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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.

Significance

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

eLife assessment

This **important** study contributes to our understanding on how prior exposure to a non-pathogenic Leptospira strain could prime the host to prevent severe leptospirosis following infection with a pathogenic strain. The work described is **solid** and broadly supports the claims, with minor weaknesses that could be addressed in future studies. The work will be of interest to scientists interested in host-pathogen interactions and leptospirosis.

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Introduction

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

The genus *Leptospira* is broadly categorized into 2 major clades P and S (Pathogens and Saprophytes) and further categorized into 4 subclades, P1, P2, S1 and S2 based on their virulence properties, growth conditions and genetic make-up (6, 7). Subclade P1 is further divided in two phylogenetically related groups named P1+ (high-virulence pathogens, established pathogenic species, e.g. *L. interrogans*) and P1-(low-virulence pathogens, phenotypically not well-characterized) (8). *Leptospira* survives in moist conditions and are free-living organisms naturally found in soil and water bodies (9, 10). The spread of infection occurs through contaminated water contact with breached skin or mucosal surfaces (11). Saprophytic strains of *Leptospira,* such as *L. biflexa* (S1), are unable to establish disease due to the lack of certain virulence factors (7) and have been found in natural environments around the world alongside pathogenic serovars (6, 11, 12). Moreover, *L. biflexa* exhibits certain niche-specific adaptations that allow them to persist in both environmental and host setting (13, 14).

Our previous studies (15, 16) demonstrated that *L. biflexa* triggers a robust innate immune response in mice during the acute phase of infection. This raised the question of whether saprophytic *Leptospira* induced immune responses could confer any degree of resistance or immune memory that could suppress a subsequent challenge with a pathogenic serovar of *Leptospira*. Answering that question was the main goal of the current study.

Materials and Methods

Animals

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

Bacteria

Non-pathogenic *Leptospira biflexa* serovar Patoc (LB) belonging to subclade S1 was purchased from ATCC and grown in EMJH media. Pathogenic *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 (LIC) belonging to subclade P1+ (high-virulence pathogens) (6, 8) 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 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 (n=3) was the naive control which received PBS (PBS^1), Group 2 (n=4) was inoculated with 10^8 *L. biflexa* once at 6 weeks (LB^1), Group 3 (n=4) received PBS for 2 weeks followed by challenge with 10^8 *L. interrogans* at 8 weeks (PBS^1LIC^1), and Group 4 (n=4) was 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 (n=3) was the naive control which received PBS (PBS^2), Group 2 (n=4) received 10^8 *L. biflexa* IP at 6 and 8 weeks (LB^2), Group 3 (n=4) received PBS for 2 weeks followed by challenge with 10^8 *L. interrogans* at 10 weeks (PBS^2LIC^2), and Group 4 (n=4) was 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 for flow cytometric analysis was collected from mice after euthanasia only in the double exposure study given that all mice consistently survived challenge.

Leptospira detection through qPCR

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

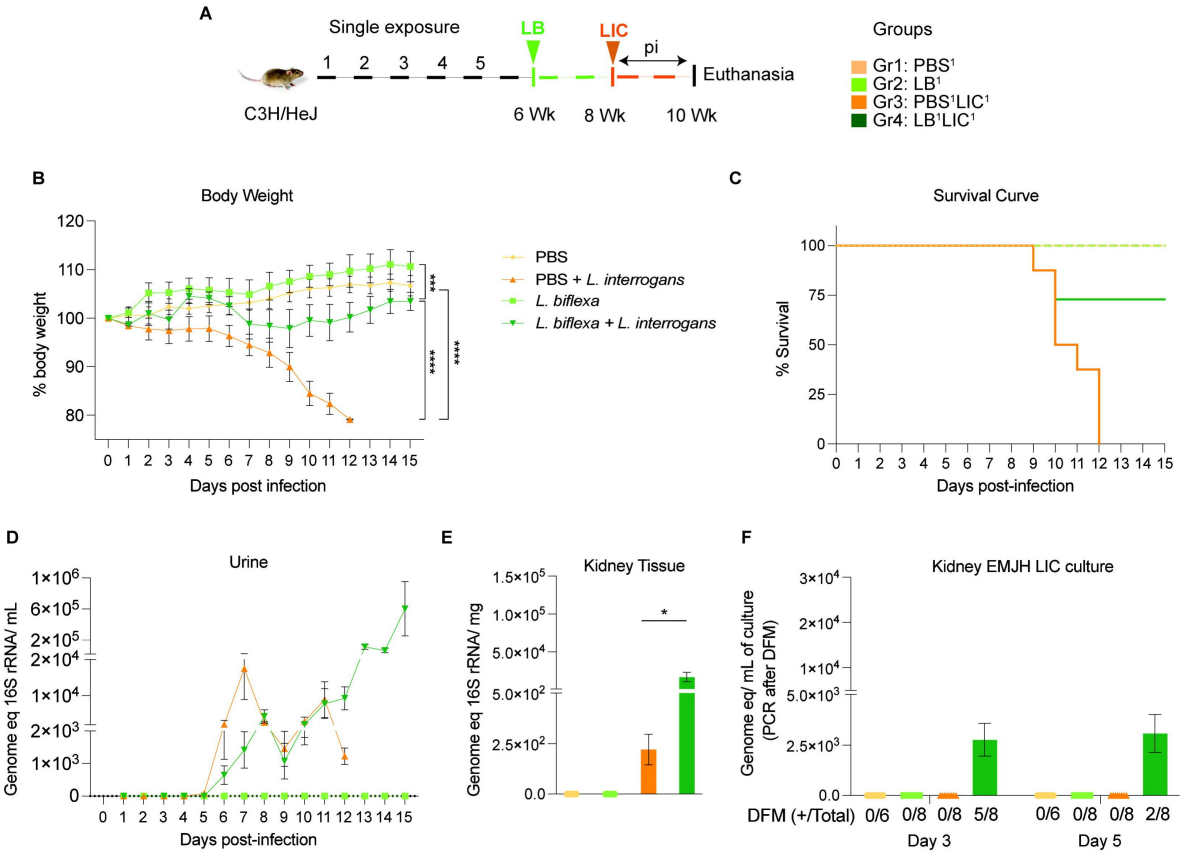

Figure 1

Weight loss, kidney colonization, shedding in urine and survival to challenge with *Leptospira interrogans* following a single exposure to *L. biflexa*.

