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BACTERIAL INFECTIONS

Sex Matters: Male Hamsters Are More Susceptible to Lethal Infection with Lower Doses of Pathogenic Leptospira than Female Hamsters

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ABSTRACT A somewhat contradictory published body of evidence suggests that sex impacts severity outcomes of human leptospirosis. In this study, we used an acute animal model of disease to analyze leptospirosis in male and female hamsters infected side by side with low but increasing doses of *Leptospira interrogans* serovar Copenhageni. We found that male hamsters were considerably more susceptible to leptospirosis, given that only 6.3% survived infection, whereas 68.7% of the females survived the same infection doses. In contrast to the females, male hamsters had high burdens of *L. interrogans* in kidney and high histopathological scores after exposure to low infection doses (~10^3 bacteria). In hamsters infected with higher doses of *L. interrogans* (~10^4 bacteria), differences in pathogen burdens as well as cytokine and fibrosis transcript levels in kidney were not distinct between sexes. Our results indicate that male hamsters infected with *L. interrogans* are more susceptible to severe leptospirosis after exposure to lower infectious doses than females.

KEYWORDS *Leptospira interrogans*, acute leptospirosis, hamster, sex, acute, lethal, male

Leptospirosis is an emerging widespread zoonotic disease caused by pathogenic spirochetes of the genus *Leptospira*. The infection commonly occurs through direct contact with infected urine or indirectly through contaminated water (1). Caused by over 250 serovars of *Leptospira* spp., which are hosted mainly by rodents, leptospirosis shows clinical manifestations in humans that vary from flu-like symptoms to multiorgan failure (2). Morbidity estimates are ~1 million cases a year worldwide, with a 5 to 10% mortality rate (1, 3).

Differences in susceptibility to inflammatory responses between males and females have been noted for a number of years, and sex is now accepted as a risk factor for infectious and autoimmune diseases (4–6). Although evidence that women are more susceptible to leptospirosis was reported in the past (7), more recent clinical and seroepidemiological evidence suggests that the incidence of human leptospirosis is higher in males than in female adults and children (8–11). Furthermore, its severe clinical signs requiring hospitalization are also more frequently observed in men (12, 13).

Most studies focused on the identification of motility factors, lipopolysaccharide (LPS) biosynthesis, and outer membrane proteins using animal models of acute leptospirosis have been done with male hamsters; a few studies used females (14–23). The U.S. National Institutes of Health (NIH) established new guidelines to enhance the reproducibility of scientific results that mandate the analysis of the effect of sex differences in cell and animal studies (24, 25). The goal of our study was to infect male

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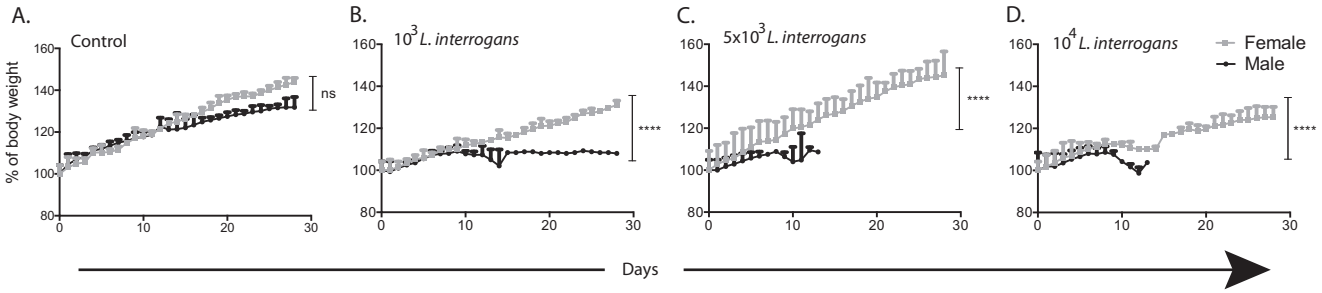

FIG 1 Body weights of male and female Golden Syrian hamsters infected with *L. interrogans*. Groups of hamsters were infected intraperitoneally with increasing doses of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (10^3, 5×10^3, or 10^4 bacteria); control groups were inoculated with PBS. Body weight measurements (grams) were recorded for 28 days postinfection and normalized to 100% on day 0. Asterisks indicate significant differences between males and females by a Mann-Whitney U exact test. ****, $P < 0.0001$; ns, not significant ($P = 0.2873$). The number of animals was 4 per group per sex (total, $n = 32$ hamsters). Data are representative of results from two experiments.

and female hamsters side by side with low but increasing doses of the same strain of *Leptospira interrogans* serovar Copenhageni, Fiocruz, and analyze differences in pathophysiology and disease severity.

