Exposure to live saprophytic *Leptospira* before challenge with a pathogenic serovar prevents severe leptospirosis and promotes kidney homeostasis

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ABSTRACT

Previous studies demonstrated that *Leptospira biflexa*, a saprophytic species, triggers innate immune responses in the host during early infection. This raised the question of whether these responses could suppress a subsequent challenge with pathogenic *Leptospira*. We inoculated male C3H/HeJ mice with a single or a double dose of *L. biflexa* before challenge with a pathogenic serovar, *L. interrogans* ser. Copenhageni FioCruz (LIC). Pre-challenge exposure to *L. biflexa* did not prevent LIC dissemination and colonization of the kidney. However, it rescued weight loss and mouse survival thereby mitigating disease severity. Unexpectedly, there was correlation between rescue of overall health (weight gain, higher survival, lower kidney fibrosis) and higher shedding of LIC in urine. This stood in stark contrast to the *L. biflexa* unexposed LIC challenged control. Immune responses were dominated by increased frequency of B cells and effector T helper (CD4+) cells in spleen, as well as significant increases in serologic IgG2a. Our findings suggest that exposure to live saprophytic *Leptospira* primes the host to develop Th1 biased immune responses that prevent severe disease induced by a subsequent challenge with a pathogenic species. Thus, hosts exposed to live saprophytic *Leptospira* before challenge with a pathogenic serovar may withstand LIC infection with far better outcomes. Furthermore, a status of homeostasis may have been reached after kidney colonization that helps LIC complete its enzootic cycle.

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46 **Significance**

47 Previous evidence of host innate immunity induced by live saprophytic *Leptospira* led us to
48 posit that these responses might mitigate leptospirosis severity upon a subsequent challenge
49 with a pathogenic serovar. In this study, we validated our hypothesis. This is important for
50 development of novel strategies to control leptospirosis and for understanding the
51 epidemiologic risk factors of this and other infectious diseases transmitted by direct contact
52 between pathogen and host. Unexpectedly, these studies also show that there is a correlation
53 between kidney health after *L. interrogans* infection (less fibrosis) and higher shedding of this
54 spirochete in urine. This suggests that a status of homeostasis may be reached after kidney
55 colonization by *L. interrogans* that helps the spirochete fulfill its enzootic cycle.

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68 **Introduction**

69 Leptospirosis, a neglected re-emerging enzootic spirochetal disease, affects millions of
70 people worldwide causing an overall mortality rate of 65000 per year (1). In addition, it causes
71 serious health problems in animals of agricultural interest which leads to substantial economic
72 losses mostly in tropical and subtropical countries. Assessing the true severity of leptospirosis
73 can be incredibly challenging, especially when early diagnosis is difficult due to nonspecific
74 symptoms overlapping with other illnesses (2). Recent outbreaks of both human and canine
75 leptospirosis in New York and California in 2020 to 2022 (3, 4) underscores the need for
76 development of effective strategies to control this disease. Although serovar-specific vaccines
77 are available for animals and at least one is available for humans, no broadly effective vaccine is
78 available for either (5). The absence of an effective cross-protective vaccine candidate increases
79 the risk of disease re-emergence on a global scale. Efforts to use leptospiral surface antigens in
80 various vaccine formulations have shown limited success in conferring protection against
81 leptospiral dissemination and shedding, as well as severe disease. *Leptospira'* immune evasion
82 strategies contribute to the complexities of finding good vaccine candidates.

83 The genus *Leptospira* is broadly categorized into 3 major groups: saprophytic,
84 intermediate and pathogenic based on their virulence properties and genetic make-up (6, 7).
85 *Leptospira* survives in moist conditions and are free-living organisms naturally found in soil and
86 water bodies (8, 9). The spread of infection occurs through contaminated water contact with
87 breached skin or mucosal surface (10). Saprophytic strains of *Leptospira,* such as *L. biflexa*, are
88 unable to establish disease due to the lack of certain virulence factors (7) and have been found

89 in natural environments around the world alongside pathogenic and intermediate serovar (6,
90 10, 11). Moreover, *L. biflexa* exhibits certain niche-specific adaptations that allow them to
91 persist in both environmental and host setting (12, 13).

92 Our previous studies (14, 15) demonstrated that *L. biflexa*, a non-pathogenic strain of
93 *Leptospira*, triggers a robust innate immune response in the host during the acute phase of
94 infection. This raised the question of whether saprophytic *Leptospira* induced immune
95 responses could confer any degree of resistance or immune memory that could suppress a
96 subsequent challenge with a pathogenic serovar of *Leptospira*. Answering that question was the
97 main goal of the current study.

98

99 **Materials and Methods**

100 **Animals**

101 Male C3H/HeJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and
102 maintained in a pathogen-free environment in the Laboratory Animal Care Unit of the
103 University of Tennessee Health Science Center (UTHSC). All experiments were performed in
104 compliance with the UTHSC Institutional Animal Care and Use Committee, Protocol no. 19-
105 0062.

106 **Bacteria**

107 Non-pathogenic *Leptospira biflexa* serovar Patoc (LB) was purchased from ATCC and grown in
108 EMJH media. Pathogenic *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 (LIC)
109 was grown in EMJH media and subsequently passaged in hamster to maintain virulence. EMJH

culture passage 2 was used to inoculate mice (10^8) after counting *Leptospira* under a dark-field microscope (Zeiss USA, Hawthorne, NY) using a Petroff- Hausser chamber.

Infection of mice and study design

We carried out two experiments set apart by a single or double exposure to a saprophytic serovar of *Leptospira* (*L. biflexa*) before challenge with a pathogenic serovar (*L. interrogans*). Groups of mice (n=4) were inoculated with 10^8 *Leptospira* intraperitoneally (IP) both for exposure to *L. biflexa* and for challenge with *L. interrogans*. Each experiment was reproduced once. In the single exposure study (Fig 1A), Group 1 was the naive control which received PBS (PBS^1), Group 2 was inoculated with 10^8 *L. biflexa* once at 6 weeks (LB^1), Group 3 received PBS for 2 weeks followed by challenge with 10^8 *L. interrogans* at 8 weeks (PBS^1LIC^1), and Group 4 mice were inoculated with 10^8 *L. biflexa* at 6 weeks and challenged with 10^8 *L. interrogans* at 8 weeks (LB^1LIC^1). In the double exposure study (Fig 2A), Group 1 was the naive control which received PBS (PBS^2), Group 2 received 10^8 *L. biflexa* IP at 6 and 8 weeks (LB^2), Group 3 received PBS for 2 weeks followed by challenge with 10^8 *L. interrogans* at 10 weeks (PBS^2LIC^2), and Group 4 mice were inoculated with 10^8 *L. biflexa* at 6 and 8 weeks and challenged with 10^8 *L. interrogans* at 10 weeks (LB^2LIC^2). Weight was monitored daily. Mice were euthanized 15 days after *L. interrogans* challenge or when they reached the endpoint criteria (20% body weight loss post-infection). Blood and kidney were collected at euthanasia: blood was used for quantification of anti-*Leptospira* antibody; kidney was used for quantification of *Leptospira* load (16S rRNA) and it was cultured in EMJH media for evaluation of bacterial viability. Furthermore, kidney samples were stored in 10% formalin for Hematoxylin and Eosin (H&E) staining. Spleen

