Serum cytokines associated with severity and complications of kala-azar

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Objectives: Recent clinical data suggest that severe kala-azar (or visceral leishmaniasis) is an exaggerated innate immune response mediated by inflammatory cytokines, leading to a systemic inflammatory syndrome similar to what is observed in malaria, sepsis and other diseases. We tested this hypothesis by measuring serum cytokines in individuals with kala-azar.

Methods: We compared patients with severe kala-azar (i.e. hemorrhagic manifestations, $n=38$) with patients without evidence of hemorrhage ($n=96$). We conducted a detailed clinical and laboratory evaluation, measuring serum IL-1beta, IL-6, IL-8, IL-10, IL-12, interferon-gamma, and TNF-alpha, and markers of disseminated intravascular coagulation (DIC).

Results: Infants had higher levels of inflammatory cytokines, while HIV-infected patients had lower concentrations of IL-10 and interferon-gamma. Higher levels of IL-6, interferon-gamma, and IL-8 were found among deceased patients. IL-8 and interferon-gamma were independently associated with bleeding. Several cytokines were associated with different signs of severe clinical and laboratory manifestations, including DIC. IL-6 was highly positively and independently associated with IL-1beta, IL-8, IL-10, and negatively associated with TNF-alpha. IL-1beta and TNF-alpha were also highly independently associated with disease severity.

Conclusion: In its severe form, kala-azar, a neglected tropical disease, initiates a systemic inflammatory response that leads to DIC and other manifestations. Children may have higher risk of death due to the more intense cytokine release. The data supports the notion that IL-6 is the central cytokine that is associated with lethal disease, but interferon-gamma, IL1beta, IL-8, and TNF-alpha are also involved with disease severity. Inhibition of IL-6 is a potential target of adjuvant therapy for severe or pediatric forms of this disease.

Keywords: Cytokines, Kala-azar, *Leishmania infantum*, Disseminated intravascular coagulation, Systemic inflammatory response

Introduction

Kala-azar, or visceral leishmaniasis, typically leads to fever, anemia, wasting, and hepatosplenomegaly and is cured with known therapies.[1] For unknown reasons, some patients progress with heavy bleeding, bacterial co-infections, dyspnea, edema (lower limbs or anasarca), jaundice, and/or emesis that lead to death, even when treated with recommended therapies.[2–5] Research suggests that this is not due to different parasite species, as the disease is caused by two species of protozoa of the genus *Leishmania*, obligatory parasites of mononuclear phagocytes, normally found in the spleen, liver, and bone marrow. *L. donovani* has a limited distribution in the Indian Subcontinent and East Africa, and *L. infantum* is found in Central Asia, Middle-East, Mediterranean, West Africa, and South and Central America.[6,7]

Herein, we develop a novel biological framework involving inflammatory cytokines and disease severity to explain different disease courses. A lack of proper understanding of the pathogenesis of severe disease and, therefore, inefficient therapy could explain the difficulties of treating the endemic *Leishmania* in Brazil where active surveillance and free treatment is provided, but mortality continues to rise annually.[8] Inflammatory cytokines have been found to be elevated in non-complicated kala-azar.[9] Further, patients with severe presentation of sepsis

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and malaria show marked increase of certain cytokines compared with those with milder clinical presentation. Thus, we hypothesize that inflammatory cytokines could also be more elevated in lethal kala-azar and this might provide insight into why some patients with severe disease cannot be rescued by specific treatment. This builds from observations in a range of cohorts where patients that have died from kala-azar were observed to have worse anemia, hepatitis, pneumonitis, nephritis, diarrhea, neutropenia, thrombocytopenia, and disseminated intravascular coagulation (DIC), which overlap with those clinical manifestations triggered by inflammatory cytokines.[1–5,10] Finally, since *Leishmania* organisms do not produce toxins and do not destroy parenchyma cells, it is reasonable to hypothesize that these cytokines might be strongly related, or be the cause itself, of the most severe kala-azar cases, by generating a damaging systemic inflammatory response.[11] This hypothesis would explain why, like sepsis and severe malaria, some patients do not respond to antibiotics or to chemotherapy and provide a basis for further research into anti-cytokines therapy for treating the most severe forms of kala-azar, as well as other severe systemic infectious diseases.

Materials and Methods

The Research Ethics Committee of the Federal University of Piauí approved the protocol. Written informed consent was obtained from each participant or his/her legal representative. From an ongoing open cohort study at a reference hospital in the city of Teresina, northeast Brazil, of 884 in-hospital patients, including 217 with any sort of bleeding (24.6%), we randomly selected 34 patients with spontaneous and clinically apparent skin or mucosal bleeding as manifested by petequiae, echimoses, hematomas, epistaxis, bleeding by gums, gastrointestinal or urinary tracts or any other site, and 96 patients with kala-azar, but without any hemorrhagic complications, to compare the concentrations of cytokines. Patients with clinical or laboratorial data associated with an increased risk of death or that had hemorrhagic complications were classified as having 'severe kala-azar'. The diagnosis was performed based on the typical symptomatology, including fever, anemia, weight loss, and hepatosplenomegaly plus a direct parasite detection or culture from bone marrow aspiration or a positive immunofluorescent test (Leishmaniose Humana, Biomanguinhos, Rio de Janeiro, Brazil) with dilutions of 1 : 80 or greater. Death was attributed to kala-azar by the investigators if it occurred before or during the treatment, or after unsuccessful drug therapy. To obtain corrected organ sizes, spleen and liver sizes were divided by individuals' body area. Edema was reported as any medical observation of generalized edema (anasarca) or local, such as facial, ascitis or in the lower limbs.