RESULTS

Male hamsters are more susceptible to leptospirosis than their female counterparts. Groups of hamsters were infected intraperitoneally with 10^3, 5×10^3, or 10^4 *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 bacteria and monitored for objective clinical signs of infection for 28 days. Regarding weight loss ($n = 32$ animals), female hamsters gained body weight significantly (between 20 and 40%), whereas the body weight of male hamsters increased by only 8% ($P < 0.0001$); differences in weight were also observed in noninfected controls, although the latter were not significant (Fig. 1). Regarding survival ($n = 40$ animals), female hamsters infected with 10^3 or 5×10^3 *L. interrogans* bacteria, as well as the noninfected controls, did not develop symptoms of disease, and 100% of these animals survived, whereas only 37.5% of the females infected with 10^4 bacteria survived ($P = 0.0165$ by a log rank exact test) (Fig. 2A). In contrast, none of the male hamsters infected with 5×10^3 or 10^4 *L. interrogans*

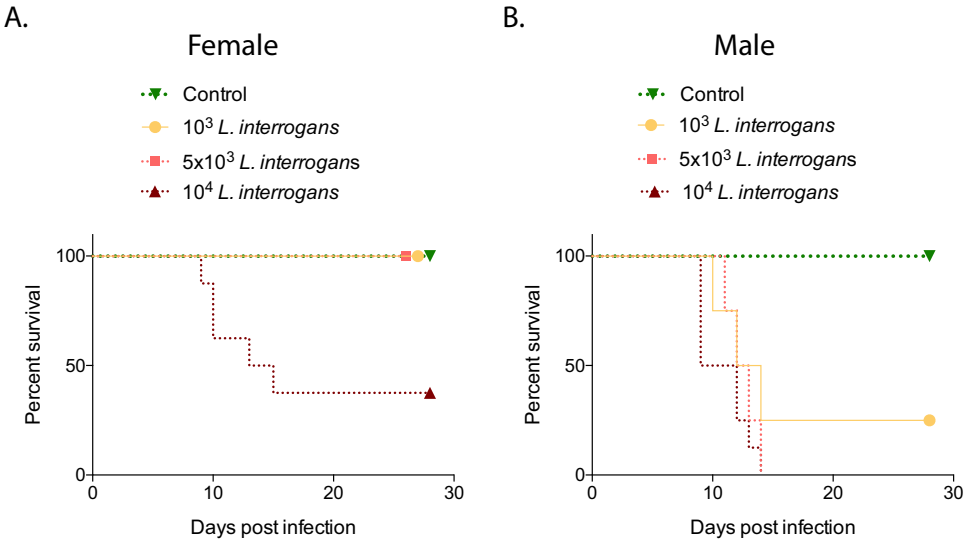

FIG 2 Percent survival of male and female hamsters after infection with increasing doses of *L. interrogans*. Groups of female (A) and male (B) hamsters were infected intraperitoneally with 10^3, 5×10^3, or 10^4 *L. interrogans* bacteria and with PBS (control) on day 0, and clinical scores were monitored for 28 days ($n = 40$ [$n = 4$ per group for infections with 10^3 and 5×10^3 bacteria and controls; $n = 8$ per group for infections with 10^4 bacteria]). Statistics were determined by a log rank exact test ($P = 0.0165$ [A] and $P = 0.0078$ [B]). Data are representative of results from three experiments.

