Title: Elevated activation of neutrophil Toll-like receptors in patients with acute severe leptospirosis: An Observational Study

Running Head: Activated neutrophils in acute leptospirosis

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ABSTRACT

1 Leptospirosis is the leading cause of zoonotic morbidity and mortality globally, yet little is
2 known about the immune mechanisms that may contribute to pathogenesis and severe disease.
3 While neutrophils are a key component of early immune responses to infection, they have been
4 associated with tissue damage and inflammation in some febrile infections. To assess whether
5 neutrophils contribute to the pathogenesis observed in severe leptospirosis, we quantitated
6 levels of neutrophil activation markers in patients with varying disease severities. Hospitalized
7 leptospirosis patients had significantly higher levels of TLR2 and TLR4 on peripheral neutrophils
8 compared to healthy controls, with the highest levels detected in patients with organ
9 dysfunction. We observed no significant differences in other neutrophil baseline activation
10 markers (CD62L and CD11b) or activation capacity (CD62L and CD11b levels following
11 stimulation) regardless of disease severity. Our results provide preliminary evidence supporting
12 the hypothesis that higher initial bacterial loads or inadequate or delayed neutrophil responses,
13 rather than TLR-driven inflammation, may drive severe disease outcomes.

Leptospirosis is a neglected zoonotic disease of global importance with over one million cases and nearly 60,000 deaths occurring annually, primarily in slum settlements worldwide.¹ In Salvador, Brazil, large epidemics of leptospirosis, usually caused by *Leptospira interrogans* serovar Copenhageni, occur during seasonal periods of heavy rainfall.^{2, 3} The bacteria penetrate skin or mucous membranes, with clinical manifestations ranging from mild to severe flu-like symptoms to multi-organ failure and death.² Notably, Weil's disease, the classic severe form of leptospirosis characterized by jaundice, acute renal failure and bleeding, and *Leptospira*-associated pulmonary hemorrhage syndrome (LPHS) have case fatalities of >10% and 50%, respectively.^{3, 4, 5}

Neutrophils are abundant innate immune cells capable of killing extracellular *Leptospira* via phagocytosis, production of reactive oxygen and nitrogen species, neutrophil extracellular traps, and specific antimicrobial peptides.^{6, 7, 8} Neutrophils at the site of infection help initiate adaptive immune responses through production of cytokines and chemokines. Despite their critical role in resolving infections, neutrophils have been associated with exacerbation of lung infections in influenza and other nosocomial infections,⁹ and could contribute to the inflammation leading to organ damage observed in 10-15% of leptospirosis cases, particularly as higher neutrophil counts have been observed in patients with more severe disease.^{2, 10, 11, 12} However, a recent study of neutrophils in hospitalized cases showed that neutrophils were not activated during acute leptospirosis despite patients having significant neutrophilia.¹³

Neutrophils recognize conserved microbial motifs through pattern recognition receptors, such as Toll-Like Receptors (TLRs), which trigger activation of antimicrobial responses and production of pro-inflammatory cytokines.¹⁴ To date, *in vitro* experiments using transfected human cell lines and healthy volunteer peripheral blood mononuclear cells (PBMCs) or whole blood have yielded contradictory results regarding the involvement of TLR2 and TLR4 in the human immune response to the leptospiral lipopolysaccharide (LPS).^{15, 16, 17, 18} A single study in patients showed neutrophils expressed TLR2 during acute leptospirosis, but did not analyze

TLR4 expression.¹³ Results from animal models of leptospirosis have yielded differing TLR responses to leptospiral LPS, with control of infection mediated by TLR2 and TLR4 in mice^{16, 17, 19, 20} and activation of TLR2 in pig cell lines.^{21, 22} A separate study demonstrated that resistant mice, but not hamsters, induced TLR2 early in infection, and when *Leptospira* and a TLR2 agonist were coinjected in hamsters, pathology and survival improved.²³ Additionally, a recent study showed mononuclear macrophages had greater capacity for killing *Leptospire*s than neutrophils and were present in larger abundance in organ infiltrates in mice with leptospirosis.²⁴ We have undertaken the current study to examine TLR expression and function on neutrophils during acute human leptospirosis to identify whether neutrophil activation is associated with organ dysfunction and severe outcomes.

