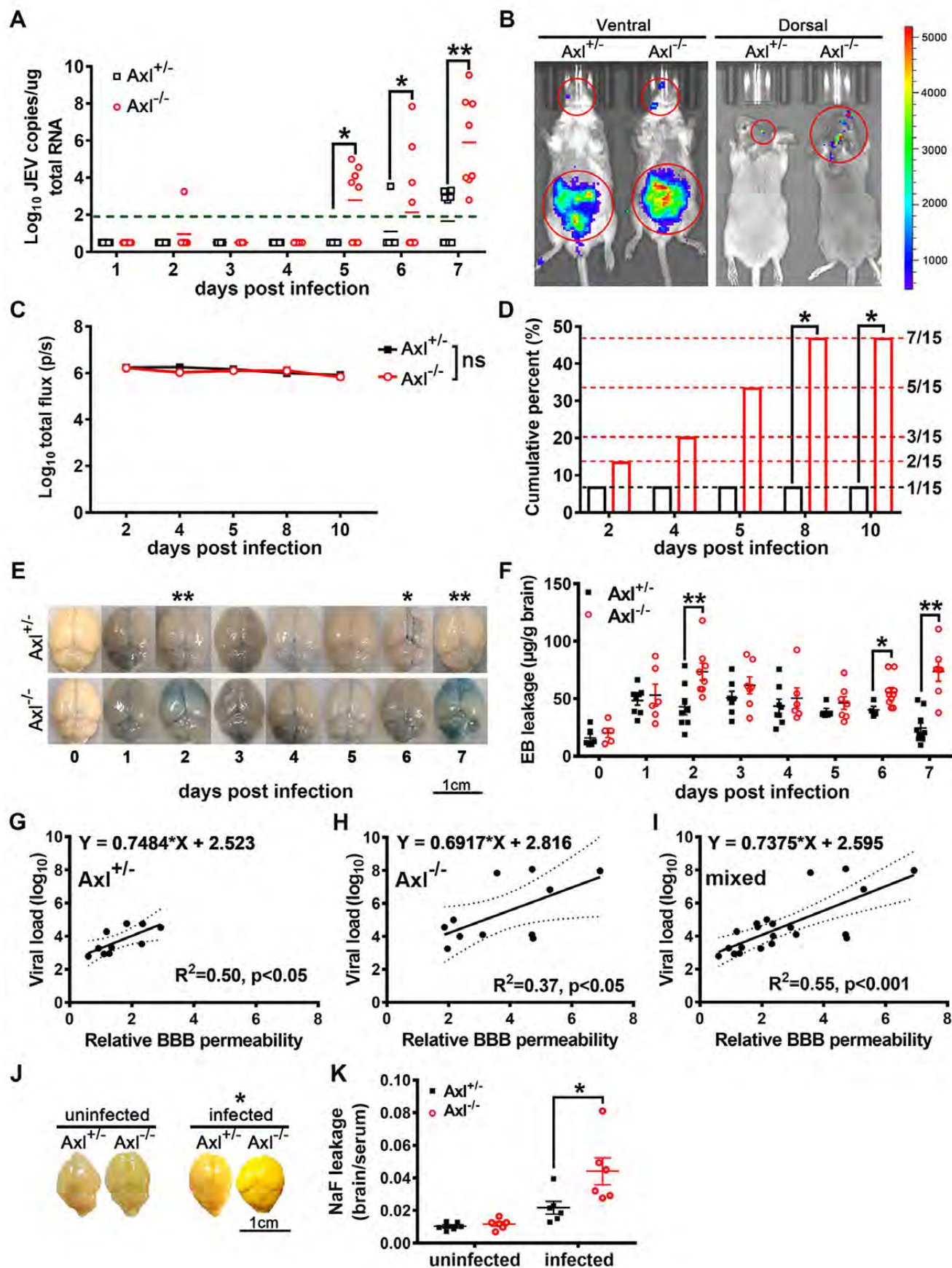
1187 cerebral cortex and hippocampus at 7 dpi, red arrow denotes neuron death, blue arrow
1188 denotes inflammatory cell infiltration, and black arrow denotes angiectasis, scale
1189 bar=100 μ m. **(E-F)** Quantification of the area of neuron death **(E)** and the area of
1190 inflammatory cell infiltration **(F)** in cerebral cortex, normalized to the average of
1191 vehicle-treated mice, n=5, and the data are compared by unpaired t test. **(G)** Viral load
1192 in mouse brains at 7 dpi, green dashed line denotes the limit of detection, n=5, and the
1193 data are compared by Mann-Whitney test. **(H)** Quantification of the endogenous IgG
1194 leakage into brain parenchyma at 7 dpi, n=5, and the data are compared by unpaired t
1195 test. **(I)** Representative image delineating endogenous IgG (green) leakage into brain
1196 parenchyma at 7 dpi. Claudin-5 (red) is used to position BBB, DAPI is used to show
1197 nucleus, scale bar=20 μ m. **(J)** Representative image depicting brain claudin-5,
1198 occludin, and ZO-1 (red), CD31 (microvascular endothelial cell marker, green), and
1199 DAPI (nucleus, blue), scale bar=20 μ m. **(K)** Quantification of the fluorescent intensity
1200 of claudin-5, occludin, and ZO-1 in brain, n=5, the data are compared by unpaired t
1201 test. **(L-N)** Four-week-old wild-type BALB/c mice receive an i.p. injection of
1202 recombinant rat IL-1Ra (10 μ g per mouse, n=10) or vehicle (sterile normal saline,
1203 n=10) 1 h prior to receiving an i.p. injection of 10^5 PFU of JEV and then are treated
1204 with IL-1Ra (5 μ g per mouse) or vehicle every two days. **(L)** Survival curves, which
1205 are compared by Log-rank test. **(M)** Body weight changes, which are compared by
1206 two-way ANOVA. **(N)** Scores of the infection signs, which are compared by Rank
1207 sum test. All the data are expressed as the means $\pm$ SEM, * denotes $p<0.05$, and **
1208 denotes $p<0.01$; each result is the representative of three independent experiments.

