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 C3H/HeJ mice with a single or a double dose of *L. biflexa* before challenge with a pathogenic serovar, *L. interrogans* serovar 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 marker ColA1) and higher shedding of LIC in urine. This stood in contrast to the *L. biflexa* unexposed LIC challenged control. Immune responses were dominated by increased frequency of 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, mice exposed to live saprophytic *Leptospira* before facing a pathogenic serovar may withstand 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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47 **Significance**

48 Previous evidence of host innate immunity induced by live saprophytic *Leptospira* in mice led us
49 to posit that these responses might mitigate leptospirosis severity upon a subsequent challenge
50 with a pathogenic serovar. In this study, we validated our hypothesis. This is important for
51 development of novel strategies to control leptospirosis and for understanding the
52 epidemiologic risk factors of this and other infectious diseases transmitted by direct contact
53 between pathogen and host. Unexpectedly, these studies also show that there is a correlation
54 between kidney health after *L. interrogans* infection (less fibrosis marker ColA1) and higher
55 shedding of this spirochete in urine. This suggests that a status of homeostasis may be reached
56 after kidney colonization by *L. interrogans* that helps the spirochete fulfill its enzootic cycle.

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69 **Introduction**

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

84 The genus *Leptospira* is broadly categorized into 2 major clades P and S (Pathogens and
85 Saprophytes) and further categorized into 4 subclades, P1, P2, S1 and S2 based on their
86 virulence properties, growth conditions and genetic make-up (6, 7). Subclade P1 is further
87 divided in two phylogenetically related groups named P1+ (high-virulence pathogens,
88 established pathogenic species, e.g. *L. interrogans*) and P1- (low-virulence pathogens,

89 phenotypically not well-characterized) (8). *Leptospira* survives in moist conditions and are free-
90 living organisms naturally found in soil and water bodies (9, 10). The spread of infection occurs
91 through contaminated water contact with breached skin or mucosal surfaces (11). Saprophytic
92 strains of *Leptospira,* such as *L. biflexa* (S1), are unable to establish disease due to the lack of
93 certain virulence factors (7) and have been found in natural environments around the world
94 alongside pathogenic serovars (6, 11, 12). Moreover, *L. biflexa* exhibits certain niche-specific
95 adaptations that allow them to persist in both environmental and host setting (13, 14).
96 Our previous studies (15, 16) demonstrated that *L. biflexa* triggers a robust innate
97 immune response in mice during the acute phase of infection. This raised the question of
98 whether saprophytic *Leptospira* induced immune responses could confer any degree of
99 resistance or immune memory that could suppress a subsequent challenge with a pathogenic
100 serovar of *Leptospira*. Answering that question was the main goal of the current study.
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103 **Materials and Methods**
104 **Animals**
105 Male C3H/HeJ mice (n=6-8/group) were purchased from The Jackson Laboratory (Bar Harbor,
106 ME) and were maintained in a pathogen-free environment in the Laboratory Animal Care Unit
107 of the University of Tennessee Health Science Center (UTHSC). All experiments were performed
108 in compliance with the UTHSC Institutional Animal Care and Use Committee (IACUC), Protocol
109 no. 19-0062.

110 **Bacteria**

111 Non-pathogenic *Leptospira biflexa* serovar Patoc (LB) belonging to subclade S1 was purchased
112 from ATCC and grown in EMJH media. Pathogenic *Leptospira interrogans* serovar Copenhageni
113 strain Fiocruz L1-130 (LIC) belonging to subclade P1+ (high-virulence pathogens) (6, 8) was
114 grown in EMJH media and subsequently passaged in hamster to maintain virulence. EMJH
115 culture passage 2 was used to inoculate mice (10^8) after counting *Leptospira* under a dark-field
116 microscope (Zeiss USA, Hawthorne, NY) using a Petroff- Hausser chamber.

117 **Infection of mice and study design**

118 We carried out two experiments set apart by a single or double exposure to a saprophytic
119 serovar of *Leptospira* (*L. biflexa*) before challenge with a pathogenic serovar (*L. interrogans*).
120 Groups of mice were inoculated with 10^8 *Leptospira* intraperitoneally (IP) both for exposure to
121 *L. biflexa* and for challenge with *L. interrogans*. Each experiment was reproduced once. In the
122 single exposure study (Fig 1A), Group 1 (n=3) was the naive control which received PBS (PBS^1),
123 Group 2 (n=4) was inoculated with 10^8 *L. biflexa* once at 6 weeks (LB^1), Group 3 (n=4) received
124 PBS for 2 weeks followed by challenge with 10^8 *L. interrogans* at 8 weeks (PBS^1LIC^1), and Group
125 4 (n=4) was inoculated with 10^8 *L. biflexa* at 6 weeks and challenged with 10^8 *L. interrogans* at 8
126 weeks (LB^1LIC^1). In the double exposure study (Fig 2A), Group 1 (n=3) was the naive control
127 which received PBS (PBS^2), Group 2 (n=4) received 10^8 *L. biflexa* IP at 6 and 8 weeks (LB^2), Group
128 3 (n=4) received PBS for 2 weeks followed by challenge with 10^8 *L. interrogans* at 10 weeks
129 (PBS^2LIC^2), and Group 4 (n=4) was inoculated with 10^8 *L. biflexa* at 6 and 8 weeks and
130 challenged with 10^8 *L. interrogans* at 10 weeks (LB^2LIC^2). Weight was monitored daily. Mice
131 were euthanized 15 days after *L. interrogans* challenge or when they reached the endpoint

132 criteria (20% body weight loss post-infection). Blood and kidney were collected at euthanasia:
133 blood was used for quantification of anti-*Leptospira* antibody; kidney was used for
134 quantification of *Leptospira* load (16S rRNA) and it was cultured in EMJH media for evaluation
135 of bacterial viability. Furthermore, kidney samples were stored in 10% formalin for Hematoxylin
136 and Eosin (H&E) staining. Spleen for flow cytometric analysis was collected from mice after
137 euthanasia only in the double exposure study given that all mice consistently survived
138 challenge.

139 ***Leptospira* detection through qPCR**

140 Isolation of genomic DNA from blood, urine and kidney were carried out using NucleoSpin
141 tissue kit (Clontech, Mountain View, CA) according to the manufacturers' instructions.
142 *Leptospira* 16S rRNA primers (Forward- CCCGCGTCCGATTAG and Reverse-
143 TCCATTGTGGCCGAACAC) and TAMRA probe (CTCACCAAGGCGACGATCGGTAGC) were used for
144 detection of *Leptospira* genus using qPCR with a standard curve of 10^5 to 1 *L. interrogans* (17,
145 18). Similarly, qPCR was performed with kidney tissues placed in EMJH to grow live *Leptospira*
146 after culturing for 5 days and visually quantified under a dark field microscope (20X, Zeiss USA,
147 Hawthorne) on d3 and d5 post culture inoculation.