131 for flow cytometric analysis was collected from mice after euthanasia only in the double
132 exposure study given that all mice consistently survived challenge.
133
134 ***Leptospira* detection through qPCR**
135 Isolation of genomic DNA from blood, urine and kidney were carried out using NucleoSpin
136 tissue kit (Clontech, Mountain View, CA) according to the manufacturers' instructions.
137 *Leptospira* 16s rRNA primers (Forward- CCCGCGTCCGATTAG and Reverse-
138 TCCATTGTGGCCGAACAC) and TAMRA probe (CTCACCAAGGCGACGATCGGTAGC) were used for
139 *Leptospira* detection using qPCR with a standard curve of 10^5 to 1 *Leptospira* (16, 17). Similarly,
140 qPCR was performed with kidney tissues placed in EMJH to grow live *Leptospira* after culturing
141 for 5 days and visually quantified under a dark field microscope (20X, Zeiss USA, Hawthorne) on
142 d3 and d5 post culture inoculation.
143 **RNA isolation and RT-PCR**
144 Kidneys were stored in RNA later after euthanasia. RNeasy mini kit (Qiagen) was used to extract
145 total RNA from kidney tissue according to the manufacturer's specifications. RNA purity was
146 measured using a Nanodrop instrument (Thermo Scientific) at A260/280 ratio. cDNA was
147 prepared using cDNA reverse transcription kit (Applied Biosystems). ColA1 primers (Forward-
148 TAAGGGTACCGCTGGAGAAC, Reverse- GTTCACCTCTCTCACCAGCA), TAMRA probe
149 (AGAGCGAGGCCTTCCCGGAC) and β-actin primers (Forward- CCACAGCTGAGAGGGAAATC,
150 Reverse- CCAATAGTGATGACCTGGCCG), TAMRA probe (GGAGATGGCCACTGCCGCATC) were
151 purchased from Eurofins Genomics.

152 **Histopathology by H&E staining**

153 Kidney tissues were fixed in formalin buffer. Histopathology was performed at the Histology
154 Department, UT Methodist University Hospital, Memphis, TN. Digital scanning of inflammatory
155 cells infiltration was measured by taking images of an average of 5 fields per sample under 20x
156 magnification. Images were captured after digitally scanning the H&E slides using Panoramic
157 350 Flash III (3D Histech, Hungary) and CaseViewer software.

158 **ELISA**

159 Leptospiral extract for *Leptospira biflexa* and *Leptospira interrogans* were prepared as
160 described previously (18). Briefly, *Leptospira* was cultured in EMJH media and once confluency
161 was observed, cells were centrifuged to obtain a pellet. This pellet was then incubated with
162 BugBuster® solution (1mL) at RT in a shaker incubator (100 rpm) for 20 min and homogenized
163 by vortexing. Stocks were stored at -20°C. This whole-cell extract of *Leptospira* was then diluted
164 in 1X sodium carbonate coating buffer. Nunc MaxiSorp flat-bottom 96 well plates (eBioscience,
165 San Diego, CA) were coated with 10^7-10^8 bacteria per well with leptospiral extract at 4°C
166 overnight. Cells were washed using 1X PBST the following day and blocked for 1 h using 1% BSA
167 solution, followed by another wash with 1X PBST. Serum samples (1:100) were added to the
168 antigen coated wells and incubated at 37°C for 1 h, washed twice with 1X PBST, followed by
169 HRP conjugate secondary anti-mouse- IgG1, IgG2a and IgG3 (1:10000) which was incubated for
170 30 mins. After washing the plate 3 times with 1X PBST the color was developed using TMB
171 Sureblue followed by Stop solution before the absorbance was measured at OD 450 nm using
172 an ELISA plate reader (Molecular Devices Spetramax).

173 **Flow cytometry**

174 Spleens were chopped into small pieces and macerated to prepare single cell suspensions on
175 the same day of euthanasia to avoid loss of cell viability. RBC lysis was performed using ACK
176 lysis buffer (Gibco). AO/PI dual staining was used to count live/dead cells on a Luna counter
177 (Logos Biosystems, South Korea). 10^6 cells were seeded in a 96 well microtiter plate after
178 washing with 1X PBS twice. Blocking was performed with anti-mouse CD16/32 antibody (1:100),
179 followed by 20 minutes incubation on ice. Fluorochrome-conjugated antibodies (Table S1) were
180 used to stain specific cell surface markers after 30 mins incubation in the dark at 4°C. Freshly
181 prepared flow staining buffer was used for washing stained cells. Cells were fixed using 4%
182 Paraformaldehyde for 10-15 mins at room temperature. Beads were stained using specific
183 fluorochrome conjugated antibodies and used for compensation, while FMO prepared with
184 spleen cells simultaneously were used for gating controls. Cells were resuspended in flow
185 staining buffer and the BioRad ZE5 cell analyzer was used for data acquisition. Data analysis was
186 done using Flow Jo software. Gating strategy used for immunophenotyping of spleen cells is
187 provided in supplementary Fig S1.

188 **Statistical analysis**

189 One-way ANOVA with Tukey's multiple comparison test and unpaired t-test with Welch's
190 correction were used to analyze differences between experimental groups. GraphPad prism
191 software was used to plot graphs; a value of $p<0.05$ is considered significant.

192

193 **Results**

194 **Exposure to saprophytic *Leptospira* before infection with a pathogenic serovar prevents**
195 **disease and increases survival of C3H-HeJ mice.** We inoculated adult C3H-HeJ male mice with a
196 single dose of *L. biflexa* two weeks before challenge with *L. interrogans* (LB^1LIC^1) at 8 weeks (Fig
197 1A) and measured a significant rescue of weight loss over a period of 15 days as compared to
198 mice infected at 8 weeks that did not receive *L. biflexa* (PBS^1LIC^1) ($p<0.0001$); unchallenged
199 control mice that received *L. biflexa* (LB^1) or PBS (PBS^1) gained weight throughout the
200 corresponding 15 days (Fig 1B). Survival curves were generated after the mice reached the
201 following endpoint criteria: 20% weight loss or 15 days post challenge with *L. interrogans* or 15
202 days post inoculation with *L. biflexa*/PBS for the controls (Fig 1C). All mice infected with *L.*
203 *interrogans* (PBS^1LIC^1) reached the 20% weight loss endpoint criteria between d9 and d12 post
204 infection. In contrast, 3/4 (75%) of the mice that received one dose of *L. biflexa* before
205 challenge with *L. interrogans* (LB^1LIC^1) survived and gained significant body weight (Fig 1B)
206 which was similar to the naïve control that received only PBS. Analysis of bacterial
207 dissemination was done by qPCR of the *L. interrogans* 16S gene in genomic DNA purified from
208 blood, kidney tissue and urine. We also found that a single exposure to *L. biflexa* before
209 challenge did not prevent dissemination of pathogenic *L. interrogans* in blood (Fig S2A) or
210 shedding in urine (Fig 1D), or kidney colonization (Fig 1E). Culture of kidney in EMJH media (Fig
211 1F) showed presence of ~ 2500 motile, morphologically intact *L. interrogans* under dark field
212 microscopy which was confirmed by 16S qPCR both on d3 and d5 post culture of kidney
213 collected from LB^1LIC^1 mice; kidney from PBS^1, LB^1 and PBS^1LIC^1 did not produce positive
214 cultures by dark field microscopy or 16S qPCR.

