Immune-Pathological Correlates of Disease Severity in New World Kala-Azar: The Role of Parasite Load and Cytokine Profiles

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Article

Immune-Pathological Correlates of Disease Severity in New World Kala-Azar: The Role of Parasite Load and Cytokine Profiles

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Abstract: Introduction: Kala-azar is a protracted disease caused by the protozoan *Leishmania infantum* (zoonotic) or *L. donovani* (anthroponotic), transmitted by sandflies. It presents with fever, anemia, and hepatosplenomegaly, potentially progressing to hemorrhages, secondary infections, and death. Its pathogenesis is linked to an exaggerated cytokine response. **Patients and Methods:** We studied 72 hospitalized patients, analyzing clinical data and outcomes in relation to *L. infantum* DNA load in blood and bone marrow, and plasma concentrations of IL-1β, IL-6, IL-8, IL-10, IL-12, TNF-α, and TGF-β. **Results:** Cytokine levels were elevated. *L. infantum* kDNA in blood and bone marrow were strongly correlated and increased with disease duration. Higher parasite loads were observed in men, adults, and HIV-infected patients, and were significantly associated with mortality. IL-6 was independently linked to sepsis. In multivariate analysis, IL-12 was the only cytokine inversely associated with blood parasite load. **Conclusions:** Parasite load, but not cytokine levels, correlated with disease severity, suggesting additional mechanisms drive progression. IL-12 appears to limit parasitemia, indicating a weak, persistent adaptive immune response that is ultimately overwhelmed by a progressive, inefficient, and inflammatory innate response.

Keywords: kala-azar; visceral leishmaniasis; *Leishmania infantum*; pathogenesis; cytokines; immunity; parasitism; mortality

1. Introduction

Kala-azar, or visceral leishmaniasis, is a lethal parasitic disease with protracted symptoms. Most patients report low-grade fever. Patients also report inappetence, diarrhea, and weight loss and appear malnourished and anemic, with enlarged spleen and liver [1]. Jaundice, vomiting, and emaciation, signs of bleeding and, together with bacterial infections, are frequent and may lead to death [2,3]. The disease is commoner and is more lethal at the extremes of age [4,5]. Immunosuppressed patients, are also at higher risk [6,7].

Two species of protozoa are the cause of disease. *Leishmania donovani* is the agent in East Africa and South Asia and is restricted to humans. Currently, *L. donovani* kala-azar has been put under control in South Asia through a set of well-coordinated control measures [8]. *L. infantum* causes the disease among humans and other mammals in Central and Western Asia, the Mediterranean Basin, and the Americas. Differently of *L. donovani* kala-azar, the incidence of *L. infantum* kala-azar remains untouched by control measures [9]. Transmission occurs mainly by the bite of several species of sandflies. The flagellar promastigotes develop in the insects and in culture media. After the infective

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bite, promastigotes are phagocytized by neutrophils and monocytes. They lose the flagella and survive as amastigotes in macrophages distributed in the spleen, in the liver Kupffer cells, bone marrow, lymph nodes [10–12]. They circulate in the blood inside monocytes and neutrophils in very low concentration [13,14].

There are no known *Leishmania* virulence factors that can directly harm mammal host cells or tissues and cause disease [3]. Instead, they lead to the sickness through host response as long as they surpass the host's innate and acquired defenses and progressively multiply, generating the typical signs and symptoms and, eventually, death [15]. As it happens with other infectious diseases, systemic inflammation is triggered by pro-inflammatory cytokines while regulatory cytokines limit inflammation and presumably immunity [11,16]. This wave of cytokines is associated with hemorrhagic manifestations, and bacterial infections [17].

How precisely the parasites and the host are intertwined to develop the lethal disease phenotype has been investigated but is still a matter of conjecture [18]. Evaluations of the role of the host showed that cytokines such as IL-6, IL-8, and INF-g, sCD14 might be involved in more life-threatening disease [17,19]. Similarly, the larger the *L. infantum* load, the more severe the disease is [20,21]. Interestingly, the *L. infantum* genome explains around 80% of the mortality of human patients, suggesting a complex parasite interaction with host response [22]. However, it is not known how the interplay of parasites and host molecules is for generating the outcome of death even after prompt diagnosis and treatment. Mortality remains 10% over the years and, it is increasing in some parts of the world [23]. Therefore, this study was developed to shed light on the connections between *L. infantum* burden, a set of host molecules, life-threatening kala-azar, and immunity, in order to better understand the pathogenesis of severe kala-azar.

2. Materials and Methods

2.1. Patients

All kala-azar patients were treated at the "Natan Portella" Tropical Disease Institute in Teresina, Brazil, and confirmed kala-azar cases by the presence of *L. infantum* amastigotes on bone marrow smears or the presence of promastigotes in culture were included in this study. The study was performed on a sample of 72 patients, sequentially admitted with available clinical information taken by a single physician and with cryopreserved plasma, blood, and bone marrow samples obtained before treatment.

2.2. Medical Data

The following parameters of VL patients were analyzed: age, sex, history of vomiting, somnolence, inappetence, diarrhea, abdominal pain, cough, shortness of breath, oliguria, and presence of fever > 39 °C, pallor, irritability, edema, bleeding, jaundice, dyspnea, lung X-ray abnormalities, urinary tract infection, renal failure, pneumonia, sepsis, HIV-1 infection, spleen and liver size, and complete blood count. Fever was present in all but one patient and, therefore, was not analyzed. The registered bleeding variables included any history of hemorrhagic manifestations on admission or during hospital stay, such as gingival or gastrointestinal bleeding, hematoma at venoclysis site, petechiae, and bruise. The variables relative to hemorrhages were collapsed into two variables: "reported bleeding" and "detected bleeding". The first was informed by the patients and the second was observed by the medical team. A patient was taken as having pneumonia when typical symptoms and signs of pneumonia were present in the X-ray. Bacterial cultures were routinely requested, but many patients were already receiving antibiotic therapy. Hyperventilation, tissue hypoperfusion, venous saturation, oliguria, and altered consciousness defined sepsis. Infections were divided into two categories: "sepsis" and "any bacterial infection". "Sepsis" refers to those patients who fulfilled the criteria described above, while "any bacterial infection" described the diagnosis of any type of bacterial infection.

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Since the number of deaths was too small to detect statistically significant clinical associations with severe disease, the score system software Kala-Cal® was also used as a proxy of disease gravity. The system uses data such as edema, jaundice, dyspnea, HIV-coinfection, vomiting, bacterial infections, and hemorrhages, and, indeed, it was significantly associated with mortality. The software has been published elsewhere [5] and can be easily reached at https://www.sbmt.org.br/kalacal/. In synthesis, disease severity was evaluated by six variables: "death", "chance of death > 10% by using Kala-Cal®", "reported bleeding", "detected bleeding", "sepsis", and "any bacterial infection".