Between July 2013 and August 2014, we identified patients with suspected leptospirosis through active surveillance at a state-run infectious disease hospital in Salvador, Brazil. We confirmed 15 (79%) of 19 serially recruited cases, using at least one of the criteria described: seroconversion or 4-fold rise in titer in paired sera samples or titer $\geq 1:800$ in single sera sample measured by serum microagglutination test (MAT), positive blood qPCR (*Leptospira* load/mL blood), or positive blood culture.^{3, 25, 26} We gathered clinical data during patient interviews and from hospital charts and collected paired venous blood samples from patients during acute illness (n=18 within 72h of hospital admission and n=1 within 96h) and paired convalescence samples from four individuals not lost to follow-up (32-57 days post-admission). Additionally, we obtained samples from four healthy Brazilian adults. We categorized the 15 confirmed patients into two groups based on organ dysfunction: Severe group “S” patients with evidence of either renal dysfunction (oliguria [<500 mL urine/day) or anuria (<50 mL urine/day) within 24 h of hospital admission]) and/or lung injury during hospitalization (mechanical ventilation and/or requiring oxygen, ≥ 250 mL blood in lungs or endotracheal tube, and/or respiration rate >38 /min), while Acute group “A” patients met none of these criteria (Table 1).

We performed assays on a BD Aria II 3-laser flow cytometer. For TLR markers, we stained fresh, heparinized whole blood on the day of collection (stored <4 h at room temperature [RT]) for 30 min with CD15-AlexaFluor647 (W6D3), TLR2-FITC (TL2.1; eBiosciences, Waltham, MA, USA), and TLR4-PE (HTA125; eBiosciences). For activation marker quantification, we stimulated (0.05 µg *E. coli* LPS, 0.5 µg Pam3CSK4, or 0.3 µg CpG; InvivoGen, San Diego, CA, USA) and labeled (CD15-AlexaFluor647 [W6D3], CD11b-APC-Cy7 [ICRF44], and CD62L-FITC [DREG56]) 100 µL whole blood as previously described.²⁷ We washed with cold FACS buffer (FB = PBS + 2% FBS + 0.1% NaN₃), lysed for 10-15 min in 1 mL freshly prepared 1x BD lysing solution in ddH₂O (Becton Dickinson, São Paulo, Brazil), and fixed in 4% paraformaldehyde for 10 min at RT. We washed fixed cells with 400 µL cold FB, resuspended in 400 µL FB, and incubated at 4°C until analyzed on the flow cytometer (within 24h). We prepared cells, isotype controls, and compensation beads (to determine gating) for every experiment using the Becton Dickinson Flow Cytometry Staining support protocols. We determined the Median Fluorescent Intensity (MFI) from 50,000 events within the granulocyte population after subtraction of isotype control levels, and defined a marker's MFI Fold-Change as MFI (stimulated with TLR agonists LPS, Pam3, or CpG) divided by MFI (medium only).

Precise mechanisms leading to high case fatality in leptospirosis patients are currently unknown. To assess whether the neutrophilia observed in leptospirosis patients was associated with increased neutrophil activation and relevant for multi-organ dysfunction, we characterized the neutrophil response during acute infection. We found no significant difference in the abundance of neutrophils or other circulating immune cell types between patients with (S; n=5) or without (A; n=10) renal/lung dysfunction (Table 1). We quantified *Leptospira* load (*Leptospira* genome/mL blood) using a standard TaqMan qPCR protocol targeting the *lipL32* gene or 16S rRNA gene for amplification.²⁵ Severe patients (S) trended higher for *Leptospira* loads, suggesting organ dysfunction correlated with greater pathogen burden (Table 1).