215 In the double exposure study, mice were inoculated with 2 bi-weekly doses of *L. biflexa*
216 two weeks before challenge with *L. interrogans* (LB^2LIC^2) at 10 weeks in comparison with the
217 respective controls (Fig 2A). As expected, mice infected at 10 weeks with LIC that did not
218 receive *L. biflexa* (PBS^2LIC^2) lost ~ 11 % of weight on d11 post challenge and did not recover (Fig
219 2B). In contrast, mice that received a double dose of *L. biflexa* two weeks before challenge at 10
220 weeks with *L. interrogans* (LB^2LIC^2) lost a maximum of 5% weight on d10 and recovered fully by
221 d15 post infection; unchallenged control mice that received *L. biflexa* (LB^2) or PBS (PBS^2) gained
222 weight throughout the 15 days (Fig 2B). Survival curves generated after the mice reached
223 endpoint criteria (Fig 2C) show that all experimental groups survived LIC infection. Analysis of
224 bacterial dissemination showed that a double exposure to *L. biflexa* before challenge did not
225 prevent dissemination of pathogenic *L. interrogans* in blood (Fig S2B), or shedding in urine (Fig
226 2D), or kidney colonization (Fig 2E). Culture of kidney in EMJH media (Fig 2F) showed presence
227 of 5,000-10,000 motile, morphologically intact *L. interrogans* on d3 and d5 post culture of
228 kidney collected from PBS^2LIC^2 mice in contrast to 1,000 to 2,500 live *L. interrogans* observed in
229 culture from kidney collected from LB^2LIC^2 mice which was confirmed by 16S qPCR; kidney from
230 PBS^2 and LB^2 mice did not produce positive cultures by dark field microscopy or 16S qPCR.
231
232 ***L. biflexa* exposure before challenge with *L. interrogans* mitigates renal histopathological**
233 **changes.** As expected, H&E staining of histological slices of all kidneys from mice challenged
234 with *L. interrogans* in both single and double exposure experiments (PBS^1LIC^1 and PBS^2LIC^2)
235 showed signs of inflammation with increased immune cell infiltration (Fig 3A and 3C) in this
236 organ, targeted by the pathogen for colonization. In contrast, H&E staining of kidney slices from

237 the groups of mice exposed to *L. biflexa* before *L. interrogans* challenge (LB^1LIC^1 and LB^2LIC^2)
238 showed reduced immune cell infiltration. We also measured expression of a marker (ColA1) for
239 kidney fibrosis. In both experiments, kidneys from mice challenged with *L. interrogans* (PBS^1LIC^1
240 and PBS^2LIC^2) had significantly higher expression of ColA1 as compared to the controls; in
241 contrast, kidneys from mice challenged with *L. interrogans* after exposure to *L. biflexa* (LB^1LIC^1
242 and LB^2LIC^2) were not different than the controls (Figs 3B and 3D).

243 In addition, we were able to collect kidneys from experimental mice subjected to the
244 double exposure of *L. biflexa* because they all survived subsequent challenge with *L.*
245 *interrogans*. As such, we did a comparative gross morphological analysis between the 4 groups
246 (Fig S3). We found that kidney from LB^2LIC^2 mice maintained their normal gross anatomy and
247 coloration as did kidneys from control mice (PBS^2 and LB^2), in contrast to kidneys from mice
248 challenged with *L. interrogans* that were not previously exposed to *L. biflexa* (PBS^2LIC^2).

249

250 **Serologic IgG responses to *L. interrogans* were significantly higher in mice pre-exposed to *L.***
251 ***biflexa* before challenge with *L. interrogans***. In both single and double *L. biflexa* exposure
252 experiments we measured anti-*L. interrogans* IgG1, IgG2a and IgG3 in serum collected 2 weeks
253 after challenge with *L. interrogans* (Figs 4A and 4B). In both experiments, IgG1 was generally
254 low and IgG2a as well as IgG3 were generally high in groups infected with *L. interrogans*.
255 Although differences between groups (PBS^2v PBS^2LIC^2 and $LB^{1/2}$ v $LB^{1/2}LIC^{1/2}$) were significant,
256 differences between the LIC infected groups ($PBS^{1/2}LIC^{1/2}$ v $LB^{1/2}LIC^{1/2}$) were not significant for
257 IgG1 and for IgG3 in contrast to IgG2a ($p=0.001$ for single exposure and $p=0.0095$ for double
258 exposure).

259

260 **Exposure to non-pathogenic *L. biflexa* before pathogenic *L. interrogans* challenge provided a**
261 **significant immune cell boost with an increase in overall B and helper T cell frequencies in**
262 **spleen as well as an effector response**. We immunophenotyped the spleen cells from the mice
263 subjected to double *L. biflexa* exposure because the animals survived to the term of the
264 experiment. Mice in the single exposure experiment met endpoint criteria before the term of
265 the experiment and thus we were not able to process spleen for immunophenotyping. In the *L.*
266 *biflexa* double exposure experiment, we found increased frequencies in B cells (Fig 5A) and
267 helper T cells (Fig 5D), as well as decreased frequencies in cytotoxic T cells (Fig 5E) in mice
268 challenged with LIC (PBS^2LIC^2 and LB^2LIC^2) in comparison to the respective negative controls
269 (PBS^2 and LB^2). In addition, differences between CD4+ helper T cells and CD8+ cytotoxic T cells
270 were significant in LIC challenged mice that received *L. biflexa* (p=0.006, Fig 5D and p=0.0056
271 Fig 5E). No differences were observed in NK cells between any of the groups. Further T cell
272 subset typing (Fig 6) showed that frequency of early effector CD4+ T helper cells (Fig 6B, CD44-
273 CD62L-) and effector T helper cells (Fig 6C, CD44+CD62L-) were mostly significantly increased
274 when compared between the LIC challenged groups and the respective controls (except PBS^2 v
275 PBS^2LIC^2 early effectors) and that frequency of early effector and effector T helper cells were
276 higher in the LB^2LIC^2 group than PBS^2LIC^2. No major changes were measured in memory CD4+ T
277 helper cells (Fig 6D, CD44+CD62L+). In the CD8+ cytotoxic T cell subsets, we measured
278 significant decreases in frequency of naïve T cells between LIC infected groups and the
279 respective controls (Fig 6E, CD62L+CD44-) and this was replicated in the CD8+ cytotoxic

280 memory except that differences with the LIC infected groups were not significant (Fig 6H,
281 CD44+CD62L+).
282
283 **Discussion**
284 Understanding host immune responses to *Leptospira* infection is crucial for advancing our
285 ability to develop new control measures for leptospirosis (18-23). Given their widespread
286 presence in nature, the likelihood of humans or animals getting exposed to non-pathogenic
287 serovars of *Leptospira* is likely high (9, 10). Our previous studies showed innate immunity
288 engagement during saprophytic *L. biflexa* infection (14, 15). In addition, *L. biflexa* extracts can
289 be used to detect *Leptospira*-specific antibody in up to 67% of serum from patients with
290 clinically confirmed leptospirosis (18) which points to a high degree of immunodominant cross-
291 reactive epitopes between *L. biflexa* and pathogenic *Leptospira*. Thus, we hypothesized that
292 these highly cross-reactive immunodominant epitopes may also induce cross-protective
293 immune responses. The objective of the current study was to assess whether exposure to a live
294 saprophytic serovar of *Leptospira* provides any heterologous cross-species protection against a
295 subsequent challenge with a pathogenic serovar in a mammalian host.
296 In the initial analysis of pathogenesis (Fig 1, Fig 2, Fig S2) we observed that prior
297 exposure to one or two doses of saprophytic *L. biflexa* rescues weight loss in mice challenged
298 with pathogenic *L. interrogans* at 8 weeks (LB^1LIC^1) and at 10 weeks (LB^2LIC^2), respectively.
299 Weight gain correlated with survival (75% survival in LB^1LIC^1 versus 0% survival of the PBS^1LIC^1
300 group) in the single exposure experiment, where we expected all mice infected at 8 weeks with
301 pathogenic *L. interrogans* (PBS^1LIC^1) to irreversibly lose weight and meet endpoint criteria for

302 euthanasia before the 2 week term of the experiment (21, 24). Loss of mice due to irreversible
303 weight loss is not expected if mice are infected with LIC at 10 weeks of age (16), as observed in
304 the *L. interrogans* control group in the double exposure experiment (PBS^2LIC^2). In both
305 experiments, mice exposed to *L. biflexa* before challenge with LIC, produced evidence of *L.*
306 *interrogans* dissemination in blood, shedding in urine and kidney colonization of the kidney.