148 **RNA isolation and RT-PCR**

149 Kidneys were stored in RNA later after euthanasia. RNeasy mini kit (Qiagen) was used to extract
150 total RNA from kidney tissue according to the manufacturer's specifications. RNA purity was
151 measured using a Nanodrop instrument (Thermo Scientific) at A260/280 ratio. cDNA was
152 prepared using cDNA reverse transcription kit (Applied Biosystems). ColA1 primers (Forward-
153 TAAGGGTACCGCTGGAGAAC, Reverse- GTTCACCTCTCTCACCAGCA), TAMRA probe

154 (AGAGCGAGGCCTTCCCGGAC) and β-actin primers (Forward- CCACAGCTGAGAGGGAAATC,
155 Reverse- CCAATAGTGATGACCTGGCCG), TAMRA probe (GGAGATGGCCACTGCCGCATC) were
156 purchased from Eurofins Genomics.

157 **Histopathology by H&E staining**

158 Kidney tissues were fixed in formalin buffer. Histopathology was performed at the Histology
159 Department, UT Methodist University Hospital, Memphis, TN. Digital scanning of inflammatory
160 cell infiltration was measured by taking images of ~ 5 fields per sample under 20x
161 magnification. Images were captured after digitally scanning the H&E slides using Panoramic
162 350 Flash III (3D Histech, Hungary) and CaseViewer software.

163 **ELISA**

164 Leptospiral extract for *Leptospira biflexa* and *Leptospira interrogans* were prepared as
165 described previously (19). Briefly, *Leptospira* was cultured in EMJH media and once confluency
166 was observed, cells were centrifuged to obtain a pellet. This pellet was then incubated with
167 BugBuster® solution (1mL) at RT in a shaker incubator (100 rpm) for 20 min and homogenized
168 by vortexing. Stocks were stored at -20°C. This whole-cell extract of *Leptospira* was then diluted
169 in 1X sodium carbonate coating buffer. Nunc MaxiSorp flat-bottom 96 well plates (eBioscience,
170 San Diego, CA) were coated with extracts prepared from 10^7-10^8 bacteria per well and
171 incubated at 4°C overnight. Cells were washed using 1X PBST the following day and blocked for
172 1 h using 1% BSA solution, followed by another wash with 1X PBST. Serum samples (1:100)
173 were added to the antigen coated wells and incubated at 37°C for 1 h, washed twice with 1X
174 PBST, followed by HRP conjugate secondary anti-mouse- IgG1, IgG2a and IgG3 (1:10000) which
175 was incubated for 30 mins. After washing the plate 3 times with 1X PBST the color was

176 developed using TMB Sureblue followed by Stop solution before the absorbance was measured
177 at OD 450 nm using an ELISA plate reader (Molecular Devices Spetramax).

178 **Flow cytometry**

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

193 **Statistical analysis**

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

197

198 **Results**

199 **Exposure to saprophytic *Leptospira* before infection with a pathogenic serovar prevents**
200 **disease and increases survival of C3H-HeJ mice.** We inoculated adult C3H-HeJ male mice with a
201 single dose of *L. biflexa* two weeks before challenge with *L. interrogans* (LB^1LIC^1) at 8 weeks (Fig
202 1A) and measured a significant rescue of weight loss over a period of 15 days as compared to
203 mice infected at 8 weeks that did not receive *L. biflexa* (PBS^1LIC^1) ($p<0.0001$); unchallenged
204 control mice that received *L. biflexa* (LB^1) or PBS (PBS^1) gained weight throughout the
205 corresponding 15 days (Fig 1B). Survival curves were generated after the mice reached the
206 following endpoint criteria: 20% weight loss or 15 days post challenge with *L. interrogans* or 15
207 days post inoculation with *L. biflexa*/PBS for the controls (Fig 1C). All mice infected with *L.*
208 *interrogans* (PBS^1LIC^1) reached the 20% weight loss endpoint criteria between d9 and d12 post
209 infection. In contrast, 3/4 (75%) of the mice that received one dose of *L. biflexa* before
210 challenge with *L. interrogans* (LB^1LIC^1) survived and gained significant body weight (Fig 1B)
211 which was similar to the naïve control that received only PBS. Analysis of bacterial
212 dissemination was done by qPCR of the *Leptospira* 16S gene in genomic DNA purified from
213 blood, kidney tissue and urine. Of note, although 16S rRNA primers can amplify *L. biflexa* 6h
214 post infection (20), we processed the tissue samples 30 days or 45 days post *L. biflexa* exposure.
215 We also found that a single exposure to *L. biflexa* before challenge did not prevent
216 dissemination of pathogenic *L. interrogans* in blood (Fig S2A) or shedding in urine (Fig 1D), or
217 kidney colonization (Fig 1E). Culture of kidney in EMJH media showed presence of ~ 2500
218 motile, morphologically intact *L. interrogans* under dark field microscopy which was confirmed
219 by 16S qPCR (Fig 1F) both on d3 and d5 post culture of kidney collected from LB^1LIC^1 mice;

220 kidney from PBS^1, LB^1 and PBS^1LIC^1 did not produce positive cultures by dark field microscopy or
221 16S qPCR.
222 In the double exposure study, mice were inoculated with 2 bi-weekly doses of *L. biflexa*
223 two weeks before challenge with *L. interrogans* (LB^2LIC^2) at 10 weeks in comparison with the
224 respective controls (Fig 2A). As expected, mice infected at 10 weeks with LIC that did not
225 receive *L. biflexa* (PBS^2LIC^2) lost ~ 11 % of weight on d11 post challenge and did not recover (Fig
226 2B). In contrast, mice that received a double dose of *L. biflexa* two weeks before challenge at 10
227 weeks with *L. interrogans* (LB^2LIC^2) lost a maximum of 5% weight on d10 and recovered fully by
228 d15 post infection; unchallenged control mice that received *L. biflexa* (LB^2) or PBS (PBS^2) gained
229 weight throughout the 15 days (Fig 2B). Survival curves generated after the mice reached
230 endpoint criteria (Fig 2C) show that all experimental groups survived LIC infection. Analysis of
231 bacterial dissemination showed that a double exposure to *L. biflexa* before challenge did not
232 prevent dissemination of pathogenic *L. interrogans* in blood (Fig S2B), or shedding in urine (Fig
233 2D), or kidney colonization (Fig 2E). Culture of kidney in EMJH media showed presence of 5,000-
234 10,000 motile, morphologically intact *L. interrogans* on d3 and d5 post culture of kidney
235 collected from PBS^2LIC^2 mice in contrast to 1,000 to 2,500 live *L. interrogans* observed in culture
236 from kidney collected from LB^2LIC^2 mice which was confirmed by 16S qPCR (Fig 2F); kidney from
237 PBS^2 and LB^2 mice did not produce positive cultures by dark field microscopy or 16S qPCR.
238
239 ***L. biflexa* exposure before challenge with *L. interrogans* mitigates renal histopathological**
240 **changes.** As expected, H&E staining of histological slices of all kidneys from mice challenged
241 with *L. interrogans* in both single and double exposure experiments (PBS^1LIC^1 and PBS^2LIC^2)

242 showed signs of inflammation with increased immune cell infiltration (Fig 3A and 3C). In
243 contrast, H&E staining of kidney slices from the groups of mice exposed to *L. biflexa* before *L.*
244 *interrogans* challenge (LB^1LIC^1 and LB^2LIC^2) showed reduced immune cell infiltration. We also
245 measured expression of a marker (ColA1) for kidney fibrosis. In both experiments, kidneys from
246 mice challenged with *L. interrogans* (PBS^1LIC^1 and PBS^2LIC^2) had significantly higher expression
247 of ColA1 as compared to the controls; in contrast, kidneys from mice challenged with *L.*
248 *interrogans* after exposure to *L. biflexa* (LB^1LIC^1 and LB^2LIC^2) were not different than the
249 controls (Figs 3B and 3D).