2.3. DNA Isolation, Purity, and Standardization

Bone marrow and blood samples were collected both in heparinized and in citrated tubes. Isolated parasites were stored in liquid nitrogen. DNA isolation was performed by the QIAmp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's instructions with 200.0 µL of bone marrow or blood. The purity and DNA concentration was evaluated in a spectrophotometer (NanoDrop ND-1000 spectrophotometer; Thermo Fisher Scientific, Waltham, MA). DNA samples were normalized to a concentration of 5 ng/µL, concentrated or diluted with distilled water when necessary.

2.4. Quantitative PCR

Quantitative PCR was based on the TaqMan probe to quantify *L. infantum* on blood and bone marrow accurately. The target sequence for *L. infantum* detection consisted of FAM – TTT TGA ACG GGA TTT CTG – MGB-NFQ (GenBank AF169140). Specific primers based on kinetoplast DNA consisted of 5′ – GGC GTT CTG CAA AAT CGG AAA A – 3′ (forward) and 5′ - CCG ATT TTT GGC ATT TTT GGT CGA T – 3′ (reverse), (Applied Biosystems, Foster City, CA, USA) were used (Zacarias et al.,2017). A standard curve was constructed using 10-fold serially diluted *L. infantum* DNA corresponding to 10^4 to 1 parasite per reaction.

Albumin was selected as the housekeeping gene to equalize the parasite count in the bone marrow. The number of parasites was expressed as a ratio to the number of human nucleated cells. The primers were 5′ - GCT GTC ATC TCT TGT GGG CTG T – 3′(forward) and 5′ – ACT CAT GGG AGC TGC TGG TTC – 3′ (reverse). The probe was VIC- GG AGA GAT TTG TGT GGG CAT GAC A –TAMRA (GenBank NG009291) (Verma et al., 2010). The standard curve was constructed using 10-fold serially diluted human cells DNA corresponding to 2 x 10^4 to 2 human nucleated cells per reaction.

Amplification and detection were performed in StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Cycling parameters were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles at 95°C for 15 s and 60°C for 1 minute. Standards, samples, and negative controls were analyzed in duplicates. The threshold cycle (Ct) value was plotted by the standard curve. The cut-off between reactions was 20%, and no deviation proportions were considered.

2.5. Plasma Cytokines

The plasma specimens were stored at - 20°C. Serum IL-1β, IL-6, IL-8, IL-10, TNF-α, IL-12 were measured using a high-sensitivity multiplex inflammatory cytokine panel by cytometric bead array (CBA), plus cytokines IL-17 and TGF-β, which were measured independently by CBA-*flex* set (BD Biosciences, San Jose, CA, USA), on the BDFAcs Array (BD Biosciences, San Jose, CA, USA), following the manufacturer's instructions. Measurements of each sample were performed in duplicate, and the average of the two measurements was used. Standard curves were derived from the cytokine standards supplied with the kit 10-fold dilution. The lower limits of detection for specific analyses ranged from 0.40 pg/mL for IL-8, 0.04 pg/mL for IL-1β, 0.04 pg/mL for IL-6, 0.97 pg/mL for IL-10, 0.03 pg/mL for TNF-α, 0.06 pg/mL for IL-12, and 0.05 pg/mL for TGF-β, based on standards curve dilution.

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2.6. Statistical Analysis

Proportions and 95% confidence intervals were calculated for the clinical and demographic variables. The median and interquartile intervals, as well as the means, were calculated for kDNA loads. The Kolmogorov–Smirnov test compared the plasma cytokines with the standard values taken from the literature [24]. The Wilcoxon rank-sum test compared blood and bone marrow kDNA load according to demographic and clinical data. Similarly, the test was used to analyze the concentration of cytokines. Pearson's correlation test evaluated the correlation between death, "risk of death > 10% by Kala-Cal®", "reported bleeding", "detected bleeding" "sepsis", and "any bacterial infection". The Spearman's correlation test was used to test the correlation between the natural logarithm of blood and bone marrow kDNA burdens and their correlation with the time of disease, as indicated by the time with fever. Finally, simple and multiple linear and quantile regression were applied to test whether the cytokines predicted the blood and bone marrow kDNA load. The statistical package Stata/IC 15.1 (College Station, TX) was used to analyze the data.

3. Results

3.1. Study Population

Table 1 shows the characteristics of the study population. Forty-two patients were male (58.3%) and 30 female (41.7%). The median age was 7.5 and the mean was 15.2 years. Seventeen participants (23.6%) were under two, 22 (30.6%) were under four, and 61 were children under 15. Six were older than 40 (8.3%). Among the 70 who were tested for HIV, 13 were positive (18.6%). Four (5.6%) patients died. Male sex was associated with HIV infection ($p = 0.032$).

Table 1. Characteristics of the study population.

Characteristic	Number (%)	95% CI[1]
Sex		
Male	42 (58.3)	46.1; 69.85
Female	30 (41.7)	30.2; 53.89
Age groups (Years)		
<2	17 (23.6)	14.0; 35.0
2<4	5 (6.9)	2.2; 15.4
4<15	22 (30.6)	20.2; 42.5
15<40	22 (30.6)	20.2; 42.5
40+	6 (8.3)	3.1; 17.3
HIV[2] (number, %)	13 (18.6)	10.3; 30.0
Deaths (number, %)	4 (5.6)	1.5; 13.61
Chance of death > 10% by Kala-Cal®	25 (34.7)	23.9; 46.9
Hemorrhages or infections	31 (43.7)	31.9; 56.0
Reported bleeding	4 (5.6%)	1.5; 13.6
Detected bleeding	15 (20.8)	12.2; 32.0
Sepsis	10 (14.1)	7.0; 24.4
Any bacterial infection	23 (31.9)	21.4; 44.0

[1]95% confidence interval. [2]Only 70 patients tested for HIV infection.

3.2. Clinical Findings

Table 1 also shows the clinical characteristics of the study population. The mean Kala-Cal® chance of death was 12.1%, and the median was 6.5. Twenty-five patients had a chance of death of at least 10% (34.7%). More than 40% had hemorrhages or infections. Fifteen patients had "reported bleeding" manifestation (20.8%). Ten patients filled the criteria for sepsis (14.1%). Four patients had some sort of bleeding at hospital stay e.g., "detected bleeding" (5.6%). Any bacterial infection was detected in 23 (31.9%) patients. Mortality was associated with "detected bleeding" ($p = 0.014$) and

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with sepsis syndrome ($p = 0.008$), as well as with any bacterial of infection ($p = 0.052$). The six markers of disease severity were significantly and positively correlated, except "death" with "reported bleeding" and "probability of death > 10%" with "sepsis" or "any bacterial infection", which were positively but not statistically correlated. The supplemental table informs the correlation matrix between these variables.