Sera and plasma were collected at admission. Immediately after collection in a flask with citrate (with the exception for fibrin degradation products that came with its own anticoagulant in the dosage kit), plasma samples were frozen at $-80°C$, and use up to 6 months after collection. Sera were stored at $-20°C$. All patients were tested for HIV. Other tests were fibrinopeptide-A (Imunoclone FPA ELISA; American Diagnostica Inc., Stamford, CT, USA), fibrinogen degradation products (ActiScreen™ XL-FDP; American Diagnostica Inc.), D-dimer (Imunoclone D-Dimer ELISA; American Diagnostica Inc.), fibrinogen (Human Fibrinogen ELISA Quantitation Kit; Gen Way Biotech Inc., San Diego, CA, USA), and C-reactive protein (Array 360 System Beckman; Abbott Laboratories, North Chicago, IL, USA). Serum levels of the cytokines IL-1beta, IL-2, IL-6, IL-10, IL-12p70, IL-8, TNF-alpha, and IFN-gamma were analyzed by flow cytometry from (Cytometric Bead Array Human Inflammatory Cytokines Kit Assay; BD Biosciences, Rockville, MD, USA, and Cytometric Bead Array Human IFN-γ Flex Set; BD Biosciences). The flow cytometric assay was performed and analyzed by a single operator, and standard curves were derived from cytokine standards.

Statistical analysis was performed with the Stata/SE® 10.0 (StataCorp. LP, College Station, TX, USA) statistical software. The Spearman analysis was applied to detect correlation between continuous variables with sparse data. Wilcoxon and the Kruskal–Wallis tests were used to compare medians of variables with a nonparametric distribution. To determine which of the variables had independent associations, linear multivariate regression analysis (MRA) and logistic MRA with a backward elimination procedure were performed. The normality of distributions was examined and confirmed with kurtosis and skewness tests.

Results

The distribution of age and gender was similar between patients who had evidence of bleeding disorders and those without. Ninety-five percent of those with hemorrhage were already bleeding when they were admitted at the hospital. Among the patients bleeding 6/38 (15.8%) died, while only 2/96 (2.1%) died among those without evidence of hemorrhage. Twelve patients were infected with HIV, but there was no difference among these patients with respect to the bleeding status. The presence of bleeding was irrespective of the length of symptoms (Table 1).

Association of cytokines with death, age, and HIV infection

Higher serum levels of cytokines among patients who died as compared to survivors were found for IL-6, interferon-gamma, and IL-8 (Fig. 1). Children
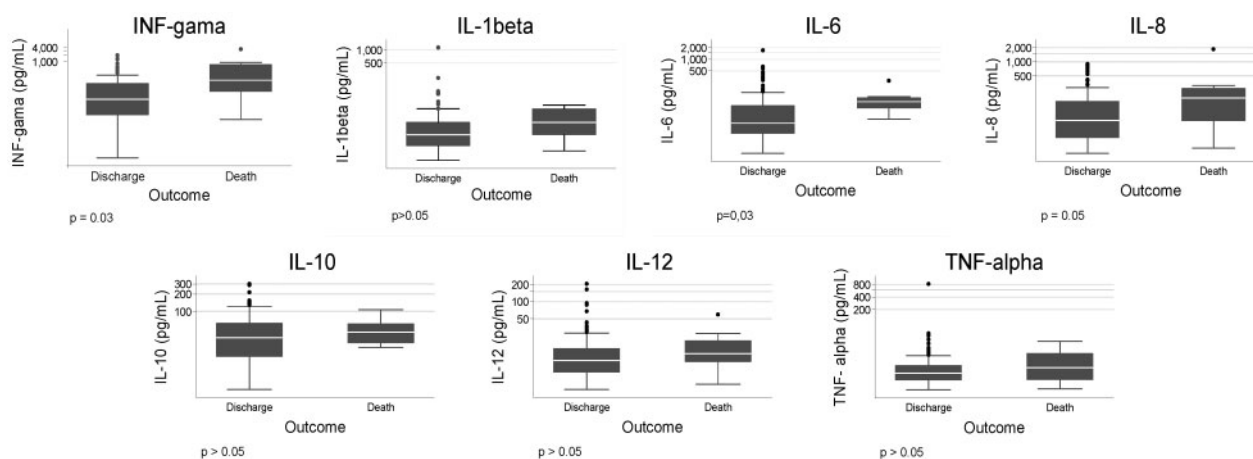
Figure 1 Box plots of the serum concentration of cytokines in patients with kala-azar according to the occurrence of death.