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

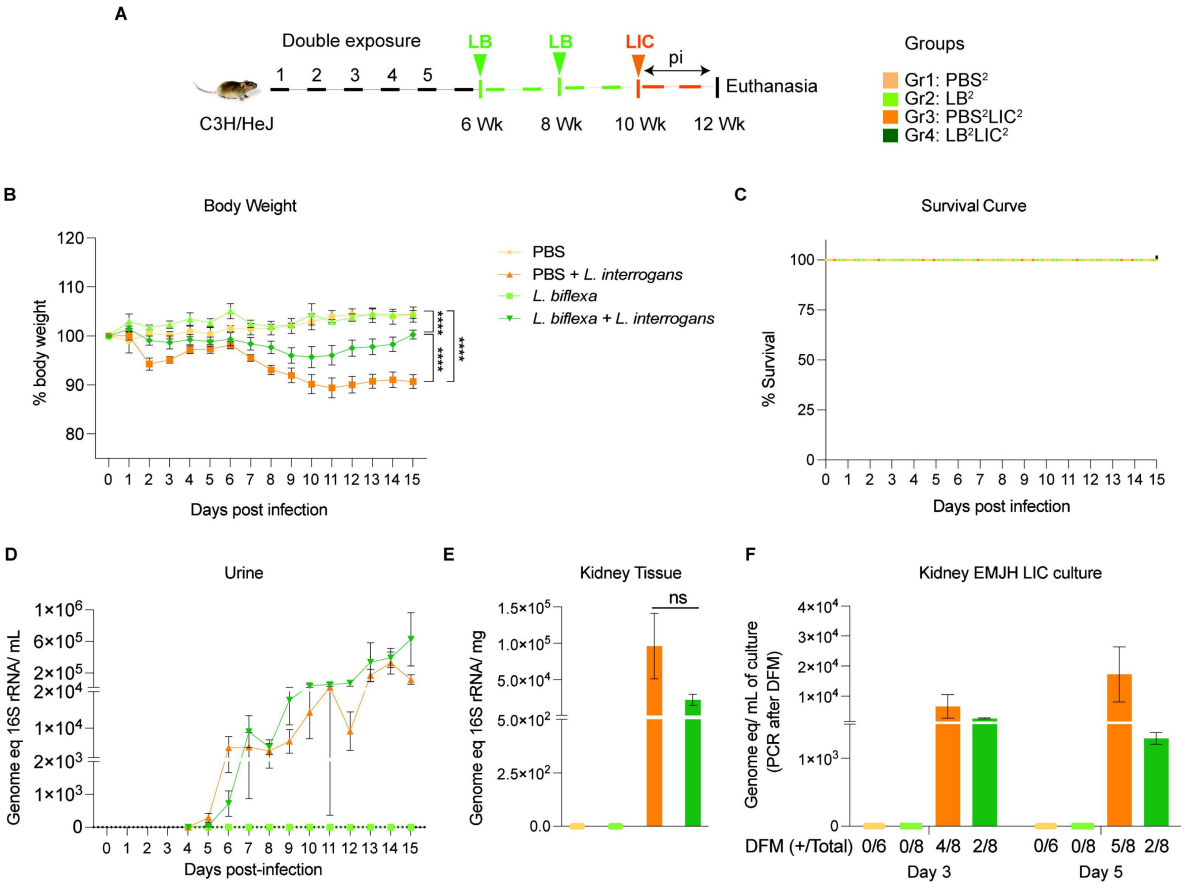

Figure 2

Weight loss, kidney colonization, shedding in urine and survival to challenge with *Leptospira interrogans* following a double exposure to *L. biflexa*.

Male C3H/HeJ mice were inoculated twice with 10^8 *L. biflexa* at 6 and 8 weeks, and at 10 weeks they were challenged with 10^8 *L. interrogans* ser Copenhageni FioCruz (LIC). A) experimental layout; B) body weight measurements (%) acquired for 15 days post challenge with LIC; C) mouse survival within the 15 days post challenge with LIC; D) 16s rRNA qPCR quantification of live LIC in urine; E) 16s rRNA qPCR quantification of *Leptospira* burden in kidney tissue harvested on d15 post challenge with LIC and F) 16s rRNA qPCR from kidney EMJH cultures containing live *Leptospira* previously observed by dark field microscopy (DFM). DFM Positive culture from the total data is represented in numbers under the graph. Statistical analysis was performed by ordinary one-way ANOVA followed by Tukey's multiple comparison correction between challenged groups and their respective controls,*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. N = 6-8 mice per group. Data represents two independent experiments.

RNA isolation and RT-PCR

Kidneys were stored in RNA later after euthanasia. RNeasy mini kit (Qiagen) was used to extract total RNA from kidney tissue according to the manufacturer's specifications. RNA purity was measured using a Nanodrop instrument (Thermo Scientific) at A260/280 ratio. cDNA was prepared using cDNA reverse transcription kit (Applied Biosystems). ColA1 primers (Forward-TAAGGGTACCGCTGGAGAAC, Reverse-GTTCACCTCTCTCACCAGCA), TAMRA probe (AGAGCGAGGCCTTCCCGGAC) and β-actin primers (Forward-CCACAGCTGAGAGGGAAATC, Reverse-CCAATAGTGATGACCTGGCCG), TAMRA probe (GGAGATGGCCACTGCCGCATC) were purchased from Eurofins Genomics.

Histopathology by H&E staining

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

ELISA

Leptospiral extract for *Leptospira biflexa* and *Leptospira interrogans* were prepared as described previously (19). Briefly, *Leptospira* was cultured in EMJH media and once confluency was observed, cells were centrifuged to obtain a pellet. This pellet was then incubated with BugBuster® solution (1mL) at RT in a shaker incubator (100 rpm) for 20 min and homogenized by vortexing. Stocks were stored at -20°C. This whole-cell extract of *Leptospira* was then diluted in 1X sodium carbonate coating buffer. Nunc MaxiSorp flat-bottom 96 well plates (eBioscience, San Diego, CA) were coated with extracts prepared from 10^7-10^8 bacteria per well and incubated at 4°C overnight. Cells were washed using 1X PBST the following day and blocked for 1 h using 1% BSA solution, followed by another wash with 1X PBST. Serum samples (1:100) were added to the antigen coated wells and incubated at 37°C for 1 h, washed twice with 1X PBST, followed by HRP conjugate secondary anti-mouse-IgG1, IgG2a and IgG3 (1:10000) which was incubated for 30 mins. After washing the plate 3 times with 1X PBST the color was developed using TMB Sureblue followed by Stop solution before the absorbance was measured at OD 450 nm using an ELISA plate reader (Molecular Devices Spetramax).