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

256

257 **Serologic IgG2a responses to *L. interrogans* were significantly higher in mice pre-exposed to *L.***
258 ***biflexa* before challenge with *L. interrogans*.** In both single and double *L. biflexa* exposure
259 experiments we measured anti-*L. interrogans* total IgM, total IgG, and IgG subtypes IgG1, IgG2a
260 and IgG3 in serum collected 2 weeks after challenge with *L. interrogans* (IgG subtypes shown in
261 Figs 4A and 4B). In both experiments, total IgM and IgG were significantly increased in PBS-LIC
262 and LB-LIC when compared to the respective controls, but not between PBS-LIC and LB-LIC
263 groups. Regarding IgG isotypes, IgG1 was generally low and IgG2a as well as IgG3 were

264 generally high in groups infected with *L. interrogans*. Although differences between groups
265 (PBS^2v PBS^2LIC^2 and $LB^{1/2}$ v $LB^{1/2}LIC^{1/2}$) were significant, differences between the LIC infected
266 groups ($PBS^{1/2}LIC^{1/2}$ v $LB^{1/2}LIC^{1/2}$) were not significant for IgG1 and for IgG3 in contrast to IgG2a
267 (p=0.001 for single exposure and p=0.0095 for double exposure).

268 **Exposure to non-pathogenic *L. biflexa* before pathogenic *L. interrogans* challenge induced**
269 **increased frequencies of effector helper T cells in spleen**. We immunophenotyped the spleen
270 cells from the mice subjected to double *L. biflexa* exposure because all animals survived to the
271 term of the experiment. Mice in the single exposure experiment met endpoint criteria before
272 the term of the experiment and thus we were not able to process spleen for
273 immunophenotyping. In the *L. biflexa* double exposure experiment, we measured increased
274 frequencies in B cells when LIC infected mice were compared to the respective controls, but not
275 between PBS^2LIC^2 and LB^2LIC^2 mice (Fig 5A). We measured decreased frequencies in T cells
276 when LIC infected mice were compared to the respective controls but not between PBS^2LIC^2
277 and LB^2LIC^2 mice (Fig 5B). No differences were observed in NK cells between any of the groups
278 (Fig 5C). We measured increased frequencies in helper T cells between all groups; of note,
279 PBS^2LIC^2 vs LB^2LIC^2 p=0.006 (Fig 5D). We also measured decreased frequencies in cytotoxic T
280 cells between all groups; of note PBS^2LIC^2 vs LB^2LIC^2 p=0.0056 (Fig 5E). Furthermore, T cell
281 subset typing (Fig 6) showed that frequency of early effector CD4+ T helper cells (Fig 6B, CD44-
282 CD62L-) and effector T helper cells (Fig 6C, CD44+CD62L-) were significantly increased when
283 compared between the LIC challenged groups and the respective controls (except PBS^2 v
284 PBS^2LIC^2 early effectors) and that frequency of early effector and effector T helper cells were
285 higher in the LB^2LIC^2 group than PBS^2LIC^2. No major changes were measured in memory CD4+ T

286 helper cells (Fig 6D, CD44+CD62L+). In the CD8+ cytotoxic T cell subsets, we measured
287 significant decreases in frequency of naïve T cells between LIC infected groups and the
288 respective controls (Fig 6E, CD62L+CD44-) and this was replicated in the CD8+ cytotoxic
289 memory except that differences with the LIC infected groups were not significant (Fig 6H,
290 CD44+CD62L+).

291 **Discussion**

292 Understanding host immune responses to *Leptospira* infection is crucial for advancing our
293 ability to develop new control measures for leptospirosis (19, 21-25). Given their widespread
294 presence in nature, the likelihood of humans or animals getting exposed to non-pathogenic
295 serovars of *Leptospira* is likely high (10, 11). Our previous studies showed innate immunity
296 engagement during saprophytic *L. biflexa* infection in mice (15, 16). In addition, *L. biflexa*
297 extracts can be used to detect *Leptospira*-specific antibody in up to 67% of serum from patients
298 with clinically confirmed leptospirosis (19) which points to a high degree of immunodominant
299 cross-reactive epitopes between *L. biflexa* and pathogenic *Leptospira*. Further, the current
300 hypothesis regarding evolution of *Leptospira* species is that symbiosis of *Leptospira* with
301 eukaryotes emerged from free-living ancestral species (26); in other words, pathogenic
302 *Leptospira* may have evolved from an environmental ancestor (6). Thus, we hypothesized that
303 these highly cross-reactive immunodominant epitopes may also induce cross-protective
304 immune responses. The objective of the current study was to assess whether exposure to a live
305 saprophytic serovar of *Leptospira* provides any heterologous cross-species protection against a
306 subsequent challenge with a pathogenic serovar in a mammalian host (mouse).

307 In the initial analysis of pathogenesis (Fig 1, Fig 2, Fig S2) we observed that prior
308 exposure to one or two doses of saprophytic *L. biflexa* rescues weight loss in mice challenged
309 with pathogenic *L. interrogans* at 8 weeks (LB^1LIC^1) and at 10 weeks (LB^2LIC^2), respectively.
310 Weight gain correlated with survival (75% survival in LB^1LIC^1 versus 0% survival of the PBS^1LIC^1
311 group) in the single exposure experiment, where we expected all mice infected at 8 weeks with
312 pathogenic *L. interrogans* (PBS^1LIC^1) to irreversibly lose weight and meet endpoint criteria for
313 euthanasia before the 2 week term of the experiment (23, 27). Loss of mice due to irreversible
314 weight loss is not expected if mice are infected with LIC at 10 weeks of age (17), as observed in
315 the *L. interrogans* control group in the double exposure experiment (PBS^2LIC^2). In both
316 experiments, mice exposed to *L. biflexa* before challenge with LIC, produced evidence of *L.*
317 *interrogans* dissemination in blood, shedding in urine and kidney colonization of the kidney.
318 Histological inspection of kidney slices (Fig 3) showed that exposure to a saprophytic
319 *Leptospira* before challenge supported normal structural morphology and prevented infiltration
320 of immune cells in both single and double exposure experiments; in addition, it significantly
321 reduced a fibrosis marker (ColA1) in the single exposure experiment. In the double exposure
322 experiment, differences in ColA1 fibrosis marker are not significant between the two LIC
323 infected groups because 10-week old C3H-HeJ infected with LIC are more resistant to pathology
324 resultant from infection. Our findings are intriguing as they suggest that while prior live
325 saprophytic exposure did not prevent infection or leptospiral dissemination, it may confer
326 protection against kidney fibrosis.
327 Our data also shows that prior exposure to non-pathogenic *Leptospira* before
328 pathogenic challenge, induced higher antibody titer in the serum, specifically IgG2a antibodies

329 against *L. interrogans* in both single and double exposure experiments (Fig 4). Increased IgG2a
330 response in serum is associated with induction of a Th1 biased immune response. Others have
331 recently found that saprophytic *L. biflexa* induced Th1 responses, higher T cell proliferation and
332 IFN-g producing CD4+ T cells (28). Persistent IgM and strain specific IgG responses was
333 observed during a homologous leptospiral challenge in C57BL6/J mice (22). In our study,
334 exposure to a saprophytic *Leptospira* induced antibody responses that may provide
335 heterologous protection against the pathogenic strain of *Leptospira*. This supports a promising
336 broad-spectrum efficacy. Thus, live vaccines derived from a saprophytic strain of *Leptospira*
337 could offer broader protection and overcome the limitation of serovar specificity often
338 observed with killed whole-cell vaccines based on pathogenic strains.