The chance of death calculated by Kala-Cal® was well correlated with the occurrence of death (r = 0.40, $p < 0.001$). As expected at admission, the estimated chance of death among those who survived was 8.9%, much less than chance of those who actually lately died (65.3%).

3.3. Quantity of Parasite Load and Cytokines

Table 2 depicts the blood and bone marrow *L. infantum* load, plasma cytokine concentrations, and reference values. The median blood kDNA concentration was 856.7 kDNA amastigote-equivalents/mL (AEq/mL), and the mean was 3,515.4 AEq/mL. The median bone marrow kDNA concentration was 55.7 AEq/10^6 DNA equivalents of human cells (HCEq), with IQ 3.7 – 400.7 AEq/10^6HCEq. The standard deviation was 1.7 larger than the mean blood kDNA, while the same relationship was 3.2 for bone marrow kDNA, demonstrating the higher variability of the bone marrow count. The standard values were assumed to be zero, although some asymptomatic patients may harbor minimal blood kDNA load [25–28]. Regarding the cytokines, IL-6, IL-8, IL-10, and TGF-b had medians and means much above the values of a healthy population, while IL-1b and IL-12 had a more modest increase. Despite the high plasma concentration, cytokines were not detected in a relevant proportion of patients: 28% for IL-12, 19% for IL-1b, 9% for TNF-a, and 6% for IL-6.

Table 2. Concentration of kDNA and cytokines in the plasma of patients with kala-azar.

Variables	Median	Interquartile intervals	Mean	Reference values (median)	Kolmogorov–Smirnov test (*p*-value)
Plasma kDNA (AEq[1] /mL)	856.7	145.5-3,527.9	3,515.4	0[6]	0.000
Bone marrow kDNA (AEq/10^6HCEq[2])	55.7	3.6 – 4,008	889.9	0[6]	0.000
IL-1b pg/mL	0.9	0.2 – 2.1	2.0	0.18 (0–3.66)[3]	0.000
IL-6 pg/mL	9.5	2.4 – 28.0	41.7	0 (0–0)[3]	0.000
IL-8 pg/mL	26.2	9.8 – 145.5	146.9	0 (0–0)[3]	0.000
IL-10 pg/mL	18.4	8.7 – 35	30.2	0 (0–0)[3]	0.000
IL-12 pg/mL	1.2	0.0 – 2.5	1.8	0 (0–0)[3]	0.000
TNF-a pg/mL	1.0	0.3 – 3.0	2.2	0 (0–0)[3]	0.000
TGF-b ng/mL	23.6	11.2 – 42.4	39.6	NA[4,5]	-

[1]Parasite-equivalents. [2]AEq/10^6 equivalents of human cells. [3]Kildey et al, 2014. [4]Grainger et al, 2000. [5]Not available. [6]Assumed to be zero in persons without visceral leishmaniasis.

3.4. Blood and Bone Marrow L. infantum Load

The Spearman's correlation test revealed a moderate, but statistically significant correlation between the natural logarithm of blood and the natural logarithm of bone marrow parasite load (r = 0.48, $p < 0.001$) (Figure 1). Bone marrow kDNA load without including human cell counts as the denominator in the parameter was poorly correlated with the parasite blood load (r = 0.21, $p = 0.077$).

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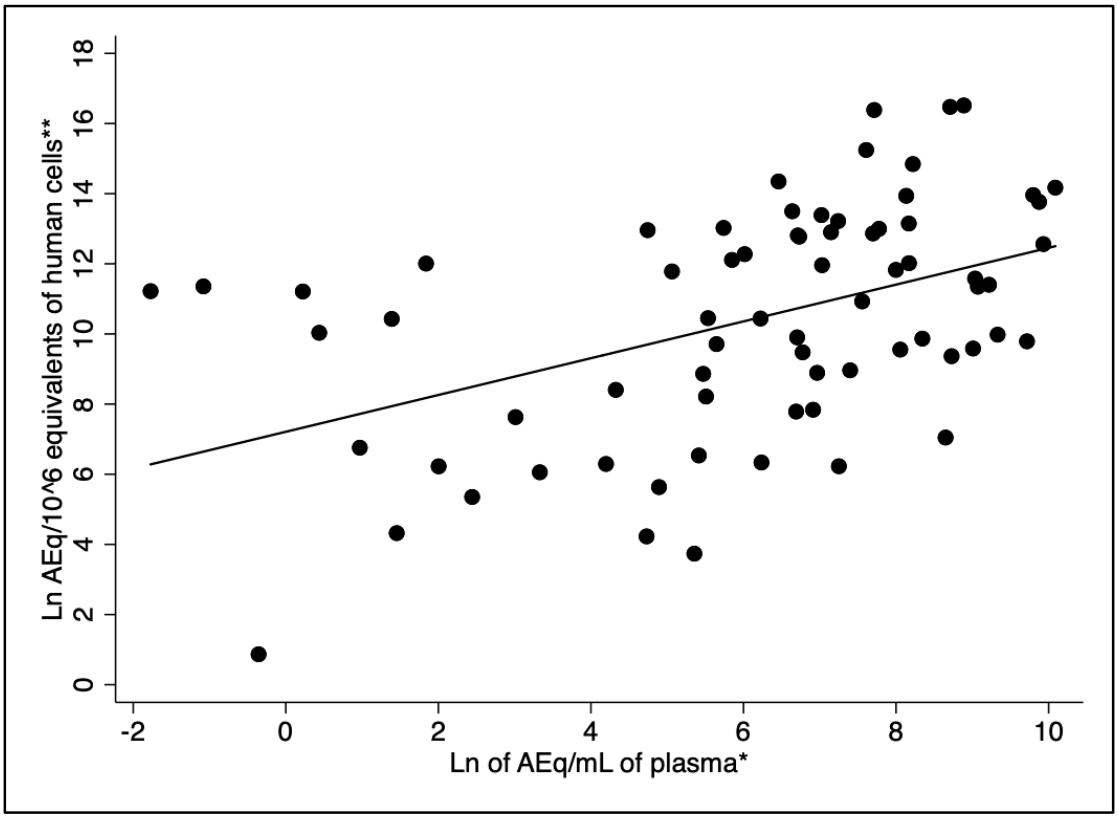

Figure 1. Correlation between plasma and bone marrow parasite load (r = 0.48, *p*-value < 0.001). Legend: *Natural logarithm of kDNA amastigote-equivalents/mL of plasma (AEq/mL). ** *Natural logarithm of AEq/10^6 DNA equivalents of human cells.

