Humanized TLR4 immunocompetent mice are more susceptible to kidney disease after infection with *Leptospira interrogans* than wild-type mice

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Abstract

Toll-Like Receptor (TLR) 4 plays a central role in the control of leptospirosis and TLR4 deficiency results in lethal infection in mice. Human TLR4 however does not recognize *Leptospira* spp which may allow for their escape from immune recognition. We hypothesized that the replacement of murine TLR4 with human TLR4 may produce an immunocompetent mouse model of sublethal leptospirosis. We infected TLR4/MD-2 humanized transgenic mice (huTLR4) developed on a C57BL/6J TLR4/MD-2 double knock-out background with a sublethal dose of *L. interrogans* serovar Copenhageni (Leptospira). We found that Leptospira disseminated to target organs of huTLR4 mice as shown by the presence of spirochetes in blood, urine and kidney after infection. We did a comparative study between huTLR4 mice and wildtype C57BL/6J (muTLR4) to find out if the differences observed in the humanized TLR4 transgenic model were due to the transgene modification or to other factors. Replacing murine TLR4 with human TLR4 resulted in considerable higher shedding of Leptospira in urine in huTLR4 mice. Regarding inflammation of the kidney, MIP-2 and iNOS were significantly decreased in huTLR4 in comparison to muTLR4, whereas TNF-α and IFN-γ were increased, but there was no difference in RANTES that was increased in both groups of infected mice. In conclusion, our data suggests that human TLR4 may mediate higher susceptibility to leptospirosis that results in kidney pathology associated with downregulation of local MIP-2, antimicrobial iNOS and RANTES which are insuficient to restrict Leptospira and reduce shedding in urine. The TLR4 humanized mouse model of sublethal leptospirosis described is a novel tool to test therapies and vaccines against this neglected re-emerging zoonotic disease.

Introduction

Leptospira spp (Leptospira) are not considered gram-negative bacteria but they produce lipopolysaccharide (LPS), a potent inflammatory cell wall component. Leptospira LPS stimulates mouse and human TLR2 through associated abundant lipoproteins (Haake and Zuckert 2015). Differences of recognition exist between mouse and human TLR4, in that the anchor of LPS in the outer membrane of Leptospira (LipidA) activates murine, but not human TLR4 (Werts, Tapping et al. 2001), (Que-Gewirth, Ribeiro et al. 2004), (Nahori, Fournie-Amazouz et al. 2005). Over the past several years we developed mouse models of sublethal leptospirosis using C3H-HeJ mice (Richer, Potula et al. 2015),(Potula, Richer et al. 2017),(Sullivan, Nair et al. 2017),(Nair, Guedes et al. 2020) by adapting the lethal model described by Pereira (Pereira, Andrade et al. 1998), Nally (Nally, Fishbein et al. 2005), and Vinetz (Viriyakosol, Matthias et al. 2006). C3H-HeJ mice have a point mutation in a toll like receptor 4 gene (*tlr4*), that results in impaired recognition of bacterial LPS (Vogel, Johnson et al. 1999) which makes them more susceptible to bacterial infection.

One of the drawbacks of C3H-HeJ is that due to the point mutation in TLR4, this mouse model is considered somewhat immunocompromised. Other groups working on the function of the host TLR4 in leptospirosis used immunocompetent C57BL/6J and its respective congenic TLR4 knockouts. They found that wild-type mice expressing competent TLR4 receptors in their immune cells are resistant to lethal infection with *L. interrogans* whereas TLR4 knockouts are susceptible, have larger numbers of Leptospira in tissues and succumbed to infection (Chassin, Picardeau et al. 2009). This suggests that the competence of the TLR4 receptor affects leptospirosis outcomes. Considering that human TLR4 does not sense the atypical Leptospira LPS (Nahori, Fournie-Amazouz et al. 2005), we hypothesized that transgenic mice in which murine TLR4 was replaced with human TLR4 (along with its co-receptor MD-2) should be more susceptible to persistent leptospirosis as compared to congenic wild-type mice expressing murine TLR4. In this study we used TLR4/MD-2 humanized

transgenic mice developed by Dr. Hajjar on a C57BL/6J TLR4/MD-2 double knock-out (ko) background (Hajjar, Ernst et al. 2012), infected with a sublethal dose of *L. interrogans* serovar Copenhageni FioCruz and evaluated signs of disease progression as we previously described (Richer, Potula et al. 2015), (Potula, Richer et al. 2017). In addition, we did a comparative experiment against the wild-type C57BL/6J. We discuss the data considering our previous studies using the C3H/HeJ mouse model.

Results

To find out whether huTLR4/MD2 C57BL/6J immunocompetent mice (huTLR4) are susceptible to sublethal infection with Leptospira, we infected groups of mice with ~10^8 cells of a culture of *L. interrogans* Copenhageni FioCruz L1-130 (passage 2) intraperitoneally. We measured weight (g) as a clinical sign of disease progression and quantified the burden of live Leptospira in fluids and tissues by qPCR (Fig 1). We found that differences in weight between infected huTLR4 mice and

