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ORIGINAL INVESTIGATION

The monocytosis during human leptospirosis is associated with modest immune cell activation states

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Abstract

Leptospirosis is a life-threatening zoonotic disease and it has been hypothesized that the innate immune system fails to control the infection through ill-characterized mechanisms. The aim of this observational study was to better evaluate the activation processes of monocytes at the early stage of the disease. Blood samples were taken from healthy donors ($n = 37$) and patients hospitalized for either non-severe ($n = 25$) or severe ($n = 32$) leptospirosis. Monocyte cell counts and phenotypes were assessed by flow cytometry. We analysed the expression of several cell activation markers: CD14, CD16, HLA-DR, CD69, TLR2, TLR4, CD11b and CD11c. Although monocyte values at admittance were not significantly different from controls, patients experienced significant monocytosis at 1.33×10^9/L ($p < 0.0001$ compared to controls: 0.56×10^9/L) during their hospital stay. This monocytosis observed during hospital stay was correlated to several surrogate markers of organ injury. Non-classical (CD14−CD16+) and intermediate (CD14+CD16+) monocyte subsets increased compared to controls ($p < 0.05$). Accordingly, classical monocyte subset (CD14+CD16−) showed decreased percentages ($p < 0.0001$). Levels of several cell surface activation molecules were decreased: HLA-DR involved in MHC class II antigen presentation, integrins CD11b and CD11c implicated in phagocytosis and cell recruitment ($p < 0.0001$). None of these parameters had a prognostic value. Results from this study showed that during acute human leptospirosis, patients experienced monocytosis with a switch toward an inflammation-related phenotype contrasted by low expression levels of markers implicated in monocyte function.

Keywords Leptospirosis · Innate immunity · Monocytes · HLA-DR · Phagocytosis

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Introduction

Leptospirosis is a zoonotic disease of global importance with a recent estimated annual incidence of approximately 1 million cases and a mortality of 6% [1]. Humans are accidental hosts of *Leptospira* bacteria that can overcome the immune system to disseminate to target organs and induce multi-organ failure [2]. The phenomenon of immune paralysis has already been demonstrated during severe sepsis and septic shock [3]. This sepsis-induced immune paralysis can be defined as the sepsis-induced defects in innate and adaptive immune cells leading to failure to control the primary infection or the acquisition of secondary hospital-acquired infections [3]. It is not known whether this pathophysiological process is also taking place during human leptospirosis.

Within innate immunity, monocytes/macrophages mediate pathogen clearance through phagocytosis, cytokine secretion and antigen presentation. During leptospirosis, many animal and in vitro studies have highlighted the ability of macrophages to produce pro- and anti-inflammatory cytokines [4]. However, the ability of monocytes/macrophages to drive phagocytosis and effective clearance of pathogenic *Leptospira* is unclear. Several studies have demonstrated that bacteria are internalized by macrophages across various species, including human cell lines [5–8]. Yet, conflicting data exist showing that pathogenic *Leptospira* may be able to resist monocyte-driven phagocytosis by evading the destruction process inside infected monocytes [5, 9, 10]. Such discrepancies in our understanding of host–pathogen interactions may be linked to bacterial strain characteristics, as well as distinct monocyte/macrophage behaviour in different inflammatory settings. Remarkably, studies in mice and zebrafish have suggested the hypothesis that leptospires may become monocyte "cargo", disseminating through the host organism within monocytes in which the destruction process had been impaired [7, 11]. In addition, it is not understood if mechanisms of leptospiral antigen processing and presentation by monocytes are altered during the infection process.

Of critical note, in vitro and animal models are not always relevant to fully grasp the pathology in humans. Cohort studies are ultimately necessary to address the clinical pathological processes. Several studies have shown significant discrepancies between the behaviour of mouse and human monocytes exposed to *Leptospira* or its components [9, 12, 13], or between human monocyte cell lines and freshly isolated mononuclear cells [14]. Such differences may hinge on differential recognition of pathogen-associated molecular patterns between species, as demonstrated for leptospiral lipopolysaccharide (LPS) which is recognized essentially by TLR2 (Toll-like receptor 2) in humans but by TLR4 in mice [4, 15, 16]. Furthermore, this may account for distinct morbidity patterns between host reservoirs and accidental hosts. Hence, further studies are needed to assess the role of monocytes in human leptospirosis.

The aim of this study was to determine the quantitative and qualitative immune phenotypic characteristics of circulating monocytes during acute human leptospirosis. We analysed the cell counts and phenotypic data related to activation, antigen presentation, phagocytic receptors and cell recruitment status in a cohort of human leptospirosis cases in La Réunion.

Materials and methods

Study design and selection of subjects

This study was approved by the local human ethic committee of tertiary teaching Hospital 'CHU de La Réunion' (protocol number R15018) and conducted according to the principles expressed in the Declaration of Helsinki. When patients were able to provide informed consent, written consent was registered prior to enrolment. When their medical conditions prevented patients form consenting, provisional consent was sought from family members and written informed consent reviewed with the patient after recovering.

Our study was conducted prospectively between January 2015 and July 2017 in three medical centres of Reunion Island: Groupe Hospitalier Est Réunion (GHER) in Saint Benoit, Gabriel Martin Hospital (CHGM) in Saint Paul and the University Hospital of St Denis. Some patients of this cohort have already been analysed for other immune parameters, as published earlier [17, 18]. The selection criteria were: age older than 18, a suspected diagnosis of acute leptospirosis in hospitalized patient further confirmed by PCR or serology, and available data for monocyte count at admittance with concomitant analysis of monocyte subsets by flow cytometry. Among leptospirosis cases, 8 patients were excluded because of incomplete monocytes data. Finally, 57 leptospirosis cases and 37 healthy age- and gender-matched controls (hospital's workers) were enrolled.

