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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102 **Materials and Methods**
103 **Animals**
104 Male C3H/HeJ mice (n=6-8/group) were purchased from The Jackson Laboratory (Bar Harbor,
105 ME) and were maintained in a pathogen-free environment in the Laboratory Animal Care Unit
106 of the University of Tennessee Health Science Center (UTHSC). All experiments were performed
107 in compliance with the UTHSC Institutional Animal Care and Use Committee (IACUC), Protocol
108 no. 19-0062.

109 **Bacteria**

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

116 **Infection of mice and study design**

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

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

138 ***Leptospira* detection through qPCR**

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

147 **RNA isolation and RT-PCR**

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

153 (AGAGCGAGGCCTTCCCGGAC) and β-actin primers (Forward- CCACAGCTGAGAGGGAAATC,
154 Reverse- CCAATAGTGATGACCTGGCCG), TAMRA probe (GGAGATGGCCACTGCCGCATC) were
155 purchased from Eurofins Genomics.
156
157
158 **Histopathology by H&E staining**
159 Kidney tissues were fixed in formalin buffer. Histopathology was performed at the Histology
160 Department, UT Methodist University Hospital, Memphis, TN. Digital scanning of inflammatory
161 cell infiltration was measured by taking images of ~ 5 fields per sample under 20x
162 magnification. Images were captured after digitally scanning the H&E slides using Panoramic
163 350 Flash III (3D Histech, Hungary) and CaseViewer software.
164 **ELISA**
165 Leptospiral extract for *Leptospira biflexa* and *Leptospira interrogans* were prepared as
166 described previously (19). Briefly, *Leptospira* was cultured in EMJH media and once confluency
167 was observed, cells were centrifuged to obtain a pellet. This pellet was then incubated with
168 BugBuster® solution (1mL) at RT in a shaker incubator (100 rpm) for 20 min and homogenized
169 by vortexing. Stocks were stored at -20°C. This whole-cell extract of *Leptospira* was then diluted
170 in 1X sodium carbonate coating buffer. Nunc MaxiSorp flat-bottom 96 well plates (eBioscience,
171 San Diego, CA) were coated with extracts prepared from 10^7-10^8 bacteria per well and
172 incubated at 4°C overnight. Cells were washed using 1X PBST the following day and blocked for
173 1 h using 1% BSA solution, followed by another wash with 1X PBST. Serum samples (1:100)
174 were added to the antigen coated wells and incubated at 37°C for 1 h, washed twice with 1X

175 PBST, followed by HRP conjugate secondary anti-mouse- IgG1, IgG2a and IgG3 (1:10000) which
176 was incubated for 30 mins. After washing the plate 3 times with 1X PBST the color was
177 developed using TMB Sureblue followed by Stop solution before the absorbance was measured
178 at OD 450 nm using an ELISA plate reader (Molecular Devices Spetramax).

179 **Flow cytometry**

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

194 **Statistical analysis**

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

198

199 **Results**

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

216 We also found that a single exposure to *L. biflexa* before challenge did not prevent
217 dissemination of pathogenic *L. interrogans* in blood (Fig S2A) or shedding in urine (Fig 1D), or
218 kidney colonization (Fig 1E). Culture of kidney in EMJH media showed presence of ~ 2500
219 motile, morphologically intact *L. interrogans* under dark field microscopy which was confirmed
220 by 16S qPCR (Fig 1F) both on d3 and d5 post culture of kidney collected from LB^1LIC^1 mice;
221 kidney from PBS^1, LB^1 and PBS^1LIC^1 did not produce positive cultures by dark field microscopy or
222 16S qPCR.

223 In the double exposure study, mice were inoculated with 2 bi-weekly doses of *L. biflexa*
224 two weeks before challenge with *L. interrogans* (LB^2LIC^2) at 10 weeks in comparison with the
225 respective controls (Fig 2A). As expected, mice infected at 10 weeks with LIC that did not
226 receive *L. biflexa* (PBS^2LIC^2) lost ~ 11 % of weight on d11 post challenge and did not recover (Fig
227 2B). In contrast, mice that received a double dose of *L. biflexa* two weeks before challenge at 10
228 weeks with *L. interrogans* (LB^2LIC^2) lost a maximum of 5% weight on d10 and recovered fully by
229 d15 post infection; unchallenged control mice that received *L. biflexa* (LB^2) or PBS (PBS^2) gained
230 weight throughout the 15 days (Fig 2B). Survival curves generated after the mice reached
231 endpoint criteria (Fig 2C) show that all experimental groups survived LIC infection. Analysis of
232 bacterial dissemination showed that a double exposure to *L. biflexa* before challenge did not
233 prevent dissemination of pathogenic *L. interrogans* in blood (Fig S2B), or shedding in urine (Fig
234 2D), or kidney colonization (Fig 2E). Culture of kidney in EMJH media showed presence of 5,000-
235 10,000 motile, morphologically intact *L. interrogans* on d3 and d5 post culture of kidney
236 collected from PBS^2LIC^2 mice in contrast to 1,000 to 2,500 live *L. interrogans* observed in culture