Flow cytometry

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

Statistical analysis

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

Results

Exposure to saprophytic *Leptospira* before infection with a pathogenic serovar prevents disease and increases survival of C3H-HeJ mice

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

In the double exposure study, mice were inoculated with 2 bi-weekly doses of *L. biflexa* two weeks before challenge with *L. interrogans* (LB^2LIC^2) at 10 weeks in comparison with the respective controls (**Fig 2A**). As expected, mice infected at 10 weeks with LIC that did not receive *L. biflexa* (PBS^2LIC^2) lost ~ 11 % of weight on d11 post challenge and did not recover (**Fig 2B**). In contrast, mice that received a double dose of *L. biflexa* two weeks before challenge at 10 weeks with *L. interrogans* (LB^2LIC^2) lost a maximum of 5% weight on d10 and recovered fully by d15 post infection; unchallenged control mice that received *L. biflexa* (LB^2) or PBS (PBS^2) gained weight throughout the 15 days (**Fig 2B**). Survival curves generated after the mice reached endpoint criteria (**Fig 2C**) show that all experimental groups survived LIC infection. Analysis of bacterial dissemination showed that a double exposure to *L. biflexa* before challenge did not prevent dissemination of pathogenic *L. interrogans* in blood (Fig S2B), or shedding in urine (**Fig 2D**), or kidney colonization (**Fig 2E**). Culture of kidney in EMJH media showed presence of 5,000-10,000 motile, morphologically intact *L. interrogans* on d3 and d5 post culture of kidney collected from PBS^2LIC^2 mice in contrast to 1,000 to 2,500 live *L. interrogans* observed in culture from kidney collected from LB^2LIC^2 mice which was confirmed by 16S qPCR (**Fig 2F**); kidney from PBS^2 and LB^2 mice did not produce positive cultures by dark field microscopy or 16S qPCR.

L. biflexa exposure before challenge with *L. interrogans* mitigates renal histopathological changes

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

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

Serologic IgG2a responses to *L. interrogans* were significantly higher in mice pre-exposed to *L. biflexa* before challenge with *L. interrogans*

In both single and double *L. biflexa* exposure experiments we measured anti-*L. interrogans* total IgM, total IgG, and IgG subtypes IgG1, IgG2a and IgG3 in serum collected 2 weeks after challenge with *L. interrogans* (IgG subtypes shown in **Figs 4A** and **4B**). In both experiments, total IgM and IgG were significantly increased in PBS-LIC and LB-LIC when compared to the respective controls, but not between PBS-LIC and LB-LIC groups. Regarding IgG isotypes, IgG1 was generally low and IgG2a as well as IgG3 were generally high in groups infected with *L. interrogans*. Although differences between groups (PBS^2v PBS^2LIC^2 and $LB^{1/2}$ v $LB^{1/2}LIC^{1/2}$) were significant, differences between the LIC infected 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 (p=0.001 for single exposure and p=0.0095 for double exposure).

Exposure to non-pathogenic *L. biflexa* before pathogenic *L. interrogans* challenge induced increased frequencies of effector helper T cells in spleen

We immunophenotyped the spleen cells from the mice subjected to double *L. biflexa* exposure because all animals survived to the term of the experiment. Mice in the single exposure experiment met endpoint criteria before the term of the experiment and thus we were not able to process spleen for immunophenotyping. In the *L. biflexa* double exposure experiment, we measured increased frequencies in B cells when LIC infected mice were compared to the respective controls, but not between PBS^2LIC^2 and LB^2LIC^2 mice (**Fig 5A**). We measured decreased frequencies in T cells when LIC infected mice were compared to the respective controls but not between PBS^2LIC^2 and LB^2LIC^2 mice (**Fig 5B**). No differences were observed in NK cells between any of the groups (**Fig 5C**). We measured increased frequencies in helper T cells between all groups; of note, PBS^2LIC^2 vs LB^2LIC^2 p=0.006 (**Fig 5D**). We also measured decreased frequencies in cytotoxic T cells between all groups; of note PBS^2LIC^2 vs LB^2LIC^2 p=0.0056 (**Fig 5E**). Furthermore, T cell subset typing (**Fig 6**) showed that frequency of early effector CD4+ T helper cells (**Fig 6B**, CD44-CD62L-) and effector T helper cells (**Fig 6C**, CD44+CD62L-) were

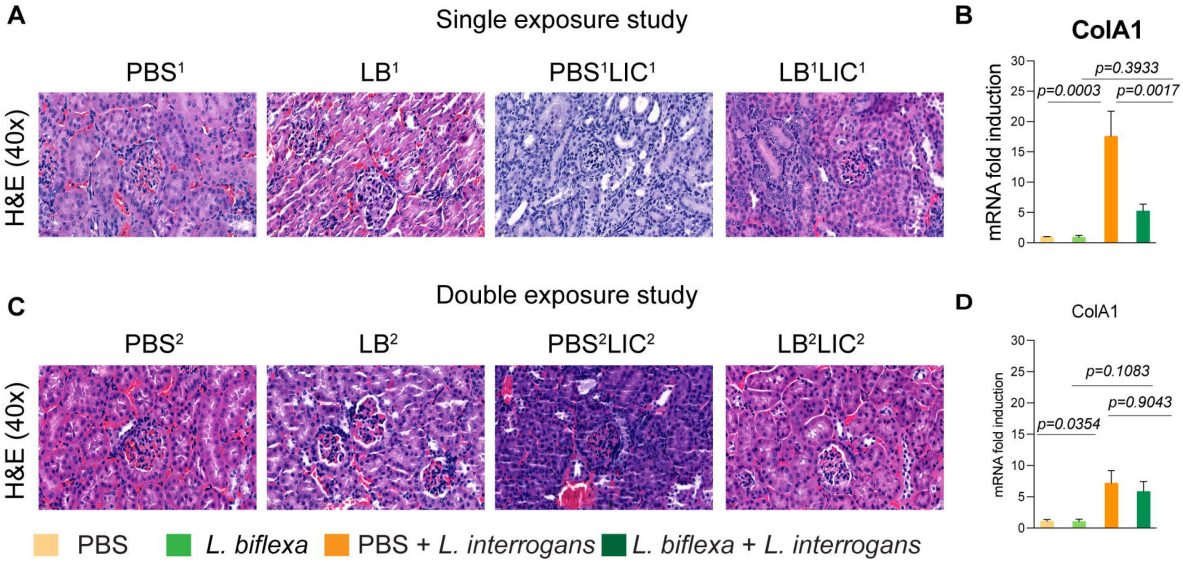

Figure 3

Kidney histopathology and quantification of renal fibrosis.

Representative H & E-stained kidney tissue sections from both single and double exposure studies are included in A and C, respectively. The images were captured at 40X magnification. B and D represent the mRNA expression of kidney fibrosis marker ColA1 by qPCR normalized to endogenous b-actin expression. Data was analyzed by ordinary one-way ANOVA followed by Tukey's multiple comparison correction between challenged groups with their respective controls; * p values are included in the graphs. Data represents one of two independent experiments.

