TLR4 competence and mouse models of leptospirosis

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Abstract

Mice are slowly being accepted as alternative models for investigation of leptospiral infection. The strain most used to analyse sublethal disease (C3H-HeJ) expresses a *tlr4* gene in its immune cells that is hyporesponsive to leptospiral LPS and thus the model is deemed immunocompromised. To help resolve this valid scientific concern we did a study in which we compared infection with *Leptospira interrogans* serovar Copenhageni Fiocruz in mice expressing fully competent *tlr4* (C3H-HeN, C57BL6) versus *tlr4* hyporesponsive mice (C3H-HeJ) over a period of two weeks. We found that the C3H-HeJ mouse was the only strain that sustained lethal infection, thus confirming the association between *tlr4* function and acute disease. We found that the two mouse strains with a fully functional *tlr4* gene (C3H-HeN and C57BL/6) also developed clinical and molecular signs of sublethal leptospirosis nearly on par with C3H-HeJ, as quantified by weight loss, survival curves, presence of *Leptospira* 16S rRNA in blood and urine, and burden of viable spirochetes in kidney. Regarding kidney markers of inflammation and fibrosis, C3H-HeN and C57BL/6 produced less IL-1 beta, iNOS and ColA1 than C3H-HeJ, which is consistent with increased resilience to infection expected of the *tlr4* competent strains. Regarding Leptospira-specific antibody, all mouse strains produced IgM and T-cell independent IgG3. Interestingly, in contrast to C57BL/6, both C3H strains produced circulating IgG1 but only C3H-HeN produced IgG2a to *L. interrogans* two weeks post infection. Thus, TLR4-competent C3H-HeN and C57BL/6 may be more appropriate mouse models of sublethal leptospirosis, whereas C3H-HeJ can be used to further establish a mouse model of lethal leptospirosis. Our data strongly suggests that TLR4 function is important but not sufficient to cause susceptibility to leptospirosis.

Keywords: C3H, mouse model, leptospirosis, TLR4, *Leptospira interrogans*

Author summary

Previous studies by different laboratories have shown C3H-HeJ to be a susceptible mouse strain to sublethal and lethal leptospirosis. The goal of our study was to evaluate if low *tlr4* responsiveness to leptospiral LPS in C3H-HeJ mice is the factor that drives susceptibility to infection with pathogenic *Leptospira*. We did a comparative study using mouse strains immunocompetent and hyporesponsive to *tlr4*. The data shows that although *tlr4* does affect the virulence of leptospirosis (only C3H-HeJ succumbed to infection), the *tlr4* competent strains (C3H-HeN and C57BL/6) also developed measurable sublethal leptospirosis. Thus, competent recognition of leptospiral-LPS by murine TLR4 plays a role but it is not sufficient to determine susceptibility to leptospirosis. Furthermore, the data suggests that T-cell dependent responses could be involved in susceptibility to leptospirosis in the C3H models of infection.

70 **Introduction**

71 Leptospirosis, an often overlooked but resurging infectious disease caused by a
72 spirochetal bacteria, is a significant global health concern, impacting millions of individuals
73 worldwide. This disease carries a high mortality rate, resulting in approximately 65,000 deaths
74 annually [1]. Furthermore, it poses a serious threat to animals of agricultural importance, leading
75 to substantial economic losses, particularly in tropical and subtropical regions [2]. Although
76 significant efforts have been made to formulate novel vaccination approaches that confer
77 enduring immunity and safeguard against various serovars, our understanding of the specific
78 immune factors contributing to host defense and disease progression remains limited [3].

79 Leptospirosis research is challenging due to the inconsistent outcomes observed in
80 leptospiral infections involving small animals, large animals and humans [4] and a plethora of
81 high and low virulence *Leptospira* serovars [5-8]. Hamsters and guinea pigs have been widely
82 used as small-animal models of acute disease, as they recapitulate the manifestations of severe
83 disease in humans. Rats have been used as a model for studying the severity of human
84 leptospirosis as they become chronically infected and shed *Leptospira* in urine many months
85 after infection [9, 10]. However, the usage of these animals in research is limited in some parts of
86 the world where the disease is endemic due to stringent animal regulations [4] and the scarcity of
87 accessible reagents for routine experiments.

88 Mouse models are extensively utilized in biomedical research because they provide a
89 versatile approach due to the accessibility of diverse immunological and genetic tools, as well as
90 a broad range of available reagents [4]. Researchers have studied the outcomes of experimental
91 leptospiral infection using various mouse models, including studies on lethal, sublethal, and
92 chronic leptospirosis manifestations [4]. For instance, C57BL/6 and BALB/c, exhibit increased

93 resilience to acute disease and have the potential to serve as models for persistent infection
94 caused by *Leptospira interrogans* [11, 12]. C3H-HeJ mice infected with *L. interrogans* develop
95 disease that can be easily monitored through measurement of clinical scores. This strain produces
96 valuable models of lethal [13-15] and sublethal disease [16], and has been used for investigating
97 inflammatory signatures of infection and necroptosis, immunology and efficacy of protection
98 studies [17-19]. Toll-like receptor (TLR) 4, the LPS receptor, was shown to play a central role in
99 the control of leptospirosis [20, 21]. One mechanism described how leptospiral-LPS activates
100 murine, but not human, TLR4 in cultured macrophages [22], and it is associated with resistance
101 to infection [23]. C3H-HeJ mice have a single amino acid substitution (aa712, P to H) within the
102 coding region of the *tlr4* gene that makes this molecule hyporesponsive to the atypical
103 *Leptospira* LPS [21, 24]. Of note, humans, also believed to be susceptible to leptospirosis
104 express a TLR4 molecule that does not sense the atypical *Leptospira* LPS [22]. Nevertheless, one
105 consistent criticism regarding the use C3H-HeJ mice is that its hyporesponsive Toll-Like
106 Receptor 4 (TLR4) qualifies these mice as immunocompromised. To help resolve this valid
107 scientific concern we did a study in which we compared infection with *L. interrogans* in mice
108 expressing fully competent *tlr4* (C57BL6, C3H-HeN) versus mice that are *tlr4* hyporesponsive
109 (C3H-HeJ).
110