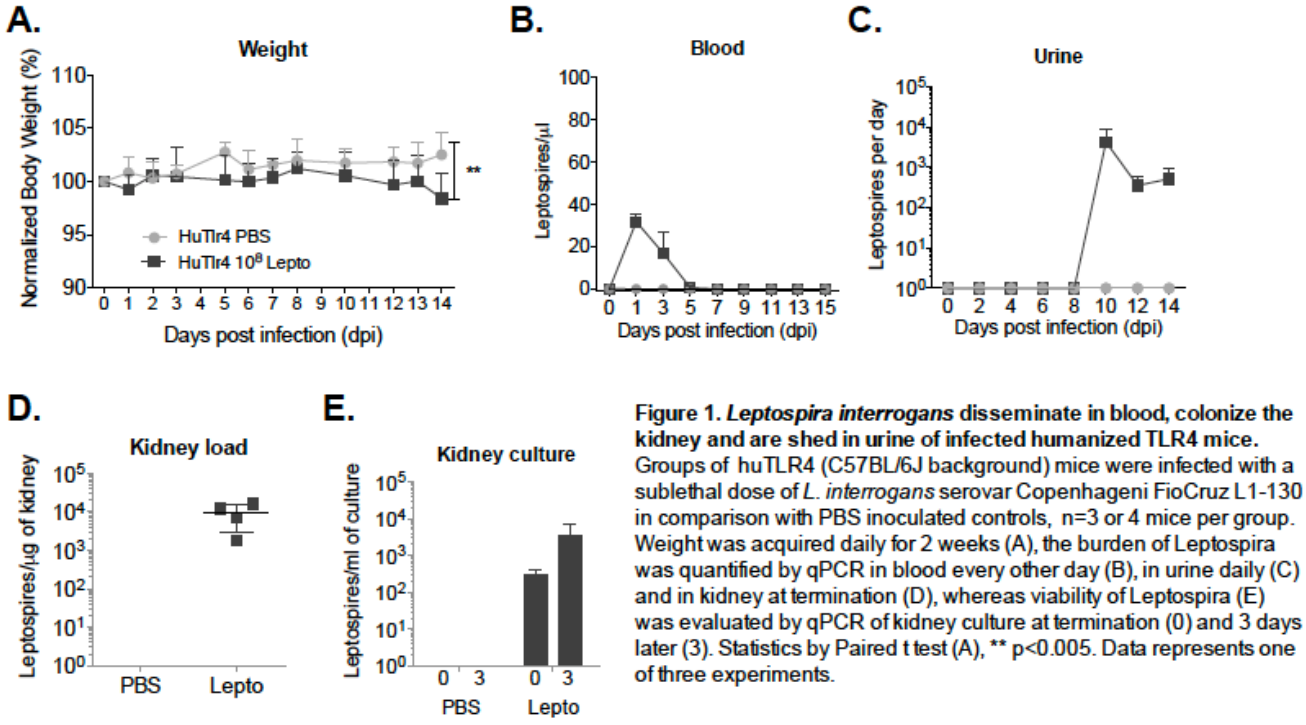

Figure 1. *Leptospira interrogans* disseminate in blood, colonize the kidney and are shed in urine of infected humanized TLR4 mice. Groups of huTLR4 (C57BL/6J background) mice were infected with a sublethal dose of *L. interrogans* serovar Copenhageni FioCruz L1-130 in comparison with PBS inoculated controls, n=3 or 4 mice per group. Weight was acquired daily for 2 weeks (A), the burden of Leptospira was quantified by qPCR in blood every other day (B), in urine daily (C) and in kidney at termination (D), whereas viability of Leptospira (E) was evaluated by qPCR of kidney culture at termination (0) and 3 days later (3). Statistics by Paired t test (A), ** $p<0.005$. Data represents one of three experiments.

uninfected controls (PBS) were significant (Fig 1A); very low numbers of Leptospira disseminated in blood, with ~30 leptospires /µl 1 day post infection (dpi) and 20/µl at 3 dpi (Fig 1B), whereas at 10 dpi ~$5x10^3$ Leptospira were shed in urine (Fig 1C). At termination 15 dpi, ~10^4 Leptospira were detected per µg of renal tissue (Fig 1D) with kidney cultures 3 days post-termination attesting to their viability (Fig 1E). These results suggest that *L. interrogans* infection leads to sublethal leptospirosis, renal colonization and stable shedding of Leptospira in urine of huTLR4 mice.

We then evaluated systemic cellular immune responses of Leptospira-infected huTLR4. Flow cytometry analysis of splenocytes from humanized TLR4 mice infected with *L. interrogans* revealed an increase of CD3+CD4+ T cells, a decrease of the proportion of CD3+CD8+ cytotoxic T cells and no difference in the percentages of CD3+ Double Negative (CD4-/CD8-) T cells as compared to the uninfected controls (Fig 2). Of the increased population of CD4+ T helper cells ~30% acquired an effector phenotype. Differences observed between CD8+ effector populations were not significant. Neither CD4+ or CD8+ memory T cells were affected by *L. interrogans* infection. We also analyzed the proportion of B cells (B220+) and found no differences between infected splenocytes and uninfected controls.

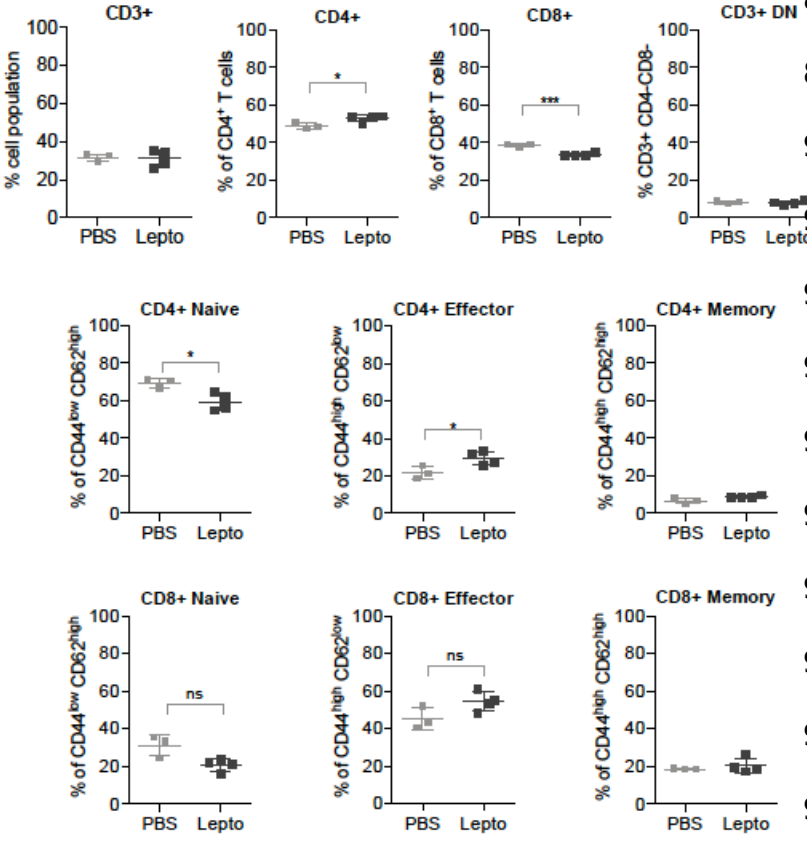

Figure 2. CD4+ effector T cell populations are increased in spleens of humanized TLR4 mice infected with *L. interrogans*. Freshly isolated splenocytes were blocked with anti-mouse CD16/CD32, incubated with the respective surface lineage markers and acquired in a LSRII flow cytometer. Data are the mean +/- SD percentage of cells for 3 or 4 mice per group. Statistics by unpaired t test with Welch's correction, * $p<0.05$ and *** $p<0.005$. Data represents one of two experiments.

To find out if replacing the murine TLR4 with human TLR4 affects the kinetics of disease progression, we compared infection in wild-type C57BL/6J (muTLR4) and humanized TLR4/MD2 transgenic mice (huTLR4). We inoculated mice intraperitoneally with ~10^8 *L. interrogans* Copenhageni FioCruz to analyze weight loss and to quantify the total amount of *Leptospira* (16s rRNA, *rrm* locus) in blood and excreted in urine (days 1-15 post infection) as well as the burden of live Leptospira in kidney at termination (d15) by qPCR (Fig 3). Compared to PBS controls, both muTLR4 and huTLR4 either lost small but significant amounts of weight (muTLR4) or did not gain weight (huTLR4) over two weeks of infection (Fig 3A). We found very low numbers of Leptospira in blood, with the sum all tested days ~100/µl in both groups of infected mice (Fig 3B). Interestingly, much higher numbers of Leptospira were excreted in urine of infected muTLR4 (~14,000 ± 2,500) and huTLR4 (~92,000 ± 34,000), with a ~1Log difference between the two groups (Fig 3C). Culture of the kidney tissue at termination showed that live Leptospira colonized the kidney of both groups of infected mice (Fig 3D).