307 Histological inspection of kidney slices (Fig 3) showed that exposure to a saprophytic
308 *Leptospira* before challenge supported normal structural morphology and prevented infiltration
309 of immune cells in both single and double exposure experiments; in addition, it significantly
310 reduced a fibrosis marker (ColA1) in the single exposure experiment. In the double exposure
311 experiment, differences in ColA1 fibrosis marker are not significant between the two LIC
312 infected groups because 10 week old C3H-HeJ infected with LIC are more resistant to pathology
313 resultant from infection. Our findings are intriguing as they suggest that while prior live
314 saprophytic exposure did not prevent infection or leptospiral dissemination, it conferred
315 protection against kidney fibrosis.

316 Our data also shows that prior exposure to non-pathogenic *Leptospira* before
317 pathogenic challenge, induced higher antibody titer in the serum, specifically IgG2a antibodies
318 against *L. interrogans* in both single and double exposure experiments (Fig 4). The increased
319 IgG2a response in serum suggests induction of a Th1 biased immune response. Others have
320 recently found that saprophytic *L. biflexa* induced Th1 responses, higher T cell proliferation and
321 IFN-γ producing CD4+ T cells (25). Persistent IgM and strain specific IgG responses was
322 observed during a homologous leptospiral challenge in C57BL6/J mice (20). In our study,
323 exposure to a saprophytic *Leptospira* induced antibody responses that provided heterologous

324 protection against the pathogenic strain of *Leptospira*. This supports a promising broad-
325 spectrum efficacy. Thus, live vaccines derived from a saprophytic strain of *Leptospira* could
326 offer broader protection and overcome the limitation of serovar specificity often observed with
327 killed whole-cell vaccines based on pathogenic strains.

328 The stark difference in the antibody titer among the *L. interrogans* infected group pre-
329 exposed to saprophytic *L. biflexa* can be attributed to the robust trafficking and differentiation
330 of B and helper T cells (CD4+) measured in spleen (Fig 5 and Fig 6). Presence of effector helper T
331 (CD4+) cells in the spleen indicate a robust cellular immune response as these cells produce
332 cytokines that play a pivotal role in activating other immune cells, including antibody-producing
333 B cells. Moreover, our findings align with another observation which further reinforces the
334 potential immunostimulatory properties of components (polar lipids) derived from saprophytic
335 *L. biflexa*, indicating that these components could play a crucial role in inducing robust B cell
336 responses (26). Induction of helper T cell responses along with dynamic transition from naïve to
337 early effector and effector without T helper memory reflects an orchestrated immune response
338 upon pathogenic challenge in the saprophytic pre-exposed group that is typical of effective
339 responses to vaccines. Previous studies have highlighted the significance of activated CD4+ T
340 cells during *Leptospira* infection in providing protective immunity to the host and mitigating the
341 severity of leptospirosis by releasing cytokines (27). Correlating induction of chemo-cytokines
342 by saprophytic *Leptospira* with subsequent adaptive immune responses, such as the activation
343 of CD4+ T cells or the production of specific antibodies, provides insights into how innate
344 immune signals drive the adaptive immune response against a pathogenic threat. It may also

345 aid in identifying key signaling molecules or pathways that could be targeted for therapeutic
346 interventions or vaccine design.

347 While other researchers have explored vaccination strategies using live attenuated or
348 mutant strains of pathogenic serovars, our approach was to utilize a live saprophytic bacterial
349 strain which is unique in the field (19, 21, 28, 29). Current vaccines based in inactivated
350 pathogenic species provide equivalent protection to the one achieved in this study (5, 30) with
351 the caveat of being serovar specific (29-31). Although our current study conclusively shows
352 protection from severe leptospirosis after heterologous challenge, it remains to be shown if
353 protection extends to multiple pathogenic serovars of *Leptospira.* Using a live saprophytic strain
354 of *Leptospira* as control strategy could pave the way for development of novel broadly effective
355 vaccines against leptospirosis. Such a vaccine could have a substantial economic impact if
356 applied to animals of agricultural interest.

357 Another interesting aspect of our current study is that it shows that exposure to a live
358 saprophytic strain of *Leptospira* provides protection against a pathogenic serovar. Thus, in the
359 real-life scenario where individuals or animals may naturally encounter a saprophytic *Leptospira*
360 species, they may develop immune responses that mitigate severe disease outcomes if the host
361 later encounters a pathogenic strain of *Leptospira*. By exploring the immune dynamics during
362 the co-exposure to different *Leptospira* serovars, this study could open avenues of research on
363 strategies that leverage natural exposure to saprophytic species to devise safe control
364 measures against leptospirosis. This concept is important for understanding the epidemiological
365 risk factors of leptospirosis and it should be applicable other infectious diseases caused by
366 direct contact between the pathogen and mucosal membranes or abraded host skin.

Importantly, we found that in mice pre-exposed to live saprophytic *Leptospira* there was a correlation between kidney health after LIC infection (less infiltration of immune cells in kidney and less fibrosis marker ColA1) and higher shedding of live LIC in urine. This suggest that a status of homeostasis was reached after kidney colonization that helps the spirochete complete its enzootic cycle. Additional research is needed to fully understand the mechanisms involved in kidney homeostasis after LIC infection.

DATA AVAILABILITY

All data generated or analyzed during this study are included in this manuscript. All relevant data are available from the authors.

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Competing Interests

The authors have no competing interests.

Author Contributions

389 MGS is responsible for the overall concept. SK, AS conducted the experiments. SK, MGS
390 designed the experiments and analyzed the data. MGS secured funding, administered the
391 project and supervised personnel. SK and MGS wrote the manuscript.
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485