111 **Material and methods**

112 **Animals.** Male, 9-10 week old C3H-HeJ, C3H-HeN, C57BL/6 mice and 7-8 week old C3H-HeJ
113 were used for this study. C3H-HeJ animals were purchased from The Jackson Laboratory (Bar
114 Harbor); C3H-HeN and C57BL/6 were purchased from Charles River (Wilmington, MA) and
115 maintained in specific pathogen-free at the Laboratory Animal Care Unit of the University of

116 Tennessee Health Science Centre, with unrestricted access to food and water. We used male
117 mice because they are more susceptible to leptospirosis [25]. We used 4 animals per group, and
118 experiments were reproduced once. All animal procedures were carried out in accordance with
119 the University of Tennessee Health Science Centre Institutional Animal Care and Use
120 Committee protocol number 22-0362.
121
122 **Bacterial strains and culture.** *L. interrogans* serovar Copenhageni strain Fiocruz L1-130
123 (henceforth LiC), frozen in -80°C, was passaged in hamster. Their kidneys were harvested and
124 cultivated in 4mL of Hornsby-Alt-Nally (HAN) media [26] with 100 µg/mL 5-fluorouracil (MP
125 Chemicals, CA) at 29°C for better growth. Passage 2 was done in Ellinghausen-McCullough-
126 Johnson-Harris (EMJH) medium supplemented with Difco *Leptospira* enrichment EMJH
127 (Becton, MD) at 28-30°C. EMJH culture passage 2 was allowed to reach the log phase of
128 growth, pelleted by centrifugation at 3,000 × *g* for 5 min, and washed and resuspended in sterile
129 1× phosphate-buffered saline (PBS) (Thermo Fisher Scientific). Next, the cells were used to
130 infect the animals after counted under a dark-field microscope (Zeiss USA, Hawthorne, NY)
131 using a Petroff- Hausser chamber as previously described [25].
132
133 **Animal infection and collection of specimens.** Mice were inoculated with 1×10^8 LiC in 200 µL
134 of endotoxin-free 1× PBS intraperitoneally (i.p.). Control mice were inoculated with the same
135 volume of endotoxin-free 1× PBS. Survival and body weight loss were monitored for 15
136 consecutive days post-infection. Mice were euthanized 15 days after post-infection or when they
137 reached the endpoint criteria (20% body weight loss post-infection). Blood, urine, and kidney
138 were collected for further analysis.

139 **Bacterial quantification.** The NucleoSpin tissue kit (Clontech, Mountain View, CA) was used
140 to purify genomic DNA from kidney, blood and urine following the manufacturer's protocol, and
141 the purified DNA was then stored at −20°C for further analysis. To quantify *Leptospira*,
142 quantitative polymerase chain reaction (qPCR) was performed using *Leptospira* 16S rRNA
143 primers (Forward: CCCGCGTCCGATTAG and Reverse: TCCATTGTGGCCGAACAC) and a
144 TAMRA probe (CTCACCAAGGCGACGATCGGTAGC) obtained from Eurofins (Huntsville,
145 AL). The results were reported as the number of *Leptospira* genome equivalents. The qPCR
146 mixture consisted of 25 μM of each primer, 250 nM of the specific probe, and 2 μL of DNA
147 sample, with a total volume of 20 μL. Duplicate reactions were performed. The amplification
148 protocol included an initial step of 10 min at 95°C, followed by 40 cycles of amplification (15 s
149 at 95°C and 1 min at 60°C). The analysis was conducted using the comparative threshold cycle
150 (CT) method. A negative result was determined if no amplification occurred.
151
152 **Measurement of *Leptospira*-specific antibody.** *Leptospira*-specific antibody levels were
153 measured using the enzyme-linked immunosorbent assay (ELISA). The process of preparing a
154 leptospiral extract for LiC was carried out following previous instructions [27]. Briefly, LiC was
155 cultured in EMJH media until it reached confluency. The bacterial cells were then separated by
156 centrifugation to form a pellet. This pellet was subjected to incubation with BugBuster® solution
157 (1mL) at room temperature in a shaker (100 rpm) for 20 min and mixed thoroughly by vortexing.
158 The resulting mixture was stored at -20°C. The whole cell extract of *Leptospira* was
159 appropriately diluted in a sodium carbonate coating buffer with a concentration of 1X. A 96-well
160 flat-bottom ELISA microtiter plate (Nunc-eBioscience) was coated overnight at 4ºC with 100 μL
161 1X sodium carbonate coating buffer whole-cell extract of *Leptospira* (10^7–10^8 bacteria per well).