3.5. Time of Disease, Blood and Bone Marrow L. infantum Load, Plasma Cytokines, and Severity

The blood and bone marrow *L. infantum* kDNA load increased with the time of disease, as estimated by the duration of fever (Figure 2). The Spearman's correlation coefficient for blood kDNA with time was 0.33 ($p < 0.005$), and for the bone marrow, it was 0.38 ($p = 0.001$). There was no correlation of any cytokine with time. At the univariate linear regression analysis of the relationship of the natural logarithm of time with fever with the natural logarithm of blood kDNA load, the probability of rejecting the model was < 0.005, and the *p*-value of the coefficient was 0.005. For predicting the natural logarithm of the bone marrow load, the probability of rejecting the model was < 0.001, and the *p*-value of the coefficient was < 0.001. There was no association of any cytokine with the time of disease. Higher chance of death and "detected bleeding" were slightly correlated with the time of fever (r = 0.23, $p = 0.056$ and r = 0.24, $p = 0.044$, respectively).

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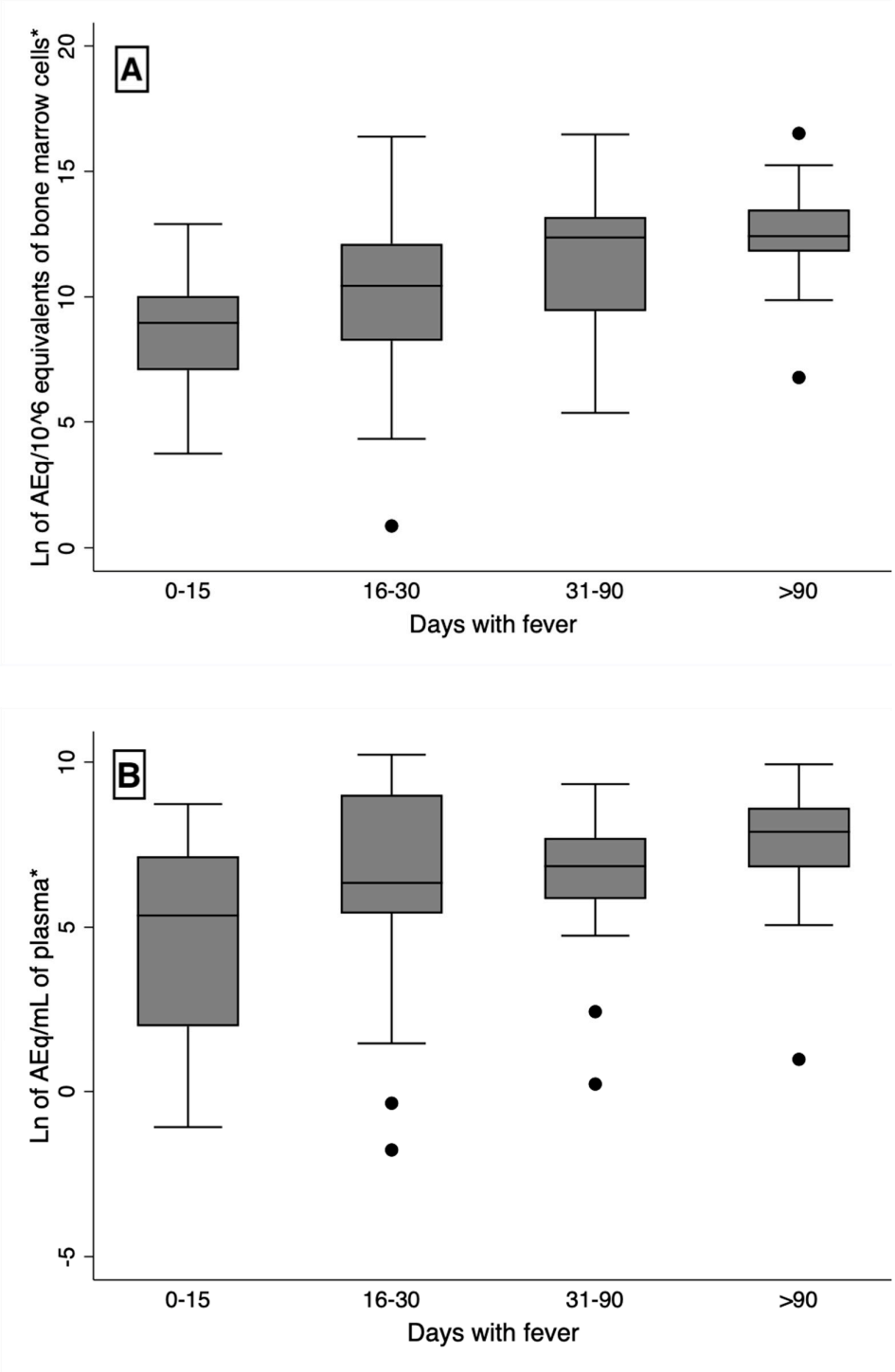

Figure 2. Increasing parasite load with time of fever. A: plasma load (r = 0.33, *p*-value < 0.005); B: bone marrow load (r = 0.38, *p*-value < 0.001).

3.6. Blood and Bone Marrow L. infantum Load, Age, Sex, HIV Infection, and Kala-Azar Severity

Table 3 shows the relationships between the *L. infantum* load with age, sex, HIV infection, and markers of kala-azar severity. Individuals with 15 years of age or older had higher blood median load ($p = 0.022$), and patients 40 years old or older had the highest blood load (median = 9,129.5 AEq/mL) (data not shown). The same happened in the bone marrow ($p = 0.043$). The blood and bone marrow

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load were higher in men, $p = 0.012$ and $p = 0.073$, respectively. HIV-infected patients had eight times higher blood load than those non-infected ($p = 0.006$). On the other hand, in bone marrow, although higher, this difference was non-significant. At the multivariate quantile regression, HIV status did not stand out, and only sex and age were associated with blood kDNA load.

Table 3. Table 2. Concentration of *Leishmania infantum* amastigotes estimated by kDNA in the blood of patients with kala-azar.