339 Differences in antibody titer among the *L. interrogans* infected group pre-exposed to
340 saprophytic *L. biflexa* can be attributed to the robust trafficking and differentiation of B and
341 helper T cells (CD4+) measured in spleen (Fig 5 and Fig 6). Presence of effector helper T (CD4+)
342 cells in the spleen indicate a robust cellular immune response as these cells produce cytokines
343 that play a pivotal role in activating other immune cells, including antibody-producing B cells.
344 Moreover, our findings align with another observation which further reinforces the potential
345 immunostimulatory properties of components (polar lipids) derived from saprophytic *L. biflexa*,
346 indicating that these components could play a crucial role in inducing robust B cell responses
347 (29). Induction of helper T cell responses along with dynamic transition from naïve to early
348 effector and effector without T helper memory reflects an orchestrated immune response upon
349 pathogenic challenge in the saprophytic pre-exposed group that is typical of effective responses
350 to vaccines. Previous studies have highlighted the significance of activated CD4+ T cells during

351 *Leptospira* infection in providing protective immunity to the host and mitigating the severity of
352 leptospirosis by releasing cytokines (30). Correlating induction of chemo-cytokines by
353 saprophytic *Leptospira* with subsequent adaptive immune responses, such as the activation of
354 CD4+ T cells or the production of specific antibodies, provides insights into how innate immune
355 signals drive the adaptive immune response against a pathogenic threat. It may also aid in
356 identifying key signaling molecules or pathways that could be targeted for therapeutic
357 interventions or vaccine design.

358 While other researchers have explored vaccination strategies using live attenuated or
359 mutant strains of pathogenic serovars, our approach was to utilize a live saprophytic bacterial
360 strain which is unique in the field (21, 23, 31, 32). We previously showed that oral delivery of a
361 probiotic strain, *Lactobacillus plantarum*, reduces the severity of leptospirosis by recruiting
362 myeloid cells (21) which suggests that a general phenomenon of trained immunity may be
363 involved. Current vaccines based in inactivated pathogenic species provide equivalent
364 protection to the one achieved in this study (5, 33) with the caveat of being serovar specific (32-
365 34). Although our current study conclusively shows protection from severe leptospirosis after
366 heterologous challenge, it remains to be shown if protection extends to multiple pathogenic
367 serovars of *Leptospira*. Using a live saprophytic strain of *Leptospira* as control strategy could
368 pave the way for development of novel broadly effective vaccines against leptospirosis. Such a
369 vaccine could have a substantial economic impact if applied to animals of agricultural interest.

370 Another interesting aspect of our current study is that it shows that exposure to a live
371 saprophytic strain of *Leptospira* provides protection against a pathogenic serovar. Thus, in the
372 real-life scenario where individuals or animals may naturally encounter a saprophytic *Leptospira*

373 species, they may develop immune responses that mitigate severe disease outcomes if the host
374 later encounters a pathogenic strain of *Leptospira*. By exploring the immune dynamics during
375 the co-exposure to different *Leptospira* serovars, this study could open avenues of research on
376 strategies that leverage natural exposure to saprophytic species to devise safe control
377 measures against leptospirosis. This concept is important for understanding the epidemiological
378 risk factors of leptospirosis and it should be applicable to other infectious diseases caused by
379 direct contact between the pathogen and mucosal membranes or abraded host skin.

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

386

387 **DATA AVAILABILITY**

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

390

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395 does not involve the official views of NIAID or NIH.
396

397 **Competing Interests**

398 MGS and SK have a relevant patent that might pose a conflict of interest.

399

400 **Author Contributions**

401 MGS is responsible for the overall concept. SK, AS conducted the experiments. SK, MGS
402 designed the experiments and analyzed the data. MGS secured funding, administered the
403 project and supervised personnel. SK and MGS wrote the manuscript.

404

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498

499 **Figure Legends:**

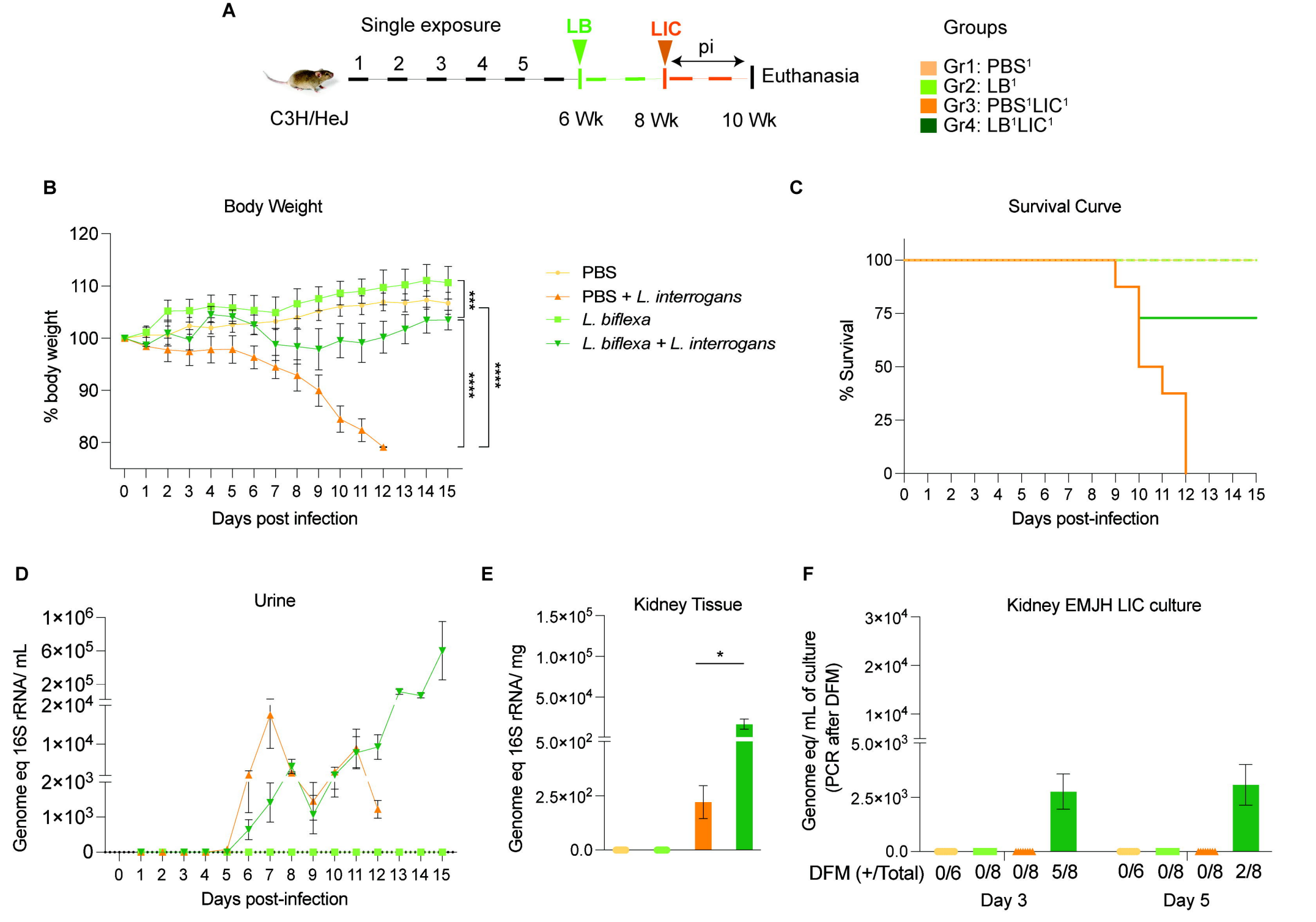
500 **Figure 1: Weight loss, kidney colonization, shedding in urine and survival to challenge with**
501 ***Leptospira interrogans* following a single exposure to *L. biflexa*.** Male C3H/HeJ mice were
502 inoculated once with 10^8 *L. biflexa* (LB) at 6 weeks and they were challenged with 10^8 *L.*
503 *interrogans* serovar Copenhageni FioCruz (LIC) at 8 weeks. A) experimental layout; B) body
504 weight measurements (%) acquired for 15 days post challenge with LIC; C) mouse survival
505 within the 15 days post challenge with LIC; D) 16s rRNA qPCR quantification of live LIC in urine;
506 E) 16s rRNA qPCR quantification of *Leptospira* burden in kidney tissue harvested on d15 post
507 challenge with LIC and F) 16s rRNA qPCR from kidney EMJH cultures containing live *Leptospira*
508 previously observed by dark field microscopy (DFM). DFM positive culture from the total data is
509 represented in numbers under the graph. Statistical analysis was performed by ordinary one-
510 way ANOVA followed by Tukey's multiple comparison correction between challenged groups
511 and their respective controls, *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$, N = 6-8 mice
512 per group. Data represents two independent experiments.