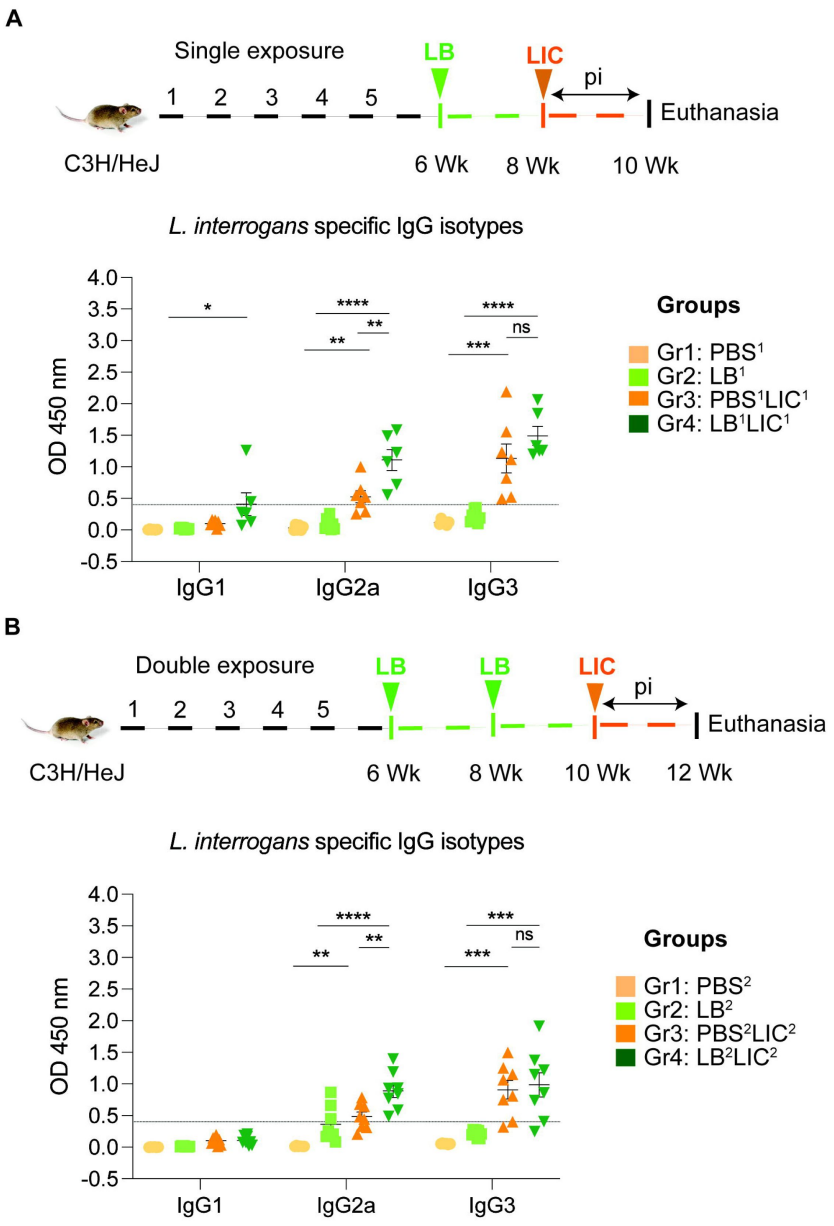

Figure 4

Detection of IgG1, IgG2a and IgG3 specific to *L. interrogans* in serum from experimental mice.

A) represents IgG isotypes specific to *L. interrogans* in 10-week serum of mice exposed once to *L. biflexa* before *L. interrogans* challenge. B) represents IgG isotypes specific to *L. interrogans* in 12-week serum of mice exposed twice to *L. biflexa* before *L. interrogans* challenge. Ordinary one-way ANOVA followed by Tukey's multiple comparison correction test was used to compare between challenged groups with their respective controls; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns= not significant; N= 6-8 mice per group. Data represents two independent experiments.

significantly increased when compared between the LIC challenged groups and the respective controls (except PBS^2 v PBS^2LIC^2 early effectors) and that frequency of early effector and effector T helper cells were higher in the LB^2LIC^2 group than PBS^2LIC^2. No major changes were measured in memory CD4+ T helper cells (**Fig 6D**, CD44+CD62L+). In the CD8+ cytotoxic T cell subsets, we measured significant decreases in frequency of naïve T cells between LIC infected groups and the respective controls (**Fig 6E**, CD62L+CD44-) and this was replicated in the CD8+ cytotoxic memory except that differences with the LIC infected groups were not significant (**Fig 6H**, CD44+CD62L+).

Discussion

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

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

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

Our data also shows that prior exposure to non-pathogenic *Leptospira* before pathogenic challenge, induced higher antibody titer in the serum, specifically IgG2a antibodies against *L. interrogans* in both single and double exposure experiments (**Fig 4**). Increased IgG2a response in serum is associated with induction of a Th1 biased immune response. Others have recently

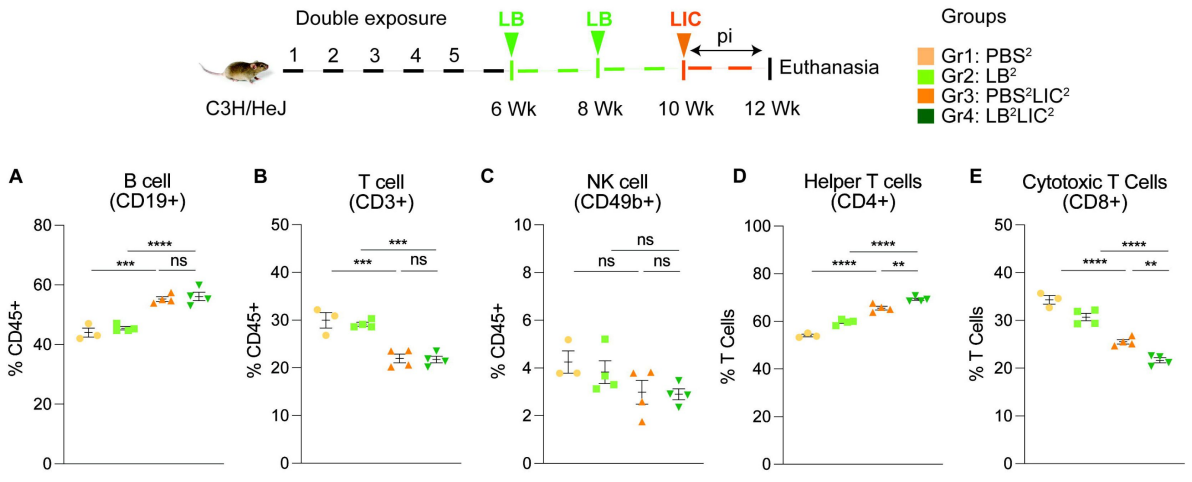

Figure 5

Frequency of lymphocytes in spleen of mice subjected to a double exposure of *L. biflexa* before challenge with *L. interrogans*.