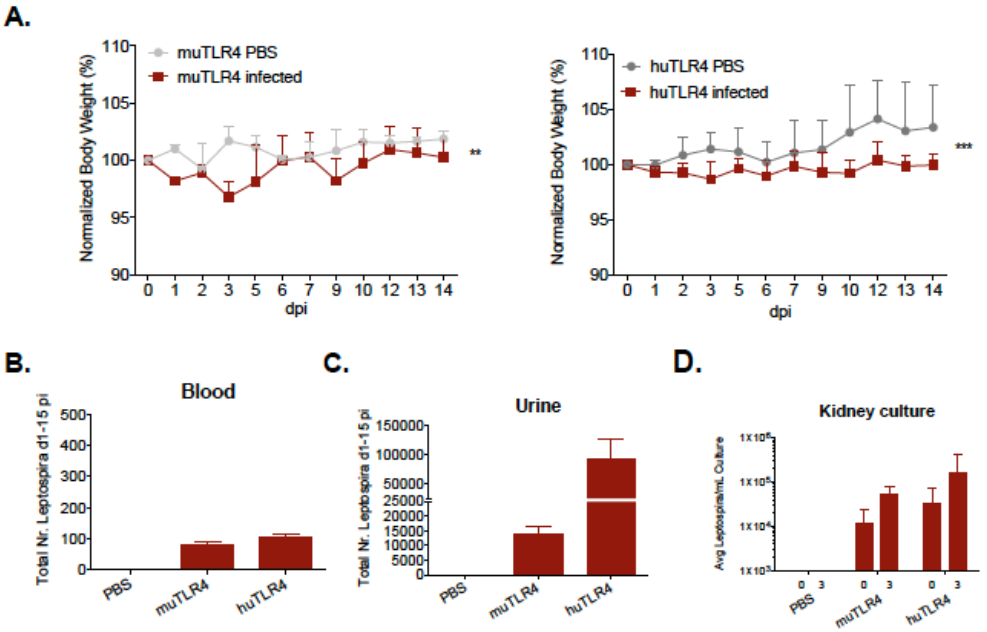

Figure 3. Higher numbers of Leptospira are excreted in urine of humanized TLR4 than wild-type mice. Quantification of weight (A) and total Leptospira in blood (B), urine (C) and in kidney culture (D). Total numbers of Leptospira in blood and urine are represented as the sum of Leptospira detected between days 1-15 post infection (B and C); kidney culture (D) is represented by the average number (nr) of Leptospira per mL of culture at termination (0) and 3 days later (3). Statistics by paired t test, ** p<0.005, *** p<0.0005.

To find out if replacing murine TLR4 with human TLR4 affects inflammation of the kidney, we analyzed the differences in inflammatory, antimicrobial and fibrosis markers in kidney tissue

collected from the two groups of infected and uninfected mice at two weeks post infection. We purified mRNA from infected and uninfected kidney and quantified a number of chemokines and cytokines (Fig 4). We found that the expression of CxCL2/MIP-2, CCL5/RANTES and IFN-γ were significantly increased in the infected kidneys of both muTLR4 and huTLR4 compared to the PBS controls; of note, TNF-α was only increased in kidney of huTLR4. Between groups of infected muTLR4 and huTLR4 mice, differences between

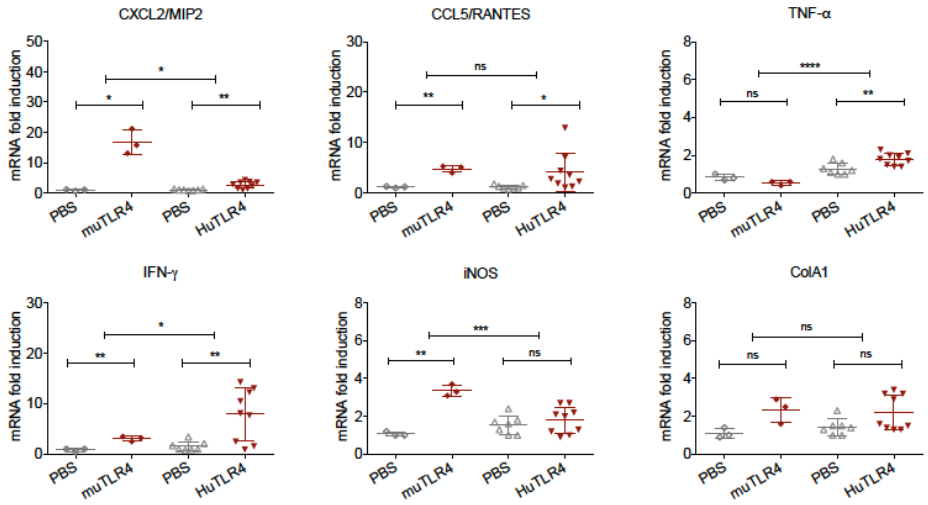

Figure 4. *L. interrogans* induce inflammation in the kidney of wild-type and humanized TLR4 mice but not antimicrobial iNOS in humanized TLR4 mice. qPCR quantification of reverse-transcribed pro-inflammatory, antimicrobial and fibrosis markers in the kidney of wildtype TLR4 (muTLR4) and humanized TLR4 (huTLR4) mice two weeks post-infection with *L. interrogans* serovar Copenhageni FioCruz, n=3 to 9 mice per group. Statistics by unpaired t test with Welch's correction. Data is representative of two experiments.

CxCL2/MIP2, TNF-α, IFN-γ and iNOS were significant: in huTLR4, MIP-2 and iNOS were decreased whereas TNF-α and IFN-γ were increased when compared to muTLR4. RANTES and ColA1 were increased in groups of infected mice compared to the respective PBS controls but differences were not significant between groups of infected mice. Furthermore, there were no differences in CxCL1/KC, IL-4, IL-6, IL-10 and IL-23 between *L. interrogans* infected and uninfected kidneys, and between infected kidneys of muTLR4 and huTLR4 mice (data not shown). The higher levels of expression of TNF-α and IFN-γ mRNA in kidney of infected huTLR4 mice suggest that *Leptospira* infection triggers increased inflammation in the kidneys of huTLR4 mice than muTLR4. Interestingly, the levels of antimicrobial iNOS were decreased in huTLR4 when compared to the wild-type muTLR4.

To get further insight into the role of TLR4 in Leptospira induced inflammation, we compared primary splenocytes from wild-type C57BL/6J mice (muTLR4) and humanized TLR4 mice (huTLR4) stimulated with 10^7/ml heat-killed *L. interrogans* and gated on the $CD11b^+$ macrophage/monocyte population using an *ex vivo* flow-cytometry based assay (Hajjar, Ernst et al. 2012) (Fig 5). We found that ~6% of CD11b+ macrophages from huTLR4 stimulated with 10^7 *Leptospira* produced TNFα in comparison to ~14% of muTLR4. This suggests that the systemic inflammatory response of huTLR4 macrophages to Leptospira is reduced compared to muTLR4, which is in agreement with the lack of sensing of Leptospira through huTLR4 (Nahori, Fournie-Amazouz et al. 2005).