162 After overnight incubation, the ELISA plate was washed with 1X PBST. The plate was blocked
163 by a blocking buffer (100 µL/well) containing 1% BSA, followed by incubation for 1 h at 37ºC.
164 After washing, serum samples (1:100) was added, and the plate was incubated for 1 h at 37ºC.
165 The unbound primary antibody was removed by vigorous washing. Next, anti-mouse secondary
166 antibodies for IgM IgG, IgG3, IgG1 or IgG2a (all from Cell signaling technology, CST)
167 conjugated with horseradish peroxidase was added, which was incubated for 30 min, followed by
168 standard color development using TMB Sureblue. Absorbance measurement was carried out at
169 OD 450 nm using Molecular Devices Spetramax.
170
171 **Expression of inflammatory and fibrosis markers.** Total RNA was extracted using the
172 RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. Complementary DNA
173 (cDNA) was synthesized from the extracted RNA using the RevertAid First Strand cDNA
174 Synthesis Kit (Thermo Fisher Scientific). The resulting first-strand cDNA served as the template
175 for reverse transcription PCR (RT-qPCR), which was performed on a QuantStudio™ 3 Real-
176 Time PCR (Applied Biosystems) using the PowerTrack™ SYBR™ Green Master Mix (Applied
177 Biosystems). Each RT-qPCR reaction (10 µL total volume) included the cDNA template and
178 specific primers. The cycling conditions were as follows: an initial step at 50°C for 2 minutes,
179 followed by denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 sec for
180 denaturation, and 60°C for 1 min for annealing/extension. A melt curve analysis was conducted
181 at the end of the amplification to confirm the specificity of the PCR products. Relative gene
182 expression levels across samples were quantified by the double delta Ct ($2^{-\Delta\Delta Ct}$) method, with
183 glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serving as the endogenous reference

184 control. The primer sequences used in this study are listed in Table S1 of the supplemental
185 material.
186
187 **Statistical analysis.** We used GraphPad Prism 10 software. The *P*-value was determined by
188 Mann-Whitney *U* test, or Two-way ANOVA with Tukey's multiple comparison test.
189
190 **Results**
191 **Weight loss, burden in blood, shedding in urine and survival after infection with *L.***
192 ***interrogans*.** Following experimental leptospiral infection (10^8 LiC), mice were monitored over a
193 15-day period (Fig 1A). In C3H-HeJ, a steady weight loss was observed beginning on day 6,
194 reaching a peak around day 9, at which point one mouse met the endpoint criteria. The remaining
195 mice recovered gradually thereafter. The overall weight loss in this group ranged from 2% to
196 21% (Fig 1D). C3H-HeN mice exhibited daily weight loss from day 1 which decreased gradually
197 until day 14. The overall weight loss in this group ranged from 7% to 16% (Fig 1E). In contrast,
198 C57BL/6 mice experienced low % of weight loss, with an overall decrease of 1% to 8%. (Fig
199 1F). The weight loss curves measured for the three strains were statistically significant
200 (p=0.0116, p<0.0001) as compared to their respective control groups (Fig 1D to F).
201
202 Quantitative PCR (qPCR) targeting the *Leptospira* 16S rRNA gene was performed to
203 analyse the dissemination of *Leptospira* in blood and urine. Blood samples were collected on
204 days 1, 3, 6, 9, 12, and 14 post-inoculation to quantify the *Leptospira* burden. Peak *Leptospira*
205 burdens were observed on day 3 in C3H-HeJ mice (~$3x10^4$), whereas the peak burden occurred
206 on day 1 in both C3H-HeN and C57BL/6 mice, ~$6\text{-}9x10^3$ (Fig 1B). *Leptospira* shedding in urine

207 was also assessed using qPCR on alternate days over a 15-day period. *Leptospira* shedding was
208 detected a low levels (~10^2) during the first week post-infection, peaking on day 10 in the second
209 week for C3H-HeN and C57BL/6 mice (~10^4), whereas in C3H-HeJ mice it peaked on day 14 at
210 ~10^6 (Fig 1C). Regarding survival, the data shows that 75% of C3H-HeJ mice survived at 15
211 days post-infection, whereas 100% of C3H-HeN and C57BL/6 mice survived during this period
212 (Fig 2).
213
214 Survival of C3H-HeJ to lethal infection was further explored by using 7-8 week old mice
215 infected with the same dose of *L. interrogans* serovar Copenhageni FioCruz (10^8) and weight
216 was monitored for 15 days. We found that 100% of the mice reached 20% of weight loss
217 between days 9-12 post infection and met endpoint criteria for euthanasia (Supplemental Fig 1).
218
219 **The burden of *L. interrogans* and viability in kidney tissue after infection**
220 To assess renal colonization by *L. interrogans*, kidney tissues from infected mice were collected
221 on the day the mice were euthanized on day 15 post-infection and analyzed for the presence of
222 *Leptospira* DNA using qPCR (Fig 3A). The bacterial burden was ~7.5×10^4 *Leptospira* per mg
223 of kidney tissue in C3H-HeJ, ~4.1×10^4 in C3H-HeN, and ~2.1×10^4 in C57BL/6 infected mice
224 (Fig 3B). Additionally, the viability of *Leptospira* was assessed by culturing kidney tissues in
225 EMJH medium at 30°C for 4 days and quantifying viable spirochetes using qPCR. On day 4 of
226 culture, the average number of viable spirochetes per 100 µL of EMJH culture was ~4.3×10^3 for
227 C3H-HeJ, ~3.1×10^3 for C3H-HeN, and ~2.2×10^3 for C57BL/6 mice (Fig 3C).
228