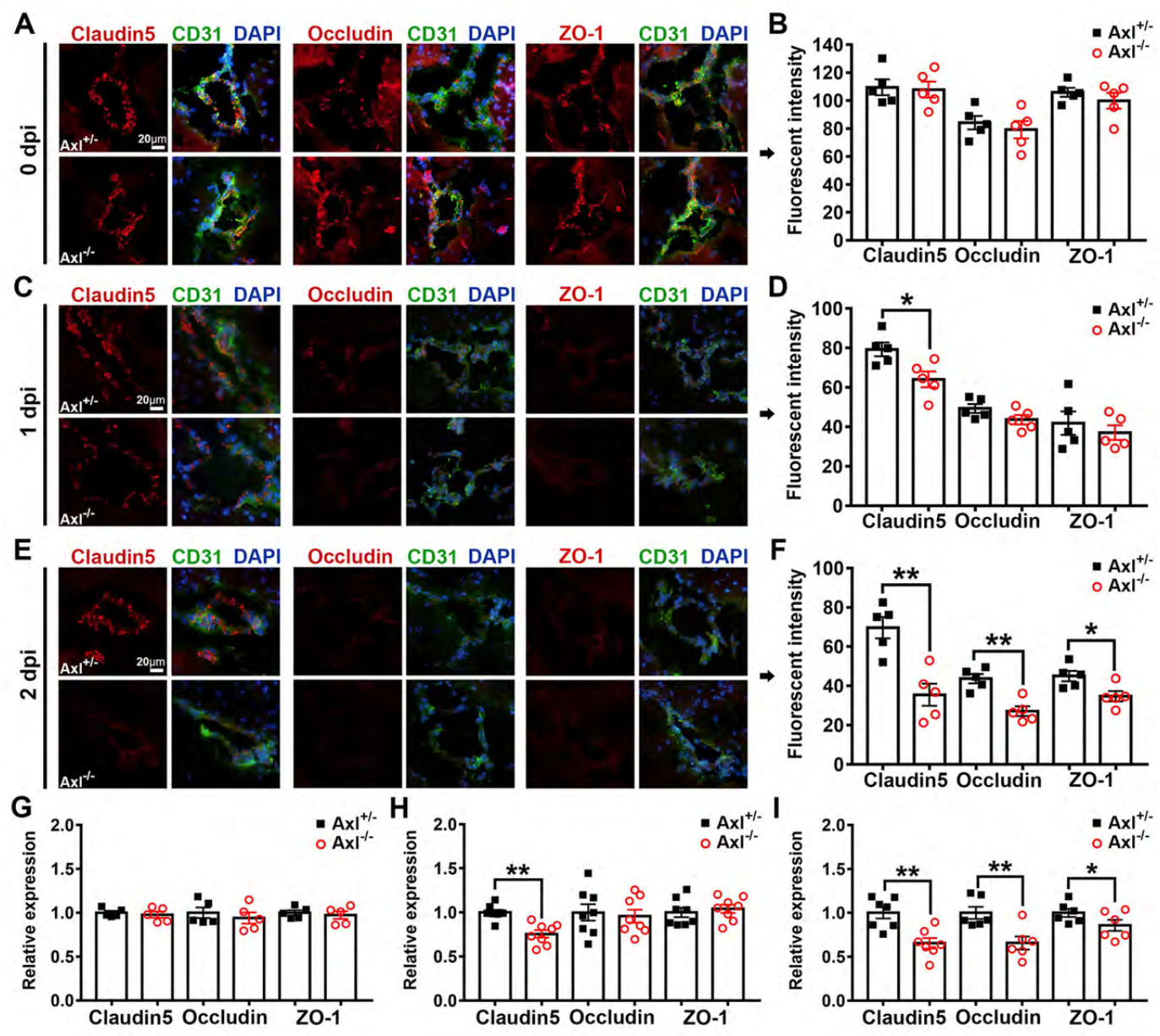
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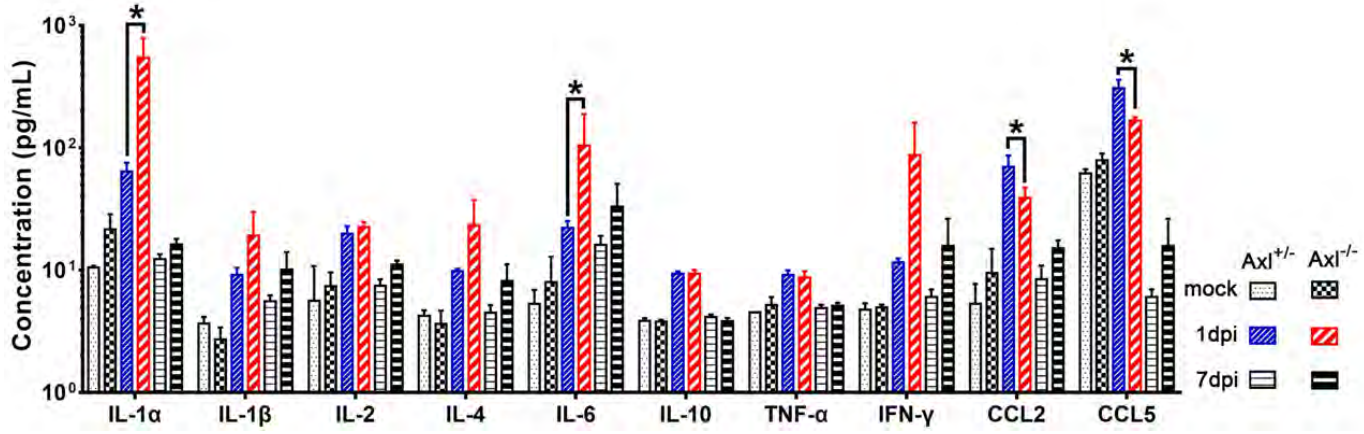
1210 **Table 1. The transcription levels of IL-1 signaling related and cell death related**
1211 **genes.**

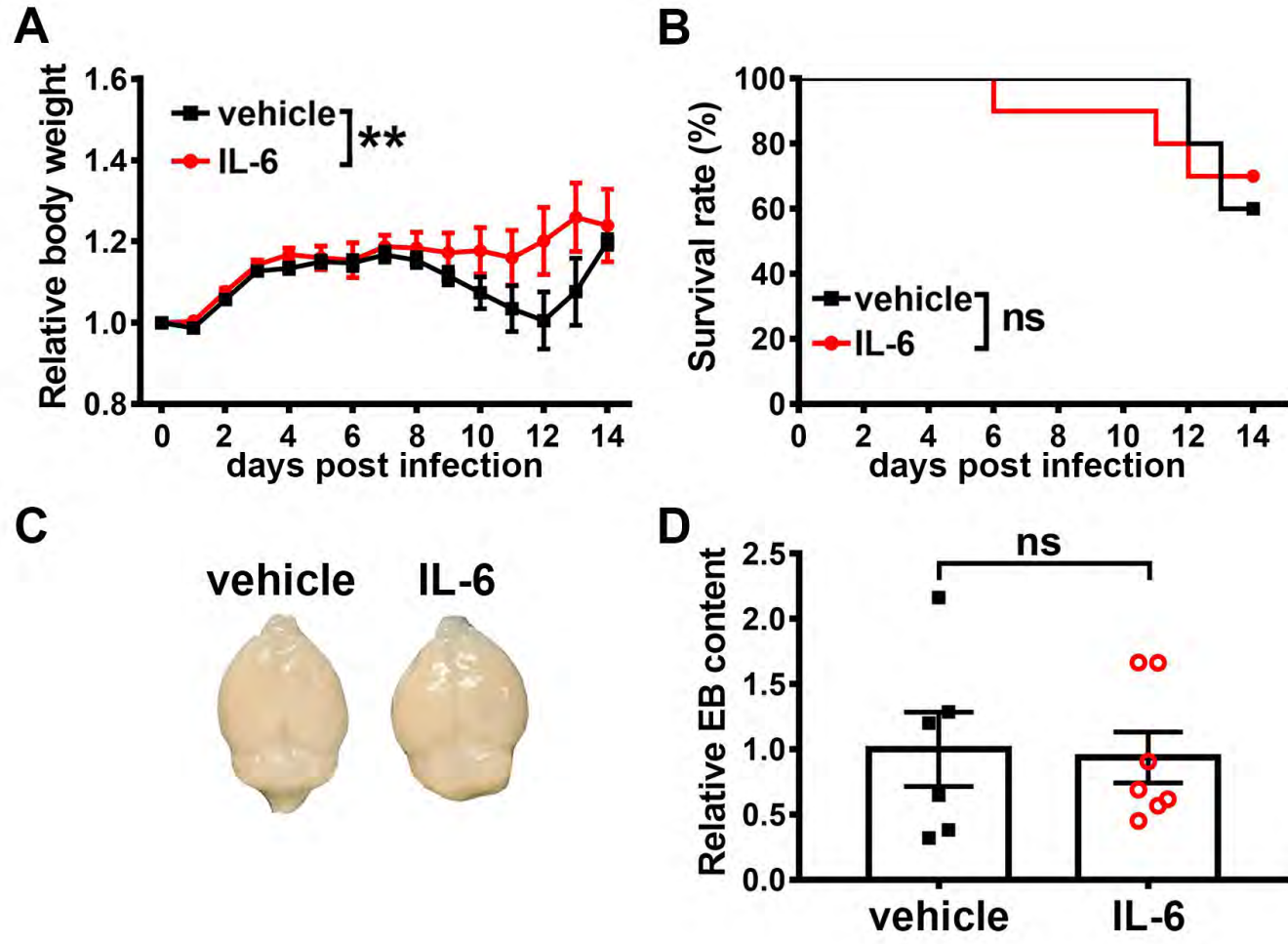


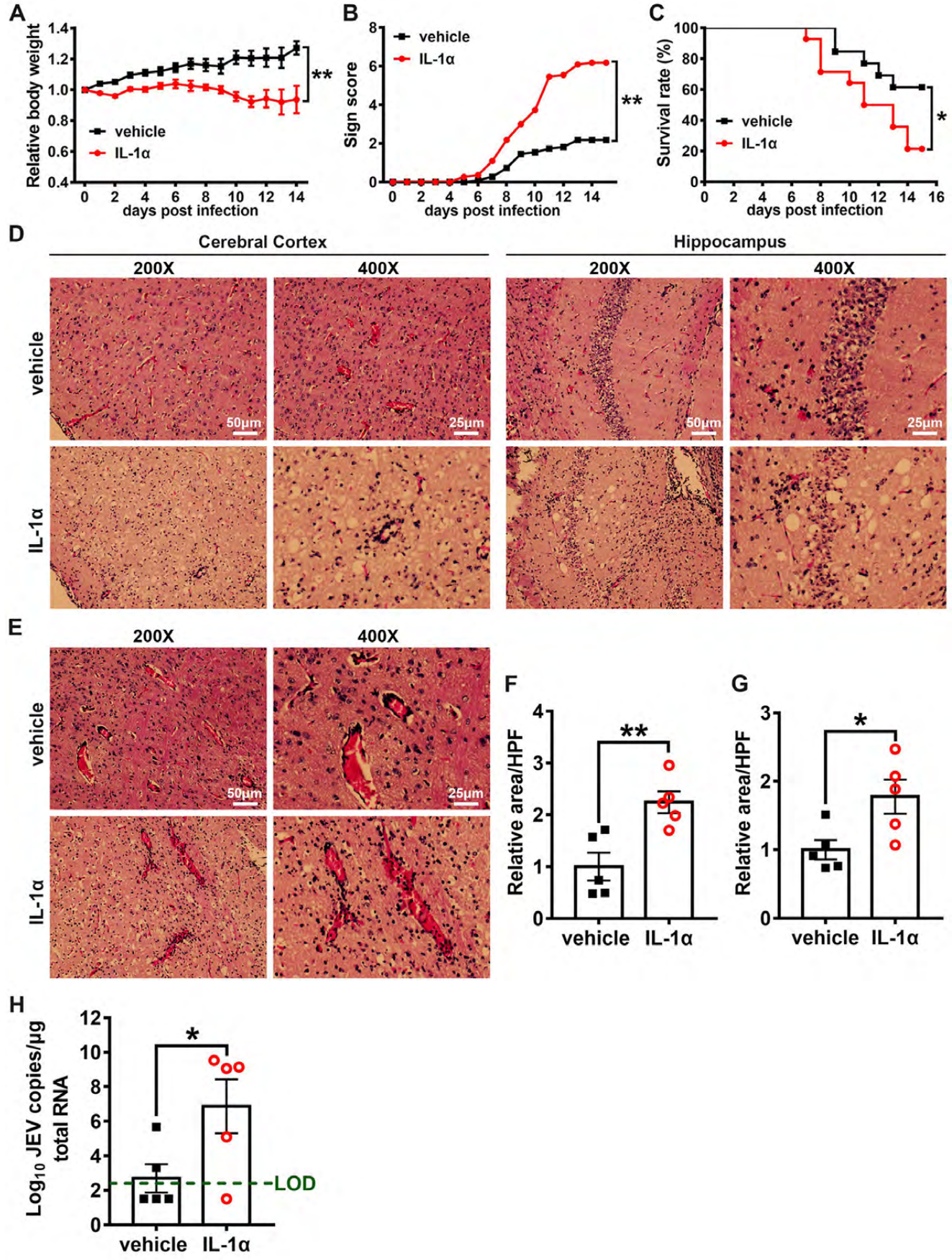