Markers of severe disease (number of patients)	Blood AEq[1] median, (mean)	*p*-value[2]	Bone marrow AEq/10^9HCEq median, (mean)	*p*-value[3]
Age (years)				
<15 (27)	508 (2,513)		27 (519)	
15+ (44)	2,679 (5,091)	**0.022**	137 (1,494)	**0.043**
Sex				
Female (30)	329 (1,580)		28 (631)	
Male (41)	1,452 (4,898)	**0.012**	90 (1,079)	**0.073**
HIV				
Yes (13)	4,207 (7,016)		56 (2,867)	
No (57)	511 (2,768)	**0.006**	35 (500)	**0.168**
Hospital outcome				
Death (4)	11,826 (11,925)		487 (605)	
Survival (67)	830 (3,020)	**0.169**	56 (907)	**0.273**
Chance of death > 10% by Kala-Cal®				
> 10% (25)	3,532 (6,617)		290 (2,280)	
<10% (46)	311 (1,866)	**<0.001**	20 (350)	**0.001**
Reported bleeding				
Yes (4)	8,855 (10,440)		89 (406)	
No (67)	830 (3,108)	**0.350**	56 (919)	**0.517**
Detected bleeding				
Yes (15)	823 (4,427)		214 (1,406)	
No (56)	876 (3,276)	**0.873**	21 (752)	**0.012**
Sepsis				
Yes (10)	888 (5,619)		247 (375)	
No (60)	1,130 (3,210)	**0.856**	34 (988)	**0.374**
Any bacterial infection				
Yes (23)	837 (3,679)		425 (466)	
No (48)	1,059 (3,439)	**0.758**	65 (1,093)	**0.722**

[1]Amastigote-equivalents/mL [2]Wilcoxon ranksum test. [3]AEq/10^6 equivalents of human cells.

The four patients who died had kDNA in the blood with a median 14.2 times higher than the survivors but non-significantly associated to death ($p = 0.169$), likely due to the small number of deceased patients. The bone marrow *L. infantum* load was also higher in the deceased in those who survived, but not statistically significant. The concentration of kDNA in the blood was 11.4 times higher in those with a chance of death estimated above 10% ($p < 0.001$). In the bone marrow, it was 14.5 times higher in those with a higher than 10% chance of death ($p = 0.001$).

The association of the two variables relative to hemorrhagic phenomena, "reported bleeding" and "detected bleeding", were not concordant regarding *L. infantum* load in the blood and in the bone marrow. Blood kDNA was ten times higher in those four patients with "detected bleeding", than in those with "reported bleeding". The opposite happened in the bone marrow: *L. infantum* load was not that high in those with "detected bleeding" but was 10 times higher in those patients with "reported bleeding" ($p = 0.12$).

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3.7. Plasma Cytokines, Age, Sex, HIV Infection, and Markers of Kala-Azar Severity

Table 4 shows the associations between age, sex, HIV infection, and the clinical manifestations of the severity of kala-azar with the seven measured plasma cytokines. There was a paucity of statistically solid associations between plasma cytokines and demographic and clinical data. IL-10 was almost two times higher in children ($p = 0.02$). IL-10 was also much higher in women than in men ($p = 0.06$). Similarly, IL-10 was the only cytokine associated with HIV infection, being significantly lower in those with HIV ($p = 0.04$). Possibly, due to the small number of deaths in the study population (four), no cytokine had any statistically significant association with death. However, the median of IL-6 was noticeably more than two times higher in those who died. The used proxy of death and disease severity, e.g., the chance of death according to the application of the Kala-Cal® software, showed no significant association with any cytokines. Only three patients with measured cytokines had "detected bleeding". However, in these three patients, IL-6 was almost four times higher ($p = 0.03$). Only IL-8 was associated with "reported bleeding" ($p = 0.04$). Sepsis syndrome had some significant associations with cytokines. IL-12 was higher in those with the syndrome ($p = 0.05$), as well as nearly significant with IL-1b ($p = 0.06$), IL-6 ($p = 0.06$), and IL-10 ($p = 0.07$). By the end of these results, the variable "any bacterial infection" was statistically associated with IL-1b ($p = 0.01$), IL-6 ($p = 0.01$), IL-8 ($p = 0.03$), IL-10 ($p = 0.04$) and TNF-*a* (p = 0.01). However, no statistically significant association would exist if the Bonferroni analysis for multiple comparisons is applied. In the multivariate linear regression, IL-6 was the only predictor of sepsis ($p = 0.04$), confirmed in a multiple quantile regression. No cytokine was found predicting the chance of death or any other clinical outcome at the *p*-value <0.05 (data of regressions not shown).

Table 4. Plasma concentration of cytokines according to demographic data, and HIV-infection, and markers of kala-azar severity.

Variables (number of patients)	IL-1b median, (mean)	*p*-value[1]	IL-6 median, (mean)	*p*-value	IL-8 median, (mean)	*p*-value	IL-10 median, (mean)	*p*-value	IL-12 median, (mean	*p*-value	TNF-a median, (mean)	*p*-value	TGF-b median, (mean)	*p*-value
Age (years)														
<15 (27)	1.1 (2.1)		14.0 (49.3)		28.8 (130.7)		23.4 (36.4)		1.4 (2.1)		1.2 (2.5)		23.1 (40.5)	
15+ (44)	0.4 (1.9)	0.18	7.8 (28.9)	0.16	23.3 (174.2)	0.95	12.8 (19.8)	*0.02*	0.6 (1.3)	0.18	0.9 (1.6)	0.52	24.5 (38.0)	0.95
Sex														
Female (30)	0.9 (1.9)		8.4 (55.9)		25.6 (118.7)		23.1 (32.6)		1.0 (2.2)		2.1 (3.1)		13.9 (40.6)	
Male (41)	1.0 (2.0)	0.95	13.6 (32.6)	0.79	26.2 (164.8)	0.99	13.6 (28.7)	0.06	1.3 (1.6)	0.92	0.9 (1.7)	0.54	28.5 (38.9)	0.25
HIV-infection														
Yes (12)	1.3 (2.7)		8.1 (13.0)		46.7 (188.1)		12.4 (14.3)		1.2 (1.7)		1.6 (1.9)		16.5 (35.9)	
No (57)	0.9 (1.9)	0.46	13.0 (48.8)	0.50	25.4 (140.5)	0.50	21.8 (34.2)	*0.04*	1.3 (1.9)	0.87	1.0 (2.3)	0.70	24.05 (40.1)	0.65
Hospital outcome														
Death (4)	1.3 (1.3)		24.7 (44.4)		48.1 (173.9)		12.0 (31.5)		1.4 (1.8)		1.2 (1.8)		18.6 (20.4)	
Survival (62)	0.9 (2.1)	0.45	9.1 (42.2)	0.44	26.4 (147.5)	0.96	18.7 (30.5)	0.44	1.2 (1.8)	0.61	1.0 (2.3)	0.84	24.1 (20.4)	0.58
Chance of death[2]														
> 10% (23)	1.0 (2.5)		15.4 (44.3)		65.2 (206.5)		16.2 (27.8)		1.1 (1.6)		1.0 (2.3)		24.5 (45.4)	
<10% (43)	0.9 (1.8)	0.33	9.5 (44.3)	0.45	24.6 (118.5)	0.10	19.1 (32.0)	0.59	1.3 (1.9)	0.68	0.9 (2.2)	0.36	22.3 (36.1)	0.53
Reported bleeding														
Yes (3)	1.6 (1.8)		34.1 (63.7)		78.6 (246.1)		34.7 (51.3)		2.0 (2.1)		0.4 (1.8)		15.7 (19.8)	
No (63)	0.9 (2.0)	0.14	8.6 (41.2)	*0.03*	24.9 (144.5)	0.16	16.6 (29.5)	0.15	1.2 (1.8)	0.44	1.0 (2.3)	0.83	23.6 (40.3)	0.51
Detected bleeding														