229 **Expression of inflammatory cytokines and signatures of fibrosis in kidney of C3H-HeJ,**
230 **C3H-HeN and C57BL/6 mice following *L. interrogans* infection.** To assess the upregulation of
231 inflammatory cytokines critical for immune responses and fibrosis markers associated with
232 kidney damage, mice were inoculated with *L. interrogans* as described in Fig 4. Kidney tissues
233 were collected and processed for mRNA expression analysis 15 days post-infection. The
234 expression levels of IL-1 beta mRNA, a key cytokine involved in inflammatory responses was
235 significantly upregulated both in C3H-HeJ and C3H-HeN mice (Fig 4B). Importantly, mRNA of
236 the fibrosis marker, inducible nitric oxide synthase (iNOS), was significantly increased in both
237 C3H-HeJ and C3H-HeN mice, but not in C57BL/6 (Fig 4C). The mRNA expression of collagen
238 A1 (ColA1) was also markedly elevated in both C3H-HeJ and C3H-HeN mice and no ColA1
239 upregulation was detected in C57BL/6 (Fig 4D). We also analysed the mRNA expression levels
240 of IFN-gamma, IL-17, IL-6, and IL-23 cytokines in all mouse strains; however, the differences
241 observed were not statistically significant.
242
243 **Serological antibody responses in C3H-HeJ, C3H-HeN and C57BL/6 at 15 days post**
244 **infection with *L. interrogans.*** Terminal blood samples were collected 15 days post-infection to
245 evaluate the levels of antibody production to *L. interrogans* (Fig 5). As expected, the three
246 strains of mice produced increased levels of anti-*Leptospira*-specific IgM (Fig 5B), and IgG (Fig
247 5C) compared to the respective uninfected controls. IgG isotyping produced the following
248 results: IgG3 was the only antibody significantly produced by the three mouse strains (Fig 5D)
249 and IgG1/IgG2a were not detected in C57BL/6 (Fig 5E and F). Significantly higher levels of
250 IgG1 were measured in C3H-HeJ than C3H-HeN compared to the respective controls (Fig 5E)
251 and IgG2a was measured in both C3H strains but was only significant in C3H-HeN (Fig 5F).

252 **Discussion**

253 We previously used the C3H-HeJ strain to develop a mouse model of experimental
254 sublethal leptospirosis [16, 25, 28]. Since resistance to lethal acute infection with *L. interrogans*
255 was associated with *tlr4* [23] and C3H-HeJ is hyporesponsive to *L. interrogans* atypical LPS,
256 there are valid concerns that this mouse model does not recapitulate a fully competent immune
257 response to this spirochete. In this study, we did a side-by-side comparative analysis using three
258 mouse strains (C3H-HeJ, C3H-HeN and C57BL/6). C3H-HeJ was the only strain that sustained
259 lethal infection thus confirming an association between a fully functional *tlr4* gene and
260 susceptibility to acute *L. interrogans* infection. Nevertheless, the two mouse strains with a fully
261 functional *tlr4* gene (C3H-HeN and C57BL/6) also developed clinical and molecular signs of
262 sublethal leptospirosis over two weeks of infection, nearly on par with C3H-HeJ. Our data
263 indicates that TLR4-competent C3H-HeN and C57BL/6 may be more appropriate mouse models
264 of sublethal leptospirosis, whereas C3H-HeJ may be good model of lethal leptospirosis.

265 We found weight loss and survival differences between C3H and C57BL/6 in that C3H
266 mice showed larger weight loss curves (~ 2-21%) than C57BL/6 mice (1-8%) throughout the 15-
267 day infection period, that in the case of C3H-HeJ, affected survival. For a choice of animal
268 models of disease, differences in weight loss in the C3H background produced clinical scores
269 that are easier to reproduce consistently. qPCR analysis of *Leptospira* 16S rRNA demonstrated
270 differences in the dynamics of *L. interrogans* dissemination in blood and urinary shedding
271 among the 3 mouse strains that culminated in a gradient of kidney colonization which was
272 highest in C3H and lowest in C57BL/6. This is consistent with the severity of systemic
273 dissemination observed for C3H strains in weight loss and survival, suggesting that overall, C3H
274 mice are more susceptible to *Leptospira* infection than C57BL/6.

275 There are numerous links between competent recognition of Leptospiral LPS by TLR4
276 and resistance to infection in mice [20-23]. Our study confirms that hyporesponsivenes to *tlr4*
277 results in lethal infection in C3H-HeJ mice (Fig 1, Fig 2 and Supplemental Figure 1). Our data
278 also shows that susceptibility to sublethal infection can be observed in C3H-HeN and C57BL/6
279 mice, which express fully competent TLR4s, although differences in measurements are more
280 readily obtained in C3H-HeN. Of note, humans, also believed to be susceptible to leptospirosis
281 express a TLR4 molecule that does not sense the atypical *Leptospira* LPS [22]. In another study
282 we found that inoculation of potentially susceptible TLR4/MD-2 humanized transgenic mice
283 with *L. interrogans* did not produce different measurements of disease progression or *Leptospira*
284 dissemination than wildtype mice [29]. This may have been related to the humanized transgenic
285 mouse background being C57BL/6. Overall, the data suggests that although TLR4 plays a role
286 in susceptibility to *Leptospira* infection, it is not the ultimate determinant as other receptors in
287 mammalian cells (ex. TLR2) likely compensate for deficient receptor binding.
288 While innate immune responses are the main immunological pathway for eliminating
289 *Leptospira*, the humoral immune response plays a vital role in effectively eradicating the bacteria
290 and expelling it from the host [30]. Our present observations revealed that LiC infection led to an
291 increase of *Leptospira*-specific IgM, IgG antibodies in the blood of the three mouse strains. This
292 was expected as several other groups have reported [16, 23, 31, 32]. However, IgG isotyping in
293 C3H models of infection produced some interesting, unexpected results for IgG3, IgG1 and
294 IgG2a. IgG3 was the only antibody significantly produced by the three mouse strains and may
295 explain high total IgG for C57BL/6 in our study. Two weeks after infection of C57BL/6, IgG1
296 was extremely low as observed by others [32]. IgG2a antibodies were not detected in C57BL/6
297 as can be explained by the absence of a gene for IgG2a [33]. However, at this early time point of