Definitions

- Definite leptospirosis case: fever or symptoms compatible with acute leptospirosis and presence of at least one of the following: positive PCR in blood and/or urine, serology titre above upper normal value in ELISA or higher than 1:400 as measured with the microscopic agglutination test (MAT).
- Severe leptospirosis was defined as death or severe organ injury during disease course: jaundice (total bilirubin > 50 µmol/L); septic shock requiring vasopressor drug administration; mechanical ventilation or oxygen

requirement; acute renal failure defined according to RIFLE 'Failure' definition [19] or requirement of extra-renal replacement therapy. Patients not fulfilling these criteria were defined as non-severe leptospirosis cases as they did not experience severe organ failure.

- Oxygen requirement was defined as use of oxygen to maintain partial pressure of oxygen (PaO_2) above 60 mmHg or transcutaneous oxygen saturation above 92%.
- Septic shock was defined according to internationally recognized criteria of ACPP/SCCM Consensus Conference Committee [20].

Variables recorded

For patients, clinical and laboratory data were recorded until the point of discharge or death. The following variables were recorded: age, gender, risk of *Leptospira* exposure (occupation, hobbies, exposure to animals, housing type and location), comorbidities or immunosuppression, initial symptoms prior to and at hospital admittance, organ injury assessment, received treatments: both antibiotics and supportive care, time from first symptoms to antibiotic onset, hospital length of stay. Usual blood testing values were also recorded: total blood cell count, liver function tests, electrolytes and creatinine, creatine phosphokinase (CPK), C-reactive protein (CRP).

Laboratory testing for leptospirosis confirmation and bacteria quantification

Biological specimens for diagnosis of leptospirosis were sampled at admittance of patients. Pathogenic *Leptospira* DNA was detected in plasma or urine using quantitative real-time PCR (qPCR) using TaqMan® Universal PCR Mastermix with primers and probe specific of a portion of 23S rRNA as target as described elsewhere [21].

For quantification of bacterial burden in plasma by PCR, serial dilutions of genomic DNA extracts from *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni were performed. These dilutions corresponded to concentrations from 4×10^6 to 2 bacteria/mL and the number of bacteria per mL in plasma samples were inferred from the cycle threshold (C_t) values of PCR according to the log-transformed standard curve, as detailed in previous reports [22].

In the eventuality of strong suspicion in PCR-negative patients, ELISA (SERION) serological testing was performed. If the detection of anti-leptospiral IgM antibodies was positive (> 50 U/mL), the testing was completed with a MAT (national reference centre, Pasteur Institute, Paris) assessing reactivity with different serovars: Australis, Automnalis, Bataviae, Canicola, Cynopterii, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Panama, Patoc, Pomona, Pyrogenes, Sejroe, and Tarassovi.

Identification of monocyte cell population subsets by flow cytometry

Blood samples for flow cytometry assay were collected only from the time of admission, in EDTA-containing tubes. Monocyte phenotypes (stained for CD14 and CD16 markers) were assessed for all patients and controls. We also investigated other cell surface markers (CD69, TLR2, TLR4, CD11b, CD11c and HLA-DR). For these markers, we included leptospirosis patients ($n = 24$) and controls ($n = 26$) recruited during the 2017 period. For each test, 100 µL of whole blood was mixed with monoclonal antibodies against different cell surface markers at a concentration of 1 µg/mL or according to provider instructions. The following antibodies were purchased: (i) from Beckman Coulter: ECD-anti-CD14 (IM2707U), FITC-anti-CD16 (IM0814U), FITC-anti-CD11b (IM0503), ECD-anti-HLA-DR (IM3636), PE-anti-CD14 (A07764), ECD-anti-CD16 (A33098); (ii) from Becton Dickinson: ECD-anti-CD11c (551077), FITC-anti-CD69 (555530); (iii) from Biolegend: FITC-anti-TLR2 (309706), PE-anti-TLR4 (312806). The appropriate isotype controls were used and to set the background staining levels.

Tubes were vortexed after adding each antibody. After 30 min of incubation at room temperature in the dark, red blood cells were lysed with Beckman ImmunoPrep™ Reagent System (Beckman Coulter ref 7546999) using TQ-Prep™ Workstation. Cytometry analysis was performed with the Becton Dickinson C6 Accuri™ flow cytometer, data extracted from BD Accuri™ C6 software version 1.0 and analysed with FlowJo® version 10.2.

Monocytes were gated according to FSC and SSC standards, and then cells were considered for further analysis according to CD14 and CD16 expression. At least 5000 cells from each sample were analysed. The mean fluorescence intensity (MFI) values and percentages of positive cells were evaluated. Three monocytes subsets were defined according to previous reports [23]: classical monocytes or M^{class} (CD14+CD16−), non-classical monocytes or M^{nc} (CD14−CD16+) and intermediate monocytes or M^{int} (CD14+CD16+). The percentages and absolute values of each subset were used for subsequent analyses. Absolute subset population values were calculated as the monocyte values from full blood count at admission multiplied by the percentage of the subset found with cytometry.

Statistics

All data were analysed for Gaussian or normal distribution using D'Agostino and Pearson normality test. Quantitative variables were expressed as means and standard deviation, or as medians and interquartile (IQR) ranges according to Gaussian or non-Gaussian distribution, respectively. Statistical significance of difference for quantitative variables between groups was determined by Student's *t* test or non-parametric Mann–Whitney *U* test depending on normality test. Qualitative variables were expressed as numbers and percentages and compared with Fisher's exact test. Repeated analyses with more than two groups were conducted by one-way repeated measures ANOVA with Sidak correction for multiple comparisons. The Spearman test was used to analyse correlations among variables. *p* values below 0.05 were considered statistically significant. Statistics were performed with GraphPad Prism™ version 6.