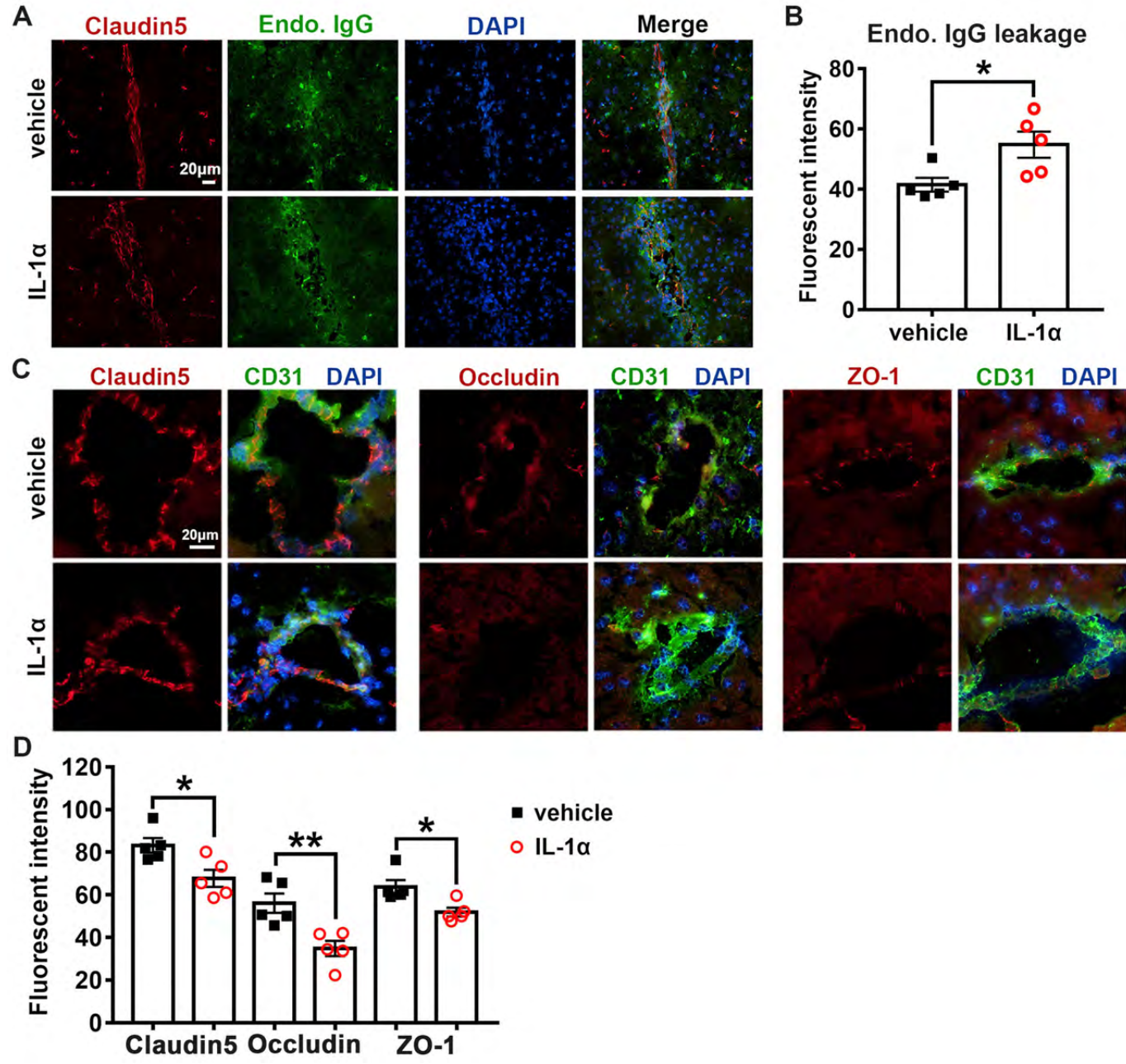


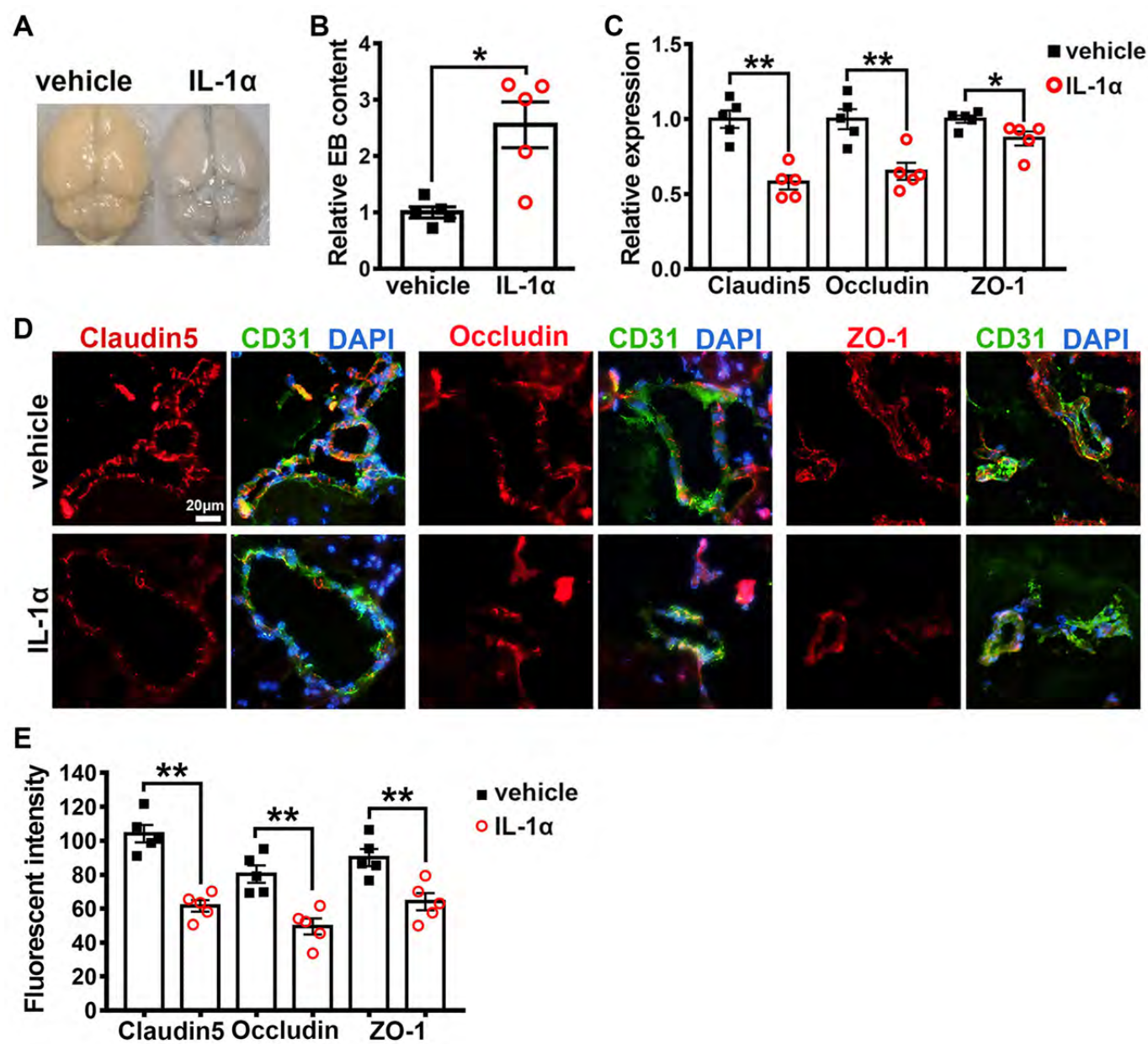


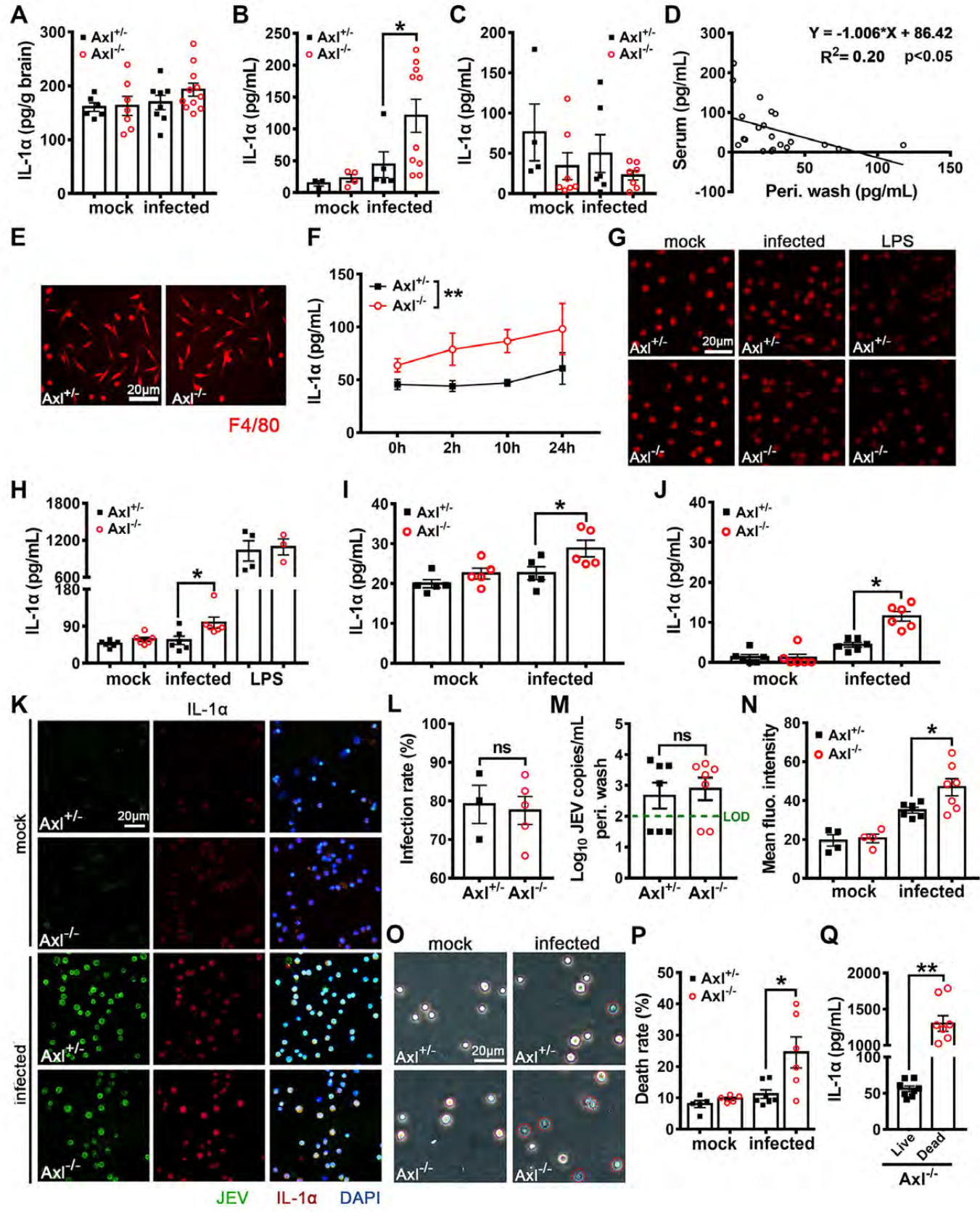


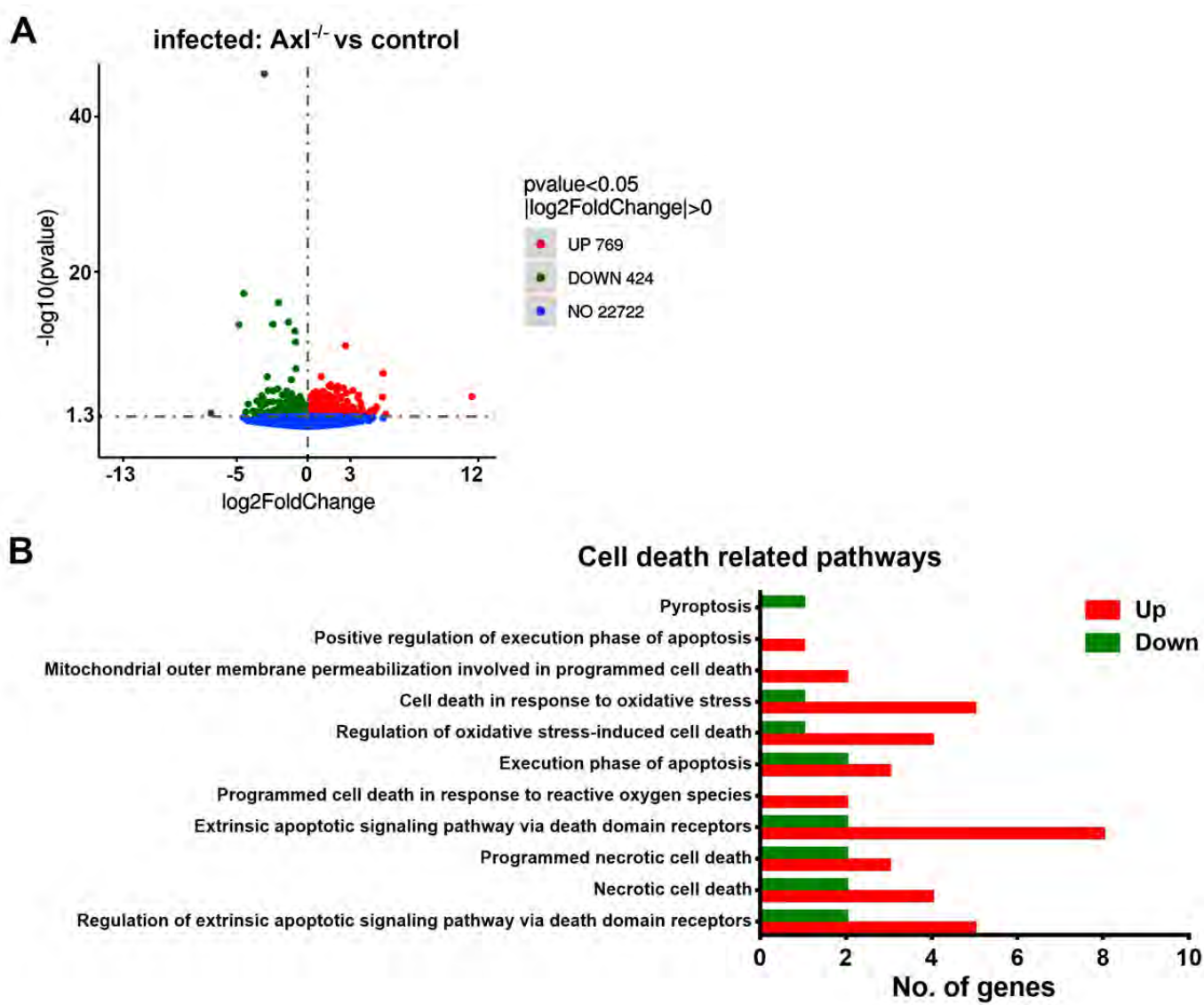


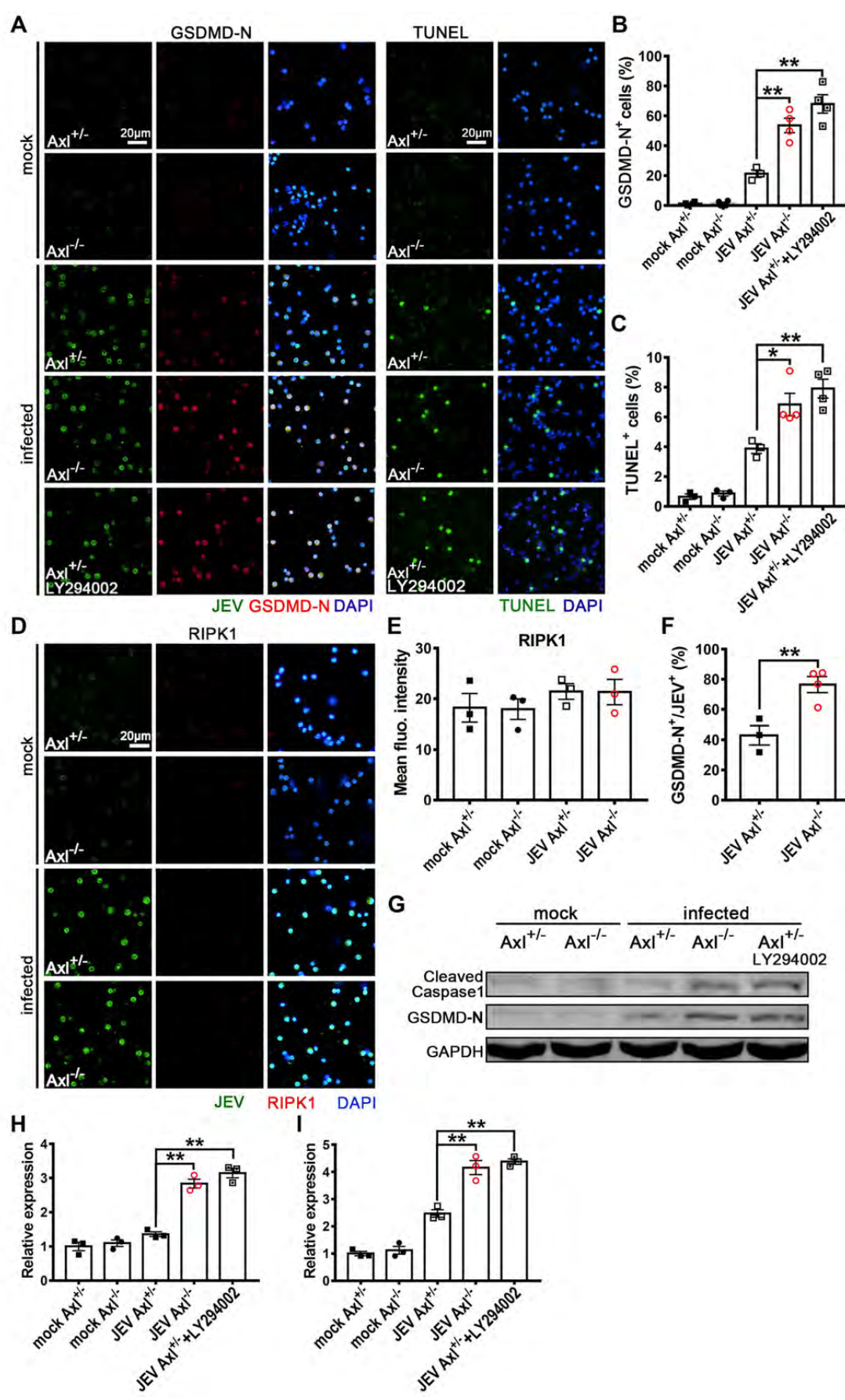


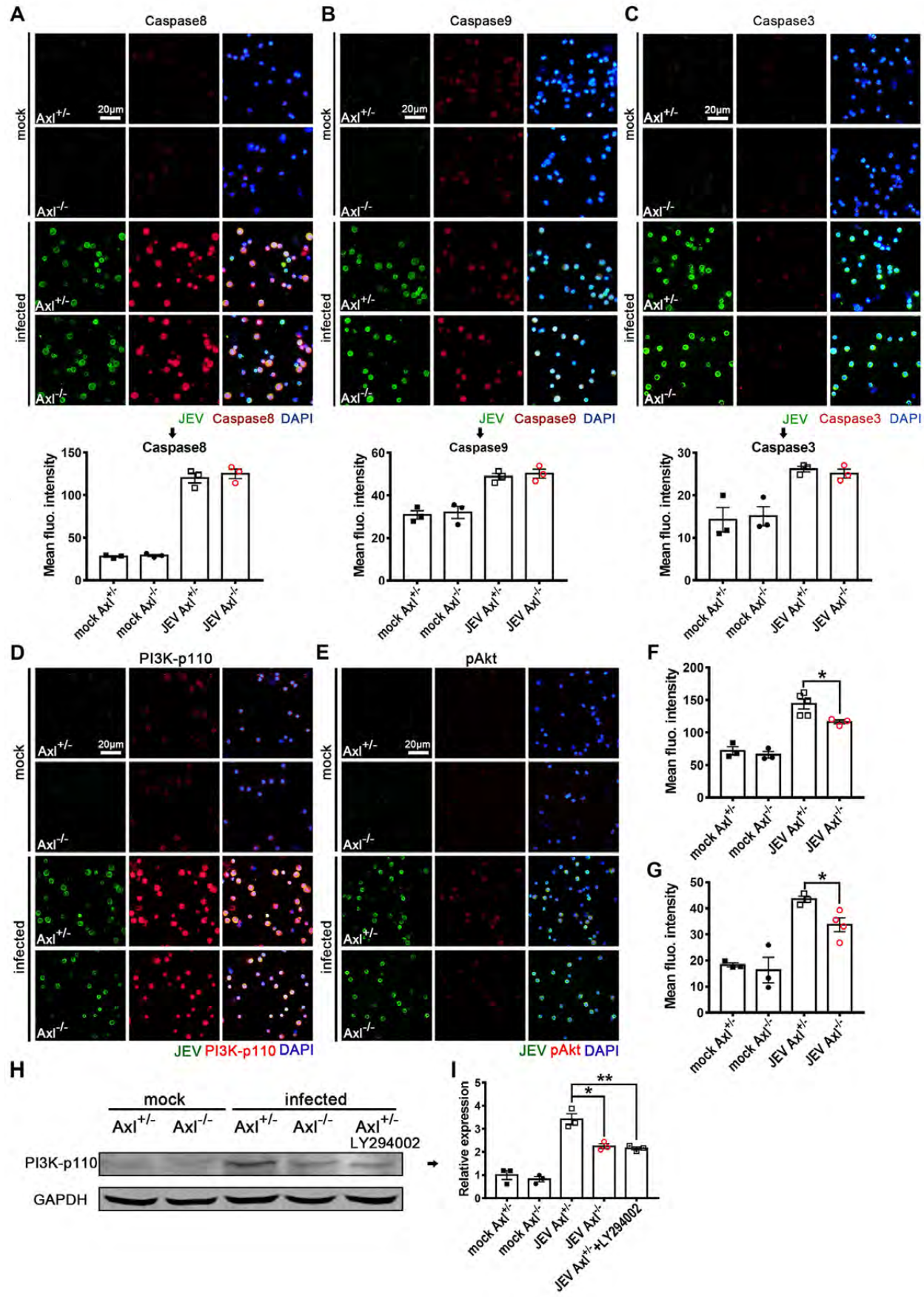


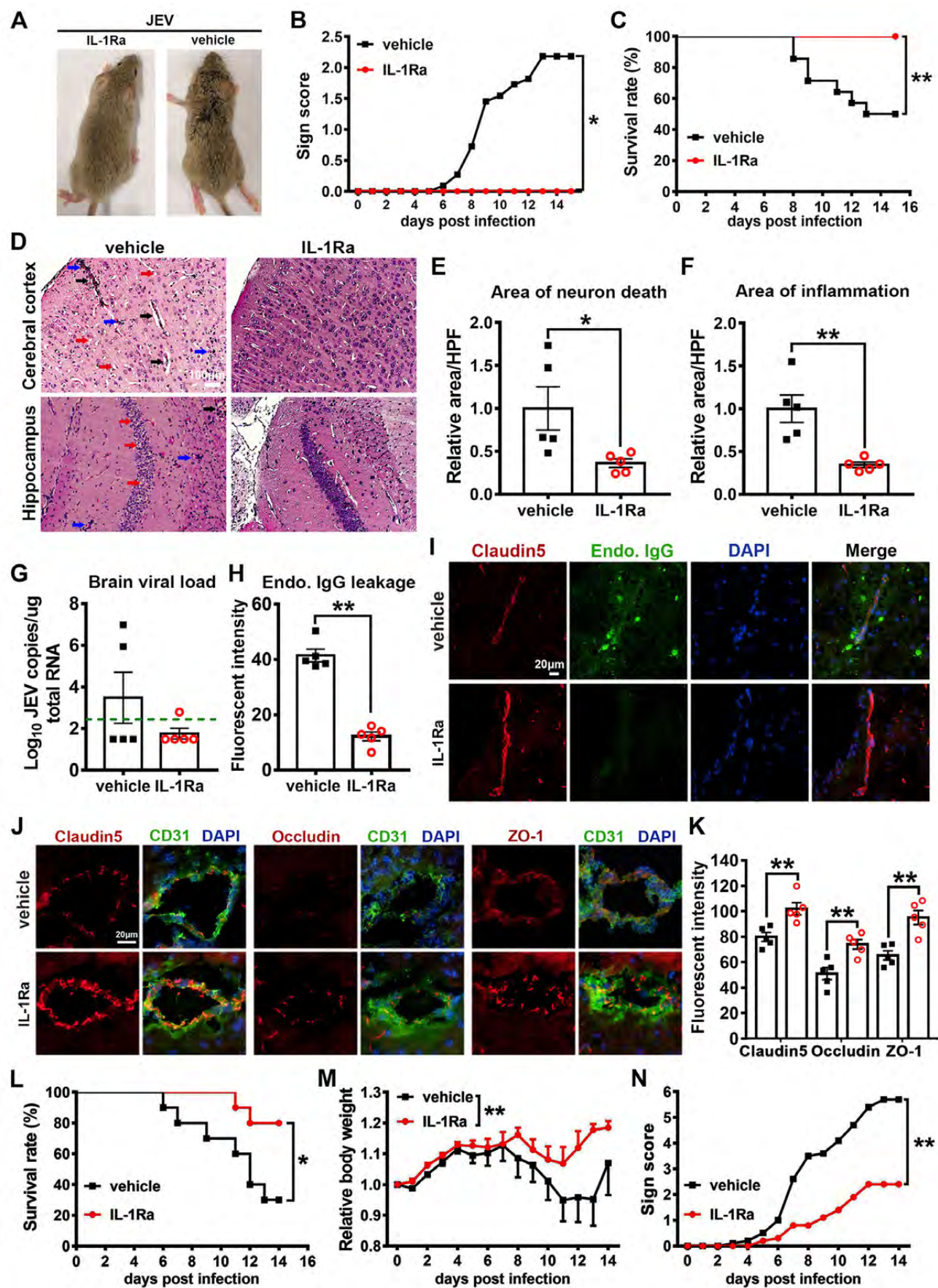