298 infection (2 weeks) significantly higher levels of IgG1 were measured in C3H-HeJ than C3H-
299 HeN, and IgG2a was measured in both C3H strains but was only significantly higher in C3H-
300 HeN. The production of Th2-dependent IgG1 antibody in C3H-HeJ and C3H-HeN mice suggests
301 a Th2-biased activation of the immune response, as shown in our previous studies for C3H-HeJ
302 [16]. Presence of Th1 dependent IgG2a in C3H-HeN mice at two weeks post infection with *L.*
303 *interrogans* suggests that this response may be driven in part by TLR4 which has been associated
304 with resistance and may explain why disease progression in this mouse is less pronounced than
305 C3H-HeJ. The data strongly suggests that the mouse genetic background affects the type of IgG
306 antibody response to pathogenic *Leptospira*; hyporesponsiveness to TLR4 may account for lower
307 levels of IgG2a in the C3H-HeJ genetic background which result from impaired Th1 responses
308 and could explain increased susceptibility to leptospirosis.
309 In conclusion, our data suggests C3H-HeN and C57BL/6 mice, both TLR4 competent
310 strains, can be used to recapitulate sublethal leptospirosis, although the C3H-HeN strain
311 produces unambiguous differences in clinical and molecular measurements of disease
312 progression, tissue pathology and bacterial dissemination. Furthermore, and considering that
313 both C3H-HeJ and humans are hyporesponsive to leptospiral LPS, the C3H-HeJ strain may be
314 more suitable for analysis of lethal leptospirosis on par with the hamster model. In addition, both
315 C3H strains can be used to investigate competent Th immune responses to pathogenic *Leptospira*
316 in early time-points of infection. Each of the mouse strains have different characteristics that can
317 be leveraged in pursuit of knowledge on immunity and host response to pathogenic *Leptospira*.
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319
320

Declaration of Competing Interest

The authors do not have competing financial interests or personal relationships that could appear to influence the work reported in this paper.

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Author contributions

MGS designed the study. OZA, SK and LMM performed the experiments and data analysis. OZA, SK and MGS wrote the paper.

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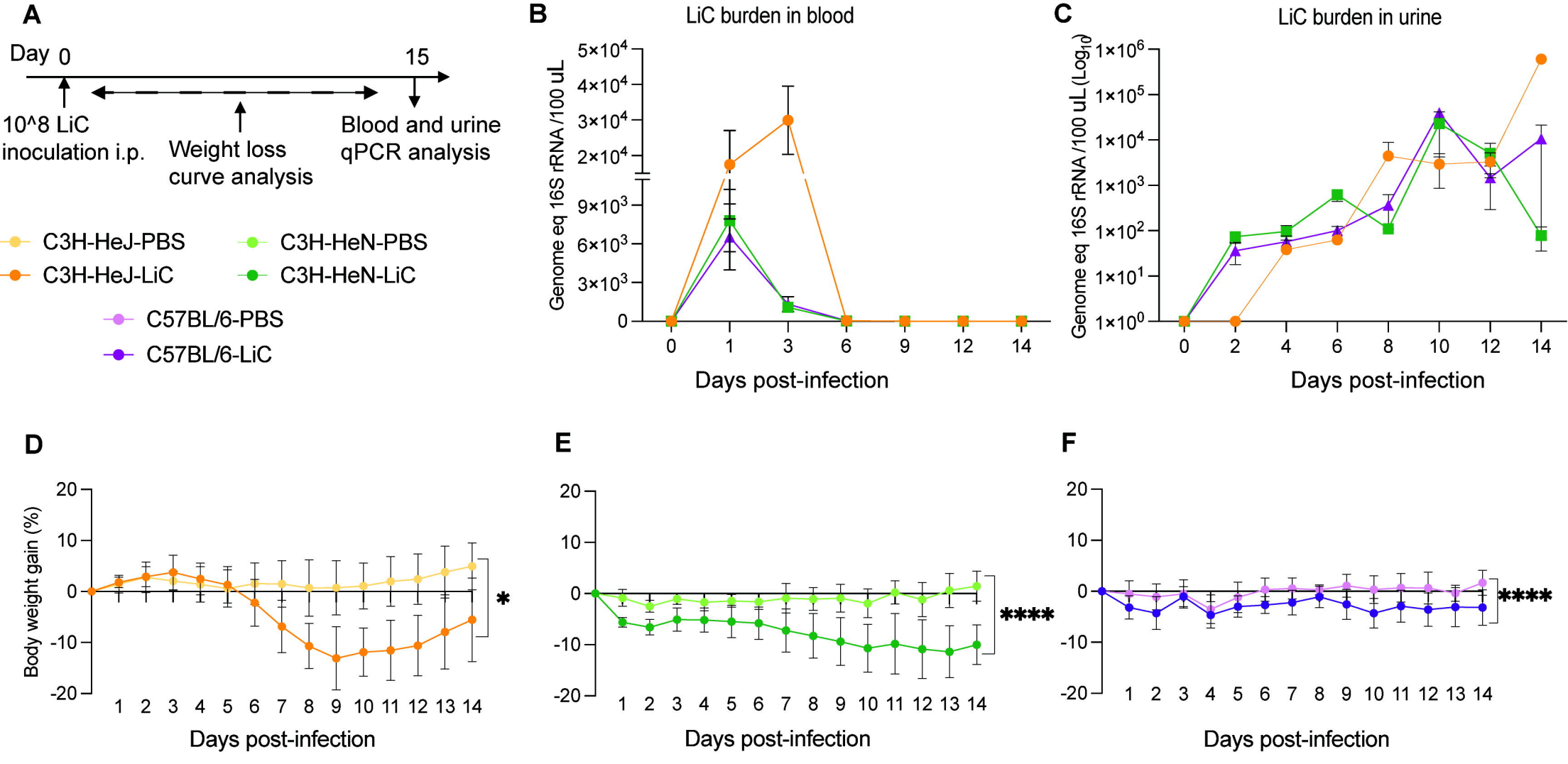

Figure 1. Weightloss and bacterial burden in blood and urine after infection. C3H-HeJ, C3H-HeN and C57BL/6 male mice (n=4/group) were inoculated IP with 1 × 10^8 *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (LiC) and with PBS (control) (A). Quantification of bacterial load in blood (B) and urine (C) was determined by real-time qPCR of the16S rRNA gene. Body weight (% change) was recorded daily for 15 days after infection (D to F). * p=0.0116, ****$p < 0.0001$ by Mann-Whitney U test. Data shown represents one of two experiments.