2 years old or less had higher concentration of IL-6 (62.6 versus 20.7; $P=0.01$) and of interferon-gamma (46.1 versus 24.8; $P=0.05$). HIV-infected patients had lower concentration of IL-10 (15.0 versus 38.1; $P=0.01$) and of interferon-gamma (3.5 versus 32.1; $P=0.05$).

Correlations of cytokines with clinical and laboratorial findings

The following signs and symptoms were found to be associated with mortality after a logistic MRA: higher body temperature, presence of bleeding, presence of sepsis, vomiting, diarrhea, edema, dyspnea, jaundice, liver, and spleen size. However, among them, only bleeding, edema, and vomiting were associated to serum cytokine concentrations. Patients with hemorrhage had higher concentrations of IL-1beta, IL-6, IL-8, and interferon-gamma, but the logistic MRA showed that only higher levels of IL-8 ($P=0.004$) and interferon-gamma ($P=0.006$) were independently associated with bleeding. Patients with edema had higher concentration of IL-6 and IL-8 and patients with vomiting had higher

concentration of IL-6 ($P=0.02$) at the uni- and MRA (Fig. 2).

Many hematological findings, liver enzymes, acute phase proteins, and kidney function abnormalities were associated with the risk of death (data not shown), but not all were correlated with serum cytokine concentrations. Figure 3 shows the significant correlations of laboratorial data with cytokines. Anemia, one of the most common signs of the disease, as measured by the hemoglobin concentration, had significant negative correlation with IL-1beta, IL-6, IL-8, and IL-10, but only IL-1beta was associated at the MRA ($P=0.049$; F-test$=0.02$; $R^2=0.12$). Neutrophil count was negatively correlated to IL-10, interferon-gamma, and TNF-alpha at the univariate analysis; IL-6 was the sole cytokine correlated to neutrophil count ($P=0.044$; F-test$=0.11$; $R^2=0.03$). Lymphocyte count displayed two patterns of association with cytokines: at 2000/µl and above, it was negatively correlated with IL-1beta, IL-8, and TNF-alpha, but bellow 2000/µl, it was positively correlated with TNF-alpha; however, there was no association found with the MRA. The liver

Table 1   Characteristics of the study population

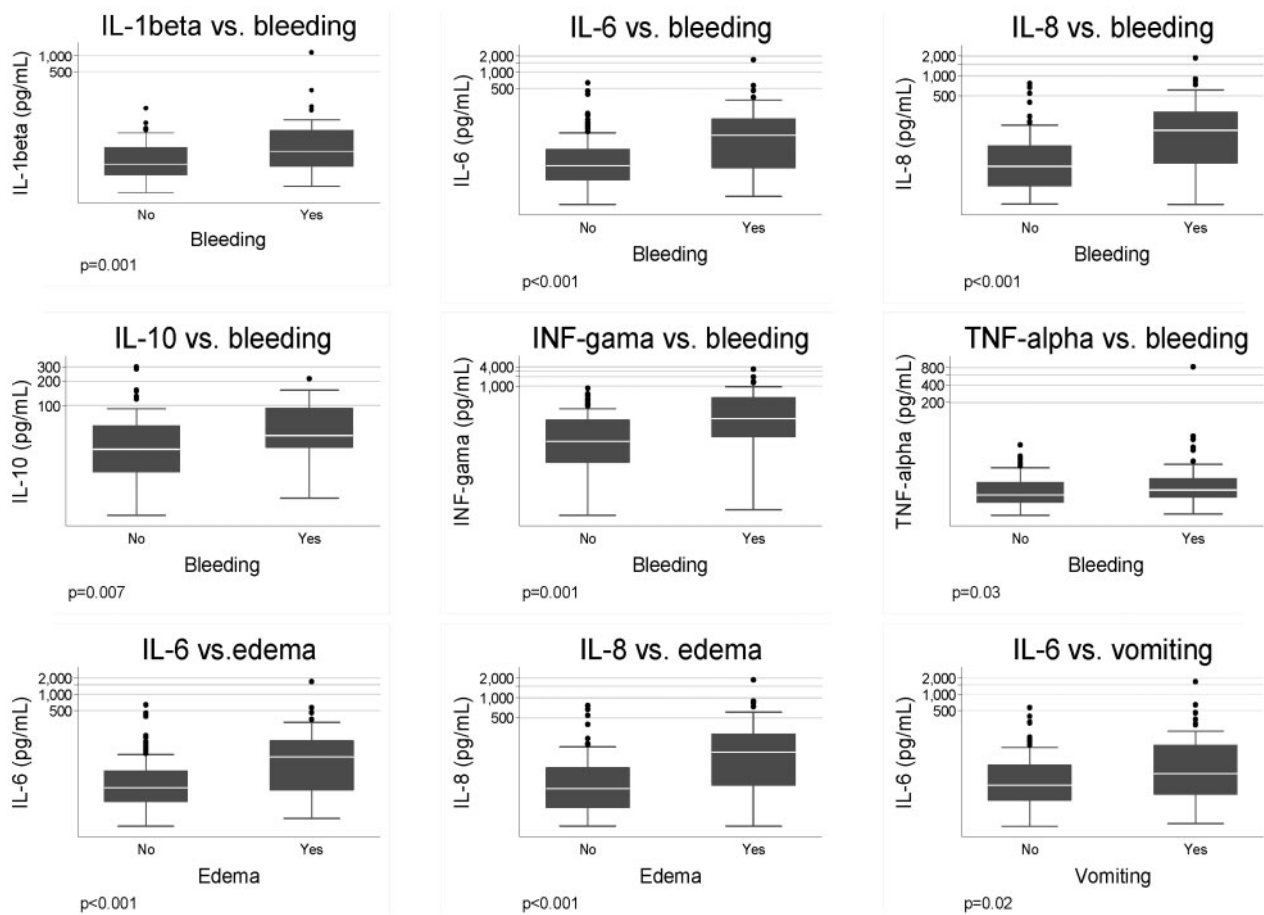| Characteristic	Hemorrhage ($n=38$), n (%)	No hemorrhage (n=96), n (%)	P value
Age (years)			0.13
<1	15 (39.5)	22 (22.9)	
1<5	6 (15.8)	23 (23.9)	
5<20	2 (5.3)	18 (18.8)	
20–40	10 (26.3)	21 (21.9)	
>40	5 (13.1)	12 (12.5)	
Gender			0.76
Male	26 (68.4)	63 (65.6)	
HIV infection			0.71
Yes*	4 (11.4)	8 (9.2)	
Outcome			0.003
Death	6 (15.8)	2 (2.1)	
Duration of symptoms days (95% IC)	73.3 (48.2–98.4)	79.6 (51.7–107.5)	0.78