486

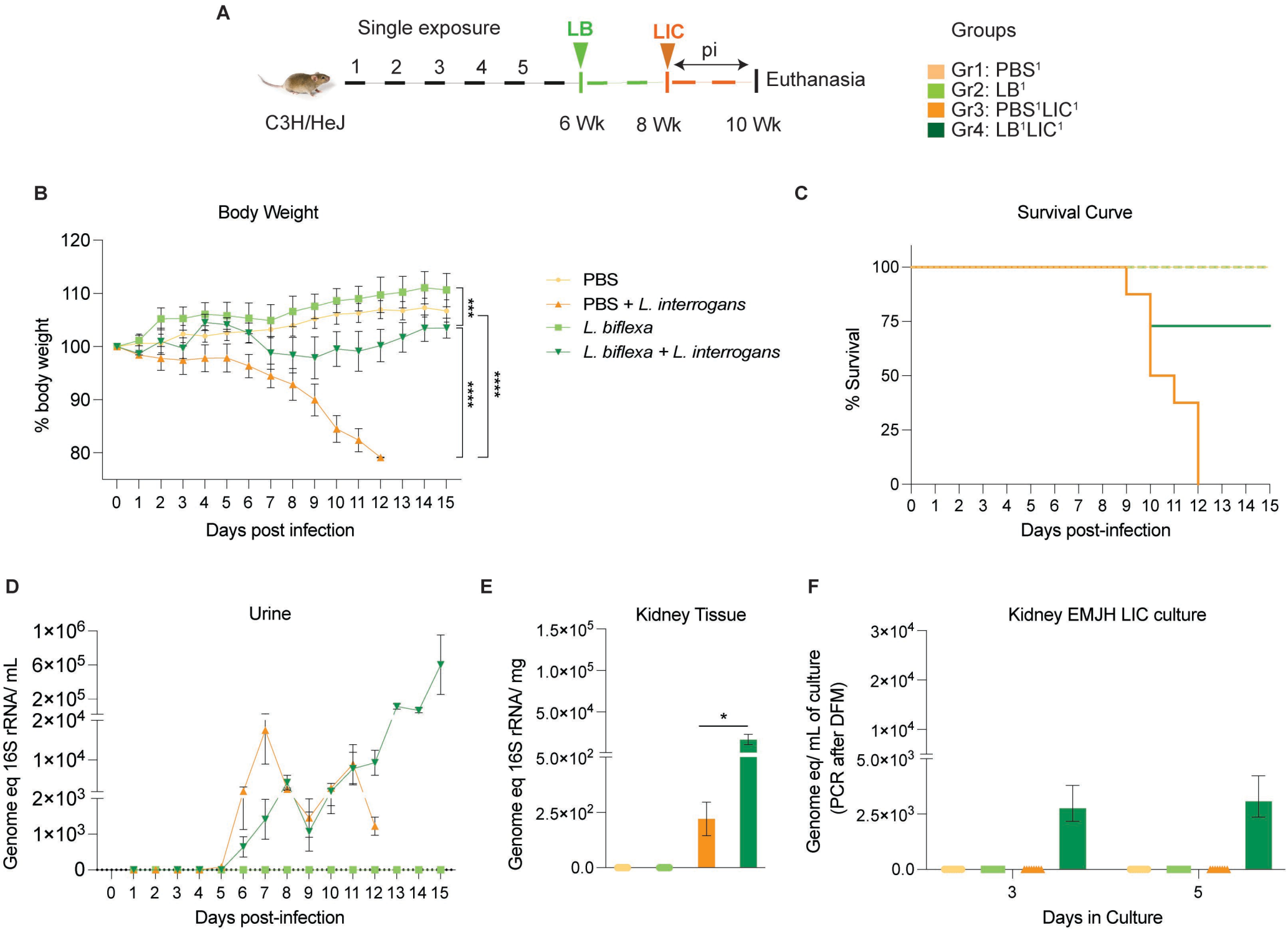
487 **Figure Legends:**

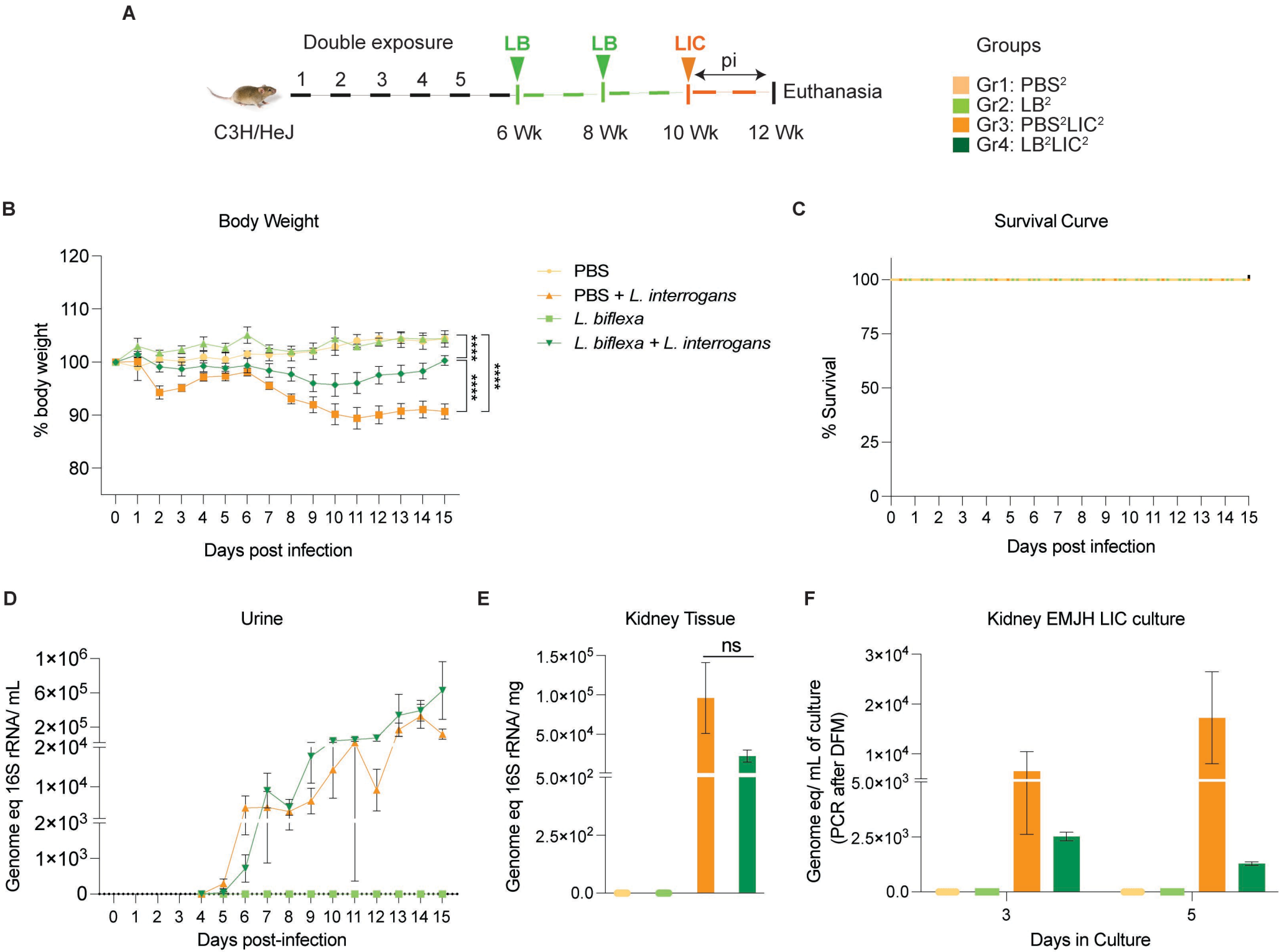
488 **Figure 1: Weight loss, kidney colonization, shedding in urine and survival to challenge with**
489 ***Leptospira interrogans* following a single exposure to *L. biflexa*.** Male C3H/HeJ mice were
490 inoculated once with 10^8 *L. biflexa* at 6 weeks and at 8 weeks they were challenged with 10^8 *L.*
491 *interrogans* ser Copenhageni FioCruz (LIC). A) experimental layout; B) body weight
492 measurements (%) acquired for 15 days post challenge with LIC; C) mouse survival within the 15
493 days post challenge with LIC; D) 16s rRNA qPCR quantification of live LIC in urine; E) 16s rRNA
494 qPCR quantification of *Leptospira* burden in kidney tissue harvested on d15 post challenge with
495 LIC and F) 16s rRNA qPCR from kidney EMJH cultures containing live *Leptospira* previously