Results

Characteristics of patients

Fifty-seven ($n = 57$) patients and 37 ($n = 37$) healthy donors were recruited for this study. The comparison between these two groups is reported in Table 1. Among the patients, 41 (72%) presented one or more risk of *Leptospira* exposure, and 46% had comorbidities, mainly tobacco/alcohol abuses and diabetes. PCR testing was positive in blood for 50 patients, in urine for 19, and 1 patient was diagnosed after serology testing only. MAT was performed in 9 patients: 5 were positive for *Leptospira interrogans* serovar Icterohaemorrhagiae (strain Verdun), 2 for serovar Canicola and 3 undetermined.

The main organ injuries at baseline corresponded to the classical hallmarks of leptospirosis as shown with

Table 1 Description and univariate analyses of clinical and laboratory features of leptospirosis patients at admission compared to healthy control subjects

	Healthy donors	Leptospirosis cases	Controls vs leptospirosis	Non-severe leptospirosis cases	Severe leptospirosis cases	Non-severe vs severe leptospirosis
Number of individuals	37	57		25	32	
Ratio M/F	33/4	54/3	NS	23/2	31/1	NS
Age (years), mean ± SD	40.14 ± 13.98	42.25 ± 14.94	NS	40.91 ± 13.24	43.29 ± 16.27	NS
Comorbidities, numbers		26		6	20	$p = 0.007$
Tobacco abuse		19		4	15	
Alcohol abuse		8		3	5	
Diabetes		4		1	3	
Cirrhosis		1		0	1	
Biological parameters, mean ± SD						
Neutrophils (10^9/L)	3.54 ± 1.61	9.21 ± 3.71	$p < 0.0001$	9 ± 3.46	9.38 ± 3.93	NS
Lymphocytes (10^9/L)	2.18 ± 0.63	0.82 ± 0.75	$p < 0.0001$	0.70 ± 0.26	0.90 ± 0.98	NS
Monocytes (10^9/L)	0.56 ± 0.18	0.73 ± 0.52	NS	0.58 ± 0.29	0.84 ± 0.62	$p = 0.056$
Platelets (10^9/L)	237 ± 50	104 ± 63	$p < 0.0001$	144 ± 55	71 ± 49	$p < 0.0001$
Creatinine (µmol/L)	81.6 ± 9.9	189.5 ± 164.8	P = 0.0002	111.6 ± 39.7	251.2 ± 197.2	$p = 0.0009$
Total bilirubin (µmol/L)	9 ± 3.9	60 ± 85	$p = 0.001$	16 ± 10	93 ± 101	$p = 0.0005$
Alkaline phosphatase (IU/L)	55 ± 24	91 ± 39	$p < 0.0001$	89 ± 42	93 ± 38	NS
AST (IU/L)	22.2 ± 6.6	127 ± 190	$p = 0.002$	78 ± 109	167 ± 231	$p = 0.08$
CPK (IU/L)	170 ± 167	2797 ± 5621	$p = 0.008$	973 ± 1660	4013 ± 6916	$p = 0.003$
CRP (mg/L)	1.5 ± 2.4	275 ± 286	$p < 0.0001$	342 ± 401	215 ± 93	NS
Plasmatic *Leptospira* load* (bact./mL), median (interquartile)		762 (207; 2866)		441 (76; 115)	1951 (272 ; 5139)	$p = 0.007$

Data are expressed as means (± SD) except for bacterial load owing to non-Gaussian distribution of data: median (1st; and 3rd quartiles). Statistics between two groups are performed with parametric unpaired *t* tests or non-parametric Mann–Whitney *U* test as appropriate for quantitative variables, and with Fisher's exact test for categorical data. *p* value inferior to 0.05 was considered significant. *Plasmatic bacterial load is inferred from plasmatic RT-qPCR C_t values according to the log-transformed standard curve

AST aspartate aminotransferase, *CPK* creatine phosphokinase, *CRP* C-reactive protein, *NS* not significant biological surrogate markers listed in Table 1. Patients were classified as non-severe ($n = 25$) and severe ($n = 32$) leptospirosis cases according to definitions mentioned in method section. Characteristics of severe and non-severe patients, as well as comparisons between the two groups are reported in Table 1. One patient deceased during intensive care unit stay. The other severe cases required supportive care as follows: 10 patients underwent transient renal replacement therapy, 10 required mechanical ventilation, and 13 were under vasopressor drugs. All patients received appropriate antibiotic therapy: mainly amoxicillin and ceftriaxone. The median time from symptoms onset to antibiotics initiation was 4 days in median (IQR 3–5). The mean length of stay in hospital was 6 days (4–8.75), and significantly longer for severe cases compared to non-severe cases: median 7 days versus 4 days ($p < 0.0001$). All patients recovered without evidence of sequelae except one harbouring persistent kidney disease.

Monocytosis accompanied leptospirosis

Monocyte cell count at baseline in patients at admittance indicated a slight increase, although it was not statistically different from controls ($p = 0.06$) as shown in Table 1. However, monocyte numbers further increased during hospitalization and reached a peak at 1.33×10^9/L median (IQR 0.74; 1.92). This monocytosis was measured for the 47 patients for whom we were able to perform at least two testings for full blood cell counts. These monocyte maximal values were above the upper limit of the normal range of 0.75×10^9/L for 75% of the patients and were significantly higher than controls (Fig. 1, $p < 0.0001$). The monocytosis was usually maximal at day 6 after symptom's onset. Monocyte cell counts returned to baseline values for patients with testing performed 1 month after discharge ($n = 10$, Supplementary Fig. 1). We further analysed the correlation between monocyte maximal values reached during hospitalization and other biological data: we observed significant correlation with several surrogate markers of infectious disease together with organ involvement (Fig. 2). Indeed, a correlation was found between maximal value of monocyte cell counts and levels of neutrophilia, thrombocytopenia, liver injury (AST, bilirubin, alkaline phosphatase), kidney injury (creatinine), as well as with hospital length of stay ($r = +0.54$, confidence interval 0.28–0.72; p value $= 0.0001$).