Note: *Only 126 tested for HIV.

Figure 2 **Box plots of the serum concentration of cytokines in patients with kala-azar according to the presence of bleeding, edema, and vomiting. Only statistically significant association results are shown.**

enzyme aspartate aminotransferase (AST) had a positive correlation with IL-1beta, IL-6, and IL-8, while, in the MRA, IL-6 was the one significantly correlated to liver damage as measured by AST ($P=0.006$; F-test$=0.049$; $R^2=0.11$). Finally, considering the acute phase proteins: C-reactive protein was positively correlated with IL-6 and interferon-gamma (but only with IL-6 in the MRA, $P<0.001$; F-test <0.001; $R^2=0.43$), while fibrinogen was correlated with IL-6 and interferon-gamma (not shown). Alkaline phosphatase, serum albumin, and glomerular filtration were not correlated to any inflammatory cytokine in this data set.

Correlations of cytokines with DIC
Platelet count was negatively correlated with IL-1beta, IL-6, IL-8, and interferon-gamma (Fig. 4). The observations that patients with any kind of bleeding had a lower platelet count ($P<0.0001$), higher concentration of plasma D-dimer ($P=0.0001$), and presence of fibrin degradation products ($P=0.009$) highlight the relationship of platelet count with markers of DIC in patients with severe kala-azar, is reinforced by the strong negative correlation between platelet count and D-dimer (data not shown) ($P<0.0001$). D-dimer was also correlated with inflammatory cytokines, as seen also in Fig. 4.

Moreover, patients with detected fibrin degradation products had higher concentration of IL-6 and interferon-gamma, by uni- and MRA (Fig. 5).

Correlation among cytokines
Figure 6 shows the mutual correlations among cytokines. All were strongly positively associated among themselves, but some particularly strong correlations were observed. These include IL-1beta and TNF-alpha (rho=0.75), IL-1beta and IL-6 (rho=0.66), IL-6 and IL-8 (rho=0.66), IL-1beta and IL-8 (rho=0.62), and IL-12 and TNF-alpha (rho=0.62). In the MRA of the natural logarithm of each cytokine as an explanatory variable to the others, all linear models were robust, with F-tests <0.0001 in all, and R^2 varying of 0.19 for interferon-gamma; 0.37 for IL-10; 0.38 for IL-12; 0.49 for IL-8; 0.65 for IL-6; 0.74 for TNF-alpha; and 0.82 for IL-1beta.