We investigated whether differences in neutrophil activation or function were associated with disease outcome by quantifying two neutrophil activation markers, CD11b ($\beta 2$ integrin) and CD62L (L-selectin), in acute and convalescent samples from patients with confirmed leptospirosis.²⁷ We detected comparable baseline frequencies (%CD15⁺ cells, Figure 1) and activation levels of neutrophils during acute infection (Figure 1A, 1B) suggesting circulating neutrophil activation does not correlate with disease severity. Unexpectedly, we observed no significant difference in the abundance of neutrophils with activation markers (CD11b, CD62L) between patients' paired acute and convalescent samples or samples from healthy adults though the healthy controls trended higher in CD62L expression (less activated) than patients lacking organ dysfunction (A) ($p=0.07$). These data suggest activated neutrophils may have migrated to sites of infection in patients and were undetectable in peripheral samples (Figure 1A, B).

We also hypothesized that greater disease severity could be associated with increased neutrophil functional activity, resulting in release of potentially damaging enzymes or reactive oxygen and nitrogen species. Therefore, we quantified neutrophil response to stimulation with agonists for TLR2/1 (Pam3CSK4), TLR4 (LPS), or TLR9 (CpG). Both patient groups showed similar activation levels (Figure 2, higher CD11b MFI fold-changes, lower CD62L MFI fold-changes) following stimulation indicating no severity-associated difference in neutrophil functional response in patients with severe infection (Figure 1C, D).

Most *in vitro* experiments on healthy human cells have shown that TLR2, not TLR4, is the primary receptor for leptospiral LPS (unlike other Gram negative bacteria), though there are reports of whole leptospires signaling through both TLR2 and TLR4 in human cell lines, whole blood, and PBMCs.^{15, 16, 17, 18} To define the roles of TLR2 and TLR4 in responding to leptospires, we quantified neutrophil TLR2 and TLR4 expression during acute, paired convalescent leptospirosis samples, and in patients with other febrile diseases. We observed elevated levels of TLR2 and TLR4 in severe (S) and acute (A) leptospirosis patients compared to healthy

volunteers and paired convalescent samples (Figure 3). Notably, TLR2 and TLR4 expression was significantly higher on neutrophils from patients with more severe leptospirosis.

To identify possible mechanisms relevant to disease severity, we compared TLR expression with clinical characteristics (Tables S1 and S2), but found no association between neutrophil TLR expression, days of symptoms, or percent neutrophils at time of hospital admission. However, higher bacterial loads in whole blood correlated independently with higher expression of both TLR2 and TLR4 (Pearson's correlation, TLR2: $r=0.810$ [0.468-0.941], $p=0.0008$ and TLR4: $r=0.636$ [0.131-0.879], $p=0.019$).

We hypothesize that higher bacterial loads from larger inocula are a key factor driving disease pathology, with the increased bacteria loads leading to elevated TLR responses. Neutrophils may have reduced activity against *Leptospira*^{13, 24, 28} and we recently demonstrated low expression of the neutrophil antimicrobial peptide, cathelicidin, correlated with fatal leptospirosis cases indicating a role for effective bactericidal response.²⁹ Protection from *Leptospira* infection is likely mediated in part through anti-leptospiral LPS antibodies,¹⁵ the production of which could be affected by neutrophil function, and our previous results showed significant decreases in antibody production in fatal cases.²⁹ These results may indicate that the elevated levels of neutrophils are associated with more severe disease^{11, 12} is due to reduced migration to sites of infection rather than aberrant activation. However, in cases of fatal human leptospirosis, autopsies have found neutrophils and other immune cells in multiple organs, though not the lungs, indicating possible direct involvement in the pathology of severe disease.^{30, 31} Other support for neutrophil involvement includes modulation of T cell, Natural Killer, and B cell responses through cytokine and chemokine production (IL-10 and IL-6), which are elevated in fatal cases of leptospirosis.^{32, 33} Thus, there is still much to learn about the role of neutrophils in the pathogenesis of leptospirosis.

In summary, this is a preliminary report describing responses of neutrophils during acute leptospirosis. We found that neutrophils expressed significantly higher TLR2 in patients with

renal and/or lung dysfunction relative to patients lacking organ failure, and that TLR2 and TLR4 expression levels correlate with bacterial loads. This is consistent with a report demonstrating neutrophils expressed TLR2 during acute leptospirosis,¹³ but in contrast to a lack of increased neutrophil activation among patients observed by Raffay et al.¹³ These contrasts could be due to differences in study populations and infecting *Leptospira* serovars. Future longitudinal analyses of neutrophil and macrophage function during the course of illness in a larger patient cohort and of cathelicidin, which has immunomodulatory and bactericidal activities, may distinguish critical contributions of innate immune cells to the resolution of human *Leptospira* infections and severe disease outcomes.