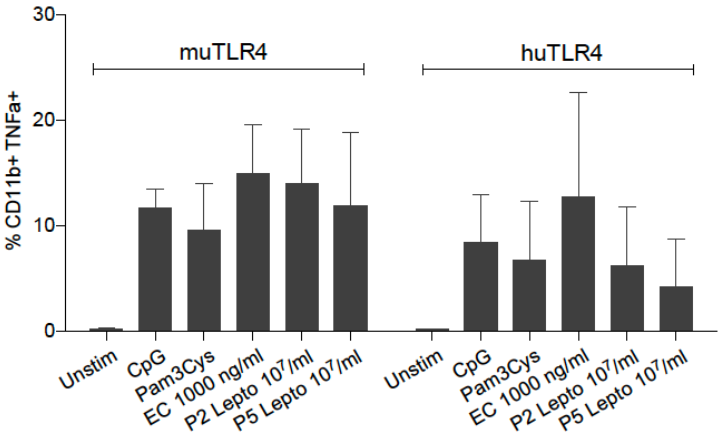

Figure 5. Spleenic macrophages of humanized TLR4 mice produce less TNF-alfa. Flow cytometric quantification of % of primary C57BL/6J mouse monocyte/macrophages producing TNF-alfa after stimulation with heat-killed *L. interrogans* ser. Copenhageni and TLR controls (CpG ~TLR9, Pam3Cys ~TLR2, EColi ~TLR4). Data is representative of two experiments.

Altogether, these results suggest that compared to wild type mice, huTLR4 respond differently to *Leptospira* infection, suggesting that TLR4 plays a species-specific role in leptospirosis, with human TLR4 being associated with higher *Leptospira* shedding and kidney inflammation.

Discussion

Our goal was to develop an immunocompetent mouse model of persistent or sublethal leptospirosis. The rationale for using humanized TLR4 transgenic mice is that, if human TLR4 does not sense the atypical *Leptospira* LPS (Werts, Tapping et al. 2001),(Que-Gewirth, Ribeiro et al. 2004),(Nahori, Fournie-Amazouz et al. 2005), these mice should be more susceptible to leptospirosis than wild-type

mice expressing murine TLR4 that do sense *Leptospira* LPS. We therefore hypothesized that C57BL/6J mice carrying the human TLR4/MD-2 receptor and co-receptor in its immune cells (huTLR4) should be more sensitive to sublethal leptospirosis than wild-type C57BL/6J (muTLR4).

We analyzed the kinetics of leptospirosis progression and colonization of the kidney between infected and uninfected humanized TLR4 mice as established previously in our laboratory for C3H-HeJ (Richer, Potula et al. 2015),(Potula, Richer et al. 2017),(Nair, Guedes et al. 2020). Compared to PBS counterparts, we found that infected huTLR4 mice did not gain weight, which is a clinical sign of disease progression, and had levels of live Leptospira in blood, urine and kidney consistent with our previous studies of sublethal infection in C3H-HeJ mice which do not sense LPS in general (Vogel, Johnson et al. 1999),(Qureshi, Lariviere et al. 1999). When we analyzed the T and B cell immune responses 2 weeks post infection with *L. interrogans* we found that compared to uninfected controls the CD3+CD4+ effector T cells proliferated, whereas B cells and memory CD4+ T cells were not affected. This is also consistent with our previous observations in C3H-HeJ mice (Richer, Potula et al. 2015),(Potula, Richer et al. 2017). These data suggest that the humanized TLR4 systemic immune response to the presence of *L. interrogans* Copenhageni is similar to that of C3H-HeJ mice. These data show that C3H-HeJ mice and humanized TLR4 C57BL6 mice expressing a “present but Leptospira-LPS-sensing-impaired” TLR4 receptor in their immune cells allow *Leptospira* to evade some immune recognition, as illustrated by the lower TNF-α response in huTLR4 compared to muTLR4 macrophages. This may cause persistent sublethal infection, kidney colonization and high shedding of Leptospira in urine.

We questioned whether the differences observed in the humanized TLR4 transgenic mice model were due to the TLR4 transgene modification or to other factors related to the C57BL/6J mouse genetic background. In our *in vivo* studies we found that both huTLR4 and muTLR4 did not gain significant amounts of weight after infection and that live *L. interrogans* disseminated in blood and colonized

the kidneys of both groups of mice. However, much higher numbers of Leptospira were excreted in urine of huTLR4 than muTLR4 mice. The numbers of Leptospira shedding in huTLR4 mice were not as high but approximate the Leptospira numbers previously detected in urine of infected C3H-HeJ mice (Richer, Potula et al. 2015). As shown by Fanton d'Andon (Fanton d'Andon, Quellard et al. 2014), this is evidence that wild-type C57BL/6J mice carrying a non-mutated TLR4 also support persistence of Leptospira which fulfils their role as host reservoirs.

Analysis of the immune mediator markers in the kidney of infected and uninfected muTLR4 mice showed that innate inflammatory chemokines MIP-2 and RANTES, cytokine IFN-γ and antimicrobial iNOS were increased in infected mice, consistent with a previous study (Fanton d'Andon, Quellard et al. 2014). Between infected and uninfected huTLR4 mice, innate inflammatory MIP-2 and RANTES were increased, whereas antimicrobial iNOS was not affected in infected mice. These data suggest that in wild-type C57BL/6J an antimicrobial response is under way that controls and limits the numbers of Leptospira shedding in urine. In humanized TLR4 mice the absence of significant antimicrobial iNOS response may allow higher numbers of Leptospira to go unchecked to shed in urine. Between groups of infected muTLR4 and huTLR4 we saw a decrease in innate inflammatory MIP-2 and antimicrobial iNOS in huTLR4, whereas differences in antimicrobial RANTES and fibrosis ColA1 were not significant. These data suggest that decreased inflammatory antimicrobial responses in the huTLR4 kidney is dependent on TLR4, with human TLR4 being more permissive of Leptospira shedding in urine.

Analysis of the cytokine panel in the kidney showed that TNF-α and IFN-γ were increased in huTLR4 compared to muTLR4. There were no significant differences in IL-4, IL-6 and IL-23 cytokines which suggests Th2, Th17 and Th follicular (respectively) were not engaged. These data suggest that a Th1 driven inflammatory response to the higher numbers of Leptospira may be induced in kidneys of huTLR4 mice compared to muTLR4.

Taken together these data suggest that the kinetics of Leptospira dissemination and disease onset in huTLR4 mice is quite similar to that observed in C3H-HeJ (Richer, Potula et al. 2015). Nevertheless, the innate immune mechanisms that lead to kidney pathology appear to be different in huTLR4 mice. It is important to note that, although much more subdued, Leptospira disseminated in wild-type C57BL6 which carry a fully functional TLR4. These data suggest that the immune response differences observed may be attributable, in part, to human TLR4 but that persistence of Leptospira is also mediated by other TLRs.

The importance of these data relates to the fact that huTLR4 transgenic mice are not immuno-compromised and by virtue of human TLR4 not sensing *Leptospira* LPS (Nahori, Fournie-Amazouz et al. 2005) they appear to be slightly more susceptible to sublethal leptospirosis than wild-type C57BL/6J, but they are not as susceptible as C3H-HeJ (Richer, Potula et al. 2015),(Potula, Richer et al. 2017),(Nair, Guedes et al. 2020) or TLR4ko mice (Chassin, Picardeau et al. 2009).