When considering the classification of organ failure as detailed in the methods, the patient group could be divided into severe ($n = 32$) and non-severe cases ($n = 25$). The two groups were equivalent in terms of age and gender proportion (Table 1). When we compared the two groups, we found significant differences for surrogate markers that were not used in the definition of severe cases. Indeed, thrombocytopenia ($p < 0.0001$), rise in CPK levels ($p = 0.003$) as well as plasmatic bacterial load ($p = 0.007$) were significantly more pronounced in the severe cases (Table 1 and Supplementary Fig. 2). Interestingly, we found a trend for increased values in the severe group concerning the number of monocytes at admission, although not statistically significant ($p = 0.056$). Moreover, we did not find any statistical differences between the two groups for the maximal monocyte values during hospitalization.

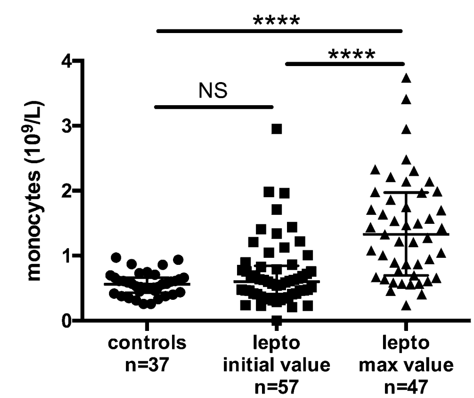


Fig. 1 Monocytosis during human leptospirosis. Monocyte values for leptospirosis patients are the value assessed at admittance to hospital—'lepto initial value'—and the maximal value reached during hospitalization—'lepto max value' (ten patients were not included in lepto max value group because they had only one evaluation of monocytes count, at admittance). Largest horizontal bars indicate the median value, upper and lower bars the IQR ranges. Comparison of the patients group and healthy donor group with non-parametric Mann–Whitney *U* test and comparison between leptospirosis groups with Wilcoxon test for matched-pairs. ****p value inferior to 0.0001

Switch in the monocyte cell subsets during leptospirosis

Three distinct monocytes subsets are currently defined according to CD14 (LPS co-receptor) and CD16 (Fcγ receptor III) cell surface expression [23]. These subsets are the classical monocytes CD14+CD16−, the non-classical monocytes CD14−CD16 + and the intermediate monocytes CD14+CD16+. For simplification purposes, these subsets are, respectively, named M^{class}, M^{nc} and M^{int}. For healthy controls, the distribution was as follows: M^{class} 80%, M^{nc} 5.5% and M^{int} 7.5% (normal ranges: 85–90%, 5% and 5–10%, respectively). The subset distribution for the leptospirosis patients was assessed at admission only and showed a significant decrease in the M^{class} proportion (69%) associated with an increase in M^{nc} (10%) and M^{int} (12%) subsets compared to controls (Fig. 3). When studying the absolute value of monocytes within each category subset, we did not find