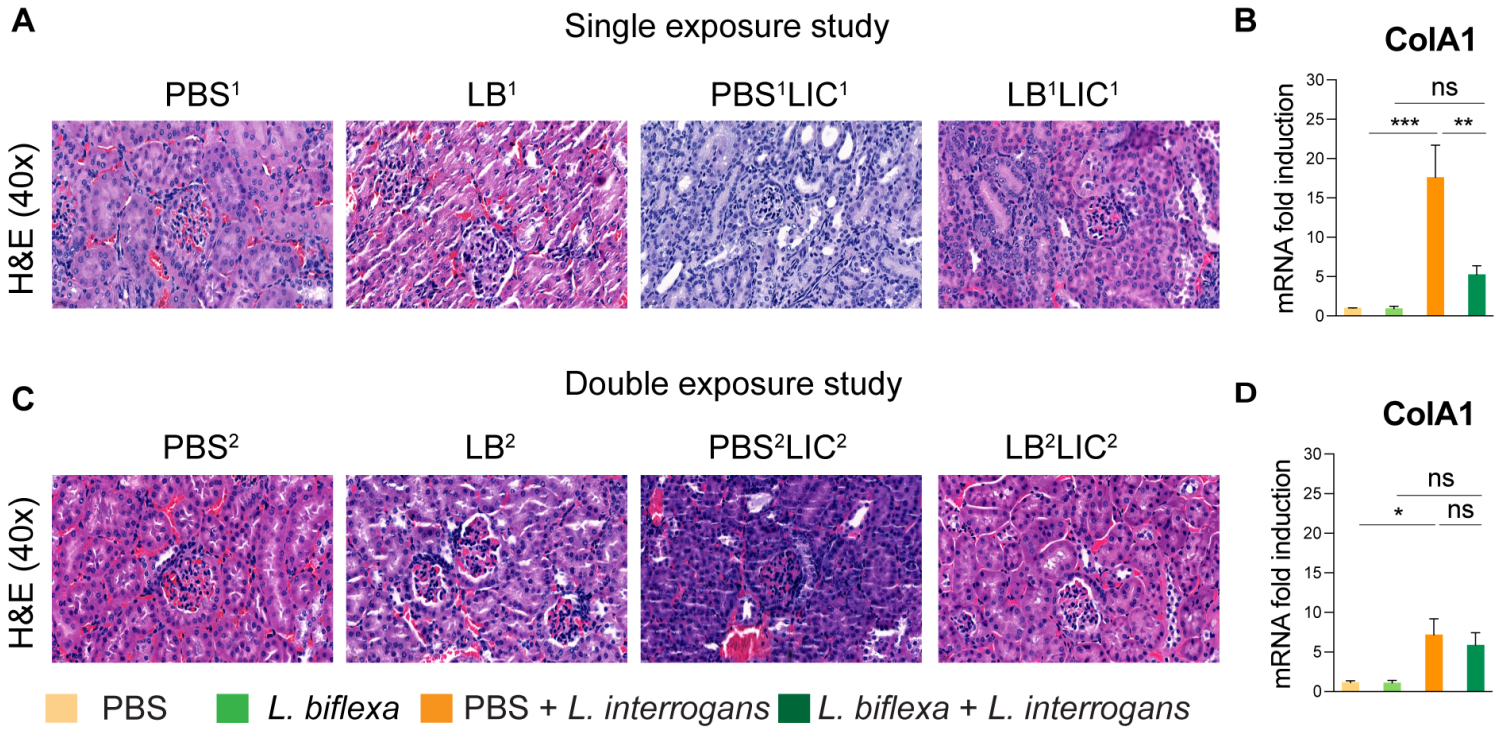
496 observed by dark field microscopy. Statistical analysis was performed by ordinary one-way
497 ANOVA followed by Tukey's multiple comparison correction between challenged groups and
498 their respective controls, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, N = 4 mice per
499 group. Data represents one of two independent experiments.
500
501 **Figure 2: Weight loss, kidney colonization, shedding in urine and survival to challenge with**
502 ***Leptospira interrogans* following a double exposure to *L. biflexa*.** Male C3H/HeJ mice were
503 inoculated twice with 10^8 *L. biflexa* at 6 and 8 weeks, and at 10 weeks they were challenged
504 with 10^8 *L. interrogans* ser Copenhageni FioCruz (LIC). A) experimental layout; B) body weight
505 measurements (%) acquired for 15 days post challenge with LIC; C) mouse survival within the 15
506 days post challenge with LIC; D) 16s rRNA qPCR quantification of live LIC in urine; E) 16s rRNA
507 qPCR quantification of *Leptospira* burden in kidney tissue harvested on d15 post challenge with
508 LIC and F) 16s rRNA qPCR from kidney EMJH cultures containing live *Leptospira* previously
509 observed by dark field microscopy. Statistical analysis was performed by ordinary one-way
510 ANOVA followed by Tukey's multiple comparison correction between challenged groups and
511 their respective controls,*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, N = 4 mice per
512 group. Data represents one of two independent experiments.
513
514 **Figure 3: Kidney histopathology and quantification of renal fibrosis.** Representative H & E-
515 stained kidney tissue sections from both single and double exposure studies are included in A
516 and C, respectively. The images were captured at 40X magnification. B and D represent the
517 mRNA expression of kidney fibrosis marker ColA1 by qPCR normalized to endogenous b-actin

518 expression. Data was analyzed by ordinary one-way ANOVA followed by Tukey's multiple
519 comparison correction between challenged groups with their respective controls; *p<0.05,
520 **p<0.01, ***p<0.001 and ns= not significant, N = 4 mice per group. Data represents one of
521 two independent experiments.
522
523 **Figure 4: Detection of IgG1, IgG2a and IgG3 specific to *L. interrogans* in serum from**
524 **experimental mice.** A) represents IgG isotypes specific to *L. interrogans* in 10-week serum of
525 mice exposed once to *L. biflexa* before *L. interrogans* challenge. B) represents IgG isotypes
526 specific to *L. interrogans* in 12-week serum of mice exposed twice to *L. biflexa* before *L.*
527 *interrogans* challenge. Ordinary one-way ANOVA followed by Tukey's multiple comparison
528 correction test was used to compare between challenged groups with their respective controls;
529 *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns= not significant; N= 4 mice per group.
530 Data represents one of two independent experiments.
531
532 **Figure 5: Frequency of lymphocytes in spleen of mice subjected to a double exposure of *L.***
533 ***biflexa* before challenge with *L. interrogans*.** A to E) show B cell (CD19+), T cell (CD3+), NK cell
534 (CD49b+), Helper T cell (CD4+) and Cytotoxic T cell (CD8+) frequencies in groups of
535 experimental mice. Ordinary one-way ANOVA followed by Tukey's multiple comparison
536 correction test was used to compare between challenged groups and their respective controls;
537 **p<0.01, ***p<0.001, ****p<0.0001 and ns= not significant; N= 3-4 mice per group. Data
538 represents one of two independent experiments.
539

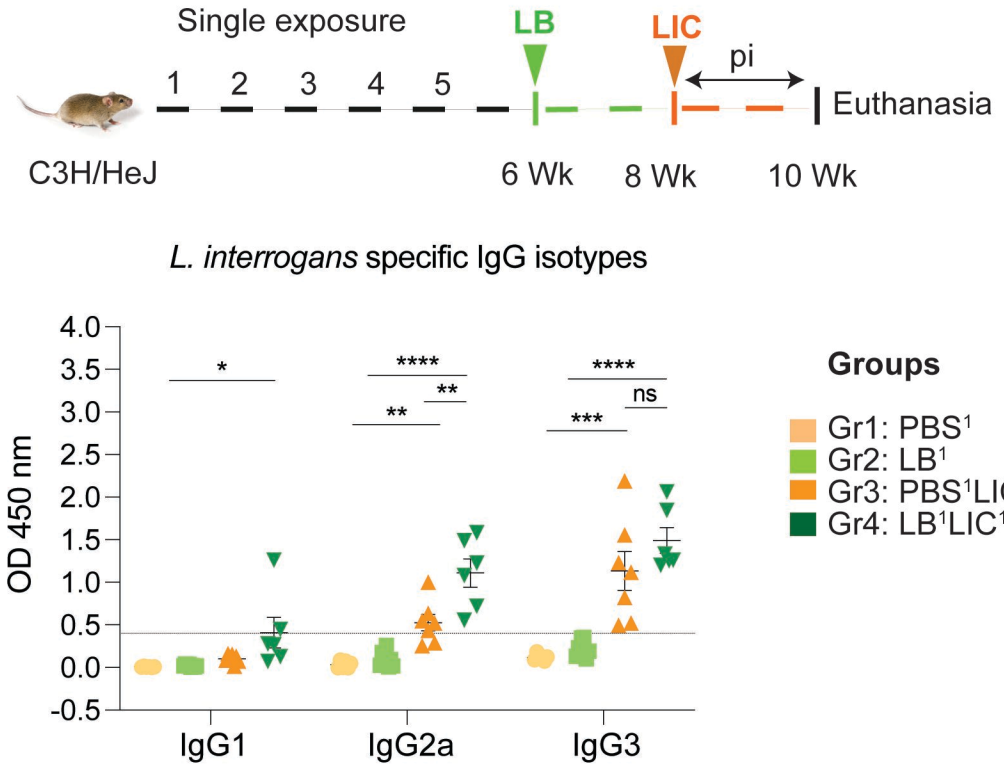
540 **Figure 6: Frequency of T cell subsets (CD62L/CD44) in spleen of mice subjected to a double**
541 **exposure of *L. biflexa* before challenge with *L. interrogans*.** A to D) represent naïve, early
542 effector, effector and memory subsets of CD4+ helper T lymphocytes, respectively. E to H)
543 represent naive, early effector, effector and memory subsets of CD8+ cytotoxic T lymphocytes,
544 respectively. Ordinary one-way ANOVA followed by Tukey's multiple comparison correction test
545 was used to compare between challenged groups and their respective controls; *$p<0.05$,
546 **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ and ns= not significant; N = 3-4 mice per group. Data
547 represents one of two independent experiments.
548
549