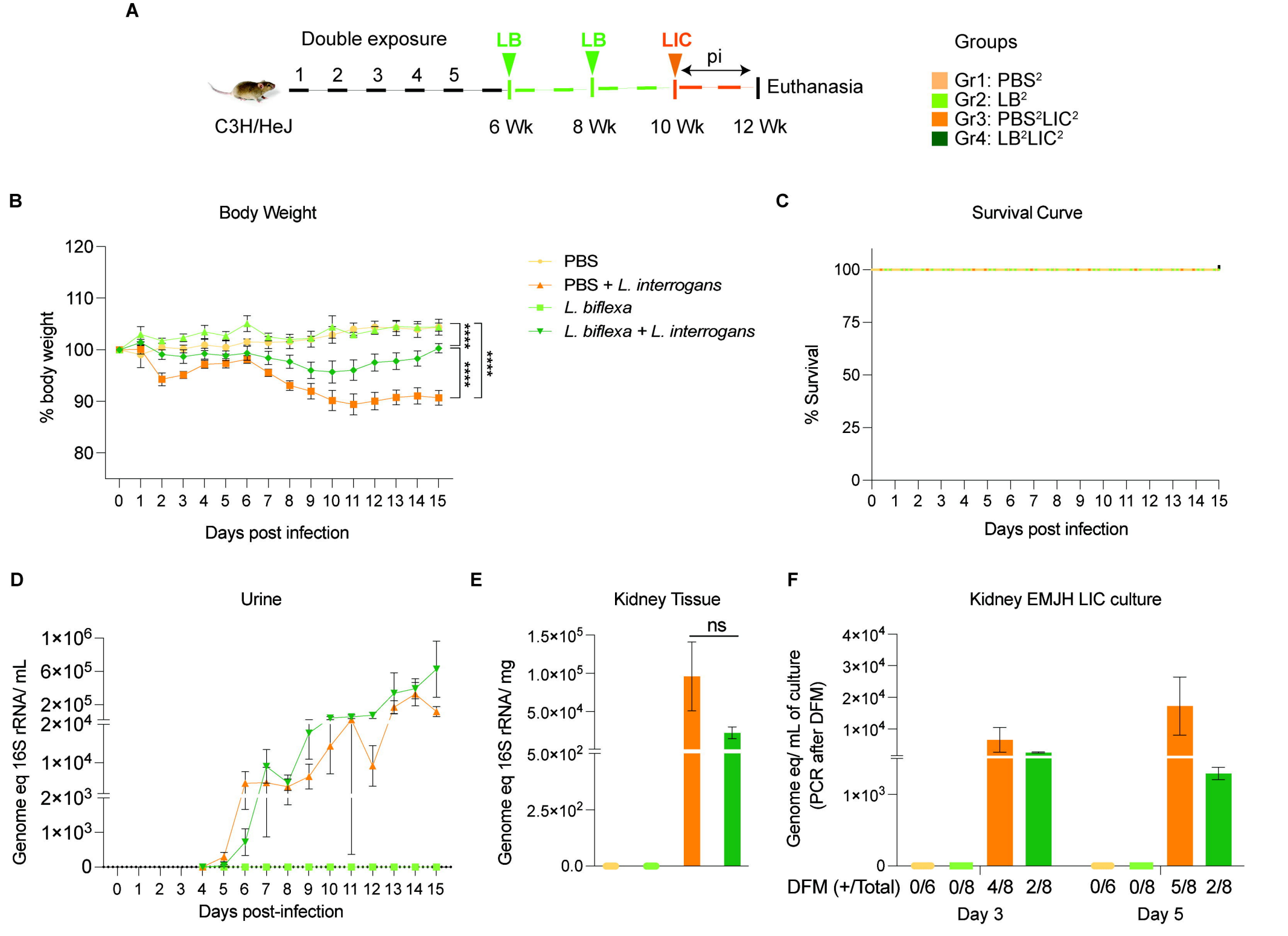
513

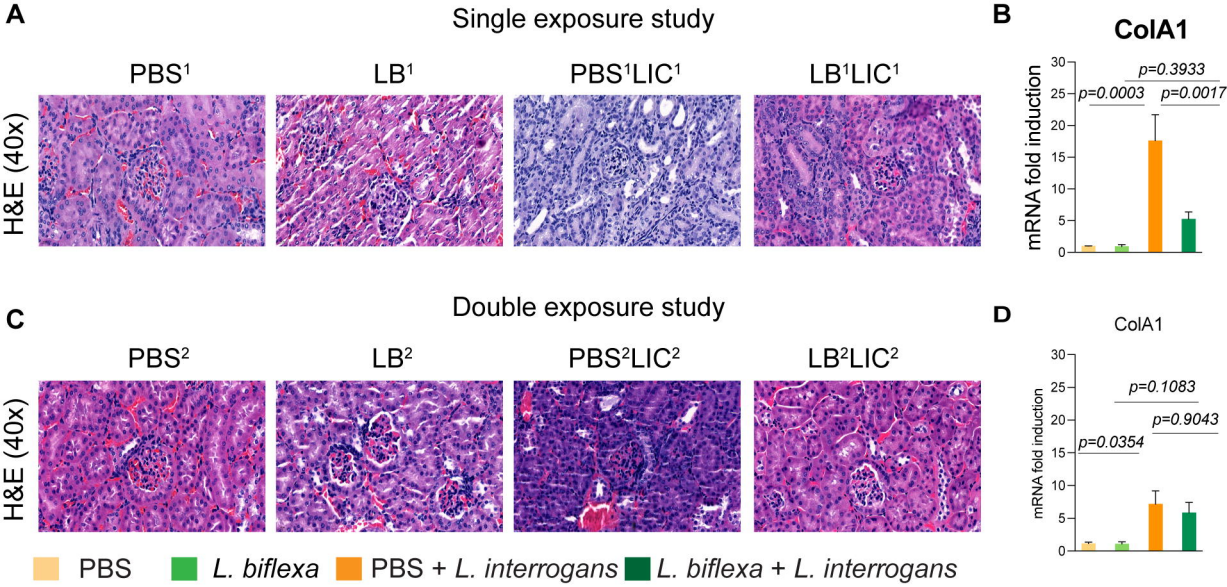
514 **Figure 2: Weight loss, kidney colonization, shedding in urine and survival to challenge with**
515 ***Leptospira interrogans* following a double exposure to *L. biflexa*.** Male C3H/HeJ mice were
516 inoculated twice with 10^8 *L. biflexa* at 6 and 8 weeks, and at 10 weeks they were challenged
517 with 10^8 *L. interrogans* ser Copenhageni FioCruz (LIC). A) experimental layout; B) body weight
518 measurements (%) acquired for 15 days post challenge with LIC; C) mouse survival within the 15
519 days post challenge with LIC; D) 16s rRNA qPCR quantification of live LIC in urine; E) 16s rRNA

520 qPCR quantification of *Leptospira* burden in kidney tissue harvested on d15 post challenge with
521 LIC and F) 16s rRNA qPCR from kidney EMJH cultures containing live *Leptospira* previously
522 observed by dark field microscopy (DFM). DFM Positive culture from the total data is
523 represented in numbers under the graph. Statistical analysis was performed by ordinary one-
524 way ANOVA followed by Tukey's multiple comparison correction between challenged groups
525 and their respective controls,*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. N = 6-8 mice
526 per group. Data represents two independent experiments.
527
528 **Figure 3: Kidney histopathology and quantification of renal fibrosis.** Representative H & E-
529 stained kidney tissue sections from both single and double exposure studies are included in A
530 and C, respectively. The images were captured at 40X magnification. B and D represent the
531 mRNA expression of kidney fibrosis marker ColA1 by qPCR normalized to endogenous b-actin
532 expression. Data was analyzed by ordinary one-way ANOVA followed by Tukey's multiple
533 comparison correction between challenged groups with their respective controls; * p values are
534 included in the graphs. Data represents one of two independent experiments.
535
536 **Figure 4: Detection of IgG1, IgG2a and IgG3 specific to *L. interrogans* in serum from**