1 **Table 1. The transcription levels of IL-1 signaling related and cell death related**
 2 **genes.**

Gene	mock		JEV	
	Axl ^{+/-}	Axl ^{-/-}	Axl ^{+/-}	Axl ^{-/-}
IL-1 α	1.00 $\pm$ 0.19	1.85 $\pm$ 0.48	3.63 $\pm$ 0.59	3.89 $\pm$ 0.77
IL-1 β	1.00 $\pm$ 0.18	1.07 $\pm$ 0.55	6.91 $\pm$ 0.84	23.55 $\pm$ 10.02*
IL-1Ra	1.00 $\pm$ 0.10	1.04 $\pm$ 0.11	15.02 $\pm$ 1.63	16.92 $\pm$ 5.71
Caspase 1	1.00 $\pm$ 0.14	0.80 $\pm$ 0.04	2.33 $\pm$ 0.08	2.03 $\pm$ 0.19
GSDMD	1.00 $\pm$ 0.11	0.93 $\pm$ 0.06	2.11 $\pm$ 0.02	2.00 $\pm$ 0.13
RIPK1	1.00 $\pm$ 0.05	0.94 $\pm$ 0.05	1.05 $\pm$ 0.02	1.02 $\pm$ 0.06
BIRC3	1.00 $\pm$ 0.05	0.94 $\pm$ 0.08	0.69 $\pm$ 0.02	0.84 $\pm$ 0.03**
Caspase 8	1.00 $\pm$ 0.07	0.95 $\pm$ 0.05	2.19 $\pm$ 0.11	2.17 $\pm$ 0.11
Caspase 9	1.00 $\pm$ 0.01	0.96 $\pm$ 0.01	1.13 $\pm$ 0.04	1.10 $\pm$ 0.02
Caspase 3	1.00 $\pm$ 0.04	1.20 $\pm$ 0.08	2.37 $\pm$ 0.09	2.21 $\pm$ 0.20
BCL10	1.00 $\pm$ 0.04	0.99 $\pm$ 0.06	0.95 $\pm$ 0.02	1.08 $\pm$ 0.03*
Noxol	1.00 $\pm$ 0.06	1.17 $\pm$ 0.11	0.46 $\pm$ 0.05	0.80 $\pm$ 0.04**
PI3KIP1	1.00 $\pm$ 0.09	0.88 $\pm$ 0.12	0.48 $\pm$ 0.03	0.72 $\pm$ 0.12*
WDR91	1.00 $\pm$ 0.03	0.92 $\pm$ 0.02	0.87 $\pm$ 0.02	1.00 $\pm$ 0.03*
TRIB3	1.00 $\pm$ 0.34	0.41 $\pm$ 0.11	0.60 $\pm$ 0.05	1.15 $\pm$ 0.24*

3 The transcription levels of specific genes are normalized to the average of mock Axl^{+/-}
 4 macrophages, and are expressed as mean $\pm$ SEM. Data between two groups are
 5 analyzed by unpaired t test. * denotes p <0.05, and ** denotes p <0.01, JEV: Axl^{-/-} vs

6 $Ax1^{+/-}$, n=5.