237 from kidney collected from LB^2LIC^2 mice which was confirmed by 16S qPCR (Fig 2F); kidney from
238 PBS^2 and LB^2 mice did not produce positive cultures by dark field microscopy or 16S qPCR.
239
240 ***L. biflexa* exposure before challenge with *L. interrogans* mitigates renal histopathological**
241 **changes.** As expected, H&E staining of histological slices of all kidneys from mice challenged
242 with *L. interrogans* in both single and double exposure experiments (PBS^1LIC^1 and PBS^2LIC^2)
243 showed signs of inflammation with increased immune cell infiltration (Fig 3A and 3C). In
244 contrast, H&E staining of kidney slices from the groups of mice exposed to *L. biflexa* before *L.*
245 *interrogans* challenge (LB^1LIC^1 and LB^2LIC^2) showed reduced immune cell infiltration. We also
246 measured expression of a marker (ColA1) for kidney fibrosis. In both experiments, kidneys from
247 mice challenged with *L. interrogans* (PBS^1LIC^1 and PBS^2LIC^2) had significantly higher expression
248 of ColA1 as compared to the controls; in contrast, kidneys from mice challenged with *L.*
249 *interrogans* after exposure to *L. biflexa* (LB^1LIC^1 and LB^2LIC^2) were not different than the
250 controls (Figs 3B and 3D).
251 In addition, we were able to collect kidneys from experimental mice subjected to the
252 double exposure of *L. biflexa* because they all survived subsequent challenge with *L.*
253 *interrogans*. As such, we did a comparative gross morphological analysis between the 4 groups
254 (Fig S3). We found that kidney from LB^2LIC^2 mice maintained their normal gross anatomy and
255 coloration as did kidneys from control mice (PBS^2 and LB^2), in contrast to kidneys from mice
256 challenged with *L. interrogans* that were not previously exposed to *L. biflexa* (PBS^2LIC^2).
257

258 **Serologic IgG2a responses to *L. interrogans* were significantly higher in mice pre-exposed to *L.***
259 ***biflexa* before challenge with *L. interrogans***. In both single and double *L. biflexa* exposure
260 experiments we measured anti-*L. interrogans* total IgM, total IgG, and IgG subtypes IgG1, IgG2a
261 and IgG3 in serum collected 2 weeks after challenge with *L. interrogans* (IgG subtypes shown in
262 Figs 4A and 4B). In both experiments, total IgM and IgG were significantly increased in PBS-LIC
263 and LB-LIC when compared to the respective controls, but not between PBS-LIC and LB-LIC
264 groups. Regarding IgG isotypes, IgG1 was generally low and IgG2a as well as IgG3 were
265 generally high in groups infected with *L. interrogans*. Although differences between groups
266 (PBS^2v PBS^2LIC^2 and $LB^{1/2}$ v $LB^{1/2}LIC^{1/2}$) were significant, differences between the LIC infected
267 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
268 (p=0.001 for single exposure and p=0.0095 for double exposure).
269
270 **Exposure to non-pathogenic *L. biflexa* before pathogenic *L. interrogans* challenge induced**
271 **increased frequencies of effector helper T cells in spleen**. We immunophenotyped the spleen
272 cells from the mice subjected to double *L. biflexa* exposure because all animals survived to the
273 term of the experiment. Mice in the single exposure experiment met endpoint criteria before
274 the term of the experiment and thus we were not able to process spleen for
275 immunophenotyping. In the *L. biflexa* double exposure experiment, we measured increased
276 frequencies in B cells when LIC infected mice were compared to the respective controls, but not
277 between PBS^2LIC^2 and LB^2LIC^2 mice (Fig 5A). We measured decreased frequencies in T cells
278 when LIC infected mice were compared to the respective controls but not between PBS^2LIC^2
279 and LB^2LIC^2 mice (Fig 5B). No differences were observed in NK cells between any of the groups

280 (Fig 5C). We measured increased frequencies in helper T cells between all groups; of note,
281 PBS^2LIC^2 vs LB^2LIC^2 p=0.006 (Fig 5D). We also measured decreased frequencies in cytotoxic T
282 cells between all groups; of note PBS^2LIC^2 vs LB^2LIC^2 p=0.0056 (Fig 5E). Furthermore, T cell
283 subset typing (Fig 6) showed that frequency of early effector CD4+ T helper cells (Fig 6B, CD44-
284 CD62L-) and effector T helper cells (Fig 6C, CD44+CD62L-) were significantly increased when
285 compared between the LIC challenged groups and the respective controls (except PBS^2 v
286 PBS^2LIC^2 early effectors) and that frequency of early effector and effector T helper cells were
287 higher in the LB^2LIC^2 group than PBS^2LIC^2. No major changes were measured in memory CD4+ T
288 helper cells (Fig 6D, CD44+CD62L+). In the CD8+ cytotoxic T cell subsets, we measured
289 significant decreases in frequency of naïve T cells between LIC infected groups and the
290 respective controls (Fig 6E, CD62L+CD44-) and this was replicated in the CD8+ cytotoxic
291 memory except that differences with the LIC infected groups were not significant (Fig 6H,
292 CD44+CD62L+).
293
294 **Discussion**
295 Understanding host immune responses to *Leptospira* infection is crucial for advancing our
296 ability to develop new control measures for leptospirosis (19, 21-25). Given their widespread
297 presence in nature, the likelihood of humans or animals getting exposed to non-pathogenic
298 serovars of *Leptospira* is likely high (10, 11). Our previous studies showed innate immunity
299 engagement during saprophytic *L. biflexa* infection in mice (15, 16). In addition, *L. biflexa*
300 extracts can be used to detect *Leptospira*-specific antibody in up to 67% of serum from patients
301 with clinically confirmed leptospirosis (19) which points to a high degree of immunodominant

302 cross-reactive epitopes between *L. biflexa* and pathogenic *Leptospira*. Further, the current
303 hypothesis regarding evolution of *Leptospira* species is that symbiosis of *Leptospira* with
304 eukaryotes emerged from free-living ancestral species (26); in other words, pathogenic
305 *Leptospira* may have evolved from an environmental ancestor (6). Thus, we hypothesized that
306 these highly cross-reactive immunodominant epitopes may also induce cross-protective
307 immune responses. The objective of the current study was to assess whether exposure to a live
308 saprophytic serovar of *Leptospira* provides any heterologous cross-species protection against a
309 subsequent challenge with a pathogenic serovar in a mammalian host (mouse).