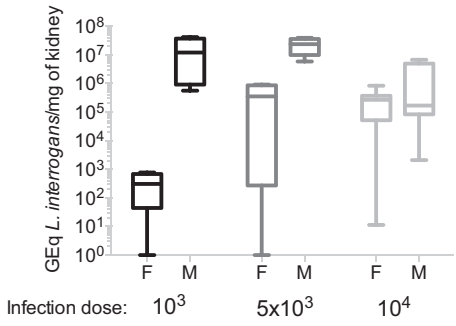

FIG 3 Quantification of *L. interrogans* DNA in kidney. DNA was quantified by real-time PCR targeted to the *Leptospira* 16S rRNA gene purified from kidneys from infected male and female hamsters ($n = 29$); controls ($n = 8$) were negative for *Leptospira* 16S rRNA (not shown). Data are representative of results from three experiments. GEq, genome equivalents; F, female; M, male.

bacteria survived, whereas 25% of the males infected with 10^3 bacteria as well as the noninfected controls survived ($P = 0.0078$ by a log rank exact test) (Fig. 2B). Overall, 1/16 male hamsters (6.3%) and 11/16 female hamsters (68.8%) survived equivalent infection doses.

Pathogen burden is higher in kidneys of male hamsters infected with lower doses of *Leptospira*. Kidneys of infected male and female hamsters that met endpoint criteria ($n = 29$ animals) were collected at termination, and the presence of leptospiral DNA was detected by PCR. Male hamsters had higher (~2 to 4 logs) genome equivalents of *L. interrogans* in the kidney than the females in the respective infected groups, except for infections with 10^4 bacteria (Fig. 3). The viability of *L. interrogans* in the kidney was analyzed by dark-field microscopy of Ellinghausen-McCullough-Johnson-Harris (EMJH) medium cultures of kidney tissue collected at termination; 100% of the infected male kidneys and 75% of the infected female kidneys had viable *L. interrogans* bacteria in culture.

Histopathological scores. Histopathological analysis of kidneys from hamsters infected with 10^3, 5×10^3, and 10^4 bacteria ($n = 10$ males and $n = 10$ females), stained with hematoxylin and eosin (H&E) and Masson's trichrome, showed that males had increased interstitial nephritis with infiltrates of mononuclear cells (~50%) and increased interstitial collagen deposition (~30%) compared with female hamsters (~27% and 17%, respectively).

Kidney function. Blood collected from euthanized hamsters previously infected with the highest dose of *L. interrogans* (10^4 bacteria) was used to determine the concentration of creatinine in serum by an enzyme-linked immunosorbent assay (ELISA) ($n = 14$, including controls). Levels of creatinine (Fig. 4) were ~2-fold higher in serum from male hamsters (mean for infected hamsters, 6.99 ng/μl; mean for controls, 2.84 ng/μl) than in the respective groups of females (mean for infected hamsters, 3.36 ng/μl; mean for controls, 2.16 ng/μl), although differences between males and females were not significant.

Gene expression of inflammatory and fibrosis markers in kidney. Kidneys collected from euthanized hamsters previously infected with 10^4 *L. interrogans* bacteria were used to determine levels of mRNA transcripts of the proinflammatory markers CCL3, CxCL10, tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), and interleukin-6 (IL-6); the anti-inflammatory marker IL-10; and the fibrosis marker ColA1 ($n = 15$, including controls). Three of the five proinflammatory markers, CxCL10, TNF-α, and IL-6, as well as the anti-inflammatory marker IL-10 and the fibrosis marker ColA1 were upregulated in male kidneys compared to the respective controls (Fig. 5). Differences between infected males and females were not significant.

Antibody response and isotyping. Blood collected from euthanized hamsters was used to determine the amounts of total IgG, IgG1, and IgG2 antibodies in serum by an ELISA. No significant difference in IgG responses was observed between males and

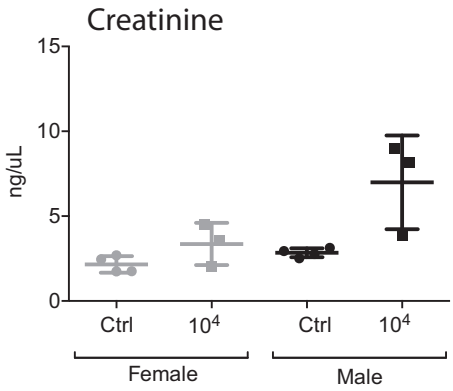

FIG 4 Kidney function. The concentration of creatinine was measured in blood collected at termination from a subset of male and female hamsters infected with 10^4 *L. interrogans* bacteria as well as from the respective controls ($n = 14$). Statistical analysis of differences between all infected and noninfected groups was performed by ordinary one-way ANOVA ($P = 0.0055$); differences between two groups determined by an unpaired *t* test with Welch's correction were not significant. Data are representative of results from two experiments.

females in the infected groups (average optical densities at 450 nm [OD_{450}] ± standard deviations of 3.407 ± 0.485 for females and 3.765 ± 0.124 for males); IgG isotyping showed that levels of IgG2 were 2.5-fold higher than those of IgG1, without differences between sexes.