Discussion
The associations between these serum cytokine concentrations with certain clinical presentations of kala-azar of severe disease provides insights into the involvement of these proteins in lethal kala-azar, particularly IL-6, interferon-gamma, and IL-8, which are those cytokines independently directly associated with death, particularly among the young. These
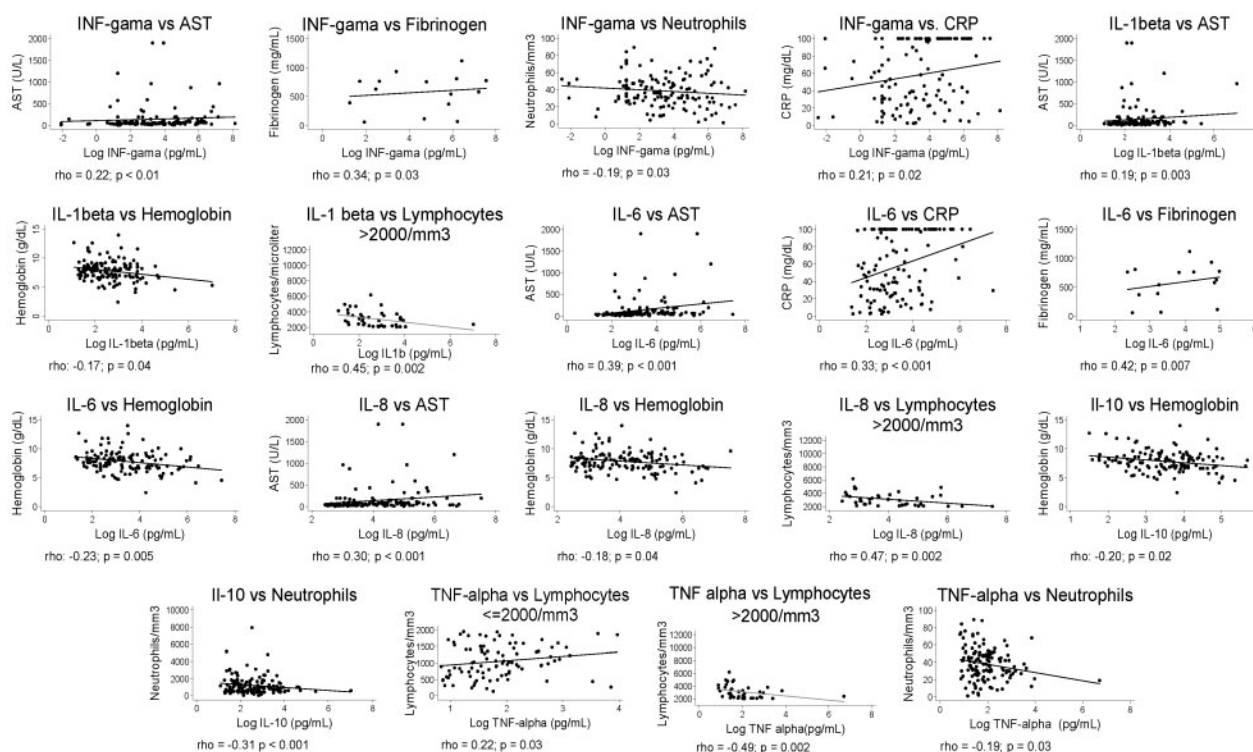
Figure 3 Correlation of serum cytokines concentration with the results of laboratory tests in patients with kala-azar.

findings also suggest that the higher mortality found among infants is due to the higher concentration of IL-6 and interferon-gamma as seen in other conditions.[12,13] IL-6 is a pleiotropic cytokine that is both produced and targeted by a variety of cell types, and thus has the capacity to induce several different intracellular signaling pathways. For example, IL-6 has a role on the pathogenesis of sepsis and rheumatoid arthritis and its main actions are promoting acute phase response together with IL-1 and on tissue factor expression, which, initiates the coagulation cascade.[14,15] interferon-gamma is produced by T- and B-cells, natural killer cells and antigen presenting cells and orchestrates many distinct cellular programs through transcriptional control over large numbers of genes.[16] Also, interferon-gamma enhances immune reactivity by increasing antigen presentation to lymphocytes via induction of HLA-DR that improves the outcome of patients with multiple organ failure following severe trauma.[17,18] The other disease inducing cytokines are IL-8, IL-1beta, and TNF-alpha. IL-8 is a chemokine[19] mostly produced by monocytes and macrophages, with the important role of recruiting
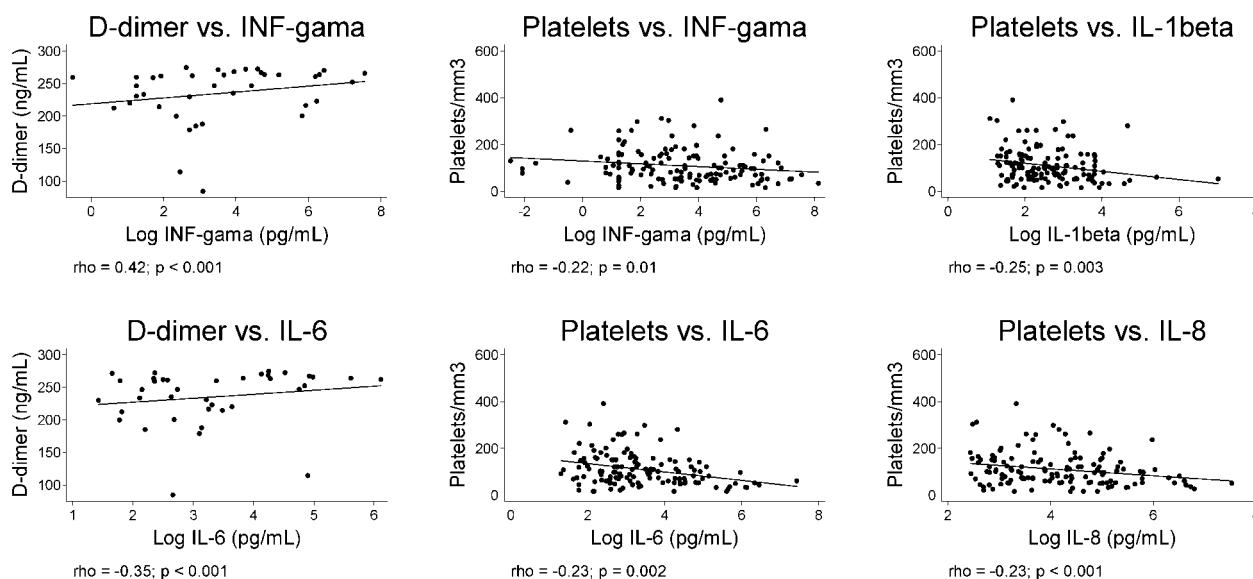