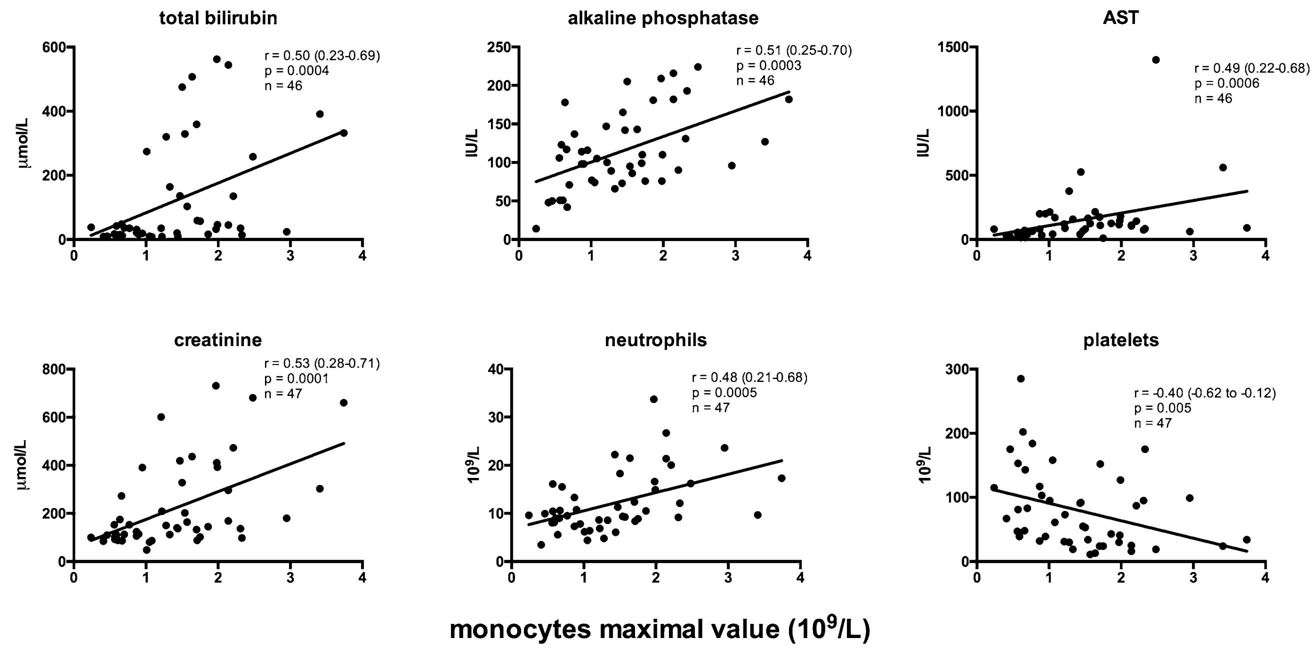


Fig. 2 Monocytosis is associated with surrogate markers of organ injuries in 47 leptospirosis cases, analyses were performed from the 47 leptospirosis cases with repeated measurement of monocyte count during hospital stay. For each biological parameter, the maximal value reached during hospital stay was used, or the minimal for the platelet count. The Spearman test was used to analyse correlations among variables, according to non-Gaussian distribution of monocyte count peak values. *p* values, correlation coefficient r values (95% confidence interval) and number of paired data analysed are indicated on the graphs. *AST* aspartate transaminase

significant differences. The percentages and absolute values of monocyte counts for each subset were not correlated to any of the surrogate markers of organ injury. Moreover, monocytes counts were not different between individuals receiving antibiotic treatment shortly after symptoms onset (<3.5 days) versus those receiving treatment more lately.

Modest levels of cell activation markers on monocytes in leptospirosis patients

We further analysed monocyte phenotypes for 24 patients and 26 controls, at the time of admission. These two groups were comparable for age and gender distribution (Table 2). We assessed cell surface expression of molecules involved in cell activation (CD69), antigen presentation (HLA-DR), LPS recognition (TLR2 and TLR4), cell recruitment and phagocytosis (complement integrins CD11b, CD11c). Analysis was performed for the whole monocyte population and not at the monocyte cell subset level given that we had limited access to blood samples. Figure 4 represents the differences between controls and leptospirosis cases. The main findings are a decreased expression of HLA-DR, CD11c, CD11b levels on monocytes in response to the infectious challenge. In contrast, the levels of TLR2, TLR4 and CD69 were not significantly different between healthy and leptospirosis groups.

We did not find any statistical differences between the two severity groups concerning percentages or absolute values of monocytes subsets. Further analyses showed no difference for the phenotypic markers (CD69, HLA-DR, CD11c, CD11b, TLRs) according to disease severity, except for CD69 for which the expression level was elevated in the severe leptospirosis group compared to non-severe patients and controls ($p<0.01$) (Supplementary Fig. 3).

Discussion

It has long been hypothesized that the innate immune response in human leptospirosis may be compromised. The aim of our work was to better assess the behaviour of monocytes in response to this infectious challenge. We demonstrated that monocyte numbers were significantly increased in blood after a few days post-infection. This was accompanied by a switch from classical, to intermediate and non-classical monocyte phenotypes in the majority of patients at admission. Further analysis suggested that despite this phenotypic switch, circulating monocytes had a decreased cell surface expression of major cell activation markers such as HLA-DR, CD11b and CD11c. This data was a clear indication that innate immune monocyte cells can detect and respond to systemic infection, but fail to further commit to a full inflammatory signature. Although we did not explore the related functions of these phenotypic data, our original observations may shed new light on the mechanisms of immune paralysis in leptospirosis.