DISCUSSION

There is evidence from clinical and seroepidemiological data that sex impacts the severity of leptospirosis, with a bias toward higher hospitalization rates for men (8–13). A comprehensive study of sex differences in clinical leptospirosis in Germany found that male patients were more likely to be hospitalized than female patients and suggested that reports on male predominance in leptospirosis may thus reflect sex-related variability in the incidence of severe disease rather than different infection rates (26). However, several reports of leptospirosis outbreaks where males and females had similar levels of exposure found no significant effects of sex differences on the development of illness (27–29).

Very few researchers have devoted resources to evaluating the question of susceptibility to severe disease related to sex in animal models. We evaluated how sex affects pathology, disease progression, and mortality after *Leptospira* infection using an acute model of leptospirosis, and we found that male hamsters are considerably more susceptible to lethal infection with pathogenic *Leptospira* than females: only 6.3% males survived infection, as opposed to 68.7% females that survived the same infectious doses. Analysis of the weights of the animals infected with increasing doses of *Leptospira* showed that males did not gain weight, as a sign of disease progression, and met endpoint criteria sooner than females exposed to the same conditions. These data are consistent with another comparative study that assessed the severity of pulmonary leptospirosis in female and male hamsters. In that study, male hamsters developed pulmonary hemorrhage after infection with *L. interrogans* serovar Hebdomadis at 120 h postinfection, whereas their female counterparts did not (30). Our results corroborate the findings of Tomizawa et al. and suggest that female hamsters exposed to the same infectious conditions as males are more resistant to the development of symptoms and develop milder disease. In this case, regarding exposure to the same infectious doses under the same conditions, sex differences appear to play an important role in the severity of leptospirosis.

Another observation from our study is that male hamsters were susceptible to low and high doses of infection, whereas the susceptibility of females increased with higher infection doses. When we quantified *Leptospira* DNA in the kidneys, male hamsters infected with 10^3 to 10^4 *Leptospira* bacteria had a consistently high number of bacteria