Figure 4 Correlation of serum cytokines concentration with platelet count and D-dimer levels.
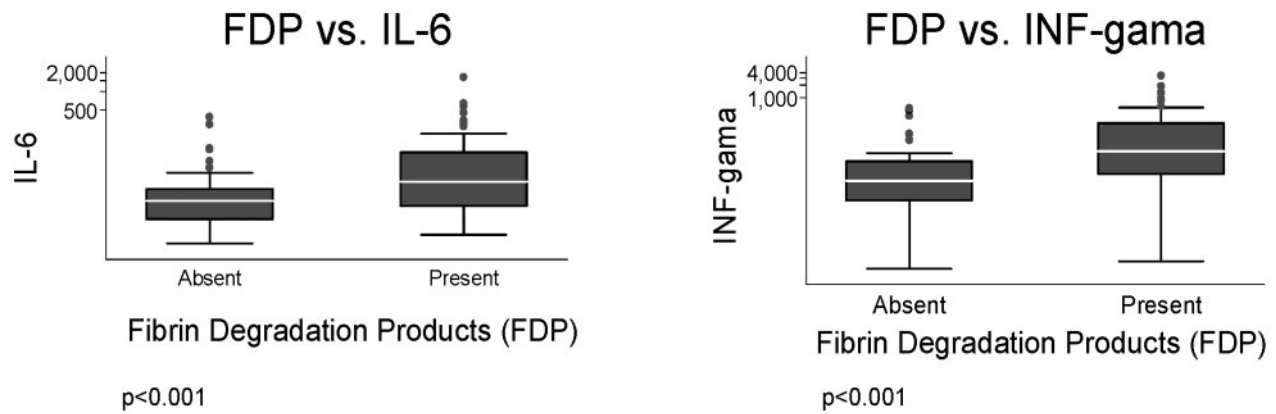
Figure 5 Box plots of the concentration of serum cytokines in patients with kala-azar according to the detection of fibrin degradation products.

leukocytes. IL-1beta is normally produced in response to infection, injury, or immunologic challenge by blood monocytes; at minimal concentrations, it causes fever, hypotension, and production of additional pro-inflammatory cytokines, such as IL-6.[20] TNF-alpha is secreted by macrophages, monocytes, neutrophils, natural killer cells, and T cells and is known to affect the growth, differentiation, survival and physiological function of a variety of different cells including macrophage-mediated cytotoxicity due to the proapoptotic effects of TNF-alpha. These cytokines can directly lead to bleeding either by inducing coagulopathy, by directly provoking thrombocytopenia or by damaging the liver of those infected. They are involved with severity not only of kala-azar but of other infectious diseases, like sepsis and *Plamodium falciparum* malaria.[21] Besides acting on kala-azar the inflammatory cytokines play a critical role on the pathogenesis of sepsis and the systemic inflammatory response syndrome) via innate immunity.[22]

Bleeding and edema were associated with high concentrations of serum cytokines and are linked to endothelium and mononuclear cells in the microvasculature.[23] Edema in severe kala-azar seems to be a consequence of both low serum albumin and capillary leakage[11] while DIC leads to bleeding.[24–27] This study shows that edema is strongly related to IL-6 and to IL-8. We hypothesize that the release of IL-6 and IL-8 by macrophages and neutrophils could induce the expression of adhesion molecules in the endothelium and could recruit leucocytes, which are precursors of vascular damage[19,28] and therefore edema.[29] Additionally, IL-6 is known to inhibit the production of albumin in the liver,[30] which might exacerbate edema.