A to E) show B cell (CD19+), T cell (CD3+), NK cell (CD49b+), Helper T cell (CD4+) and Cytotoxic T cell (CD8+) frequencies in groups of experimental mice. Ordinary one-way ANOVA followed by Tukey's multiple comparison correction test was used to compare between challenged groups and their respective controls; **p<0.01, ***p<0.001, ****p<0.0001 and ns= not significant; N= 3-4 mice per group. Data represents one of two independent experiments.

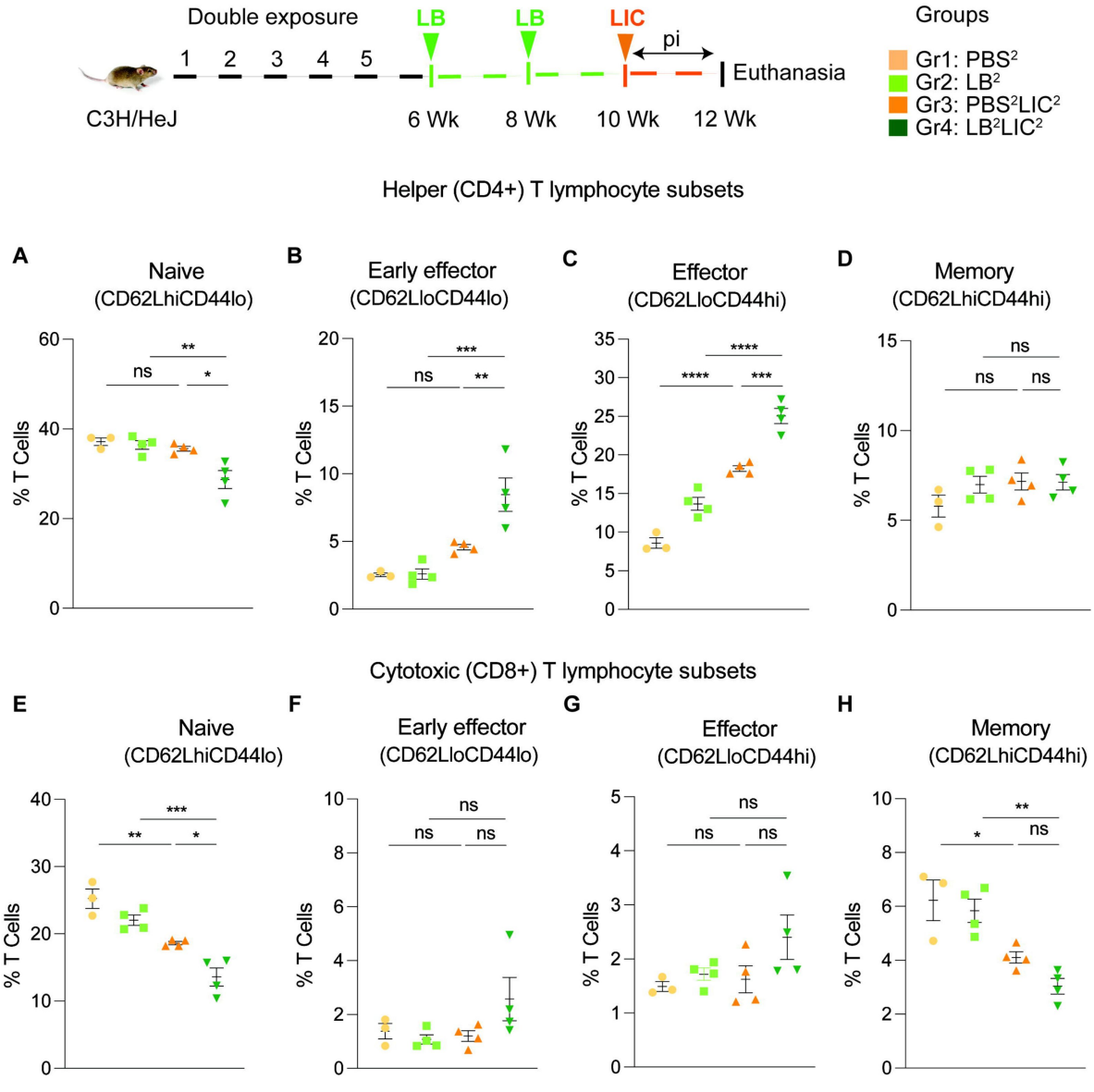

Figure 6

Frequency of T cell subsets (CD62L/CD44) in spleen of mice subjected to a double exposure of *L. biflexa* before challenge with *L. interrogans*.

A to D) represent naïve, early effector, effector and memory subsets of CD4+ helper T lymphocytes, respectively. E to H) represent naive, early effector, effector and memory subsets of CD8+ cytotoxic T lymphocytes, respectively. Ordinary one-way ANOVA followed by Tukey's multiple comparison correction test was used to compare between challenged groups and their respective controls; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns= not significant; N = 3-4 mice per group. Data represents one of two independent experiments.

found that saprophytic *L. biflexa* induced Th1 responses, higher T cell proliferation and IFN-g producing CD4+ T cells (28). Persistent IgM and strain specific IgG responses was observed during a homologous leptospiral challenge in C57BL6/J mice (22). In our study, exposure to a saprophytic *Leptospira* induced antibody responses that may provide heterologous protection against the pathogenic strain of *Leptospira*. This supports a promising broad-spectrum efficacy. Thus, live vaccines derived from a saprophytic strain of *Leptospira* could offer broader protection and overcome the limitation of serovar specificity often observed with killed whole-cell vaccines based on pathogenic strains.

Differences in antibody titer among the *L. interrogans* infected group pre-exposed to saprophytic *L. biflexa* can be attributed to the robust trafficking and differentiation of B and helper T cells (CD4+) measured in spleen (**Fig 5** and **Fig 6**). Presence of effector helper T (CD4+) cells in the spleen indicate a robust cellular immune response as these cells produce cytokines that play a pivotal role in activating other immune cells, including antibody-producing B cells. Moreover, our findings align with another observation which further reinforces the potential immunostimulatory properties of components (polar lipids) derived from saprophytic *L. biflexa*, indicating that these components could play a crucial role in inducing robust B cell responses (29). Induction of helper T cell responses along with dynamic transition from naïve to early effector and effector without T helper memory reflects an orchestrated immune response upon pathogenic challenge in the saprophytic pre-exposed group that is typical of effective responses to vaccines. Previous studies have highlighted the significance of activated CD4+ T cells during *Leptospira* infection in providing protective immunity to the host and mitigating the severity of leptospirosis by releasing cytokines (30). Correlating induction of chemo-cytokines by saprophytic *Leptospira* with subsequent adaptive immune responses, such as the activation of CD4+ T cells or the production of specific antibodies, provides insights into how innate immune signals drive the adaptive immune response against a pathogenic threat. It may also aid in identifying key signaling molecules or pathways that could be targeted for therapeutic interventions or vaccine design.