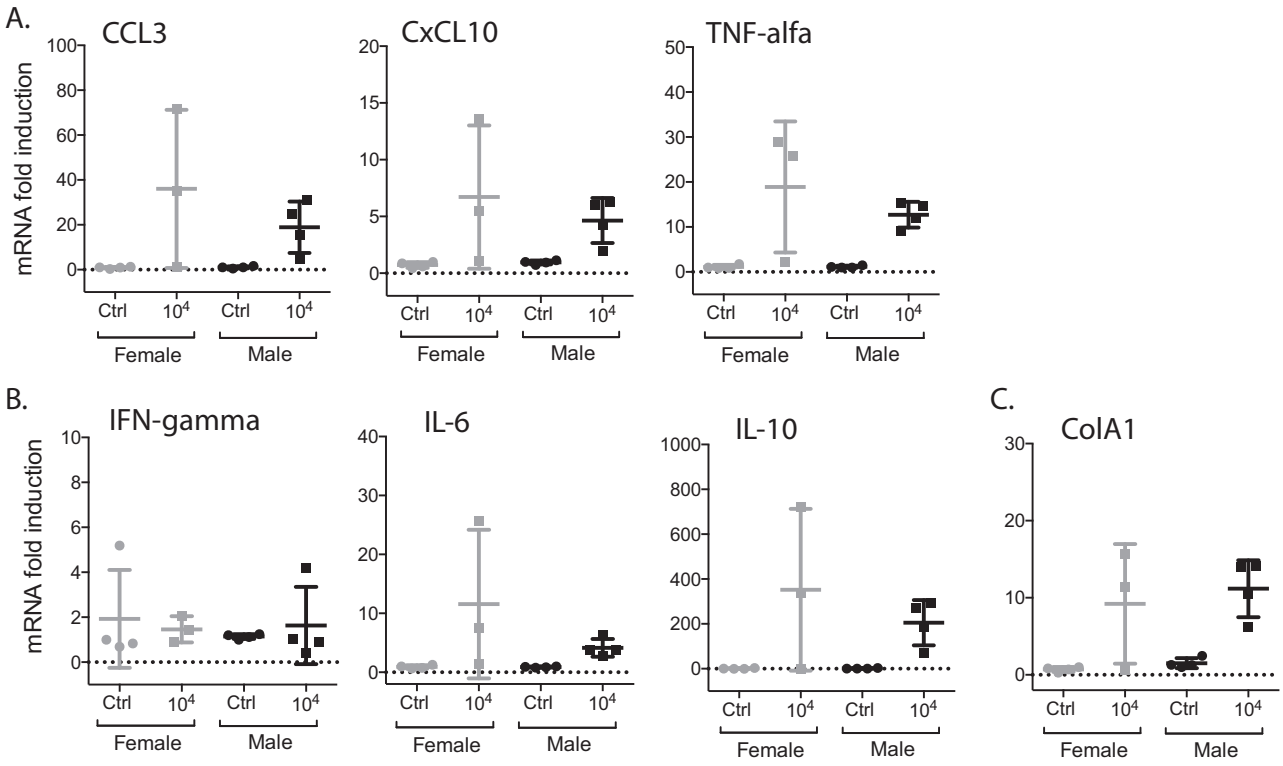

FIG 5 Quantification of levels of inflammatory and fibrosis markers in kidney. The gene expression levels of CCL3, CxCL10, TNF-α, IFN-γ, IL-6, IL-10, and ColA1 were quantified in kidney of hamsters infected with 10^4 *L. interrogans* bacteria as well as the respective controls ($n = 15$) by real-time PCR. Statistical analysis between all infected and noninfected groups was performed by ordinary one-way ANOVA (CCL3, $P = 0.0468$; CxCL10, $P = 0.0501$; TNF-α, $P = 0.0078$; IFN-γ, $P = 0.8955$; IL-6, $P = 0.0891$; IL-10, $P = 0.0425$; ColA1, $P = 0.0057$); differences between two groups were determined by an unpaired *t* test with Welch's correction (infected male and female groups [not significant], infected female and control groups [not significant], and infected male and control groups [CCL3, $P = 0.0522$; CxCL10, $P = 0.0334$; TNF-α, $P = 0.0038$; IFN-γ, $P = 0.6104$; IL-6, $P = 0.0223$; IL-10, $P = 0.0273$; ColA1, $P = 0.0121$]). Data are representative of results from two experiments.

in the kidney ($\sim 10^5$ to 10^7 bacteria per mg), whereas *Leptospira* DNA quantified in kidney from female hamsters increased exponentially with increasing infectious doses ($\sim 10^2$ to 10^6 bacteria per mg). Quantification of the levels of the inflammatory and fibrosis markers in kidneys of male hamsters infected with the highest dose of *L. interrogans* showed increased pro- and anti-inflammatory activity compared to the controls but not compared to the respective female groups. We speculate that female hamsters may be able to control lower infectious doses of *Leptospira*, but once the infectious dose reaches a certain threshold, the disease progresses with inflammatory and lethal results similar to those for males.

Our results indicate that male hamsters infected with *L. interrogans* serovar Copenhageni are susceptible to severe leptospirosis after exposure to infection doses 1 log lower than those in females. Once the critical dose is reached, females are as susceptible to infection. It remains to be determined if differences can be explained by distinctions in immune responses to pathogenic *Leptospira* between males and females that may account for the more effective control of *L. interrogans* dissemination in females early in infection.

It is noteworthy that in areas of endemicity, humans are more often exposed to lower infection doses ($\sim 10^3$ bacteria) (31), which were lethal to male hamsters, than higher doses ($>10^4$ bacteria), which were lethal to both male and female hamsters. It is possible that increased biological susceptibility to lower infectious doses contributes to aggravated outcomes of leptospirosis in males.