Besides inducing edema, IL-8, together with interferon-gamma, IL-6 and IL-1beta, up-regulates the expression of tissue factor by endothelial cells initiating the coagulation cascade that leads to DIC.[31] DIC is the primary pathway to hemorrhage, indicated by the robust association of markers of

DIC such as D-dimer and fibrinogen degradation products with IL-6 and interferon-gamma. Coagulation has also been found to induce IL-6 secretion and inflammation.[22] DIC may not be the only cause of bleeding. Indeed, liver injury indicated by alterations of a hepatic enzyme, which is common in severe kala-azar,[24,32] was independently related to IL-6 and likely is a complementary cause of bleeding. Therefore, while these proteins are up-regulated by IL-6,[33] fibrinogen appears to be down-regulated.[34] We have previously shown the association of genetic polymorphisms of another acute phase protein, mannan-binding lectin, with severe kala-azar,[35] and since this lectin activates coagulation[36,37] it can lead to DIC via MBL-dependent complement activation.[38]

Neutropenia, thrombocytopenia and anemia are not a consequence of depletion of bone marrow cellularity by *Leishmania* infiltration.[39] Rather, our data suggest that they are a consequence of systemic inflammation. For example, IL-6 was shown to be the main cytokine inducing neutropenia, yet this is not peculiar to kala-azar, since it does not occur in bacteremic neutropenic patients.[39] Thrombocytopenia appears to be potentially induced by DIC suggested by its association with the markers of DIC. Platelet count exhibited a strong association with a range of different cytokines. Anemia, the commonest sign of kala-azar, indicated by hemoglobin concentration, was associated independently with IL-1beta, IL-6, IL-8, and IL-10. This result suggests that a pathway via hepcidin exists.[40] Hepcidin is an acute phase protein released by the liver, which leads to iron-deficient anemia,[41] typical of the anemia observed in patients with kala-azar.[39] Finally, lymphocyte count in kala-azar was shown to be possibly dependent on TNF-alpha, IL-1beta, and IL-8, although not among patients with cancer.[42] A likely role of CD4 cells in the secretion of IL-10 and interferon-gamma is indicated by its lower concentration in *L. infantum*-HIV-infected patients.

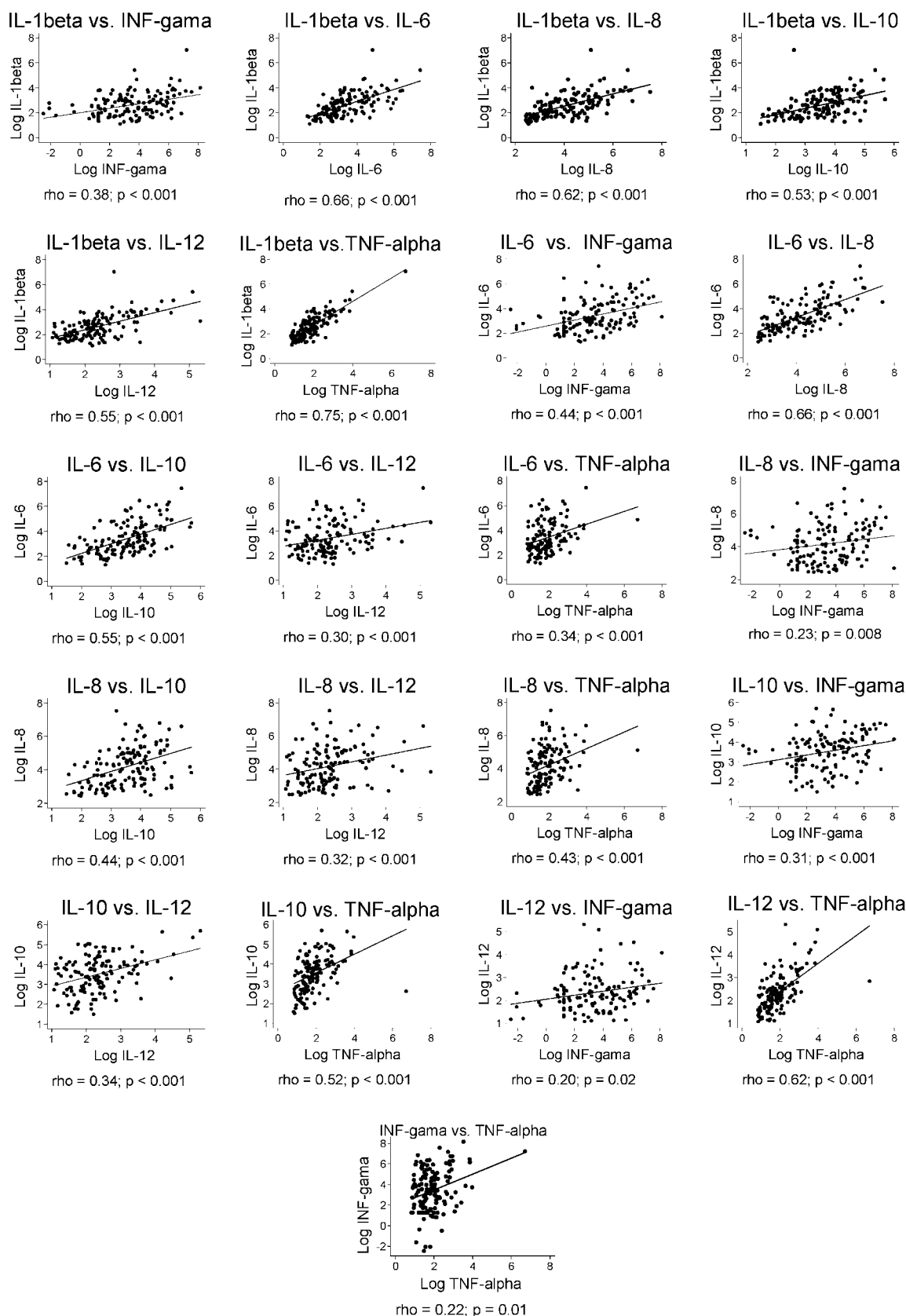These correlations between cytokines permit speculations about a dynamic network among cytokines