In conclusion, we confirmed that infection of humanized TLR4 transgenic immunocompetent mice led to a disease phenotype consistent with sublethal leptospirosis represented by differences in weight, dissemination of live Leptospira in blood, colonization and inflammation of the kidney, shedding of high numbers of Leptospira in urine and engagement of cellular immune responses.

Materials and Methods

Bacterial strains. We used *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 (henceforth *Leptospira*), culture passage 2 after hamster infection, originally isolated from a patient in Brazil. *Leptospira* was cultured as previously described (Sullivan, Nair et al. 2017) and

enumerated by dark-field microscopy (Zeiss USA, Hawthorne, NY) that was confirmed by qPCR (StepOne Plus, Life Technologies, Grand Island, NY).

Animals. 6-week old humanized TLR4/MD-2 (huTLR4) male mice were transferred from Dr Hajjar laboratory at the University of Washington to the Laboratory Animal Care Unit of the University of Tennessee Health Science Center (LACU-UTHSC) and quarantined for 3 weeks. 9-week old wild-type C57BL/6J (muTLR4) mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and acclimatized for one week at the pathogen-free environment in the LACU-UTHSC. Infections were done when mice reached 10 weeks of age.

Ethics statement. *This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the NIH.* The protocols were approved by the University of Tennessee Health Science Center (UTHSC) Institutional Animal Care and Use Committee, Animal Care Protocol Application, Permits Number 16-070 and 19-062.

Infection of mice. Intraperitoneal infection was done as described previously using a dose of ~10^8 virulent *Leptospira* in sterile PBS. Bacteria were counted in a Petroff-Hausser chamber under a dark field microscope and confirmed by qPCR. Body weights were monitored daily. Urine was also collected on a daily basis for 15 days p.i. by gently massaging the bladder area and the urine was collected into sterile aluminum foil. Blood (up to 20 µl) was collected every other day by tail nick for 15 days. At termination, kidneys were collected for qPCR.

RT-PCR and q-PCR. DNA was extracted per manufacturers' instructions from urine, blood, and kidney using a NucleoSpin tissue kit (Clontech). Quantification of *Leptospira* 16s rRNA was done using TAMRA probe and primers from Eurofins (Huntsville, AL) by real-time PCR (qPCR) (StepOne Plus). RNeasy mini kit (Qiagen) was used to extract total RNA followed by reverse transcription using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Real-time

PCR on the cDNA was performed as described (Richer, Potula et al. 2015). For RT-PCR, we used TAMRA probes specific for inducible nitric oxide synthase (iNOS), Collagen A1 (ColA1), keratinocyte-derived chemokine (KC, CxCL1), macrophage inflammatory protein 2 (MIP-2, CxCL2), RANTES (CCL5), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), IL-4, IL-6, IL-10, IL-23. β-actin was used as control for the comparative CT method (Potula, Richer et al. 2017).

Profiling of immune cell populations (T and B cells) by Flow Cytometry. Cell preparation: Spleens were harvested from mice, placed in RPMI 1640 (Mediatech, Manassas, VA), and diced with frozen slides in Hanks balanced salt solution medium (Cellgro, Manassas, VA) to produce cell suspensions. Red blood cell lysis was performed using 2 ml/spleen of 1X ACK lysing buffer (Life Technologies). Cells were then washed in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (Atlanta Biotech) and 0.09% sodium azide (Sigma-Aldrich, St. Louis, MO), followed by passage through 70-um-pore-size and 40-um-pore-size nylon filters (BD Falcon, Bedford, MA), and the cells were counted using a Luna-FL automated cell counter (Logos Biosystems, Gyunggi-Do, South Korea). Flow Cytometric Cell Staining. Cell viability was analyzed by mixing 18 ul of splenocytes with 2 ul of acridine orange-propidium iodide staining solution in a Luna-FL automated cell counter, and 3 x 10^6 cells per tube were stained as follows: cells were incubated in 0.5 ug Fc block (BD Biosciences) for 15 min at 4°C in staining buffer (Ca^{2+} - and Mg^{2+} - free PBS containing 3% heat-inactivated fetal bovine serum, 0.09% sodium azide [Sigma-Aldrich, St. Louis, MO], 5mMEDTA), washed twice, and incubated with the appropriate marker for surface staining in the dark for 30 min at 4°C. T lymphocyte staining was performed using CD3 clone 17A2 conjugated with fluorescein isothiocyanate (FITC) (1:200; BD Biosciences), CD4 clone RM4-5 conjugated with phycoerythrin (PE) (1:150; BD Biosciences), CD8 clone 53-6.7 conjugated with allophycocyanin (APC)-Cy7 (1:150; BD Biosciences), CD62L clone MEL-14 conjugated with PE-Cy7 (1:150; BD

Biosciences), CD44 clone IM7 conjugated with APC (1:150; BD Biosciences). Flow cytometric staining and analysis was done as described (Richer, Potula et al. 2015).

Splenic macrophages. Heat-killed Leptospira were prepared as follows: pellets from the culture, which were washed with 1mL PBS, were harvested by centrifugation at 12,000g and heat killed by exposure to heat for 10 min at 95°C. The supernatant was discarded, and the extract resuspended in endotoxin free PBS. Lack of motility was confirmed under a dark field microscope. Single-cell splenocyte suspensions were stimulated with 10 µg/ml CpG 1826 or Pam3CSK4, 1000 ng/ml *E. coli* O111:B4 LPS, or 10^7/ml heat-killed P2 or P5 *L. interrogans* in the presence of 10 µg/ml brefeldin A for ~4hr as described in (Hajjar, Ernst et al. 2012). Cells were then stained and 500,000 events were acquired on LSRII cytometry as described (Hajjar, Ernst et al. 2012). CpG, *E. coli* LPS and Pam3cys were used as TLR9, TLR4 and TLR2 positive controls, respectively.

Statistics. Two-tailed unpaired parametric t-test with Welch's correction was used to analyze differences between infected and non-infected groups. Weight loss differences were measured with a paired *t* Test. Statistical analysis was done using GraphPad Prism software, alpha = 0.05.

Acknowledgements

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Figure Legends

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Figure 2. CD4+ effector T cell populations are increased in spleens of humanized TLR4 mice infected with *L. interrogans*. Freshly isolated splenocytes were blocked with anti-mouse CD16/CD32, incubated with the respective surface lineage markers and acquired in a LSRII flow cytometer. Data are the mean +/- SD percentage of cells for 3 or 4 mice per group. Statistics by unpaired t test with Welch's correction, * $p<0.05$ and *** $p<0.005$. Data represents one of two experiments.