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

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

Acknowledgements

This work was supported by the National Institute of Allergy and Infectious Diseases (NIAID), United States National Institutes of Health (NIH), grant numbers R01 AI139267 (MGS), R21 AI 142129 (MGS). The content of this manuscript is totally the responsibility of the authors and does not involve the official views of NIAID or NIH.

Competing Interests

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

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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Editors

Reviewer #3 (Public Review):

Summary:

Kundu et al. investigated the effects of pre-exposure to a non-pathogenic Leptospira strain in prevention of severe disease following subsequent infection by a pathogenic strain. They utilized a single or double exposure method to the non-pathogen prior to challenge with a pathogenic strain. They found that prior exposure to a non-pathogen prevented many of the disease manifestations of the pathogen. Bacteria, however, were able to disseminate, colonize the kidneys, and be shed in the urine. This is important foundational work to describe a novel method of vaccination against leptospirosis. Numerous studies have attempted to use recombinant proteins to vaccinate against leptospirosis, with limited success. The authors provide a new approach that takes advantage of the homology between a non-pathogen and a pathogen to provide heterologous protection. This will provide a new direction in which we can approach creating vaccines against this re-emerging disease.

Strengths:

The major strength of this paper is that it is one of the first studies utilizing a live non-pathogenic strain of Leptospira to immunize against severe disease associated with leptospirosis. They utilize two independent experiments (a single and double vaccination) to

define this strategy. This represents a very interesting and novel approach to vaccine development. This is of clear importance to the field.

The authors use a variety of experiments to show the protection imparted by pre-exposure to the non-pathogen. They look at disease manifestations such as death and weight loss. They define the ability of Leptospira to disseminate and colonize the kidney. They show the effects infection has on kidney architecture and a marker of fibrosis. And they begin to define the immune response in both of these exposure methods. This provides evidence of the numerous advantages this vaccination strategy may have. Thus, this study provides an important foundation for future studies utilizing this method to protect against leptospirosis.

Weaknesses:

A direct comparison between single and double exposure to the non-pathogen is not possible with the data presented. The ages of mice infected were different between the single (8 weeks) and double (10 weeks) exposure methods, thus the phenotypes associated with LIC infection are different at these two ages. The authors state that this is expected, but do not provide a reasoning for this drastic difference in phenotypes. It cannot be determined if double-vaccination would provide an additional benefit, which is of importance to future work developing any vaccine treatment. An experiment directly comparing the two exposure methods while infecting mice at the same age would be of great relevance to and strengthen this work.

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Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *As a reviewer for this manuscript, I recognize its significant contribution to understanding the immune response to saprophytic Leptospira exposure and its implications for leptospirosis prevention strategies. The study is well-conceived, addressing an innovative hypothesis with potentially high impact. However, to fully realize its contribution to the field, the manuscript would benefit greatly from a more detailed elucidation of immune mechanisms at play, including specific cytokine profiles, antigen specificity of the antibody responses, and long-term immunity. Additionally, expanding on the methodological details, such as immunophenotyping panels, qPCR normalization methods, and the rationale behind animal model choice, would enhance the manuscript's clarity and reproducibility. Implementing functional assays to characterize effector T-cell responses and possibly investigating the microbiota's role could offer novel insights into the protective immunity mechanisms. These revisions would not only bolster the current findings but also provide a more comprehensive understanding of the potential for saprophytic Leptospira exposure in leptospirosis vaccine development. Given these considerations, I believe that after substantial revisions, this manuscript could represent a valuable addition to the literature and potentially inform future research and vaccine strategy development in the field of infectious diseases.*
>
> ***Reviewer #2 (Public Review):***
>
> *Summary:*

The authors try to achieve a method of protection against pathogenic strains using saprophytic species. It is undeniable that the saprophytic species, despite not causing the disease, activates an immune response. However, based on these results, using the saprophytic species does not significantly impact the animal's infection by a virulent species.

Strengths:

Exposure to the saprophytic strain before the virulent strain reduces animal weight loss, reduces tissue kidney damage, and increases cellular response in mice.

Weaknesses:

Even after the challenge with the saprophyte strain, kidney colonization and the release of bacteria through urine continue. Moreover, the authors need to determine the impact on survival if the experiment ends on the 15th.

Reviewer #3 (Public Review):

Summary:

Kundu et al. investigated the effects of pre-exposure to a non-pathogenic Leptospira strain in the prevention of severe disease following subsequent infection by a pathogenic strain. They utilized a single or double exposure method to the non-pathogen prior to challenge with a pathogenic strain. They found that prior exposure to a non-pathogen prevented many of the disease manifestations of the pathogen. Bacteria, however, were able to disseminate, colonize the kidneys, and be shed in the urine. This is an important foundational work to describe a novel method of vaccination against leptospirosis. Numerous studies have attempted to use recombinant proteins to vaccinate against leptospirosis, with limited success. The authors provide a new approach that takes advantage of the homology between a non-pathogen and a pathogen to provide heterologous protection. This will provide a new direction in which we can approach creating vaccines against this re-emerging disease.

Strengths:

The major strength of this paper is that it is one of the first studies utilizing a live non-pathogenic strain of Leptospira to immunize against severe disease associated with leptospirosis. They utilize two independent experiments (a single and double vaccination) to define this strategy. This represents a very interesting and novel approach to vaccine development. This is of clear importance to the field.

The authors use a variety of experiments to show the protection imparted by pre-exposure to the non-pathogen. They look at disease manifestations such as death and weight loss. They define the ability of Leptospira to disseminate and colonize the kidney. They show the effects infection has on kidney architecture and a marker of fibrosis. They also begin to define the immune response in both of these exposure methods. This provides evidence of the numerous advantages this vaccination strategy may have. Thus, this study provides an important foundation for future studies utilizing this method to protect against leptospirosis.

Weaknesses:

Although they provide some evidence of the utility of pretreatment with a non-pathogen, there are some areas in which the paper needs to be clarified and expanded.

The authors draw their conclusions based on the data presented. However, they state the graphs only represent one of two independent experiments. Each experiment utilized 3-4 mice per group. In order to be confident in the conclusions, a power analysis needs to be done to show that there is sufficient power with 3-4 mice per group. In addition, it would be important to show both experiments in one graph which would inherently increase the power by doubling the group size, while also providing evidence that this is a reproducible phenotype between experiments. Overall, this weakens the strength of the conclusions drawn and would require additional statistical analysis or additional replicates to provide confidence in these conclusions.