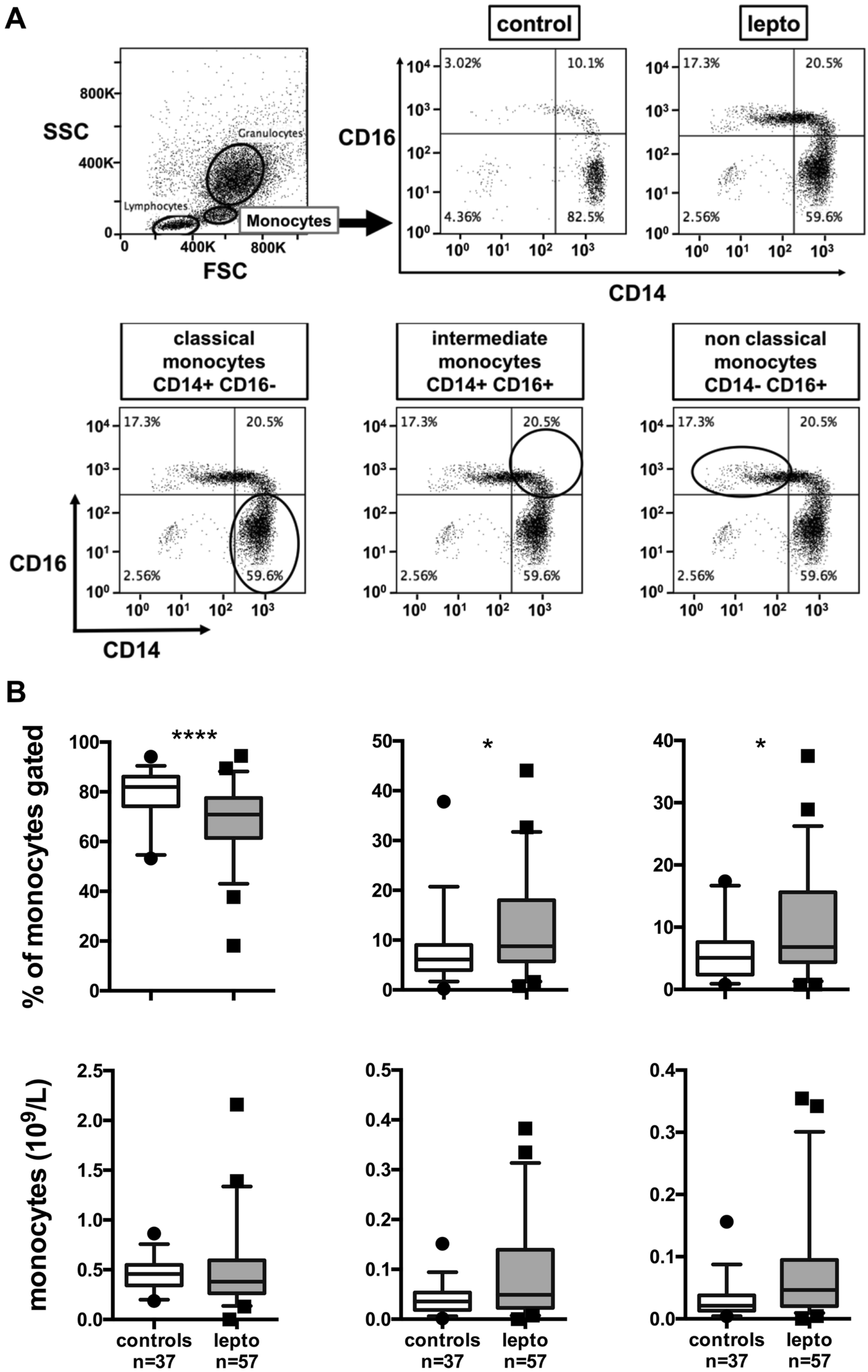
Our patient cohort is representative of worldwide leptospirosis epidemiology, not just Reunion Island; with a predominance of young males and clinical presentation of life-threatening features [1, 2, 24]. However, our cohort mortality (taking into account ICU and non-ICU cases) was 2%, which is lower than the recent worldwide estimate of 6% [1]. Of note, it has been recently reported that ICU mortality in Reunion Island is in fact rather low (6%) due to availability of prompt modern resuscitation techniques [25]. Serovar identification was available for only a few patients, but was in line with local ecological data reporting a large predominance of *L. interrogans* Icterohaemorrhagiae [24, 26]. Our study focused on hospitalized cases, which can be considered as a bias. Arguably, data from out-patients would be interesting to complete our results. However, in Reunion island more than 85% of diagnosed and definite leptospirosis cases are hospitalized regardless of severity [24]. Thus, we considered that the overall contribution of the immune system would be best assessed by a comparison of non-severe and severe forms of hospitalized cases as defined by organ impairment and tissue injury.

Previous studies which analysed total blood count, helped to describe the underlying process of neutrophilia and lymphopenia [17, 27, 28]. Our study is one of the first to investigate monocytosis in human leptospirosis. This rise was delayed of a few days when compared to the event of the early neutrophilia as reported previously [16, 26]. Interestingly, monocyte increase was associated with several hallmarks of leptospirosis, such as liver and kidney failure, as well as platelet drop [17, 27]. However, the levels of monocytosis did not differ significantly between severity groups. To our knowledge, this is the first indication that monocytes are responding to the infectious challenge, but nothing is known about the possible mechanisms by which monocyte precursors may be recruited in order to explain this rise in the blood circulation.

The heterogeneity and subset-specific contribution of blood monocytes to infectious disease is an important research field. The current nomenclature firmly established in 2010 defined three subsets according to their relative expression of CD14 and CD16 [23]. In the healthy population, the usual distribution encompasses 85–90% classical monocytes (CD14+CD16−), 5% intermediate monocytes (CD14+CD16+) and 5–10% non-classical monocytes (CD14−CD16+) [29]. Each subset has defined phenotypic and functional characteristics related to their primary effector function: M^{class} are described as phagocytic, M^{nc} patrol and survey the endothelium, while M^{int} primarily process and present antigens for T cell activation [29]. Subsets exhibit differential contributions to disease pathogenesis in atherosclerosis, infection and auto-immunity.

During severe bacterial infections and septic shock, there is evidence for expansion of the CD16+ monocytes [30] as well as an increase in either the M^{nc} and M^{int} compartment [31, 32] or only the M^{int} [33]. Nevertheless, the prognostic value of this increase has never been addressed. To the best of our knowledge, the demonstration of M^{nc} and M^{int} expansion during leptospirosis is novel. The subset distribution in healthy controls studied here was in line with the established normal values. In comparison, leptospirosis patients had significantly increased their proportions of M^{nc} and M^{int}, by 10% and 12%, respectively. Akin to previous studies in septic shock, we did not find a correlation between disease severity and monocyte increase (defined by death in septic shock studies, and organ impairment here). Overall our results demonstrate that intermediate and non-classical monocytes percentages increase during sepsis associated with *Leptospira* infection, albeit without evidence of a prognostic value. According to the presumed functions of monocyte subsets [29], a decrease of M^{class} could be associated with less phagocytosis, and an increase of M^{int} and M^{nc} could suggest more ROS production and T cell activation/proliferation. Moreover, the M^{int} and M^{nc} subsets are possibly more important in the diapedesis process towards the infectious site. However, our study did not address selectively these functions and does not permit to draw further conclusions regarding the consequences of monocyte subset switch during human leptospirosis. Collectively, the increase in monocyte counts and the subset phenotypic data suggest that monocytes are reacting to the infectious challenge during human leptospirosis.