Figure 3. Higher numbers of Leptospira are excreted in urine of humanized TLR4 than wild-type mice. Quantification of weight (A) and total Leptospira in blood (B), urine (C) and in kidney culture (D). Total number of Leptospira in blood and urine are represented as the sum of Leptospira detected between days 1-15 post infection (B and C); kidney culture (D) is represented by the average number (nr) of Leptospira per mL of culture at termination (0) and 3 days later (3). Statistics by paired t test, ** $p<0.005$, *** $p<0.0005$.

Figure 4. *L. interrogans* cause inflammation in the kidney of wild-type and humanized TLR4 mice but not antimicrobial iNOS in humanized TLR4 mice. qPCR quantification of reverse-transcribed pro-inflammatory, antimicrobial and fibrosis markers in the kidney of wildtype TLR4 (muTLR4) and humanized TLR4 (huTLR4) mice two weeks post-infection with *L. interrogans* serovar Copenhageni FioCruz, n=3 to 9 mice per group. Statistics by unpaired t test with Welch's correction. Data is representative of two experiments.

Figure 5. Splenic macrophages of humanized TLR4 mice produce less TNF-α. Flow cytometry quantification of % of primary C57BL/6J mouse splenic macrophages producing TNF-α after stimulation with heat-killed *L. interrogans* serovar Copenhageni (Passage 2 and Passage 5 Lepto) and TLR controls (CpG ~TLR9, Pam3Cys ~TLR2, *E. coli* LPS ~TLR4). Data presented are combined results from two separate experiments.

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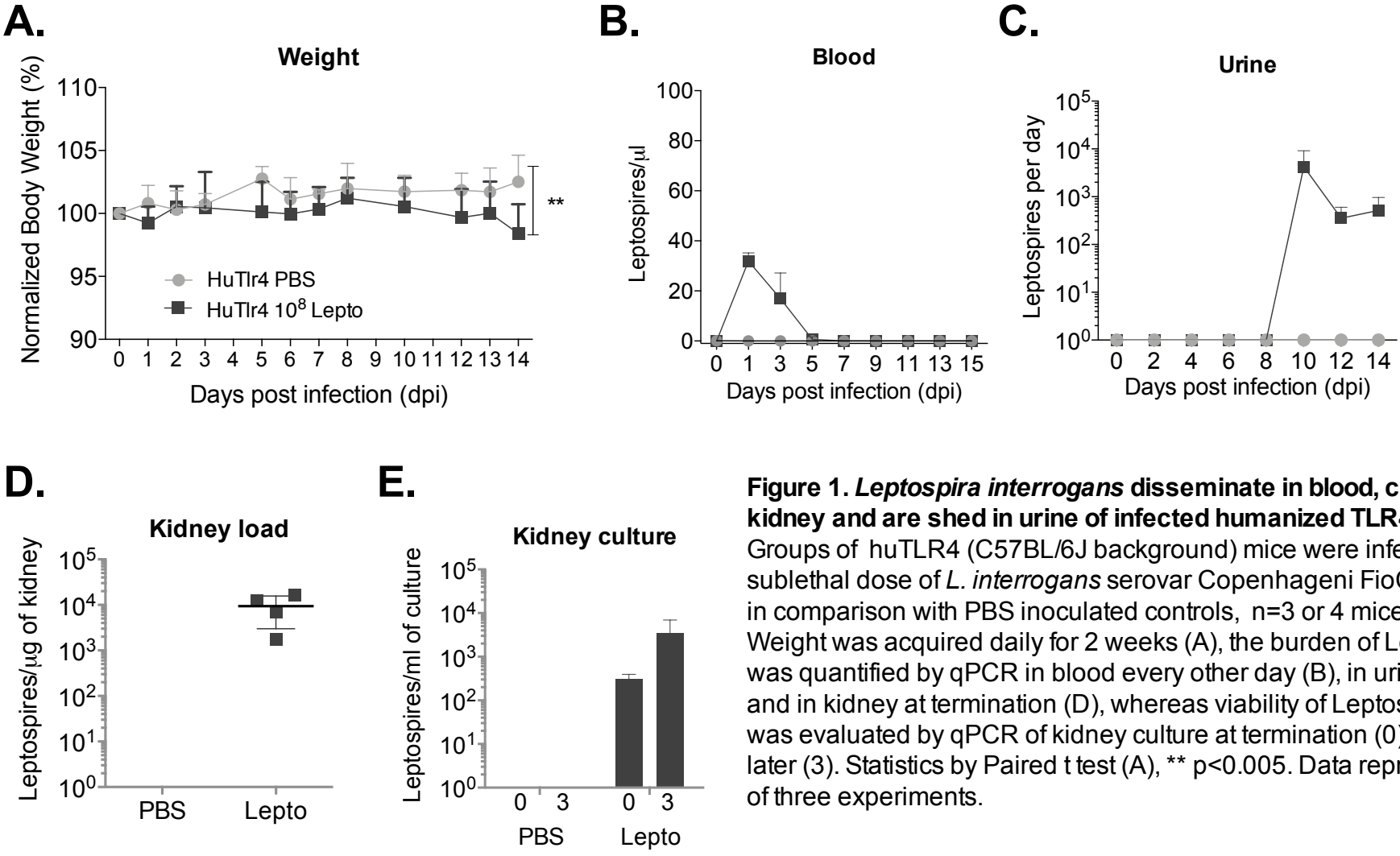

Figure 1. *Leptospira interrogans* disseminate in blood, colonize the kidney and are shed in urine of infected humanized TLR4 mice. Groups of huTLR4 (C57BL/6J background) mice were infected with a sublethal dose of *L. interrogans* serovar Copenhageni FioCruz L1-130 in comparison with PBS inoculated controls, n=3 or 4 mice per group. Weight was acquired daily for 2 weeks (A), the burden of Leptospira was quantified by qPCR in blood every other day (B), in urine daily (C) and in kidney at termination (D), whereas viability of Leptospira (E) was evaluated by qPCR of kidney culture at termination (0) and 3 days later (3). Statistics by Paired t test (A), ** $p<0.005$. Data represents one of three experiments.