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REFERENCES

1. Costa F, Hagan JE, Calcagno J, Kane M, Torgerson P, Martinez-Silveira MS, Stein C, Abela-Ridder B, Ko AI, 2015. Global Morbidity and Mortality of Leptospirosis: A Systematic Review. *PLoS Negl Trop Dis* 9: e0003898.
2. Ko AI, Goarant C, Picardeau M, 2009. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. *Nat Rev Microbiol* 7: 736-47.
3. Gouveia EL, Metcalfe J, de Carvalho AL, Aires TS, Villasboas-Bisneto JC, Queirroz A, Santos AC, Salgado K, Reis MG, Ko AI, 2008. Leptospirosis-associated severe pulmonary hemorrhagic syndrome, Salvador, Brazil. *Emerg Infect Dis* 14: 505-8.
4. Vieira SR, Brauner JS, 2002. Leptospirosis as a cause of acute respiratory failure: clinical features and outcome in 35 critical care patients. *Braz J Infect Dis* 6: 135-9.
5. Marotto PC, Nascimento CM, Eluf-Neto J, Marotto MS, Andrade L, Sztajn bok J, Seguro AC, 1999. Acute lung injury in leptospirosis: clinical and laboratory features, outcome, and factors associated with mortality. *Clin Infect Dis* 29: 1561-3.
6. Murgia R, Garcia R, Cinco M, 2002. Leptospire are killed in vitro by both oxygen-dependent and -independent reactions. *Infect Immun* 70: 7172-5.
7. Annika Linde GHL, Javier Abello and Tonatiuh Melgarejo, 2013. Clinical Relevance of Cathelicidin in Infectious Disease. *Journal of Clinical Cell Immunology* S13: 003.
8. Scharrig E, Carestia A, Ferrer MF, Cedola M, Pretre G, Drut R, Picardeau M, Schattner M, Gomez RM, 2015. Neutrophil Extracellular Traps are Involved in the Innate Immune Response to Infection with *Leptospira*. *PLoS Negl Trop Dis* 9: e0003927.
9. Pillai PS, Molony RD, Martinod K, Dong H, Pang IK, Tal MC, Solis AG, Bielecki P, Mohanty S, Trentalange M, Homer RJ, Flavell RA, Wagner DD, Montgomery RR, Shaw AC, Staeheli P, Iwasaki A, 2016. Mx1 reveals innate pathways to antiviral resistance and lethal influenza disease. *Science* 352: 463-6.

189 10. De Silva NL, Niloofa M, Fernando N, Karunanayake L, Rodrigo C, De Silva HJ,
190 Premawansa S, Handunnetti SM, Rajapakse S, 2014. Changes in full blood count
191 parameters in leptospirosis: a prospective study. *Int Arch Med* 7: 31.

192 11. Craig SB, Collet TA, Wynwood SJ, Smythe LD, Weier SL, McKay DB, 2013. Neutrophil
193 counts in leptospirosis patients infected with different serovars. *Trop Biomed* 30: 579-83.

194 12. Craig SB, Graham GC, Burns MA, Dohnt MF, Smythe LD, McKay DB, 2009.
195 Haematological and clinical-chemistry markers in patients presenting with leptospirosis:
196 a comparison of the findings from uncomplicated cases with those seen in the severe
197 disease. *Ann Trop Med Parasitol* 103: 333-41.

198 13. Raffray L, Giry C, Vandroux D, Kuli B, Randrianjohany A, Pequín AM, Renou F, Jaffar-
199 Bandjee MC, Gasque P, 2016. Major Neutrophilia Observed in Acute Phase of Human
200 Leptospirosis Is Not Associated with Increased Expression of Granulocyte Cell
201 Activation Markers. *PLoS One* 11: e0165716.