310 In the initial analysis of pathogenesis (Fig 1, Fig 2, Fig S2) we observed that prior
311 exposure to one or two doses of saprophytic *L. biflexa* rescues weight loss in mice challenged
312 with pathogenic *L. interrogans* at 8 weeks (LB^1LIC^1) and at 10 weeks (LB^2LIC^2), respectively.
313 Weight gain correlated with survival (75% survival in LB^1LIC^1 versus 0% survival of the PBS^1LIC^1
314 group) in the single exposure experiment, where we expected all mice infected at 8 weeks with
315 pathogenic *L. interrogans* (PBS^1LIC^1) to irreversibly lose weight and meet endpoint criteria for
316 euthanasia before the 2 week term of the experiment (23, 27). Loss of mice due to irreversible
317 weight loss is not expected if mice are infected with LIC at 10 weeks of age (17), as observed in
318 the *L. interrogans* control group in the double exposure experiment (PBS^2LIC^2). In both
319 experiments, mice exposed to *L. biflexa* before challenge with LIC, produced evidence of *L.*
320 *interrogans* dissemination in blood, shedding in urine and kidney colonization of the kidney.

321 Histological inspection of kidney slices (Fig 3) showed that exposure to a saprophytic
322 *Leptospira* before challenge supported normal structural morphology and prevented infiltration
323 of immune cells in both single and double exposure experiments; in addition, it significantly

324 reduced a fibrosis marker (ColA1) in the single exposure experiment. In the double exposure
325 experiment, differences in ColA1 fibrosis marker are not significant between the two LIC
326 infected groups because 10-week old C3H-HeJ infected with LIC are more resistant to pathology
327 resultant from infection. Our findings are intriguing as they suggest that while prior live
328 saprophytic exposure did not prevent infection or leptospiral dissemination, it may confer
329 protection against kidney fibrosis.

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

342 Differences in antibody titer among the *L. interrogans* infected group pre-exposed to
343 saprophytic *L. biflexa* can be attributed to the robust trafficking and differentiation of B and
344 helper T cells (CD4+) measured in spleen (Fig 5 and Fig 6). Presence of effector helper T (CD4+)
345 cells in the spleen indicate a robust cellular immune response as these cells produce cytokines

346 that play a pivotal role in activating other immune cells, including antibody-producing B cells.
347 Moreover, our findings align with another observation which further reinforces the potential
348 immunostimulatory properties of components (polar lipids) derived from saprophytic *L. biflexa*,
349 indicating that these components could play a crucial role in inducing robust B cell responses
350 (29). Induction of helper T cell responses along with dynamic transition from naïve to early
351 effector and effector without T helper memory reflects an orchestrated immune response upon
352 pathogenic challenge in the saprophytic pre-exposed group that is typical of effective responses
353 to vaccines. Previous studies have highlighted the significance of activated CD4+ T cells during
354 *Leptospira* infection in providing protective immunity to the host and mitigating the severity of
355 leptospirosis by releasing cytokines (30). Correlating induction of chemo-cytokines by
356 saprophytic *Leptospira* with subsequent adaptive immune responses, such as the activation of
357 CD4+ T cells or the production of specific antibodies, provides insights into how innate immune
358 signals drive the adaptive immune response against a pathogenic threat. It may also aid in
359 identifying key signaling molecules or pathways that could be targeted for therapeutic
360 interventions or vaccine design.

361 While other researchers have explored vaccination strategies using live attenuated or
362 mutant strains of pathogenic serovars, our approach was to utilize a live saprophytic bacterial
363 strain which is unique in the field (21, 23, 31, 32). We previously showed that oral delivery of a
364 probiotic strain, *Lactobacillus plantarum*, reduces the severity of leptospirosis by recruiting
365 myeloid cells (21) which suggests that a general phenomenon of trained immunity may be
366 involved. Current vaccines based in inactivated pathogenic species provide equivalent
367 protection to the one achieved in this study (5, 33) with the caveat of being serovar specific (32-

368 34). Although our current study conclusively shows protection from severe leptospirosis after
369 heterologous challenge, it remains to be shown if protection extends to multiple pathogenic
370 serovars of *Leptospira*. Using a live saprophytic strain of *Leptospira* as control strategy could
371 pave the way for development of novel broadly effective vaccines against leptospirosis. Such a
372 vaccine could have a substantial economic impact if applied to animals of agricultural interest.