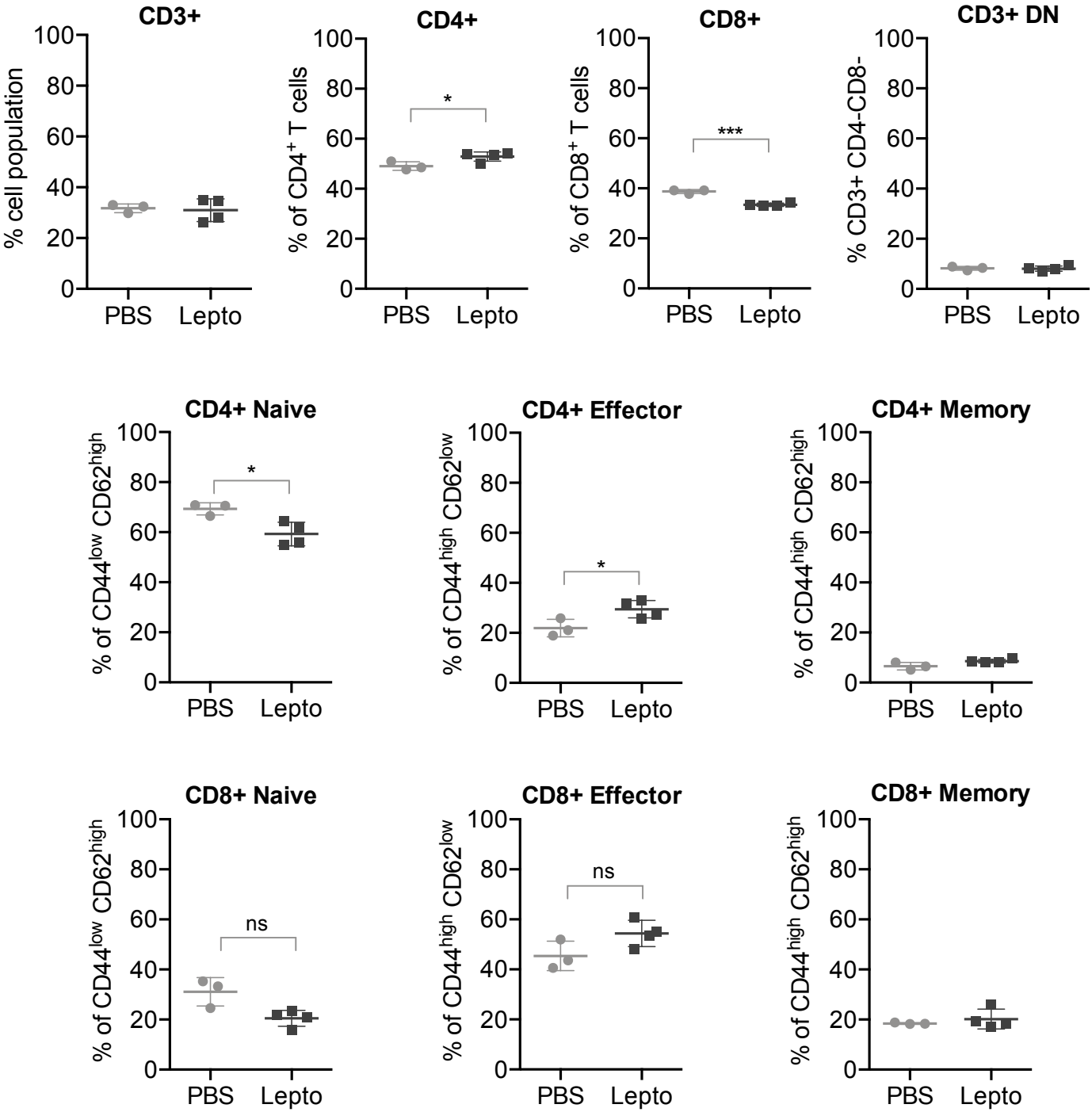

Figure 2. CD4+ effector T cell populations are increased in spleens of humanized TLR4 mice infected with *L. interrogans*. Freshly isolated splenocytes were blocked with anti-mouse CD16/CD32, incubated with the respective surface lineage markers and acquired in a LSRII flow cytometer. Data are the mean +/- SD percentage of cells for 3 or 4 mice per group. Statistics by unpaired t test with Welch's correction, * $p<0.05$ and *** $p<0.005$. Data represents one of two experiments.

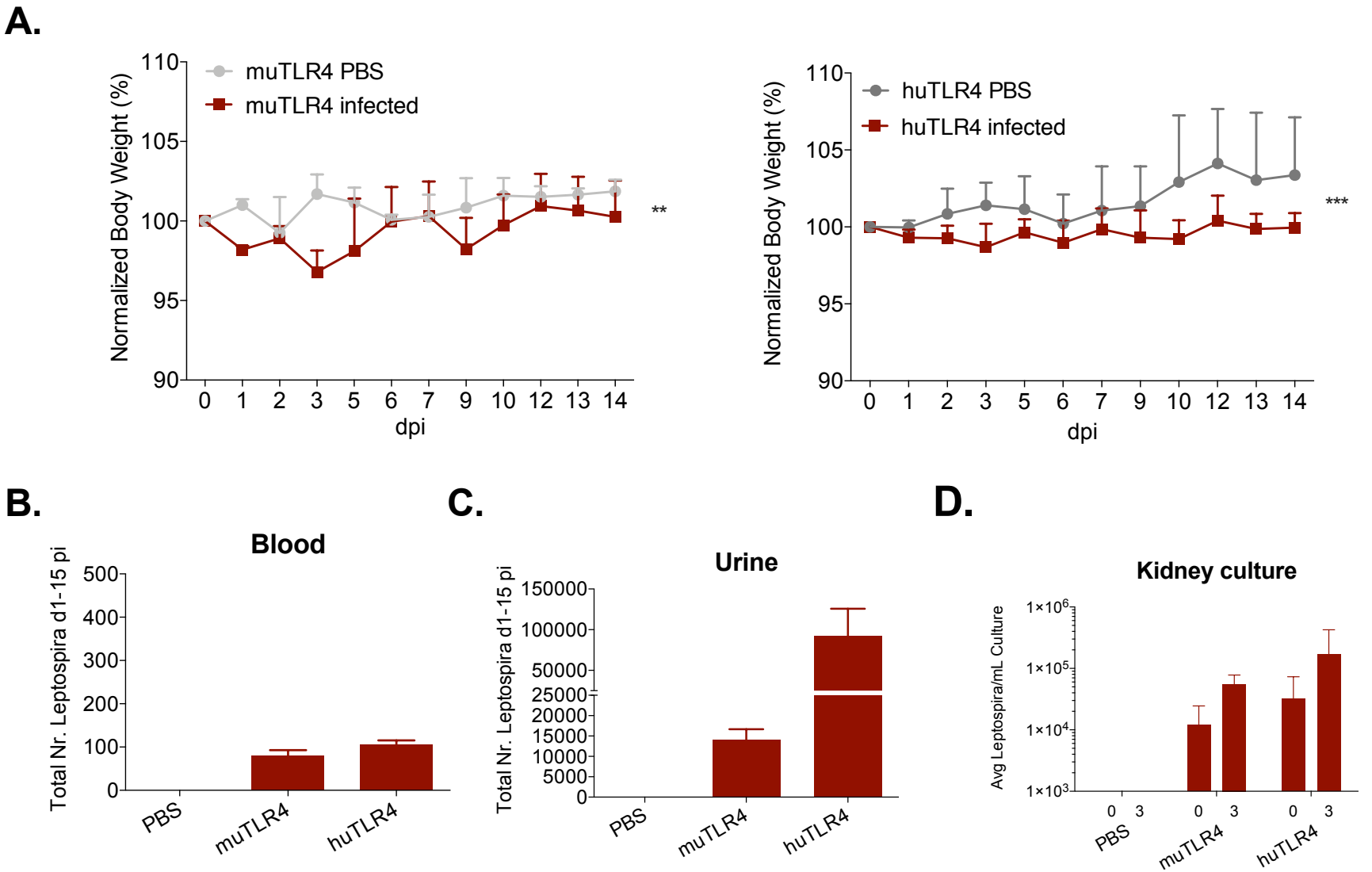

Figure 3. Higher numbers of Leptospira are excreted in urine of humanized TLR4 than wild-type mice. Quantification of weight (A) and total Leptospira in blood (B), urine (C) and in kidney culture (D). Total number of Leptospira in blood and urine are represented as the sum of Leptospira detected between days 1-15 post infection (B and C); kidney culture (D) is represented by the average number (nr) of Leptospira per mL of culture at termination (0) and 3 days later (3). Statistics by paired t test, ** $p<0.005$, *** $p<0.0005$.