202 14. Futosi K, Fodor S, Mocsai A, 2013. Neutrophil cell surface receptors and their
203 intracellular signal transduction pathways. *Int Immunopharmacol* 17: 638-50.

204 15. Zuerner RL, 2015. Host response to leptospira infection. *Curr Top Microbiol Immunol*
205 387: 223-50.

206 16. Nahori MA, Fournie-Amazouz E, Que-Gewirth NS, Balloy V, Chignard M, Raetz CR,
207 Saint Girons I, Werts C, 2005. Differential TLR recognition of leptospiral lipid A and
208 lipopolysaccharide in murine and human cells. *J Immunol* 175: 6022-31.

209 17. Werts C, Tapping RI, Mathison JC, Chuang TH, Kravchenko V, Saint Girons I, Haake
210 DA, Godowski PJ, Hayashi F, Ozinsky A, Underhill DM, Kirschning CJ, Wagner H,
211 Aderem A, Tobias PS, Ulevitch RJ, 2001. Leptospiral lipopolysaccharide activates cells
212 through a TLR2-dependent mechanism. *Nat Immunol* 2: 346-52.

- 213 18. Goris MG, Wagenaar JF, Hartskeerl RA, van Gorp EC, Schuller S, Monahan AM, Nally
214 JE, van der Poll T, van 't Veer C, 2011. Potent innate immune response to pathogenic
215 leptospira in human whole blood. PLoS One 6: e18279.
- 216 19. Viriyakosol S, Matthias MA, Swancutt MA, Kirkland TN, Vinetz JM, 2006. Toll-like
217 receptor 4 protects against lethal *Leptospira interrogans* serovar icterohaemorrhagiae
218 infection and contributes to in vivo control of leptospiral burden. Infect Immun 74: 887-
219 95.
- 220 20. Chassin C, Picardeau M, Goujon JM, Bourhy P, Quellard N, Darche S, Badell E,
221 d'Andon MF, Winter N, Lacroix-Lamande S, Buzoni-Gatel D, Vandewalle A, Werts C,
222 2009. TLR4- and TLR2-mediated B cell responses control the clearance of the bacterial
223 pathogen, *Leptospira interrogans*. J Immunol 183: 2669-77.
- 224 21. Guo Y, Fukuda T, Donai K, Kuroda K, Masuda M, Nakamura S, Yoneyama H, Isogai E,
225 2015. Leptospiral lipopolysaccharide stimulates the expression of toll-like receptor 2 and
226 cytokines in pig fibroblasts. Anim Sci J 86: 238-44.
- 227 22. Guo Y, Fukuda T, Nakamura S, Bai L, Xu J, Kuroda K, Tomioka R, Yoneyama H, Isogai
228 E, 2015. Interaction between Leptospiral Lipopolysaccharide and Toll-like Receptor 2 in
229 Pig Fibroblast Cell Line, and Inhibitory Effect of Antibody against Leptospiral
230 Lipopolysaccharide on Interaction. Asian-Australas J Anim Sci 28: 273-9.
- 231 23. Zhang W, Zhang N, Xie X, Guo J, Jin X, Xue F, Ding Z, Cao Y, 2016. Toll-Like Receptor
232 2 Agonist Pam3CSK4 Alleviates the Pathology of Leptospirosis in Hamster. Infect
233 Immun 84: 3350-3357.
- 234 24. Chen X, Li SJ, Ojcius DM, Sun AH, Hu WL, Lin X, Yan J, 2017. Mononuclear-
235 macrophages but not neutrophils act as major infiltrating anti-leptospiral phagocytes
236 during leptospirosis. PLoS One 12: e0181014.