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

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

389

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

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

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502

503

504 **Figure Legends:**

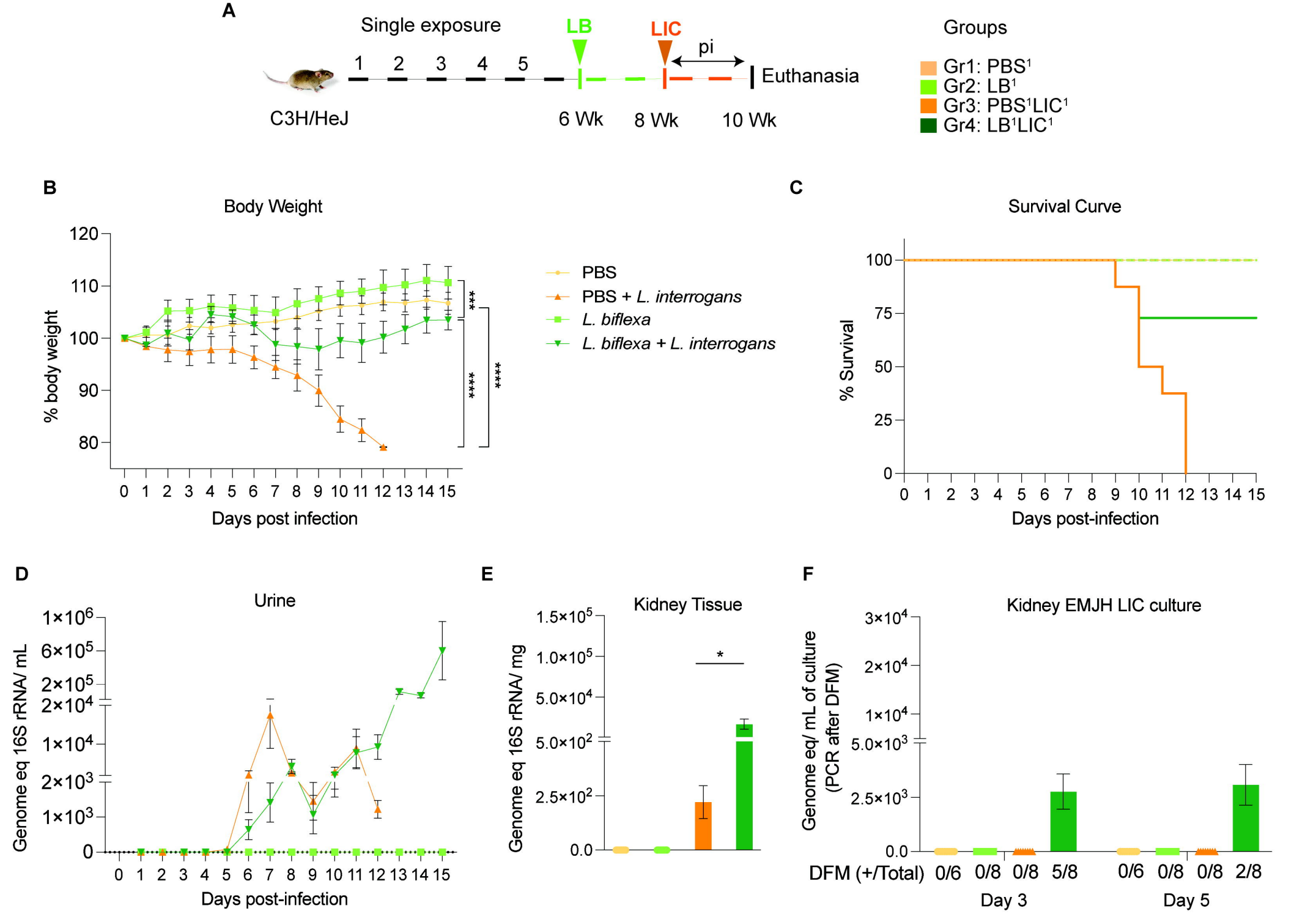
505 **Figure 1: Weight loss, kidney colonization, shedding in urine and survival to challenge with**
506 ***Leptospira interrogans* following a single exposure to *L. biflexa*.** Male C3H/HeJ mice were
507 inoculated once with 10^8 *L. biflexa* (LB) at 6 weeks and they were challenged with 10^8 *L.*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