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Yes (13)	0.9 (2.8)		17.2 (24.9)		88.5 (242.7)		16.1 (24.1)		2.0 (2.5)		2.1 (2.2)		42.1 (59.8)	
No (53)	1.0 (1.9)	0.50	8.6 (46.6)	0.42	24.6 (126.2)	*0.04*	21.8 (32.1)	0.57	1.2 (1.7)	0.13	1.0 (2.3)	0.66	20.9 (34.3)	0.09
Sepsis														
Yes (10)	1.6 (1.9)		23.0 (137.3)		110.2 (229.9)		36.9 (37.3)		1.9 (2.5)		2.4 (2.5)		18.3 (31.9)	
No (56)	0.8 (2.0)	0.06	8.1 (25.3)	0.06	24.7 (134.7)	0.21	16.1 (29.3)	0.07	0.9 (1.7)	*0.04*	0.9 (2.2)	0.17	25.7 (40.7)	0.41
Any bacterial infection														
Yes (22)	1.6 (2.4)		25.2 (83.1)		67.0 (203.9)		30.5 (40.6)		1.9 (2.1)		2.4 (2.8)		18.9 (33.0)	
No (44)	0.7 (1.8)	*0.01*	7.2 (21.9)	0.01	22.6 (121.8)	*0.03*	16.0 (25.5)	*0.04*	0.9 (1.7)	0.11	0.8 (2.0)	*0.01*	29.5 (42.5)	0.24

[1]Wilcoxon ranksum test. [2]Kala-Cal®.

3.8. Regression Analysis Between L. infantum Load and Plasma Cytokines

In the univariate linear regression analysis, IL-12 negatively and significantly predicted blood kDNA levels (p = 0.002). Similarly, TNF-α also showed a negative and significant prediction (p = 0.018). However, when controlling for IL-12, the effect of TNF-α was no longer significant. In the multivariate linear regression, only IL-12 remained a significant predictor of blood kDNA load (Figure 3); no other cytokines were associated. Finally, no cytokine predicted kDNA levels in the bone marrow. The models adjusted R^2 and pseudo R^2, respectively, were low, indicating that the studied cytokines were poor predictors of *L. infantum* load.

```
      Source |       SS           df       MS      Number of obs   =        67
-------------+----------------------------------   F(6, 60)        =      2.50
       Model |  117.471559         6  19.5785932   Prob > F        =    0.0315
    Residual |  469.194716        60  7.81991193   R-squared       =    0.2002
-------------+----------------------------------   Adj R-squared   =    0.1203
       Total |  586.666275        66  8.88888295   Root MSE        =    2.7964

------------------------------------------------------------------------------
     lnkdnap |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         il1 |   .1333955   .1391706     0.96   0.342    -.1449871    .4117781
         il6 |   .0007843   .0027095     0.29   0.773    -.0046356    .0062042
        il10 |   .0131416   .0089001     1.48   0.145    -.0046613    .0309444
        il12 |  -.5212569   .2334427    -2.23   0.029    -.9882118   -.054302
         tnf |  -.0179996   .1736329    -0.10   0.918    -.3653172    .329318
         tgf |   .0096413   .0077135     1.25   0.216     -.005788    .0250705
       _cons |   6.048545   .5614512    10.77   0.000     4.925476    7.171615
------------------------------------------------------------------------------
```

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```
Iteration  1:  WLS sum of weighted deviations =   70.22267

Iteration  1: sum of abs. weighted deviations =    70.3925
Iteration  2: sum of abs. weighted deviations =  70.224981
Iteration  3: sum of abs. weighted deviations =  69.962491
Iteration  4: sum of abs. weighted deviations =   69.63792
Iteration  5: sum of abs. weighted deviations =  69.483973
Iteration  6: sum of abs. weighted deviations =  69.439905

Median regression                                   Number of obs =         67
  Raw sum of deviations 76.24166 (about 6.9122553)
  Min sum of deviations  69.4399                    Pseudo R2     =     0.0892