MATERIALS AND METHODS

Animals and ethics statement. Adult male and female Golden Syrian hamsters (*Mesocricetus auratus*) ($n = 40$; 8 weeks old) were obtained from Charles River Laboratory. The animals were housed

in groups of same-sex pairs in an animal biosafety level 2 (ABSL-2) pathogen-free environment in the Laboratory Animal Care Unit of the University of Tennessee Health Science Center (UTHSC). This study was carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* of the NIH (32). The protocol was approved by the UTHSC Institutional Animal Care and Use Committee Animal Care Protocol Application (permit number 16-070).

Bacterial strains and culture. *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 was cultivated in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with BD Difco *Leptospira* enrichment EMJH medium at 30°C. The culture (passage 4) was allowed to reach the log phase of growth, pelleted by centrifugation at 3,000 × *g* for 5 min, and washed and resuspended in sterile 1× phosphate-buffered saline (PBS). The cells were counted by dark-field microscopy (Zeiss USA, NY) using a Petroff-Hausser chamber.

Experimental infection. Groups of animals were injected intraperitoneally with 10^3 ($n = 8$), 5×10^3 ($n = 8$), or 10^4 ($n = 16$) *Leptospira* bacteria in 1 ml 1× PBS. Noninfected controls ($n = 8$) were injected with an equal volume of sterile 1× PBS. Survival and body weight were monitored for 28 days postinfection. Animals were scored for signs of clinical illness (weight loss of >10%, loss of interest in food or water, prostration, and ruffled fur) and euthanized by isoflurane overdose when they reached the endpoint criteria or at 28 days postinfection. Blood from euthanized animals was collected in EDTA by cardiac puncture. After dissection, one of the kidneys was collected and stored in 1 ml RNAlater (Sigma-Aldrich) for molecular bioassays and histopathology, and the other kidney was placed in EMJH medium for culture of *Leptospira* (as described in reference 33). Cultures were checked for viable bacteria for up to 30 days. Tissues were not collected from three hamsters that succumbed to infection before reaching endpoint criteria.

qPCR. DNA was extracted from kidneys by using a tissue kit (NucleoSpin). *Leptospira* bacteria were quantified by using a 6-carboxytetramethylrhodamine (TAMRA) probe and primers (Eurofins) for *Leptospira* 16S rRNA by quantitative PCR (qPCR). A standard curve obtained from serial 5-fold dilutions of known numbers of *Leptospira* bacteria was used for absolute quantification. Results were expressed as the number of *Leptospira* genome equivalents per milligram of kidney tissue DNA. Total RNA was extracted from kidney tissues by using an RNeasy minikit and transcribed by using a high-capacity cDNA reverse transcription kit. cDNA was subjected to PCR using SYBR green and primers for CCL3 (MIP-1α), CxCL10 (IP-10), TNF-α, IFN-γ, IL-6, IL-10, and ColA1, as described previously (34, 35). PCR data are reported as relative increases in mRNA transcript levels using β-actin as an internal control. Each qPCR was carried out with 2 μl of cDNA or genomic DNA (gDNA) in a 20-μl final volume following gene-specific amplification programs. The specificity of SYBR green-based qPCR assays was verified by the melting temperature (T_m) of the amplicon, as calculated by the instrument software. Results were validated only when threshold cycle (C_T) values were below the limit value of 40 cycles and with acceptable reproducibility between qPCR replicates (35).

Histopathology. Kidney tissues were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) and Masson's trichrome. Histopathology was empirically quantified by scoring interstitial inflammation and collagen deposition under a bright-light microscope (36).

Measurement of levels of antibodies and creatinine in blood. Levels of total IgG and IgG isotypes specific for *L. interrogans* as well as creatinine present in serum were measured by an ELISA using anti-hamster IgG(H+L), IgG1, and IgG2 (Southern Biotech) and a creatinine assay kit (Sigma-Aldrich). For the determination of IgG levels, the whole-cell sonicate of *L. interrogans* serovar Copenhageni strain Fiocruz was used as the antigen.

Statistical tests. Differences between two groups were analyzed by a nonparametric Mann-Whitney U exact test (Fig. 1) and by a parametric unpaired *t* test with Welch's correction (Fig. 4 and 5). Differences in survival (Fig. 2) were determined by using a log rank exact test. Differences between four groups were analyzed by ordinary one-way analysis of variance (ANOVA) (Fig. 4 and 5). Data analysis was done by using GraphPad Prism 7 software. Statistical significance was set to a *P* value of <0.05.

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