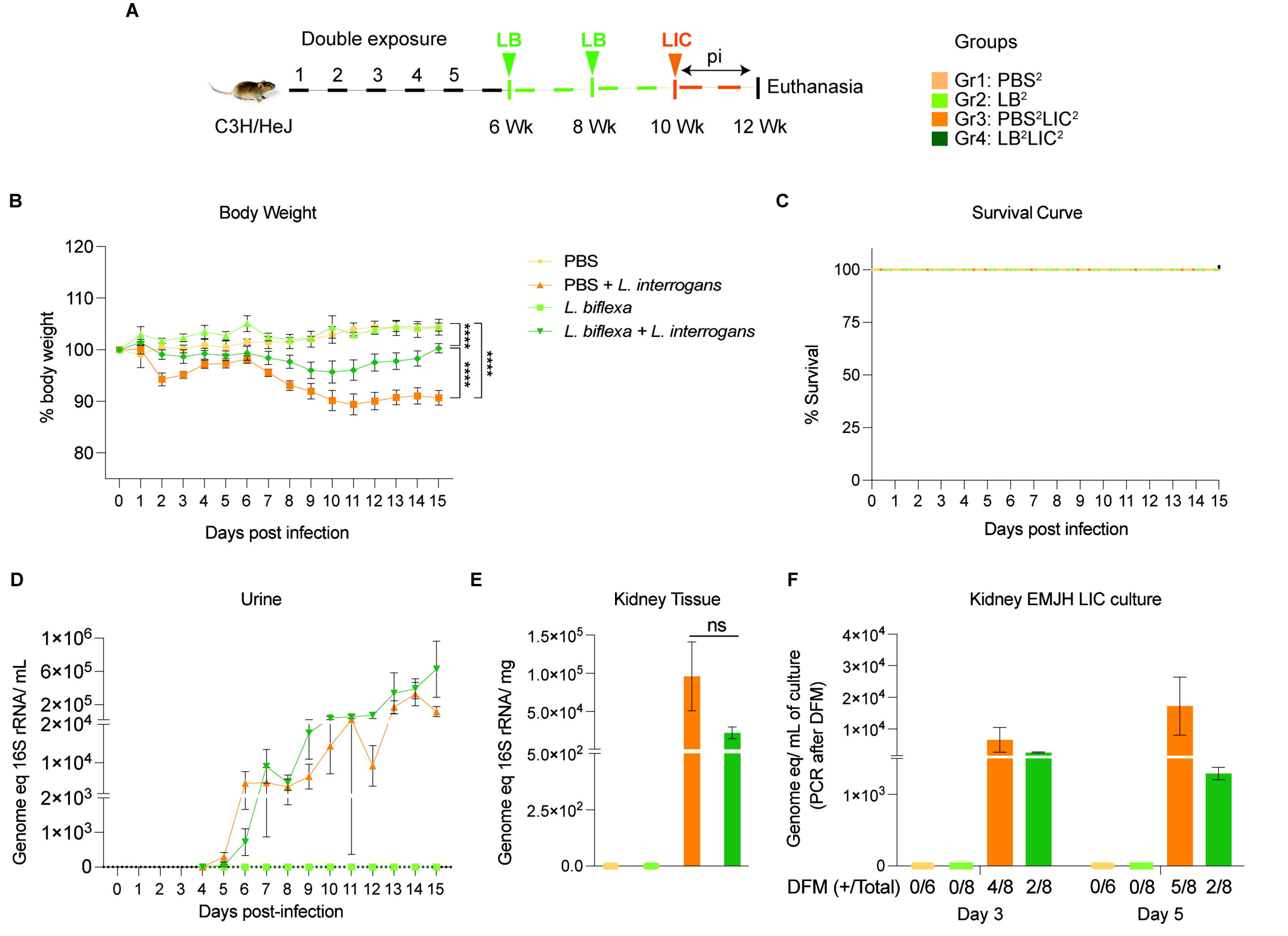
518

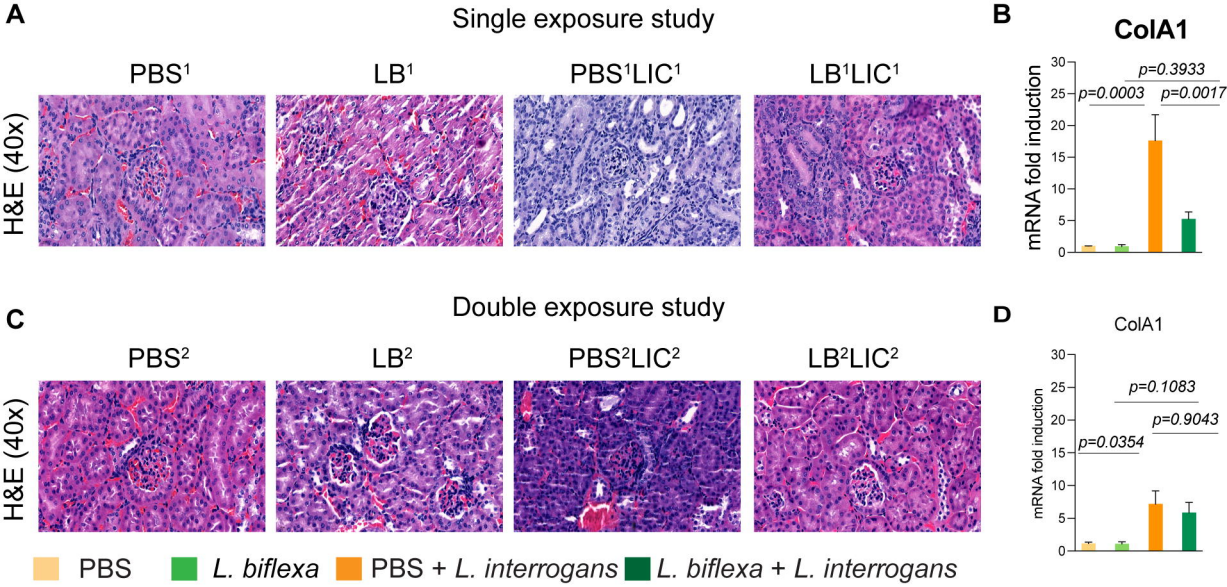
519 **Figure 2: Weight loss, kidney colonization, shedding in urine and survival to challenge with**
520 ***Leptospira interrogans* following a double exposure to *L. biflexa*.** Male C3H/HeJ mice were
521 inoculated twice with 10^8 *L. biflexa* at 6 and 8 weeks, and at 10 weeks they were challenged
522 with 10^8 *L. interrogans* ser Copenhageni FioCruz (LIC). A) experimental layout; B) body weight
523 measurements (%) acquired for 15 days post challenge with LIC; C) mouse survival within the 15