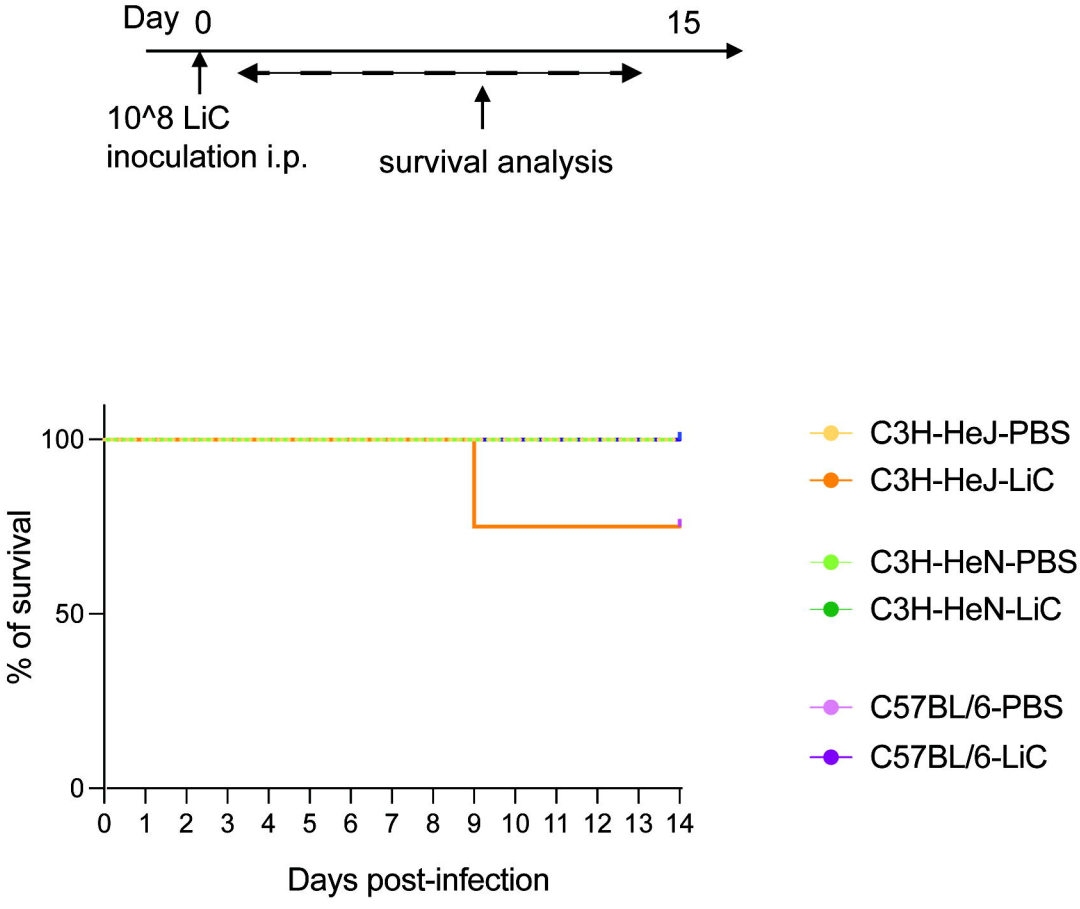

Figure 2. Survival of C3H-HeJ, C3H-HeN and C57BL/6 after infection. Male mice (n=4/group) were inoculated IP with 1 × 10^8 *L. interrogans* serovar Copenhageni Fiocruz L1-130 (LiC) and PBS (control), and the percentage of mice that survived infection for 15 days was recorded. Data represents one of two experiments.