- 237 25. Stoddard RA, Gee JE, Wilkins PP, McCaustland K, Hoffmaster AR, 2009. Detection of
238 pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the
239 LipL32 gene. *Diagn Microbiol Infect Dis* 64: 247-55.
- 240 26. Smythe LD, Smith IL, Smith GA, Dohnt MF, Symonds ML, Barnett LJ, McKay DB, 2002.
241 A quantitative PCR (TaqMan) assay for pathogenic *Leptospira* spp. *BMC Infect Dis* 2:
242 13.
- 243 27. Qian F, Guo X, Wang X, Yuan X, Chen S, Malawista SE, Bockenstedt LK, Allore HG,
244 Montgomery RR, 2014. Reduced bioenergetics and toll-like receptor 1 function in human
245 polymorphonuclear leukocytes in aging. *Aging (Albany NY)* 6: 131-9.
- 246 28. Wang B, Sullivan J, Sullivan GW, Mandell GL, 1984. Interaction of leptospires with
247 human polymorphonuclear neutrophils. *Infect Immun* 44: 459-64.
- 248 29. Lindow JC, Wunder EA, Jr., Popper SJ, Min JN, Mannam P, Srivastava A, Yao Y,
249 Hacker KP, Raddassi K, Lee PJ, Montgomery RR, Shaw AC, Hagan JE, Araujo GC,
250 Nery N, Jr., Relman DA, Kim CC, Reis MG, Ko AI, 2016. Cathelicidin Insufficiency in
251 Patients with Fatal Leptospirosis. *PLoS Pathog* 12: e1005943.
- 252 30. Arean VM, 1962. The pathologic anatomy and pathogenesis of fatal human leptospirosis
253 (Weil's disease). *Am J Pathol* 40: 393-423.
- 254 31. Sitprija V, Evans H, 1970. The kidney in human leptospirosis. *Am J Med* 49: 780-8.
- 255 32. Jaillon S, Galdiero MR, Del Prete D, Cassatella MA, Garlanda C, Mantovani A, 2013.
256 Neutrophils in innate and adaptive immunity. *Semin Immunopathol* 35: 377-94.
- 257 33. Reis EA, Hagan JE, Ribeiro GS, Teixeira-Carvalho A, Martins-Filho OA, Montgomery
258 RR, Shaw AC, Ko AI, Reis MG, 2013. Cytokine response signatures in disease
259 progression and development of severe clinical outcomes for leptospirosis. *PLoS Negl*
260 *Trop Dis* 7: e2457.

262 **TABLES**263 **Table 1. Laboratory and clinical findings at admission for patients with confirmed**264 **leptospirosis (n=15)**

	No organ dysfunction		Organ dysfunction		
	(Acute [A]) ¹		(Severe [S]) ²		
	Median (IQR)		Median (IQR)		
DEMOGRAPHICS	No.	or No. (%)	No.	or No. (%)	<i>P</i> -value ³
Sex (Male)	10	8 (80%)	5	5 (100%)	0.524
Age (Years)	10	30.0 (18.8-39.8)	5	31.0 (23.5-39.5)	0.799
CLINICAL PRESENTATION					
Days of illness ⁴	10	6.5 (5.0-7.3)	5	6.0 (3.5-6.5)	0.170
Jaundice	10	7 (70%)	5	5 (100%)	0.506
CLINICAL LABORATORY ⁵					
Hematocrit (%)	10	33.6 (31.5-40.6)	5	35.4 (34.7-37.8)	0.699
Leukocyte count (1000/μL)	10	11.8 (9.8-22.4)	5	15.0 (7.8-16.9)	0.633
Absolute neutrophil count (1000/μL)	10	9.7 (7.6-17.1)	5	13.2 (6.7-16.7)	0.859
% Lymphocytes	10	14.5 (8.8-16.3)	5	7.0 (5.5-12.0)	0.239
Platelet count (1000/μL)	10	121.5 (53.5-224.8)	5	65.0 (27.0-132.5)	0.240
Serum creatinine (mg/dL)	10	1.7 (1.5-4.7)	5	3.3 (1.7-7.7)	0.355
Blood urea nitrogen (mg/dL)	10	65.5 (36.5-97.8)	5	68.0 (51.5-162.5)	0.592
Total serum bilirubin (mg/dL)					
Bilirubin direct	6	5.1 (0.9-10.8)	5	2.7 (0.8-8.2)	0.459
Bilirubin indirect	6	1.0 (0.7-3.9)	5	1.0 (0.5-9.0)	1.000
Serum potassium (meq/L)	10	3.9 (3.6-4.4)	3	4.7 (4.7-5.3)	0.059