------------------------------------------------------------------------------
     lnkdnap |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         il1 |   .0930416   .1856988     0.50   0.618    -.2785408     .464624
         il6 |  -.0000258   .0040323    -0.01   0.995    -.0080943    .0080428
         il8 |  -.0011936   .0019178    -0.62   0.536    -.0050311    .0026439
        il10 |   .0099914   .0118901     0.84   0.404    -.0138008    .0337835
        il12 |  -.5488185   .3092857    -1.77   0.081    -1.167698    .0700607
         tnf |   .0911563   .2303905     0.40   0.694     -.369854    .5521667
         tgf |   .0049508   .0103891     0.48   0.635    -.0158377    .0257393
       _cons |    6.76311   .7608958     8.89   0.000     5.240561    8.285659
------------------------------------------------------------------------------
```

Figure 3. Above: output of the multivariate linear regression analysis for prediction of of plasma parasite kDNA load by plasma cytokines. Bellow: output of the multivariate quantile regression analysis for prediction of plasma parasite kDNA load by plasma cytokines.

4. Discussion

This study found that patients with kala-azar have a high inflammatory status and have signs of substantial risk of death. On the other hand, the data also shows that children, women, and persons living with HIV have a more prominent regulatory immune response. Markedly, however, IL-1b, IL-6, IL-8, IL-10, IL-12, TNF-a, and TGF-b were not, or were poorly associated with the risk of death. Moreover, while the studied cytokines did not increase with time of disease, blood and bone marrow *L. infantum* load expanded progressively. Additionally, blood and bone marrow *L. infantum* load were well correlated, and the parasite load was higher among men, adults, and HIV-infected patients. Finally, higher blood and bone marrow kDNA loads were associated with increased severity. Most plasma cytokines were not related to blood and bone marrow *L. infantum* load, but higher plasma IL-12 was independently associated with lower *L. infantum* load.

The study's weakest point was its cross-sectional design, which did not allow the identification of causal relationships, except with death. Other key cytokines and molecules, such as INF-g, were not studied. Additionally, bone marrow cytokines were not measured, and the spleen size was not regularly registered, hiding the organ's importance to the observed values. However, the findings are of interest for helping to understand the pathogenesis of life-threatening kala-azar and to highlight the progressive immunological failure of patients with kala-azar.

This study population describes the well-known male and children predominance in kala-azar caused by L. infantum [29]. HIV coinfection was higher than usual for the region and the country [30,31]. Although mortality was lower in this sample, more than one-third of the patients had a chance of death estimated over 10% and hemorrhages and bacterial infections. These findings demonstrate how severe the disease can be and stress the importance of understanding the pathogenetic mechanisms of these complications. When compared with blood donors [24], plasma cytokine concentrations where very high, confirming the role of inflammation. Indeed, previous works have already shown very high values plasma cytokines in patients in East-Africa, Brazil and India for INF-g, IL-8, TGF-b, L-10, and IL-6 [32–35].

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Children had higher concentrations of most cytokines than older patients, but only IL-10 reached statistical significance. This finding might support a previous observation and suggests a more immunotolerant state of children, independently of kala-azar [36]. Regarding sex, IL-10 was higher in females. Although another study in healthy adult individuals could not observe this difference, the present findings suggest that a sex-dependent IL-10 response in patients with kala-azar may actually exist [37].

Another previous work has shown that HIV-infected patients under antiretroviral therapy (ART) had lower IL-10 than patients, both in those patients not on ART and in those long-term non-progressors [38,39]. In the present study, IL-10 was lower in patients coinfected with HIV than in those non-coinfected, suggesting that HIV infection-attenuates the regulatory innate response in kala-azar.

Based on observational data, it has been proposed that plasma IL-6 and IL-8, INF-g, IL-27, and soluble CD14 are the major mediators of the pathogenicity of kala-azar [3,17,19]. Due to its overlapping actions with characteristics of kala-azar, such as hemorrhages, anemia, hypoalbuminemia, and hyperglobulinemia, IL-6 was proposed to be the best explanatory cytokine for the complications of kala-azar [40]. However, in the present study, neither the chance of death at hospital admission nor the occurrence of death, revealed associations of plasma cytokines with signs of severity strong enough. Indeed, when the *p*-values were corrected by the Bonferroni method for multiple comparisons, no association between clinical presentation and any of the seven studied cytokines was found. Additionally, at the multivariate linear regression, no cytokine was found predicting the chance of death. This set of data on the role of cytokines detected in the blood suggests that the core of the pathogenic phenomena that lead to severe kala-azar or death may not rely primarily on the direct effect of pro- or anti-inflammatory cytokines as previously proposed [17,19].

This study observed that the cytokine response does not change with time after the disease starts, and this finding may influence the comprehension of the framework for the pathogenesis of kala-azar. Although cytokines did not change, *L. infantum* load and disease severity increased with time. The phenomenon does not look to be determined by progressive spleen enlargement since parasite load in the bone marrow also increased similarly.

The median blood and bone marrow amounts of *L. infantum* kDNA in this sample were in the range of the concentrations previously found in Teresina and elsewhere using the same protocol [20,21,41]. As expected, blood and bone marrow kDNA concentrations correlated well, indicating that bone marrow is balanced with systemic parasitism. Men and adults had higher blood and bone marrow loads, likely due to the modulatory effect of testosterone and dihydrotestosterone on men, who are the majority of adult patients with kala-azar [42]. Interestingly, HIV infection was not independently associated with a higher load. One possible explanation is that the patients with HIV had a relapsing course of kala-azar and, therefore, were under secondary prophylaxis with liposomal amphotericin B for *L. infantum* to prevent kala-azar relapses.

The disease severity associated with *L. infantum* load is a relevant finding and deserves further discussion. Unfortunately, the study design cannot assess the direction of causality: if *L. infantum* load worsens the disease via a linkage with a specific, unknown, factor or if a broad ongoing multifactorial lymphoid disruption leads to a non-specific, progressive and generalized failure of immunity and then to higher parasite load.

The direct cytokines' contributions to the manifestations of severe have already been studied [17,19], but here, blood cytokines were also compared with blood and bone marrow *L. infantum* kDNA load, and with clinical presentation, and risk of death. IL-12 and TNF-a were found to be associated with blood *L. infantum* load, but IL-12 was the only one to be an independent predictor of blood kDNA load, and, importantly, this action was to decrease the *L. infantum* load in a dose-dependent manner. Indeed, this finding is consistent with the canonical role of innate-immunity-derived IL-12, which promotes antigen-specific Th1 response by T-cell activation, proliferation, and differentiation through secretion of INF-g [12,43–48]. Unfortunately, this finding had not been forecasted, and INF-g was not measured in the present study. However, previous two articles

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analyzed the association of plasma cytokines with the blood load of *L. donovani* kala-azar in India and Africa [49,50]. They found a positive correlation only with IL-10, TGF-b, and IL-17 but not with IFN-γ, TNF-α, IL-6, IL-4, IL-2, IL-12, and IL-22, suggesting that immunoregulatory cytokines are the primary controllers of blood parasite load in *L. donovani* kala-azar. However, although Teles et al [51], in Brazil, and van Dijk et al[35], in Uganda, also found a positive correlation with IL-10, they identified a negative correlation with IFN-γ. The difference of the data from India with the findings in Brazil and Uganda suggests that *L. infantum* and Indian *L. donovani* differ on the host control of kala-azar: while for the disease caused by *L. infantum* and by Ugandan parasites it seems to persist a more prominent role of sustained, acquired, type Th1 response, in Indian *L. donovani* innate regulatory cytokines "deal the cards" and play an absolute permissive role on parasite load.