A direct comparison between single and double exposure to the non-pathogen is not able to be determined. The ages of mice infected were different between the single (8 weeks) and double (10 weeks) exposure methods, thus the phenotypes associated with LIC infection are different at these two ages. The authors state that this is expected, but do not provide a reasoning for this drastic difference in phenotypes. It is therefore difficult to compare the two exposure methods, and thus determine if one approach provides advantages over the other. An experiment directly comparing the two exposure methods while infecting mice at the same age would be of great relevance to and strengthen this work.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

Major Comments

(1) Elucidation of Immune Mechanisms: The manuscript intriguingly suggests that exposure to saprophytic Leptospira primes the host for a Th1-biased immune response, contributing to survival and mitigation of disease severity upon subsequent pathogenic challenge. However, the underlying mechanisms remain broadly defined. A more detailed investigation into the cytokine profiles, particularly the levels of IFN-γ, IL-12, and other Th1-associated cytokines, could clarify the mechanism of Th1 bias. Moreover, exploring the role of antigen-presenting cells (APCs) in priming T cells towards a Th1 phenotype would add valuable insights.

In this study we continue to elucidate the immune mechanisms engaged by pathogenic and non-pathogenic Leptospira as a follow up to our previous work (Shetty et al, 2021 PMID: 34249775, and Kundu et al 2022 PMID 35392072). We, and others, have shown that saprophytic *L. biflexa* and pathogenic *L. interrogans* induce major chemo-cytokines associated with Th1 biased immune responses (Shetty et al. 2021; Cagliero et al. 2022; Krangvichian et al. 2023) and engage myeloid immune cells such as macrophages and dendritic cells. The role of antigen presenting cells such as dendritic cells in priming T cells and activating adaptive response is a separate question and can be addressed in the future. To further address this question, a recent mechanistic study (Krangvichian et al. 2023) showed that non-pathogenic leptospires (*L. biflexa*) promote MoDC maturation and stimulate the proliferation of IFN-γ-producing CD4+ T cells and potentially elicit a Th1-type response in mice, which also supports our current claim and it is referenced in our manuscript.

(2) Quantitative Analysis of Kidney Colonization: The manuscript reports that pre-exposure to L. biflexa did not prevent the colonization of kidneys by L. interrogans but led to a more regulated immune response and reduced fibrosis. A more nuanced quantification of bacterial loads in the kidneys, using techniques such as CFU counting or more sensitive qPCR methods, could provide a clearer picture of how saprophytic exposure affects the ability of pathogenic Leptospira to establish infection. Additionally, a

> *time-course study showing the kinetics of bacterial colonization and clearance post-infection would be informative.*

We are currently validating digital PCR to use in the future and plan to do time course studies.

> *(3) Characterization of B Cell and T Cell Responses: While the manuscript mentions increased B cell frequencies and effector T helper cell responses, specifics regarding the nature of these responses are lacking. For instance, detailing the isotype and specificity of antibodies produced, the proliferation rates of specific B and T cell subsets, and their functional capabilities (e.g., cytotoxicity, help for B cells) would significantly enrich the understanding of the immune response elicited by pre-exposure to saprophytic Leptospira.*

Indeed, additional experiments need to be conducted to flush out the immune responses engaged after pre-exposure to saprophytic *Leptospira* followed by LIC challenge.

> *(4) Comparative Analysis with Other Models of Pre-exposure: The study primarily focuses on pre-exposure to a live saprophytic Leptospira. Including a comparison with pre-exposure to killed saprophytic bacteria, or even to other non-pathogenic microbes, could help discern whether the observed protective effect is unique to live saprophytic Leptospira exposure or if it represents a more general phenomenon of trained immunity.*

Regarding the use of other non-pathogenic microbes, our lab has shown in the past that oral use of probiotic strain *Lactobacillus plantarum* (Potula et al 2017) also reduces the severity of Leptospirosis by recruiting myeloid cells. Thus, there may be a general phenomenon of trained immunity involved. We added this to the discussion.

> *(5) Assessment of Long-term Immunity: The study provides valuable insights into the short-term outcomes following saprophytic Leptospira exposure and subsequent pathogenic challenge. Extending these observations to assess long-term immunity, including memory B and T cell responses several months post-infection, would be crucial for understanding the potential of saprophytic Leptospira exposure in providing lasting protection against leptospirosis.*

Long term immunity is a complex and separate question that we plan to address later.

> *Minor Comments*
>
> *(1) Technical Specifics of Flow Cytometry Analysis: The manuscript could benefit from including more details on the flow cytometry gating strategy and the specific markers used to identify different immune cell subsets. This addition would aid in the reproducibility of the results and allow for a clearer interpretation of the immune profiling data.*

We included the technical specifics of the flow-cytometry analysis in the materials and methods section. The gating strategy (Fig S1) and the specific markers (TableS1) used to identify different immune cell subsets were incorporated in the supplementary datasheet. The cell specific markers were incorporated in the figures (Fig 5 and 6) under each representative cell subset which facilitates clarity and reproducibility of immune profiling.

> *(2) Statistical Methodology for IgG Subtyping: The analysis of IgG subtypes in response to Leptospira exposure is intriguing but would be strengthened by specifying the statistical tests used to compare IgG1, IgG2a, and IgG3 levels between groups. Additionally,*

> *discussing the biological significance of the observed differences in IgG subtype levels would provide a more comprehensive understanding of the immune response.*

We applied the ordinary One-way ANOVA test to compare the IgG subtypes between groups followed by a Tukey's multiple comparison correction analysis (included in the figure legend of Fig 4). We addressed the biological relevance of the observed differences in IgG subtype levels in the discussion section.

> *(3) Details on Animal Welfare and Ethical Approval: While the manuscript mentions compliance with institutional animal care and use committee protocols, providing the specific ethical guidelines followed, such as the 3Rs (Replacement, Reduction, Refinement), would reinforce the commitment to ethical research practices.*

This is addressed in our institutional IACUC which is approved and listed in Methods.

> *(4) Clarification of Figure Legends: Some figure legends are brief and could be expanded to more thoroughly describe what the figures show, including details on what specific data points, error bars, and statistical symbols represent.*

We updated and expanded the figure legends (Fig 1-4).

> *(5) Revision of Introduction and Background: The introduction provides a good overview of leptospirosis and the rationale behind the study. However, it could be further improved by briefly summarizing current challenges in vaccine development against leptospirosis and how understanding the immune response to saprophytic Leptospira could address these challenges.*

We revised the introduction keeping this comment in mind.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *- Perform the same challenge experiment with a hamster.*

We clarified throughout the manuscript that all the work was done using the C3H-HeJ mouse model which was developed in our lab for the purpose of measuring differences in sublethal and lethal LIC infections. We leave the experiments using hamster to the investigators that have thoroughly validated the hamster model of lethal Leptospira infection.

> *- Review the written part where it is understood that the challenge with saprophyte strain before virulence prevents the disease.*

We reviewed the manuscript to be understood that inoculation of mice with a saprophyte *Leptospira* before pathogenic challenge prevents severe leptospirosis and promotes kidney homeostasis and increased shedding of *Leptospira* in urine which is interesting. The last 2 sentences of the abstract read: "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."