A
Single exposure
LB
LIC
pi
1 2 3 4 5
Euthanasia
C3H/HeJ
6 Wk
8 Wk
10 Wk
Groups
Gr1: PBS¹
Gr2: LB¹
Gr3: PBS¹LIC¹
Gr4: LB¹LIC¹
B
Body Weight
% body weight
Days post infection
PBS
PBS + L. interrogans
L. biflexa
L. biflexa + L. interrogans
***
****
****
C
Survival Curve
% Survival
Days post-infection
D
Urine
Genome eq 16S rRNA/ mL
Days post-infection
E
Kidney Tissue
Genome eq 16S rRNA/ mg
*
F
Kidney EMJH LIC culture
Genome eq/ mL of culture (PCR after DFM)
Days in Culture

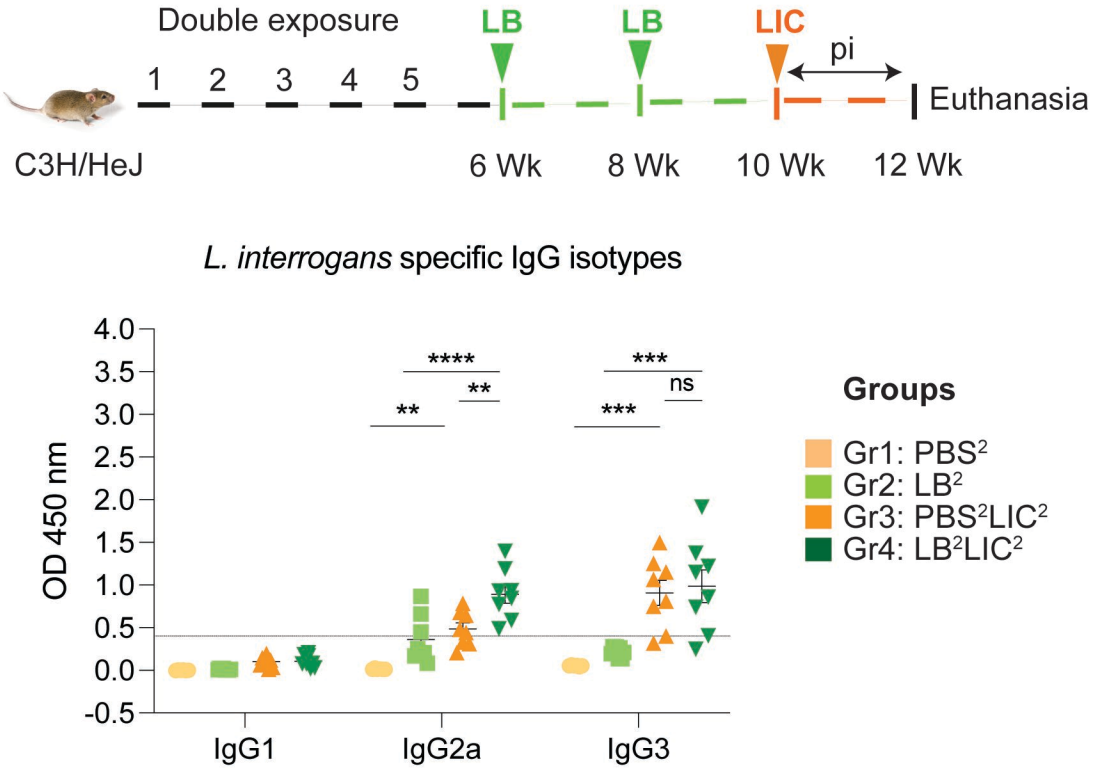

A
Double exposure
1 2 3 4 5
LB
LB
LIC
pi
Euthanasia
C3H/HeJ
6 Wk
8 Wk
10 Wk
12 Wk
Groups
Gr1: PBS²
Gr2: LB²
Gr3: PBS²LIC²
Gr4: LB²LIC²
B
Body Weight
% body weight
Days post infection
PBS
PBS + L. interrogans
L. biflexa
L. biflexa + L. interrogans
****
C
Survival Curve
% Survival
Days post infection
D
Urine
Genome eq 16S rRNA/ mL
Days post-infection
E
Kidney Tissue
ns
Genome eq 16S rRNA/ mg
F
Kidney EMJH LIC culture
Genome eq/ mL of culture (PCR after DFM)
Days in Culture

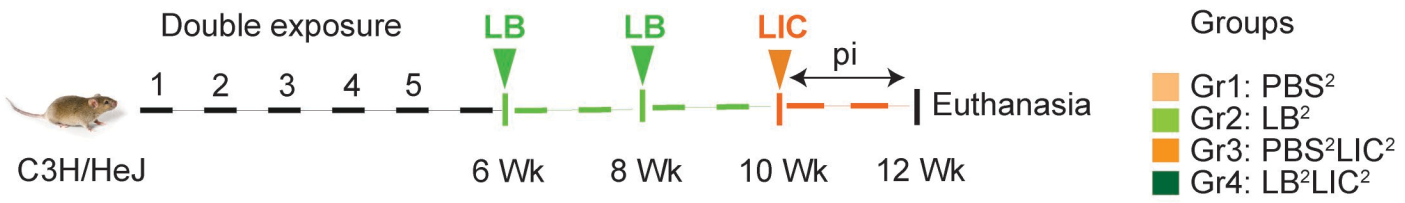

A
Single exposure study
PBS1
LB1
PBS1LIC1
LB1LIC1
H&E (40x)
B
ColA1
mRNA fold induction
ns
***
**
C
Double exposure study
PBS2
LB2
PBS2LIC2
LB2LIC2
H&E (40x)
D
ColA1
mRNA fold induction
ns
*
ns
PBS
L. biflexa
PBS + L. interrogans
L. biflexa + L. interrogans

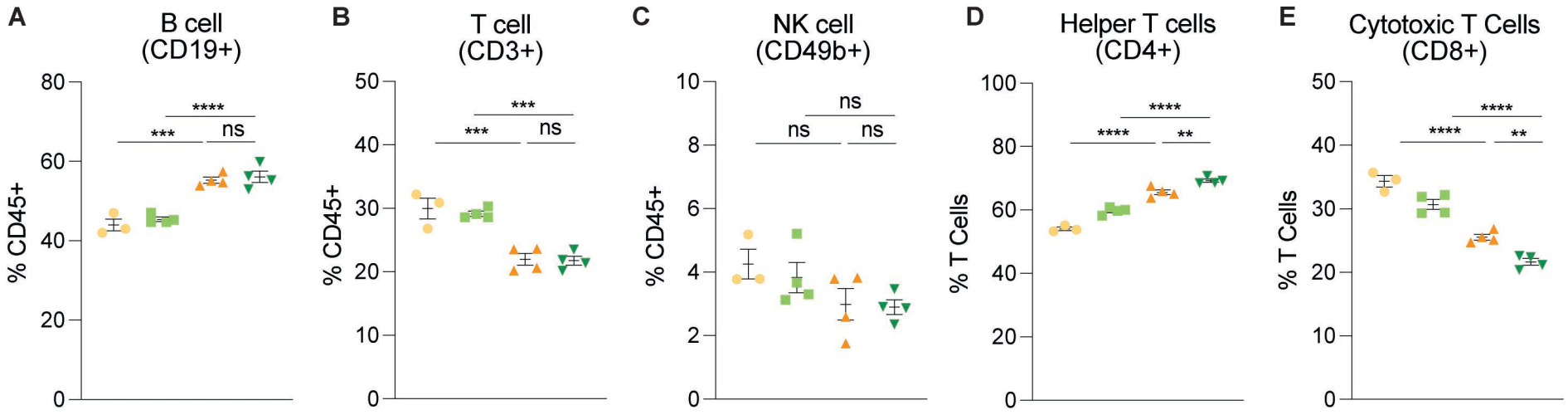
A


Single exposure
LB
LIC
pi
1
2
3
4
5
Euthanasia
C3H/HeJ
6 Wk
8 Wk
10 Wk
L. interrogans specific IgG isotypes
OD 450 nm
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
*
****
**
**
****
ns
***
IgG1
IgG2a
IgG3
Groups
Gr1: PBS1
Gr2: LB1
Gr3: PBS1LIC1
Gr4: LB1LIC1