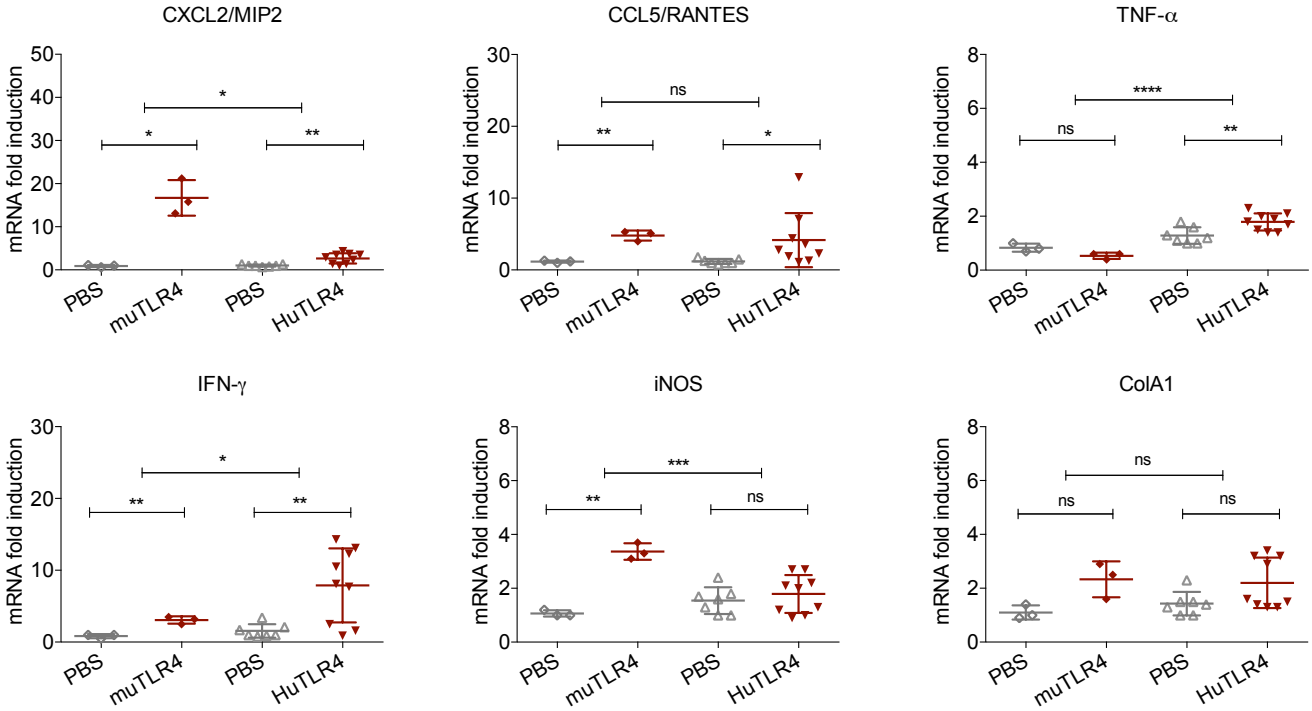

Figure 4. *L. interrogans* induce inflammation in the kidney of wild-type and humanized TLR4 mice but not antimicrobial iNOS in humanized TLR4 mice. qPCR quantification of reverse-transcribed pro-inflammatory, antimicrobial and fibrosis markers in the kidney of wildtype TLR4 (muTLR4) and humanized TLR4 (huTLR4) mice two weeks post-infection with *L. interrogans* serovar Copenhageni FioCruz, n=3 to 9 mice per group. Statistics by unpaired t test with Welch's correction. Data is representative of two experiments.

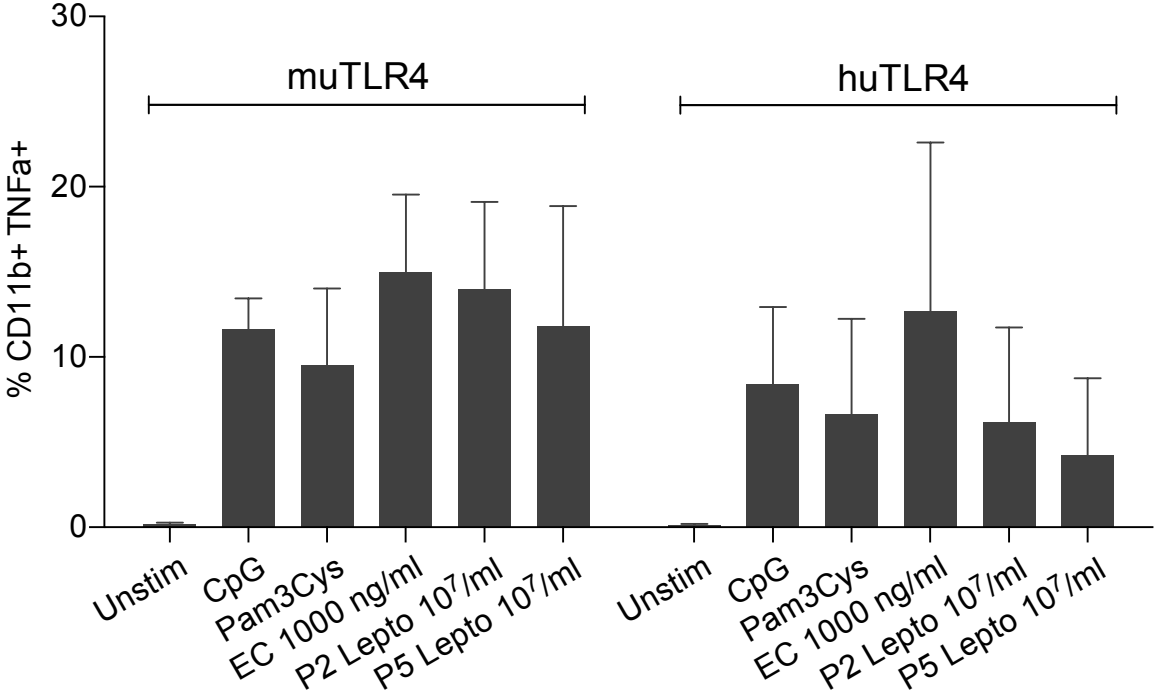

Figure 5. Spleenic macrophages of humanized TLR4 mice produce less TNF-alfa. Flow cytometric quantification of % of primary C57BL/6J mouse monocyte/macrophages producing TNF-alfa after stimulation with heat-killed *L. interrogans* ser. Copenhageni and TLR controls (CpG ~TLR9, Pam3Cys ~TLR2, EColi ~TLR4). Data is representative of two experiments.