IL-12/IL-10 interaction is the line of balance in kala-azar: at the infection site, intracellular amastigotes' molecules drive the infected macrophages for a predominant IL-12 or IL-10 synthesis [10,15]. In the majority of infections, Th1 response prevails, and stimulates T-cells to synthesize INF-g, which activate macrophages to produce free radicals that kill the amastigotes, and the infection becomes controlled [11,12,52,53]. In a small proportion of immunocompetent humans, IL-10 prevails and disrupts the macrophage IL-12 signaling to CD4+ and CD8+, blocking the secretion of INF-g and preventing macrophage activation for intracellular defense [47,53–57]. All these events may happen entirely at the innate immunity level. However, with time, acquired immunity develops. If there is a Th1-type response with T-cells secreting INF-g, memory T-lymphocytes generate *Leishmania*-specific clones, and the host becomes immune, as seen by the high proportion of persons with cellular immunity to *Leishmania* who had never developed symptoms and lived in endemic areas [25,58]. However, if a regulatory profile is maintained, parasite load increases, disease and complications appear, and the host eventually dies. This study shows that despite the dominance of IL-10 effects on *L. donovani* kala-azar, acquired immunity persistently influences parasitism in *L. infantum* and in Ugandan kala-azar.

Therefore, the IL-12 response in *L. infantum* kala-azar reported here and ING-g elsewhere suggest that an overwhelmed but enduring acquired immunity persists for a while during disease. Another reason for this hypothesis is that IL-12 is maintained for an extended period after cure, compared to the cytokines secreted after stimulation of the innate response, such as the rapid fall – one to two weeks – of the cytokines mentioned above, as previously published [35,59]. Therefore, IL-12 seems to be part of acquired immunity, not of innate immunity, since it is long-lasting, due to the memory T-cells developed after earlier antigenic priming of T-cells, while IL-1, IL-6, IL-8, IL-10, and TNF-a, albeit at higher concentrations, last only during the antigenic stimulus, as characteristically occurs with innate immunity. Nevertheless IL-10 has been described associated with T-regulatory cytokines as part of the acquired immunity, it typically falls to very low levels after the cure of kala-azar [33–35,60].

Similarly, the early fall of INF-g in the study of LIMA et al [59] and van DIJK et al [35] suggests that most plasma INF-g is originated from cells belonging to the innate immunity such as neutrophils, eosinophils, NK cells, or even T-cells in an antigen-independent process [33,61,62], not by TCR antigen-specific CD-4+ and CD8+ T-cells. Therefore, this study suggests that in kala-azar, innate and acquired immunity coexists with disease. With treatment, the hidden cellular immunity is established but relapses indicate that the parasite persists even after immunity develops and patients become asymptomatic, e.g., non-sterile immunes. This equilibrium may have advantages for both: long-lasting immunity at the cost of some chance of parasite remission and transmission.

While *L. infantum* kala-azar remains with signs of some effective defense, *L. donovani* kala-azar not. The two parasites cause remarkable phenotypic differences, nevertheless being relatively genetically similar. *L. infantum* kala-azar seems to derive from the older *L. donovani* kala-azar[63]. While *L. donovani* kala-azar is mostly transmitted among humans, *L. infantum* kala-azar is zoonotic with a breadth of mammal hosts [1]. *L. donovani* kala-azar develops in older individuals, while *L. infantum* kala-azar hits younger immunocompetent persons, primarily children, signaling its higher force of infection [29]. *L. donovani* kala-azar leads to post-kala-azar dermal leishmaniasis, which is

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rare with *L. infantum* [64]. There are other clinical and epidemiological differences between the two species, but comparative, well-controlled, head-to-head studies between the two species and places still need to be done, from genomic analysis to innate and acquired immunity and pathogenesis.

Another open question from this study is why bone marrow *L. infantum* load did not show a similar association with IL-12 or TNF-a as blood load did. One explanation is that bone marrow control of *Leishmania* parasitism has *in situ* peculiarities not captured by the measured plasma cytokines. An insightful study investigated the association between bone marrow cytokines and local *L. infantum* burden. The findings revealed that IFN-γ was associated with a reduction in bone marrow parasite load, whereas interleukin-10 (IL-10) correlated with an increase in parasite burden. Interestingly, a strong and statistically significant **positive correlation** between IFN-γ and IL-10 levels was also observed. However, IL-12 or TNF-a were not found associated with parasite burden [51]. These finding illustrates the strong opposing effects of concomitant, protective versus permissive, cytokines each one downregulating (or upregulating) the other, in the bone marrow. It was not clear if the local findings in bone marrow can be generalized for the blood.

Since cytokines are pleiotropic and redundant, with synergistic actions, it is difficult to understand their isolated role in diseases [56]. Here, the risk of death and complications were not firmly associated with specific cytokines, except the association of IL-6 with sepsis in the multivariate regression analysis. However, since sepsis is the main cause of cytokine storms [56,65], it is not valid to infer that the rise of IL-6 was due to *L. infantum* instead of the opportunistic bacteria lipopolysaccharide LPS. Remarkably, mortality and the chance of death were not associated with any plasma cytokines. Although INF-◎ was not measured in this study, it does not seem to be a candidate for kala-azar severity since its side effects are mild to the human host and do not match with the symptoms of complicated kala-azar [66]

Therefore, the question is how a microorganism without virulence factors that do not directly harm human cells or tissues would lead to death [15,18]. The progressive rise of parasite load indicates a failing acquired immunity, suggesting that a higher parasite load is a consequence and not a cause of immune failure. Two distinct mechanisms without the direct and specific action of cytokines may be pointed out to explain the global defense failure. One is T-cell exhaustion, and the other is spleen disorganization. T-cell exhaustion is a dysfunction of T-cells, mostly CD8+ cells, occurring naturally and during chronic infections and cancer [67]. The other seemingly alternative is the disorganization of lymph node and the spleen architecture, a phenomenon that disrupts the white pulp structure [68]. Both mechanisms are exacerbated in kala-azar and may hypothetically result in increasing amastigote load and in a progressive acquired immune failure and, thereafter, augmented probability of complications and death [69–71]. These two mechanisms of immune deterioration occur simultaneously in kala-azar but a clear molecular link between them has not been identified yet [72]. They are natural processes of apparently accelerated in kala-azar by an elusive systemic factor, likely linked to the prolonged and intense inflammatory status.

In summary, *L. infantum* load was associated with life-threatening kala-azar, but it is unknown how. In contrast, circulating cytokines were poorly associated with phenotypes of severe disease. As expected, Il-12 has an enduring, strong, negative effect on *L. infantum* proliferation during kala-azar. Parasite load worsened with time but not cytokines, suggesting a cytokine-independent immunological failure process that results in progressively severe disease and death. However, there are still no key host factors leading to complications and death by kala-azar. Consequently, it may only be conjectured that the immunological long-term consequences of sustained infection and inflammation, such as persistent immune activation, may lead to immune exhaustion and overall immunological disorganization.

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Data Availability Statement: Data is available through permission given directly by the authors IS and CC.

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