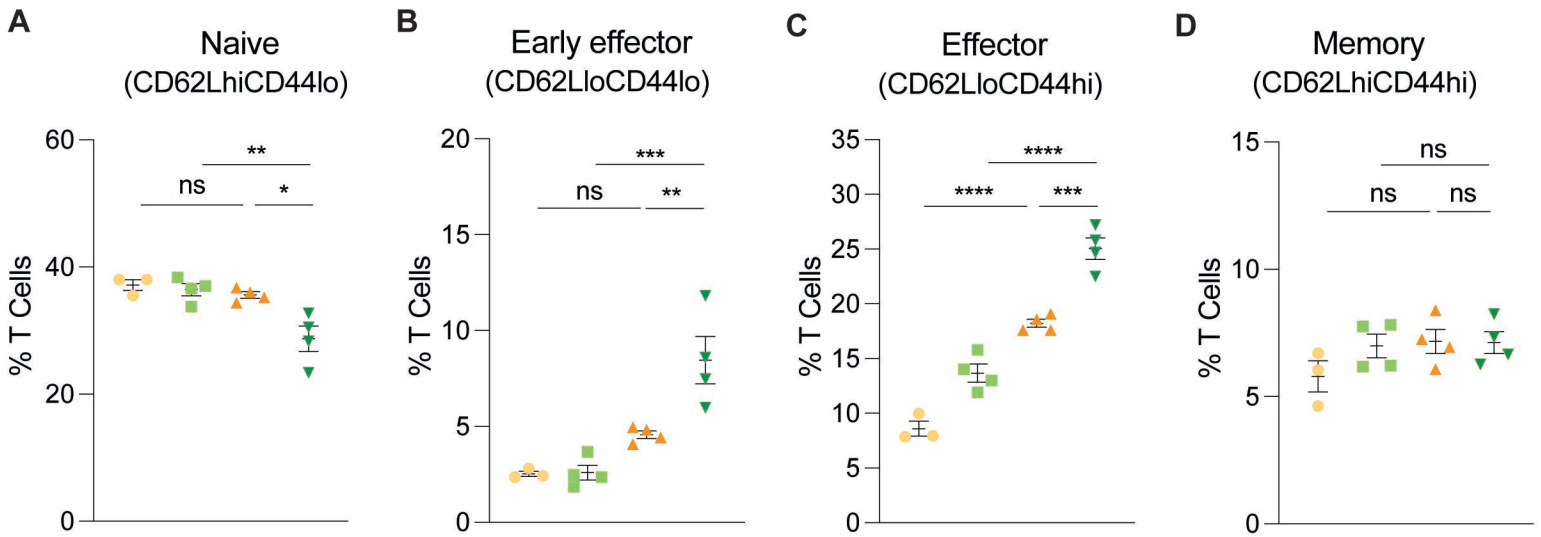
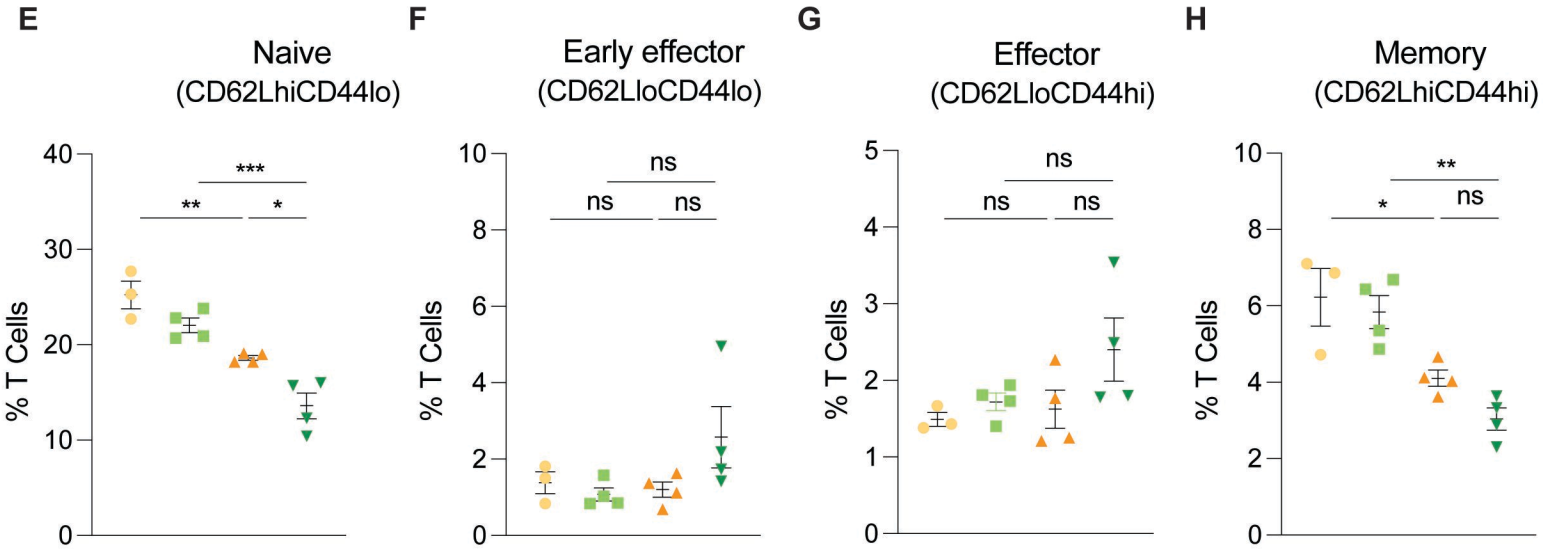
B


Double exposure
LB
LB
LIC
pi
1
2
3
4
5
Euthanasia
C3H/HeJ
6 Wk
8 Wk
10 Wk
12 Wk
L. interrogans specific IgG isotypes
OD 450 nm
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
****
**
**
***
ns
***
IgG1
IgG2a
IgG3
Groups
Gr1: PBS2
Gr2: LB2
Gr3: PBS2LIC2
Gr4: LB2LIC2


Double exposure
LB
LB
LIC
pi
1
2
3
4
5
Euthanasia
C3H/HeJ
6 Wk
8 Wk
10 Wk
12 Wk
Groups
Gr1: PBS²
Gr2: LB²
Gr3: PBS²LIC²
Gr4: LB²LIC²


A
B cell
(CD19+)
% CD45+
B
T cell
(CD3+)
% CD45+
C
NK cell
(CD49b+)
% CD45+
D
Helper T cells
(CD4+)
% T Cells
E
Cytotoxic T Cells
(CD8+)
% T Cells
ns
***
****
**

Helper (CD4+) T lymphocyte subsets



Cytotoxic (CD8+) T lymphocyte subsets