537 **experimental mice.** A) represents IgG isotypes specific to *L. interrogans* in 10-week serum of
538 mice exposed once to *L. biflexa* before *L. interrogans* challenge. B) represents IgG isotypes
539 specific to *L. interrogans* in 12-week serum of mice exposed twice to *L. biflexa* before *L.*
540 *interrogans* challenge. Ordinary one-way ANOVA followed by Tukey's multiple comparison
541 correction test was used to compare between challenged groups with their respective controls;

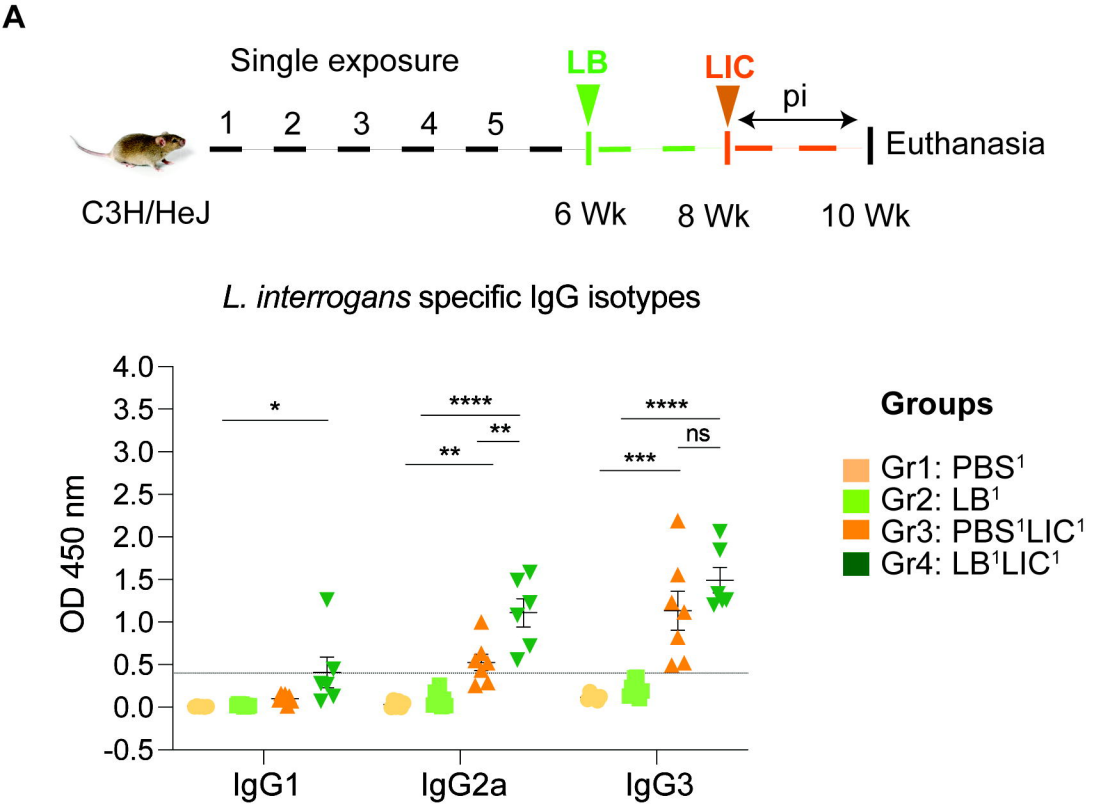
542 *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns= not significant; N= 6-8 mice per group.
543 Data represents two independent experiments.
544
545 **Figure 5: Frequency of lymphocytes in spleen of mice subjected to a double exposure of *L.***
546 ***biflexa* before challenge with *L. interrogans*.** A to E) show B cell (CD19+), T cell (CD3+), NK cell
547 (CD49b+), Helper T cell (CD4+) and Cytotoxic T cell (CD8+) frequencies in groups of
548 experimental mice. Ordinary one-way ANOVA followed by Tukey's multiple comparison
549 correction test was used to compare between challenged groups and their respective controls;
550 **p<0.01, ***p<0.001, ****p<0.0001 and ns= not significant; N= 3-4 mice per group. Data
551 represents one of two independent experiments.
552
553 **Figure 6: Frequency of T cell subsets (CD62L/CD44) in spleen of mice subjected to a double**
554 **exposure of *L. biflexa* before challenge with *L. interrogans*.** A to D) represent naïve, early
555 effector, effector and memory subsets of CD4+ helper T lymphocytes, respectively. E to H)
556 represent naive, early effector, effector and memory subsets of CD8+ cytotoxic T lymphocytes,
557 respectively. Ordinary one-way ANOVA followed by Tukey's multiple comparison correction test
558 was used to compare between challenged groups and their respective controls; *p<0.05,
559 **p<0.01, ***p<0.001, ****p<0.0001 and ns= not significant; N = 3-4 mice per group. Data
560 represents one of two independent experiments.
561
562