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *(1) Line 83: The authors refer to the classification of Leptospira by old nomenclature. The bacteria are now categorized into clades P1, P2, S1 and S2. See Vincent et al. Revisiting the taxonomy and evolution of pathogenicity of the genus Leptospira through the prism*

> *of genomics. PLoS Negl Trop Dis. 2019 May 23;13(5):e0007270. doi: 10.1371/journal.pntd.0007270. PMID: 31120895; PMCID: PMC6532842.*

We have included the categories (S1 for *L. biflexa* and P1+ for *L. interrogans*) in introduction and methods but we did not update the figures because we want to be specific about the species used in these experiments. We also include a few sentences on evolution of *Leptospira* species in discussion and reference Thibeaux 2018, Vincent 2019 and Giraud-Gatineau, 2024.

> *(2) Line 133: Please remove the extra line to be consistent with the rest of the method section format.*

We addressed all formatting issues.

> *(3) Line 137: Are these primers specific to pathogenic L. interrogans? Or do they cross react with L. biflexa? If not specific, how long does L. biflexa stick around after infection?*

The primers are specific to the genus *Leptospira*. Surdel et al. in 2022 used 16s rRNA target sequence to amplify *L. biflexa* Patoc in mice at 6 hours post infection. We did not detect any positive sample for *L. biflexa* with the 16s rRNA primer set because we do our analysis 30 days and 45 days post inoculation with *L. biflexa.* We clarified this issue in methods and results.

> *(4) Statistical analysis:*
>
> *(a) Some of your graphs have more than 4 points on them (such as Figure 4), while the legend still reads "represents one of two independent experiments". Are these actually combined replicates in the same graph? Combining them would provide strength to your conclusions throughout your manuscript and may provide stronger power for comparisons. If they are not included, why are they not included together? Please clarify what is included in each graph, and why the two experiments were not included together.*

We updated the legends with the total number of mice used in the experiment represented in the figure. Figures 1, 2, 4 and S2 contain the combined results from two independent experiments. Figures 3, 5 and 6 represent data from one of two independent experiments. For Fig 3 it would be redundant to show HE images of two experiments. Regarding Figs 5 and 6, the flow-cytometry equipment acquires data at different voltage every single time and biological samples vary between experiments even if all the markers and procedures are the same. So, we reproduce the experiment and show results from one experiment after confirming that the trend between individual experiments are the same.

> *(b) If ANOVA was used, were all columns compared to each other? Why in some graphs are "ns" labeled only for certain comparisons? I would suggest removing the "ns" comparisons and only highlighting the significant differences.*

We have incorporated the comparison analysis between control (PBS) versus the PBS-LIC, LB versus LB-LIC and PBS-LIC versus LB-LIC in both the studies although we have compared significance between all groups.

> *(5) Line 165: Bacteria were not plated, extract was plated. Perhaps you mean "extract corresponding to 107-108 bacteria"?*

We addressed it as follows: “Nunc MaxiSorp flat-bottom 96 well plates (eBioscience, San Diego, CA) were coated with extracts prepared from 107-108 bacteria per well and incubated

at 4°C overnight" …

> *(6) Line 260: The authors claim that "Exposure to non-pathogenic L. biflexa before pathogenic L. interrogans challenge provided a significant immune cell boost with an increase in overall B and helper T cell frequencies…" However, in Figure 5A, the number of B cells in both the PBS2LIC2 and the LB2LIC2 are not significantly different. Thus, the claim is not supported by the evidence provided. It appears that infection with LIC led to similar increases in B cells regardless of pretreatment.*

We rephrased that title to reflect the finding that increased differences were measured in effector Helper T cells between PBS2LIC2 and LB2LIC2 (Figs 5D and 6B, 6C) and we re-wrote this section for clarity.

> *(7) Lines 314-315: The authors claim that it protected against kidney fibrosis, however, the data only supports that only a single exposure to LB reduced levels of a marker associated with kidney fibrosis. Fibrosis was never directly measured.*

Indeed, we didn't do Mason's Trichrome stain to get supporting data for kidney fibrosis and only measured a fibrosis marker ColA1. We toned down this section: " …. it may confer protection against kidney fibrosis."

> *(8) Line 317: Authors state that pre-exposure induced higher antibodies in serum, however, this was never shown. Only an increase in IgG2a was shown. Please word this statement to make it clear total antibodies were never measured.*

We did measure total anti-*Leptospira interrogans* IgM and IgG antibodies. We added the following sentence to description of these results: "In both experiments, total IgM and IgG were significantly increased in PBS-LIC and LB-LIC when compared to the respective controls, but not between PBS-LIC and LB-LIC. Regarding IgG isotypes, IgG1…"

> *(9) Line 323: The authors state that the exposure "induced antibody responses that provided heterologous protection." There is no evidence that the protection is due to the antibody response in these experiments. In fact, they also showed that it induced increased T cell responses.*

We toned down this statement: "In our study, exposure to a saprophytic *Leptospira* induced antibody responses that may provide heterologous protection against the pathogenic strain of *Leptospira*."

> *(10) Line 328: The authors us the term "stark difference", however, only slight differences are seen.*

We toned down that statement as follows: "Differences in antibody titer among the *L. interrogans* infected…."

> *(11) Line 490: reword this sentence to provide clarity and easier to read: "inoculated once with 10^8 L. biflexa at 6 weeks and they were challenged with 10^8 L. interrogans SEROVAR Copenhageni FioCruz (LIC) at 8 weeks."*

We revised the sentence.

> *(12) Figure 1 and 2: Quantifying bacteria in culture after infection is not meaningful, as there are numerous factors that can affect the replication in culture after infection, such as how the organ perhaps was cut before placing it in culture. The comparisons in Figure*

> *2E and F therefore are not interpretable. I would suggest presenting this data as Culture Positive or Culture Negative.*

We added these data to the figure under DFM (dark field microscopy).

> *(13) Figure 3A: H&E staining often leads to different qualities of stains. But is there a better image that can be chosen for the PBS1LIC1 that provides a better comparison with the other images chosen? This is not worth repeating the experiment to get one, just make the figure look better if you have one available.*

We screened the images again but the one incorporated in the figure3A for PBS1LIC1 is the best.

> *(14) Figure 3D: I agree that the PBS-LIC treatment is significant, but please include P value, as it looks very similar to the LB-LIC group. The two LIC groups are not significantly different, so the conclusion would be pre-exposure does not mitigate renal fibrosis marker ColA in the double-exposure study.*

We included the p-values in this figure. The two LIC groups are significantly different (ColA1) in the single exposure experiment, and the in double exposure we don't expect to be able to measure ColA1 differences because the mice are older (10 wk) when we do the LIC challenge.

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