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

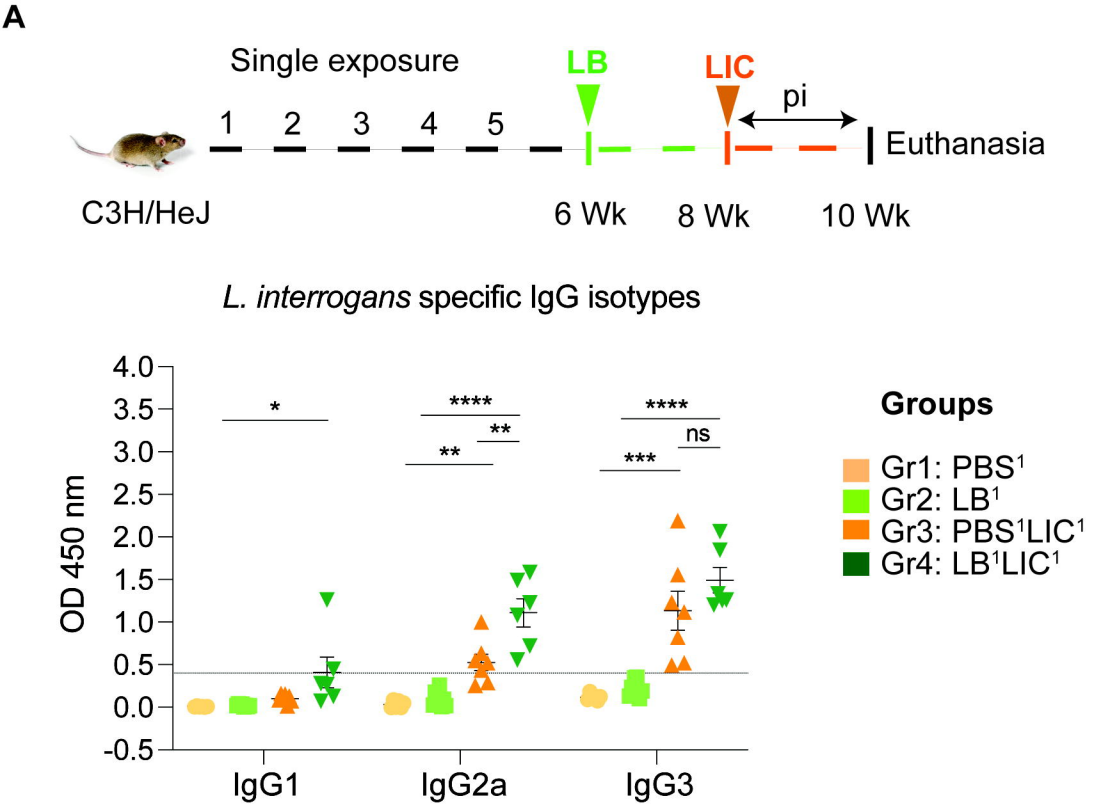
546 correction test was used to compare between challenged groups with their respective controls;
547 *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns= not significant; N= 6-8 mice per group.
548 Data represents two independent experiments.
549
550 **Figure 5: Frequency of lymphocytes in spleen of mice subjected to a double exposure of *L.***
551 ***biflexa* before challenge with *L. interrogans*.** A to E) show B cell (CD19+), T cell (CD3+), NK cell
552 (CD49b+), Helper T cell (CD4+) and Cytotoxic T cell (CD8+) frequencies in groups of
553 experimental mice. Ordinary one-way ANOVA followed by Tukey's multiple comparison
554 correction test was used to compare between challenged groups and their respective controls;
555 **p<0.01, ***p<0.001, ****p<0.0001 and ns= not significant; N= 3-4 mice per group. Data
556 represents one of two independent experiments.
557
558 **Figure 6: Frequency of T cell subsets (CD62L/CD44) in spleen of mice subjected to a double**
559 **exposure of *L. biflexa* before challenge with *L. interrogans*.** A to D) represent naïve, early
560 effector, effector and memory subsets of CD4+ helper T lymphocytes, respectively. E to H)
561 represent naive, early effector, effector and memory subsets of CD8+ cytotoxic T lymphocytes,
562 respectively. Ordinary one-way ANOVA followed by Tukey's multiple comparison correction test
563 was used to compare between challenged groups and their respective controls; *p<0.05,
564 **p<0.01, ***p<0.001, ****p<0.0001 and ns= not significant; N = 3-4 mice per group. Data
565 represents one of two independent experiments.
566
567