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)
DFM (+/Total) 0/6 0/8 0/8 5/8 0/6 0/8 0/8 2/8
Day 3
Day 5

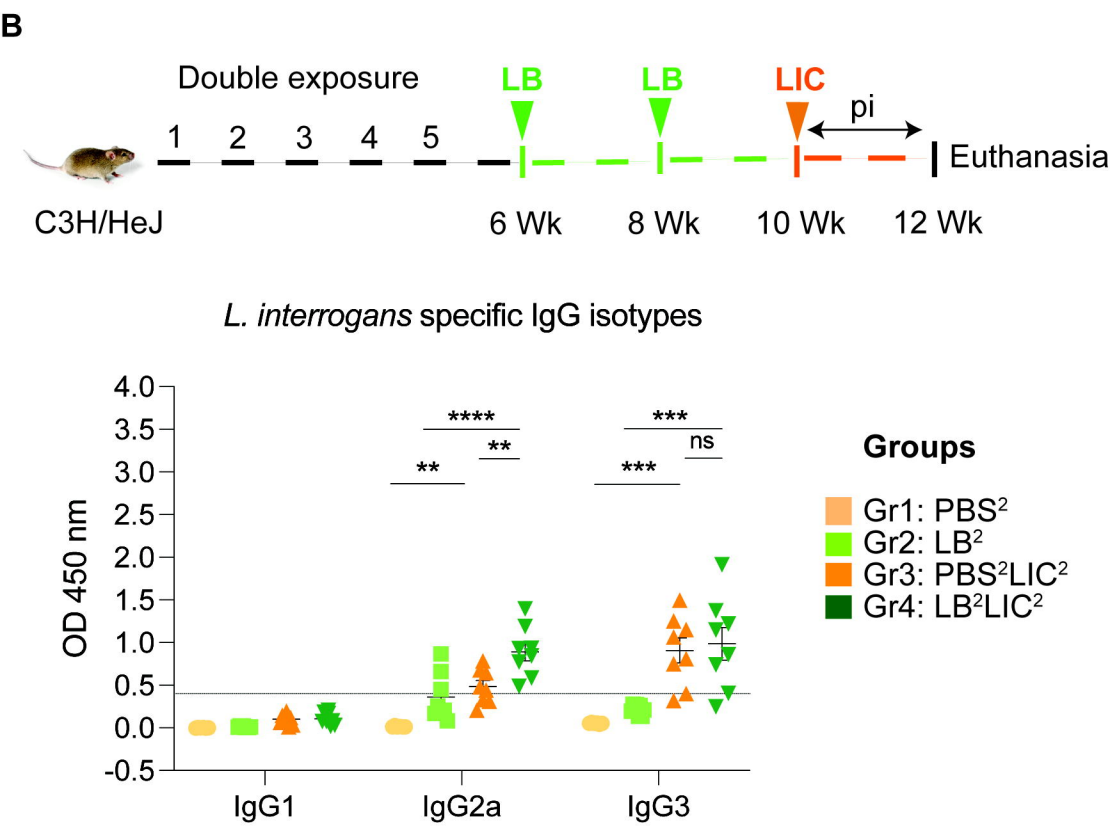

A
Double exposure
LB
LB
LIC
pi
1 2 3 4 5
Euthanasia
C3H/HeJ
6 Wk
8 Wk
10 Wk
12 Wk
Groups
Gr1: PBS²
Gr2: LB²
Gr3: PBS²LIC²
Gr4: LB²LIC²
B
Body Weight
% body weight
Days post infection
PBS
PBS + L. interrogans
L. biflexa
L. biflexa + L. interrogans
****
C
Survival Curve
% Survival
Days post infection
D
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)
DFM (+/Total) 0/6 0/8 4/8 2/8 0/6 0/8 5/8 2/8
Day 3
Day 5

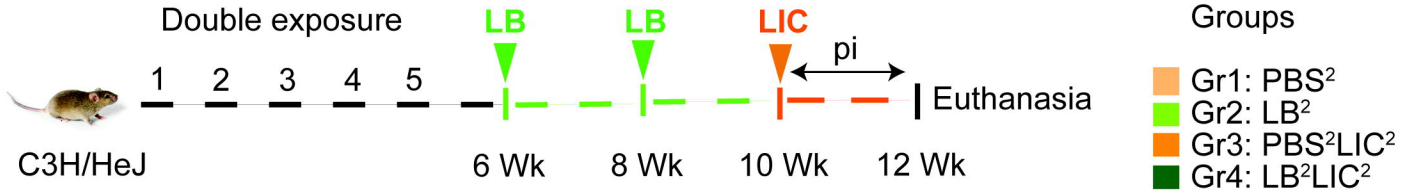

A
Single exposure study
PBS1
LB1
PBS1LIC1
LB1LIC1
H&E (40x)
B
ColA1
mRNA fold induction
p=0.3933
p=0.0003
p=0.0017
C
Double exposure study
PBS2
LB2
PBS2LIC2
LB2LIC2
H&E (40x)
D
ColA1
mRNA fold induction
p=0.1083
p=0.9043
p=0.0354
PBS
L. biflexa
PBS + L. interrogans
L. biflexa + L. interrogans

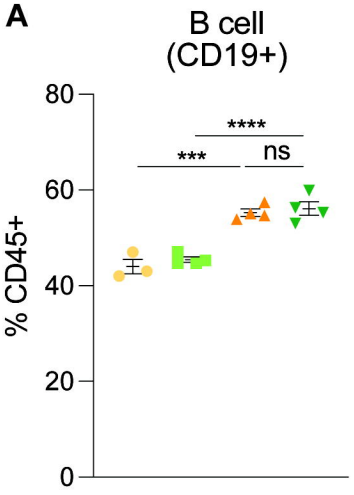
A

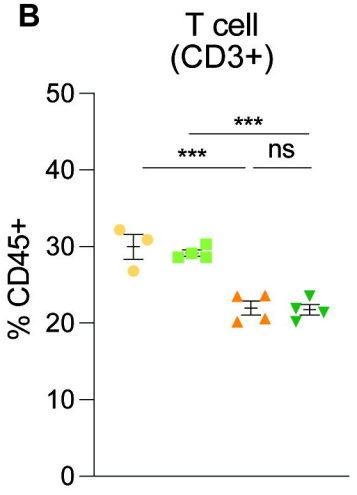

Single exposure
LB
LIC
pi
1
2
3
4
5
Euthanasia
C3H/HeJ
6 Wk
8 Wk
10 Wk
L. interrogans specific IgG isotypes
OD 450 nm
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
*
****
**
**
****
ns
***
IgG1
IgG2a
IgG3
Groups
Gr1: PBS1
Gr2: LB1
Gr3: PBS1LIC1
Gr4: LB1LIC1