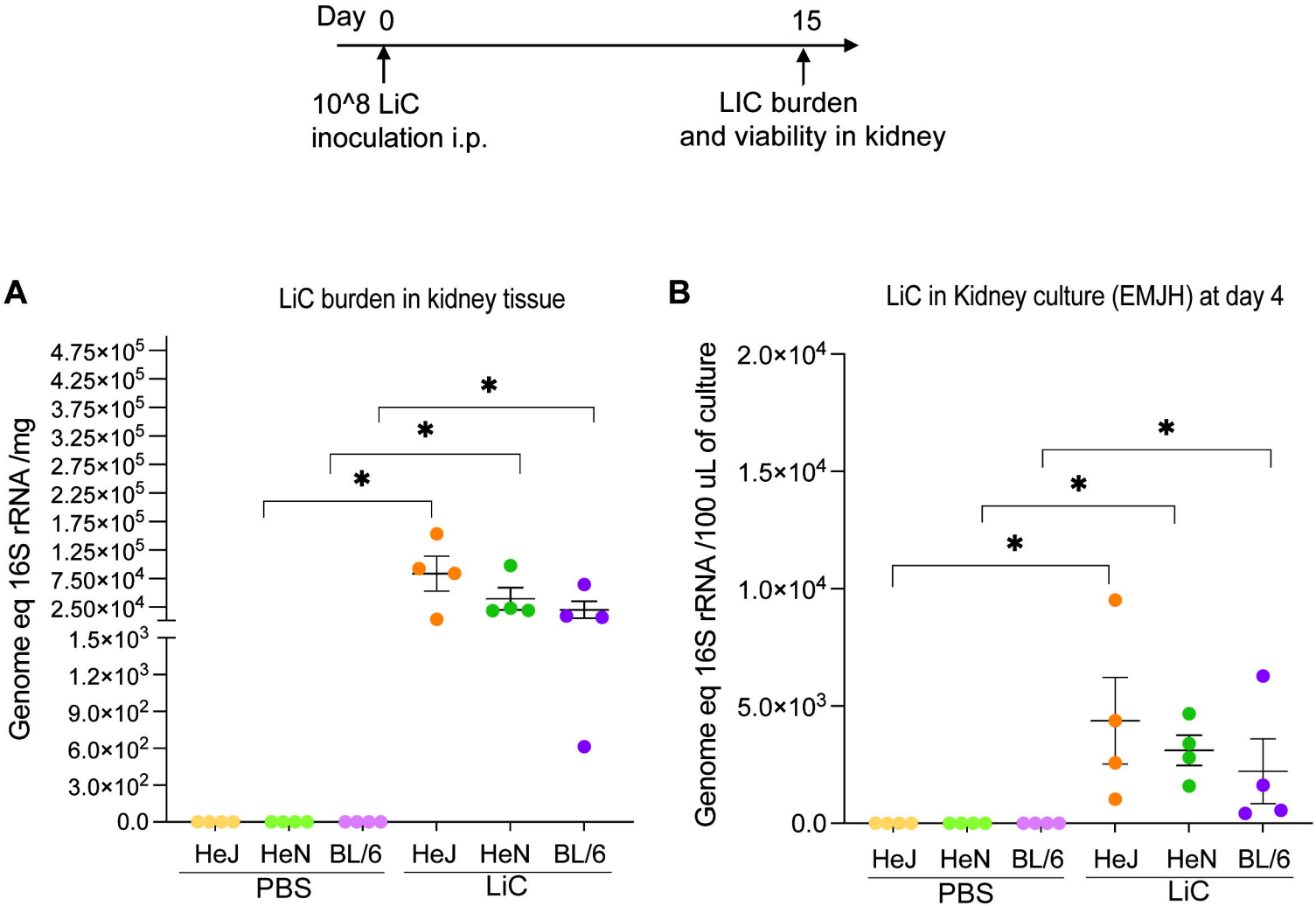

Figure 3. Burden and viability of *L. interrogans* in kidney. C3H-HeJ, C3H-HeN and C57BL/6 male mice (n=4/group) were inoculated IP with 1 × 10^8 *L. interrogans* serovar Copenhageni Fiocruz L1-130 (LiC) and with PBS (control). Kidney tissues were collected 2 weeks post infection for qPCR analysis of 16S RNA from DNA purified from kidney tissue (A) and from DNA purified from kidney culture in EMJH (B). Each dot represents a single mouse. *$p < 0.05$ was determined by Mann-Whitney *U* test. Data represents one of two experiments.