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

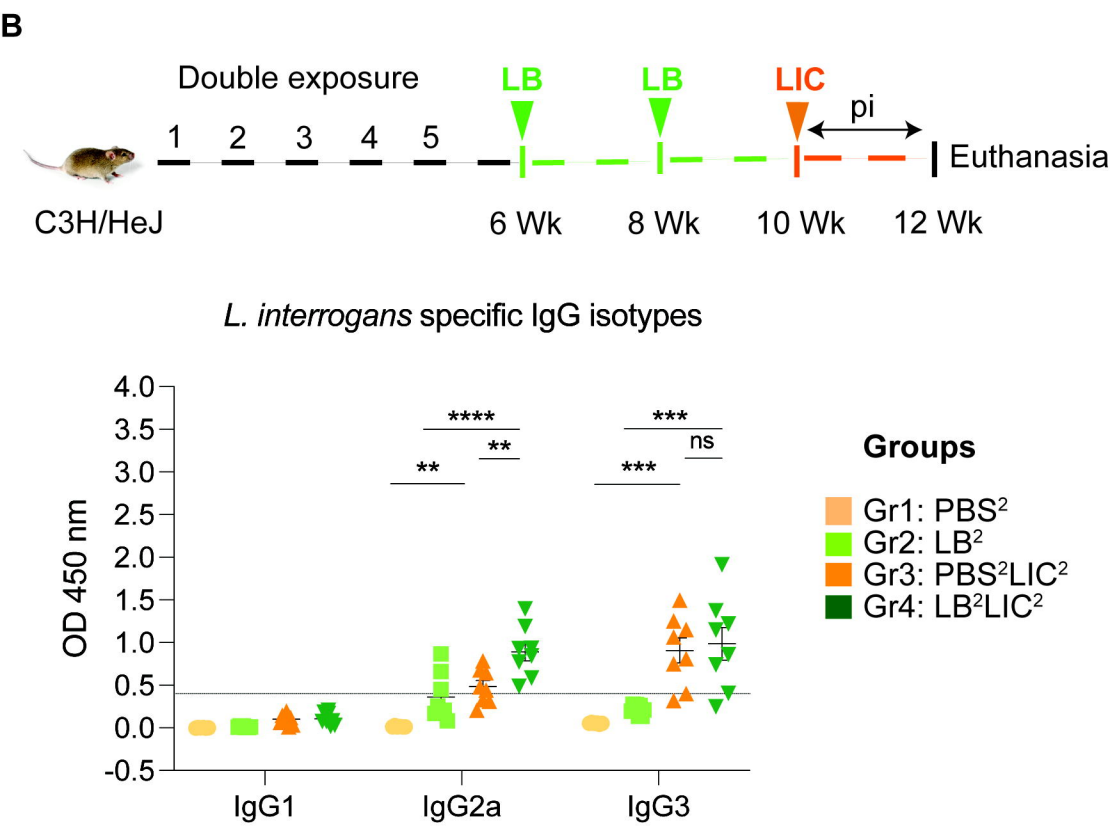

A
Double exposure
LB
LB
LIC
pi
1 2 3 4 5
Euthanasia
C3H/HeJ
6 Wk
8 Wk
10 Wk
12 Wk
Groups
Gr1: PBS²
Gr2: LB²
Gr3: PBS²LIC²
Gr4: LB²LIC²
B
Body Weight
% body weight
Days post infection
PBS
PBS + L. interrogans
L. biflexa
L. biflexa + L. interrogans
****
C
Survival Curve
% Survival
Days post infection
D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

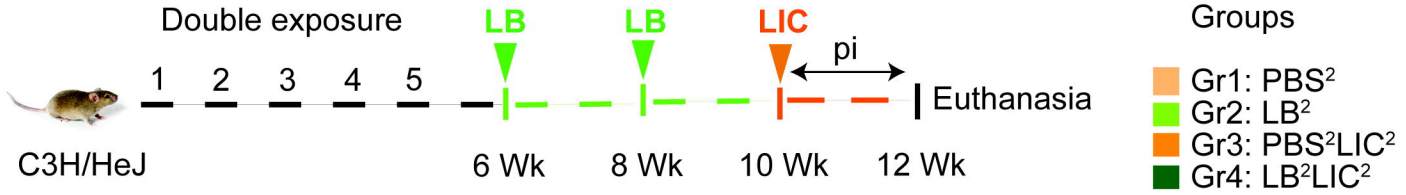

A
Single exposure study
PBS1
LB1
PBS1LIC1
LB1LIC1
H&E (40x)
B
ColA1
mRNA fold induction
p=0.3933
p=0.0003
p=0.0017
C
Double exposure study
PBS2
LB2
PBS2LIC2
LB2LIC2
H&E (40x)
D
ColA1
mRNA fold induction
p=0.1083
p=0.9043
p=0.0354
PBS
L. biflexa
PBS + L. interrogans
L. biflexa + L. interrogans

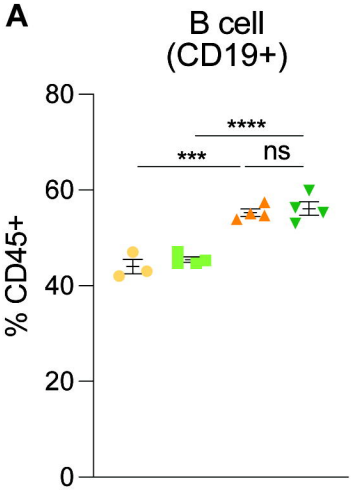
A

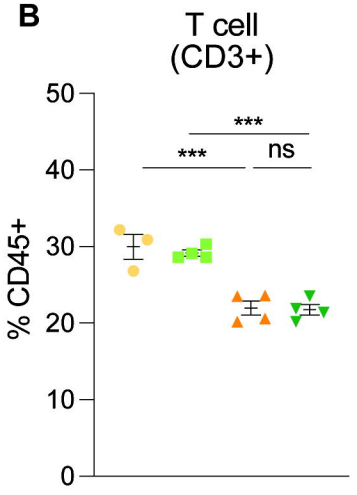

Single exposure
LB
LIC
pi
1
2
3
4
5
Euthanasia
C3H/HeJ
6 Wk
8 Wk
10 Wk
L. interrogans specific IgG isotypes
OD 450 nm
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
*
****
**
**
****
ns
***
IgG1
IgG2a
IgG3
Groups
Gr1: PBS1
Gr2: LB1
Gr3: PBS1LIC1
Gr4: LB1LIC1

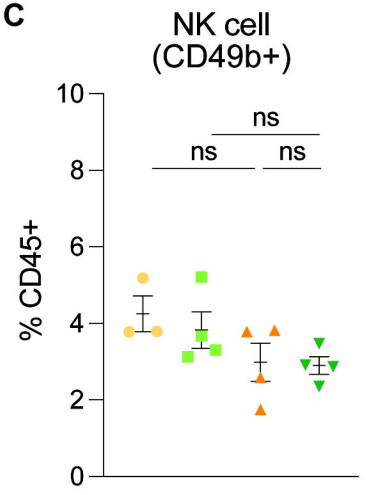
B

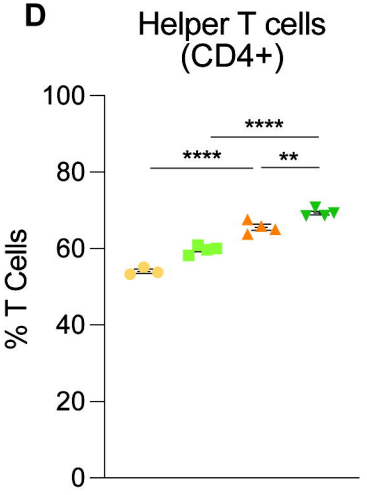

Double exposure
LB
LB
LIC
pi
1
2
3
4
5
Euthanasia
C3H/HeJ
6 Wk
8 Wk
10 Wk
12 Wk
L. interrogans specific IgG isotypes
OD 450 nm
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
****
**
**
***
ns
***
IgG1
IgG2a
IgG3
Groups
Gr1: PBS2
Gr2: LB2
Gr3: PBS2LIC2
Gr4: LB2LIC2