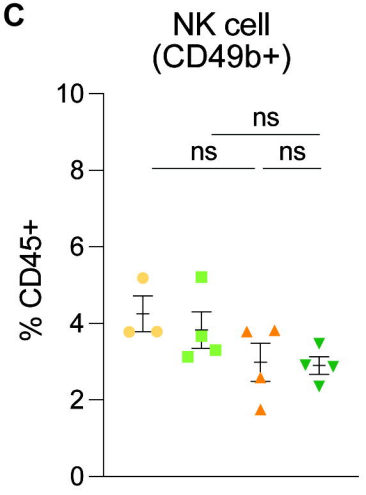
B

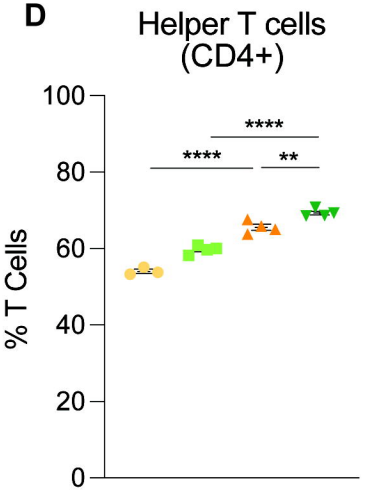

Double exposure
LB
LB
LIC
pi
1
2
3
4
5
Euthanasia
C3H/HeJ
6 Wk
8 Wk
10 Wk
12 Wk
L. interrogans specific IgG isotypes
OD 450 nm
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
****
**
**
***
ns
***
IgG1
IgG2a
IgG3
Groups
Gr1: PBS2
Gr2: LB2
Gr3: PBS2LIC2
Gr4: LB2LIC2

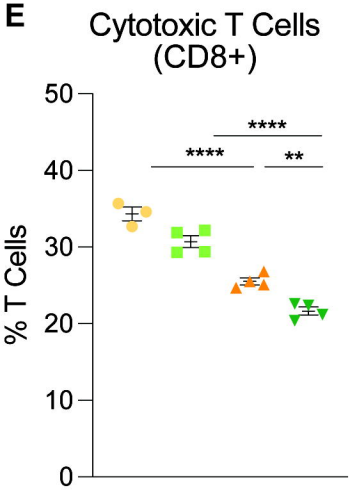

Double exposure
LB
LB
LIC
pi
1 2 3 4 5
Euthanasia
C3H/HeJ
6 Wk
8 Wk
10 Wk
12 Wk
Groups
Gr1: PBS²
Gr2: LB²
Gr3: PBS²LIC²
Gr4: LB²LIC²

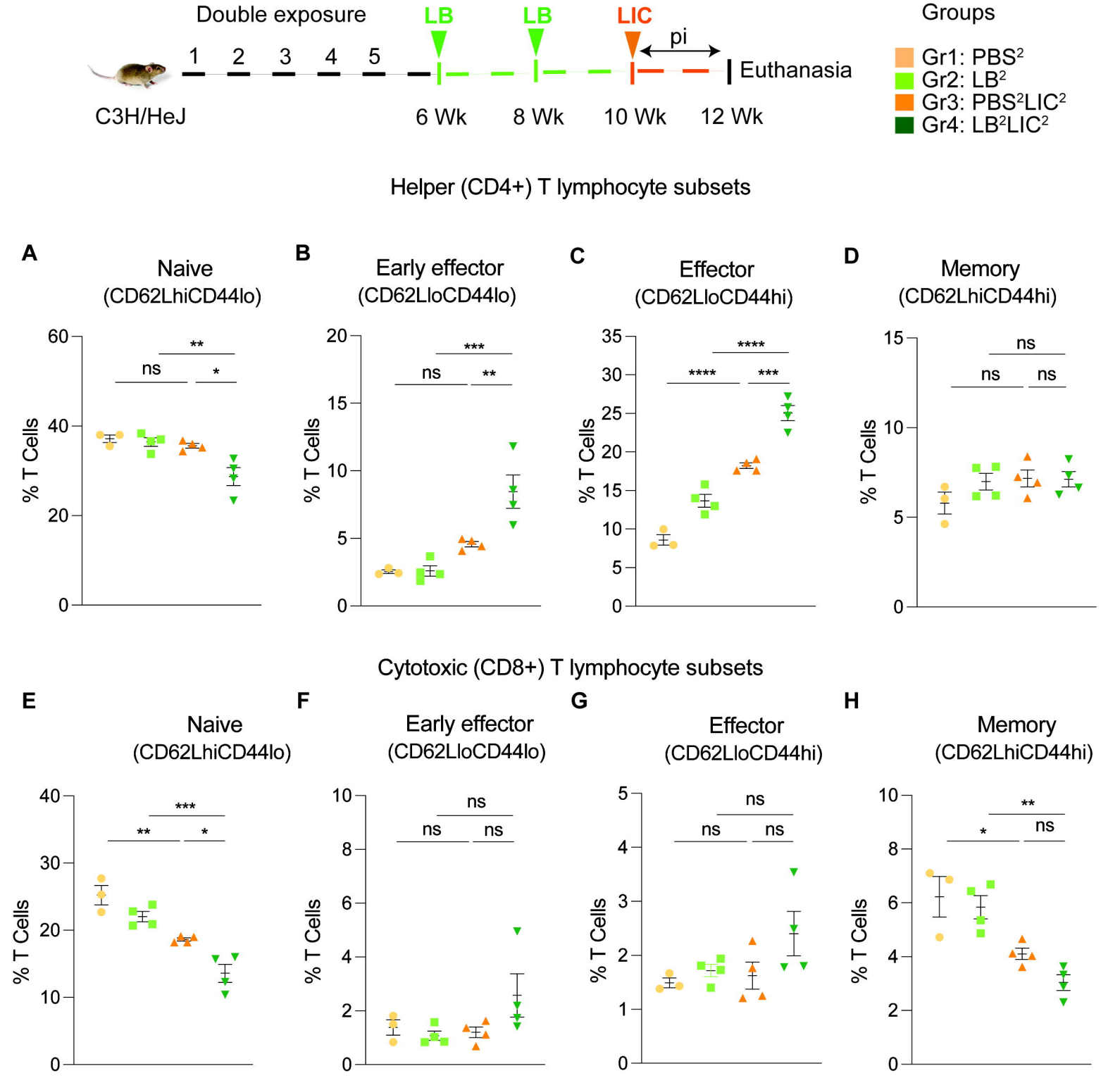

A
B cell
(CD19+)
% CD45+
0
20
40
60
80
***
****
ns


B
T cell
(CD3+)
% CD45+
0
10
20
30
40
50
***
***
ns


C
NK cell
(CD49b+)
% CD45+
0
2
4
6
8
10
ns
ns
ns


D
Helper T cells
(CD4+)
% T Cells
0
20
40
60
80
100
****
****
**


E
Cytotoxic T Cells
(CD8+)
% T Cells
0
10
20
30
40
50
****
****
**


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
Helper (CD4+) T lymphocyte subsets
A
Naive
(CD62LhiCD44lo)
B
Early effector
(CD62LloCD44lo)
C
Effector
(CD62LloCD44hi)
D
Memory
(CD62LhiCD44hi)
% T Cells
ns
*
**
***
****
Cytotoxic (CD8+) T lymphocyte subsets
E
Naive
(CD62LhiCD44lo)
F
Early effector
(CD62LloCD44lo)
G
Effector
(CD62LloCD44hi)
H
Memory
(CD62LhiCD44hi)