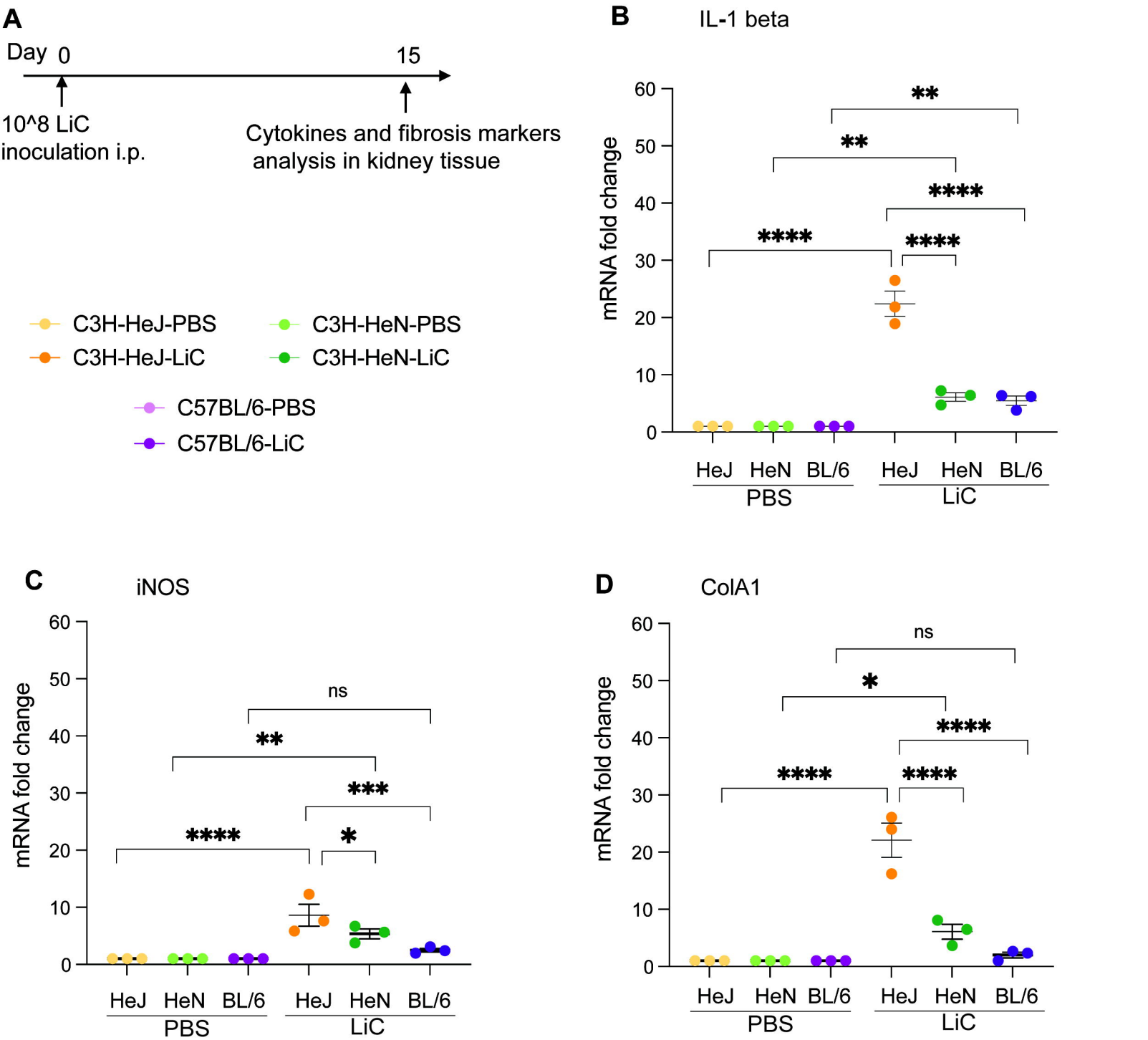

Figure 4. Inflammatory cytokines and fibrosis markers in kidney of infected mice. The expression of IL-1 beta (B), iNOS (C) and ColA1 (D) in kidney tissue were measured using RT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the calibrator gene to normalize the expression of genes. Each dot represents measurement from a single mouse. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ or ****$p < 0.0001$ was determined by two-way ANOVA followed by Tukey's multiple comparison correction test. Data shown represents one of two experiments.

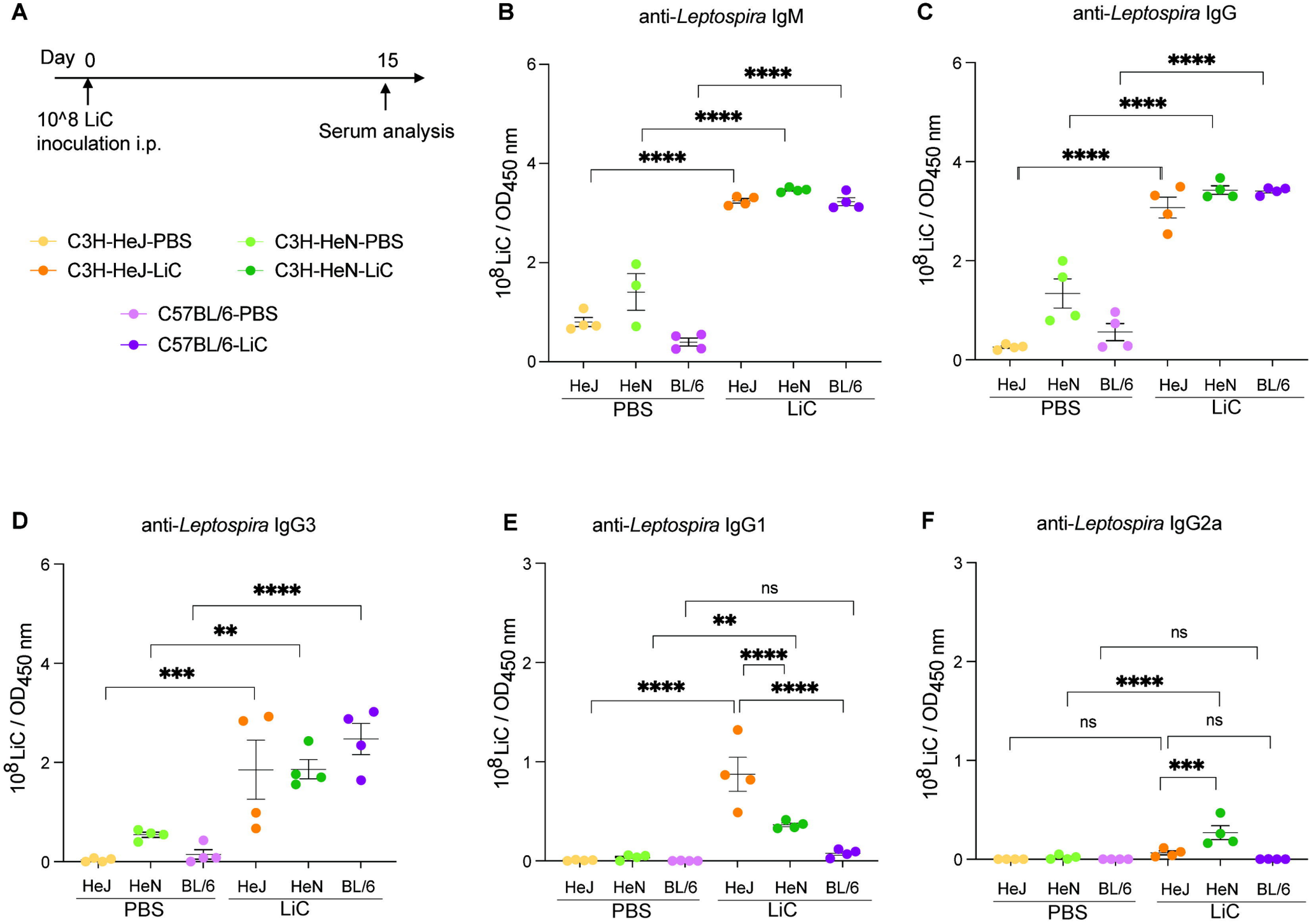

Figure 5. Antibody responses to *L. interrogans* infection. C3H-HeJ, C3H-HeN and C57BL/6 male mice (n=4/group) were inoculated IP with 1 × 10^8 *L. interrogans* serovar Copenhageni Fiocruz L1-130 (LiC) and with PBS (control) (A). Serologic leptospiral-specific IgM (B), IgG (C), IgG3 (D), IgG1 (E) and IgG2a (F) was measured using ELISA. Each dot represents a single mouse. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ or ****$p < 0.0001$ was determined by two-way ANOVA followed by Tukey's multiple comparison correction test. Data shown represents one of two experiments.