We further explored other phenotypic markers relevant to the primary function of monocytes, specifically HLA-DR. The literature has clearly indicated that reduced expression of HLA-DR during sepsis is considered as a hallmark of immunoparalysis and is associated with poor prognosis [33–36]. This decrease in HLA-DR expression may result in impairment of pro-inflammatory cytokine production and the ability to activate T cells [3]. In the present study, we observed a very low level of HLA-DR on monocytes from leptospirosis patients compared to controls; however, this was not associated with disease severity. An explanation could be the low mortality rate in our cohort, given that


A
SSC
FSC
800K
400K
0
Granulocytes
Lymphocytes
Monocytes
control
lepto
CD16
CD14
3.02%
10.1%
4.36%
82.5%
17.3%
20.5%
2.56%
59.6%
classical monocytes CD14+ CD16-
intermediate monocytes CD14+ CD16+
non classical monocytes CD14- CD16+
B
% of monocytes gated
****
*
*
monocytes (10^9/L)
controls n=37
lepto n=57

◂**Fig. 3** Switch in monocyte cell subsets in response to human leptospirosis. **a** Flow cytometry gating strategy of monocytes (FSC/SSC dot plot) and identification of monocyte subsets (CD14 and CD16 dot plots) at hospital admission time point. Representative examples are shown for one healthy control and one leptospirosis case. **b** Monocyte subset distribution was compared between controls and leptospirosis: upper panels with cell percentages, lower panels with absolute values. From left to right: M^{class}; M^{int}, M^{nc}. Box and whiskers figures represent median and 25th–75th percentiles for box, and 5th–95th percentiles for extremities. Comparisons with one-way repeated measures ANOVA with Sidak correction for multiple comparison. *, ****p values inferior to 0.05 and 0.0001, respectively

previous studies in sepsis described mortality of 30–40% [34–36]. To what extent our observation may account for immune impairment of monocytes during leptospirosis remains to be elucidated. For instance, it may be important to address the levels of intracellular pro- and anti-inflammatory cytokines, as well as reactive oxygen species to better assess innate cell effector functions. Phenotypic data during human leptospirosis pathology are scarce, with only one report indicating increased CD69 and HLA-DR expression on PBMC in the presence of leptospiral glycolipoprotein extract ex vivo [37]. These discrepancies with our results question the relevance of experiments with PBMCs from healthy individuals. Differential expression kinetics may account for such discrepancies. The inclusion of data from infected patients at different time points would be an interesting addition to future studies.

With regard to other markers, we showed no clear modification of TLR2, TLR4 and CD69 expression on monocytes from patients compared to controls. For TLRs, this is consistent with previous studies in sepsis where no modulation of expression was observed post infection [38, 39]. During leptospirosis, a study with human PBMCs demonstrated that monocytes were more likely to produce TNFα after stimulation with *Leptospira* peptidoglycans (TLR2 agonist) compared to leptospiral LPS [40]. It could thus be hypothesized that during leptospirosis human monocytes maintain their TLR expression and ability to respond to *Leptospira* cell wall components (peptidoglycan, LPS) to produce adequate inflammatory response such as TNFα secretion. Further functional analyses and intracellular stainings are needed to better assess the capacity of monocyte to express different cytokines during human leptospirosis.

Table 2 Description of healthy control subjects and leptospirosis patients who had additional flow cytometry assays at admittance

	Healthy donors	Leptospirosis cases	Controls vs leptospirosis	Non-severe leptospirosis cases	Severe leptospirosis cases
Number of individuals	26	24		8	16
Ratio M/F	24/2	23/1	NS	8/0	15/1
Age (years), mean ± SD	43.71 ± 12.95	42.58 ± 13.68	NS	40.25 ± 10.94	43.75 ± 15.1
Comorbidities, numbers		16		4	12
Tobacco abuse		12		3	9
Alcohol abuse		8		3	5
Diabetes		3		1	2
Cirrhosis		1		0	1
Biological parameters, mean ± SD					
Neutrophils (10^9/L)	3.2 ± 1	9.56 ± 3.81	$p<0.0001$	10.42 ± 4.31	9.13 ± 3.62
Lymphocytes (10^9/L)	2.15 ± 0.69	0.92 ± 1.08	$p<0.0001$	0.76 ± 0.38	1.0 ± 1.31
Monocytes (10^9/L)	0.56 ± 0.16	0.91 ± 0.63	$p=0.03$	0.77 ± 0.42	0.98 ± 0.71
Platelets (10^9/L)	223 ± 48	103 ± 52	$p<0.0001$	151 ± 30	79 ± 43
Creatinine (µmol/L)	82.3 ± 8.9	156.1 ± 141	$p=0.009$	106.8 ± 22.6	181 ± 168
Total bilirubin (µmol/L)	8.6 ± 4	83.5 ± 112.6	$p=0.002$	18.1 ± 8.8	112 ± 125
Alkaline phosphatase (IU/L)	52 ± 27	99 ± 49	$p<0.0001$	110 ± 51	95 ± 49
AST (IU/L)	20.5 ± 4.2	138 ± 259	$p=0.02$	106 ± 175	155 ± 298
CPK (IU/L)	143 ± 98	1921 ± 4237	$p=0.04$	323 ± 181	2537 ± 4894
CRP (mg/L)	1.8 ± 2.8	225 ± 87.5	$p<0.0001$	255 ± 58	210 ± 98
Plasmatic *Leptospira* load* (bact./mL), median (interquartile)		723 (387; 4125)		445 (315; 715)	2730 (427; 6262)

Data are expressed as means (± SD) except for bacterial load owing to non-Gaussian distribution of data: median (1st; and 3rd quartiles). Statistics between two groups are performed with parametric unpaired t tests or non-parametric Mann–Whitney U test as appropriate for quantitative variables, and with Fisher's exact test for categorical data. p value inferior to 0.05 was considered significant. *Plasmatic bacterial load is inferred from plasmatic RT-qPCR C_t values according to the log-transformed standard curve