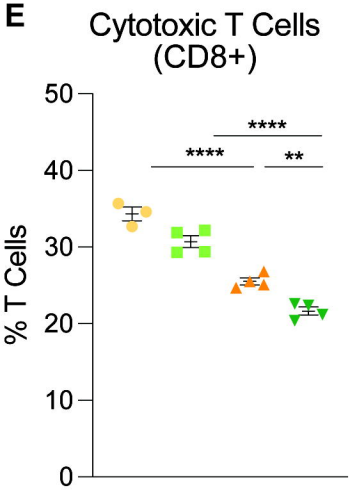

Double exposure
LB
LB
LIC
pi
1 2 3 4 5
Euthanasia
C3H/HeJ
6 Wk
8 Wk
10 Wk
12 Wk
Groups
Gr1: PBS²
Gr2: LB²
Gr3: PBS²LIC²
Gr4: LB²LIC²

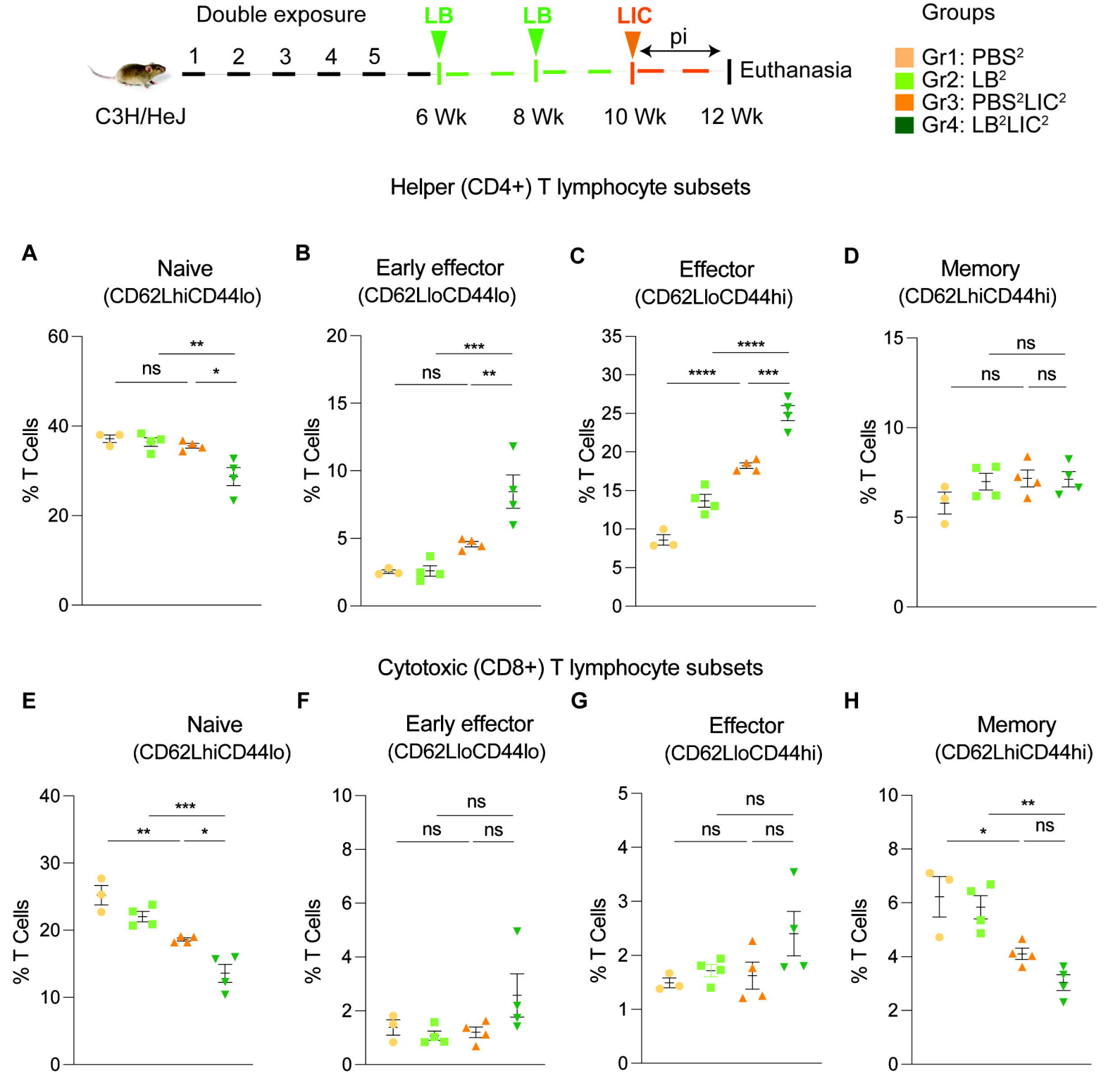

A
B cell
(CD19+)
% CD45+
0
20
40
60
80
***
****
ns


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)