COMPLICATIONS

Oliguria ⁶	10	0 (0%)	5	5 (100%)	0.0003
Pulmonary hemorrhage ⁷	10	0 (0%)	5	1 (20%)	0.333
Lung injury ⁸	10	0 (0%)	5	3 (75%)	0.022

OUTCOMES

Death	10	0 (0%)	5	1 (20%)	0.333
ICU admission	10	0 (0%)	5	2 (40%)	0.095
Mechanical ventilation	10	0 (0%)	5	2 (40%)	0.095
Dialysis	10	0 (0%)	5	4 (80%)	0.004

LABORATORY DATA

Agglutinating antibody titers

Acute phase	10	1600 (0-8000)	5	0 (0-3300)	0.220
Convalescent phase	9	3200 (50-3200)	4	3200 (3200-5600)	0.339
<i>Leptospira</i> load (Geq/mL) ⁹	9	0.0 (0-0)	4	5690 (0-17521)	0.077

265

266 ¹ Acute group did not meet the definition of the severe group.

267 ² Severe group patients showed evidence of renal dysfunction⁶ and/or lung injury⁸ during
268 hospitalization.

269 ³ P-values determined by Fischer Exact test for categorical variables or Mann-Whitney t-test for
270 continuous variables; P-value <0.05 considered significant

271 ⁴ Days of symptoms prior to hospital admission272 ⁵ Day of admission

273 ⁶ Oliguria defined by oliguria (<500mL urine/day) or anuria (<50ml urine/day) within 24h of hospital
274 admission or patient received hemodialysis

275 ⁷ Pulmonary hemorrhage defined by mechanical ventilation, ≥ 250 ml blood in lungs, or
276 endotracheal tube
277 ⁸ Lung injury defined by mechanical ventilation, ≥ 250 ml blood in lungs or endotracheal tube,
278 and/or respiration rate $>38/\text{min}$
279 ⁹ Geometric mean of *Leptospira* genomes/mL as determined by RT-qPCR
280

FIGURE LEGENDS

Figure 1. Neutrophil activation was not associated with disease severity. Expression of CD62L (A) and f CD11b (B) on neutrophils during acute leptospirosis indicate activation levels similar to those in convalescent patients and healthy controls. Results are shown as the percent of granulocytes positive for CD15 and CD62L (A) or CD11b (B) and MFI of CD62L (A) or CD11b (B) on CD15⁺ granulocytic cells. Groups are A (acute leptospirosis, no renal/lung dysfunction; n=6), S (severe acute leptospirosis, renal/lung dysfunction; n=4), C (convalescent time point; n=4), and H (healthy controls, n=4). *ns* indicates no significant differences in group comparisons (nonparametric one-way ANOVA). Means $\pm$ SEM are shown.

Figure 2. Neutrophil activation function was not associated with disease group. Cells were stimulated with TLR agonists as described. Results are represented as the means $\pm$ SEM for MFI fold-changes of CD62L (A) and CD11b (B) on CD15⁺ granulocytes. A value of “1” indicates no response to agonist stimulation relative to unstimulated controls. Groups are A (acute leptospirosis, no renal/lung dysfunction; n=6), S (severe acute leptospirosis, renal/lung dysfunction; n=4), and C (convalescent time point; n=4). *ns* indicates no significant differences in group comparisons (nonparametric one-way ANOVA). Means $\pm$ SEM are shown.

Figure 3. Increased TLR expression on neutrophils in acute leptospirosis patients with renal/lung dysfunction. (A) Increased TLR2 expression in patients with renal and/or lung dysfunction (group S). (B) TLR4 expression on neutrophils was lower than TLR2 expression but displayed a similar expression pattern across groups. Results are represented as the percent granulocytes positive for CD15 and TLR2 (A) or TLR4 (B) after isotype subtraction. Groups are A (acute leptospirosis, no renal/lung dysfunction; n=10), S (severe acute leptospirosis, renal/lung dysfunction; n=5), C (convalescent time point; n=5), and H (healthy Brazilian adults;

306 n=4). Asterisks indicate significant differences relative to group S (*, $p < 0.05$, **, $p < 0.01$). Means
307 $\pm$ SEM are shown. No other significant differences between groups were identified.

A

B

A

B