AST aspartate aminotransferase, *CPK* creatine phosphokinase, *CRP* C-reactive protein, *NS* not significant

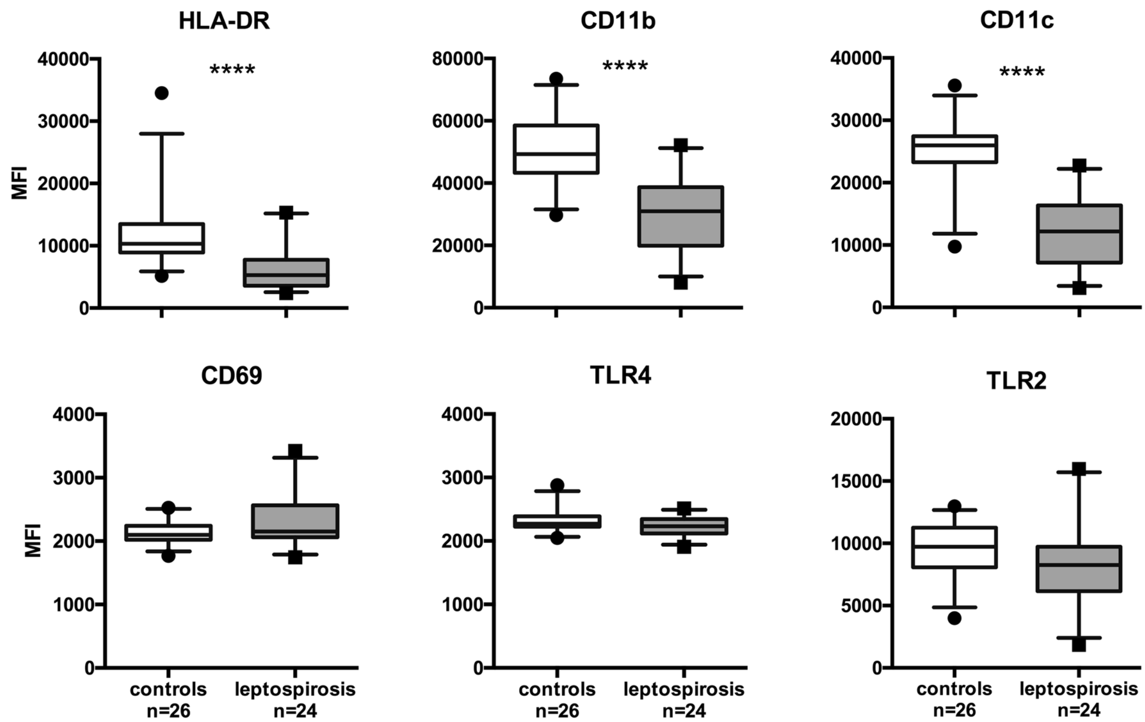


Fig. 4 Monocyte cell surface expression of HLA-DR, CD11b and CD11c is decreased during leptospirosis. Cell surface expression of different molecules determined by flow cytometry in controls (no colour) and leptospirosis patients (grey colour) at hospital admission. *MFI* mean fluorescence intensity. Box and whiskers figures represent median and 25th–75th percentiles for box, and 5th–95th percentiles for extremities. Comparisons with non-parametric Mann–Whitney test. ****p values inferior to 0.0001

For CD69, we observed a slight increased expression in the severe leptospirosis subgroup, but there was no clear increase when we globally compared patients versus controls. This relative failure to upregulate CD69 expression at the cell surface of monocytes remains unexpected. Indeed, CD69 is best known as an early biomarker of immune cell activation, and ex vivo data have already indicated increased levels of CD69 on monocytes stimulated by *Leptospira* [37].

We did not observe an increase in the expression of beta two integrins: CD11b and CD11c on monocytes in response to the infection. These molecules are major complement opsonic phagocytic receptors also involved in cell recruitment and diapedesis. Many studies have reported the rise of these integrins on monocyte cell surface during inflammatory conditions, especially during bacterial infections such as sepsis [39, 41, 42].

Overall, the descriptive nature of our study has several limitations and failed to address further the possible mechanistic implications of our findings. For example, other studies have shown a genuine activation state of monocytes in leptospirosis and leading to a pro-coagulant state [43]. The number of innate immune monocyte receptors studied herein is limited and other key effectors molecules signalling downstream from these receptors should also be considered for further studies.

In conclusion, our data and also taking into account previous ex vivo and in vitro studies support the view that monocytes can sense the bacteria plausibly through peptidoglycan/LPS/TLR interactions and are engaged in an innate immune response against the infectious challenge [14, 15, 37, 44]. We observed the canonical switch to M^{int} and M^{nc} which are subsets thought to be involved in the diapedesis process at the level of vascular beds to migrate to the infectious sites. However, are our original data further suggest that monocytes fail to commit to a full activation state in human leptospirosis. We hypothesized that they may be impaired in the capacity to present antigens, to instruct the adaptive immune cells and to phagocytose opsonized bacteria at the infectious site.

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Data availability The datasets generated during and/or analysed during the current study are available in the FlowRepository public database, URL: https://flowrepository.org/id/RvFrfom7jwL1SHc4nTFg2epdGcDpHc3qwtsGSOPoDm6ofWkmfjfQTm22c2UcA1At.

Compliance with ethical standards

Conflict of interest The authors have no competing interest to declare.

Ethical approval This study was approved by the local human ethic committee of tertiary teaching Hospital 'CHU de La Réunion' (protocol number R15018) and conducted according to the principles expressed in the Declaration of Helsinki. Informed consent was obtained from all individual participants included in the study.

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