Figure 6 Mutual correlation of serum cytokines in patients with kala-azar.

in severe kala-azar. The regression analyses allowed identifying the dependency of each cytokine on another, which opened the window for deeper inferences on their relationships. For instance, IL-12 seems to be a strongly linked to TNF-alpha, while TNF-alpha and IL-1beta show a strong relationship. We hypothesize that they are secreted by the same type of cells, possibly parasitized macrophages, stimulated by the same pathogen-associated molecular patterns,[43–45] very likely in the spleen, bone

marrow, and liver. IL-1beta is strongly controlled by IL-6 in kala-azar, leading to anemia, one of the commonest sign of kala-azar and a predictor of death. Together with IL-6, IL-1beta controls the secretion of IL-8. The tight correlation between IL-1beta, IL-6, and IL-8 also suggests a particular interplay between them leading to death with bleeding and edema. A possible pathway would be the production of IL-1beta and IL-6 by macrophages, inducing the secretion of IL-8,[46] and hence, endothelial activation with further secretion of IL-1beta and IL-6. Both IL-6 and IL-8, albeit mostly the former, were linked to interferon-gamma. Interferon-gamma is an important disease causing factor as seen by its independent association with death, bleeding, DIC, liver involvement, thrombocytopenia, neutropenia, and the acute phase response indicated by C-reactive protein secretion, nevertheless its protective effect for *Leishmania*, as a component of acquired immunity. This dual action of interferon-gamma suggests that the molecules identified in the sera are rather part of the harmful excessive innate response secreted by neutrophils, eosinophils, and natural killer T-cells.[9] IL-6 appears to play a central role in the pathogenesis of severe kala-azar, not only because it is directly and independently associated with a range of pathological manifestations such as edema, DIC, neutropenia, liver damage (as seen by AST), and acute phase response, but also due to its induction of several other disease causing cytokines such as IL-1beta, interferon-gamma, and IL-8. Besides possibly down-regulating TNF-alpha, it is strongly related to IL-10 secretion, therefore establishing a link with the innate response with the acquired immunity in kala-azar patients, via the T-regulatory response. Indeed, IL-10 production by splenic $CD4^+CD25^-$ $Foxp3^-$ T cells seems to lead to the immunoparalysis that allow parasite proliferation and infection success.[43–45,47,48] One hypothesis is that an initial localized parasite proliferation initiates an innate response inhibiting the effective Th1 response via IL-10 control of IL-12,[49] and by the inhibition of an effective innate response by TNF-alpha via IL-6. These mechanisms may be the cause of severe disease when a high parasite burden is progressively reached, leading to an exaggerated release of pathogen-associated molecular pattern that triggers the lethal innate immune response of kala-azar. Progression to severity appears to occur as indicated by the known effect of time on the chance of death. However, it seems to be a very slow process, since we found no effect of time on bleeding. Moreover, the effect of time on mortality is very late. Taken together, these data suggest that most patients reach an early plateau of higher risk; thereafter, the increase in the chance of death would be due only to the accumulation of daily repeated chances of complications and death.

Limitations

Some points related to the study design must be addressed. While a larger sample would have been attractive, it is uncommon to study such a number of severe kala-azar patients. Since almost all the patients with hemorrhage were already bleeding when they entered in the hospital (and the same is true for other symptoms), it was not possible to develop a longitudinal study to clarify a cause–effect relationship. While scientifically attractive, it would be unethical to admit patients without bleeding and then to wait the development of hemorrhage just to observe a causal effect of cytokine sera concentration.

Regarding causality, the only possible detectable temporal relationship with cytokines and disease was death, and HIV infection, and therefore, all the other associations shall not be taken as causal. Consequently, while IL-6, IL-8, and interferon-gamma can be inferred as being part of a causal pathway to death, or, at least, as predictors of mortality, the other associations of cytokines with symptoms and signs should be taken only as markers of severity. Which of the clinical and pathogenic alterations are the immediate causes of death cannot be ascertained in this study. However, since with the exception of shock, the manifestations of severe kala-azar are similar to those of sepsis and systemic infection where inflammatory cytokines play a critical role, multiple organ failure and bleeding are the main consequences of inflammation that lead to death.[22,50,51] Finally, an interesting future analysis would expand upon the limitation of being able to stratify patients based on 'severity', perhaps by using metrics such as parasite load.

Conclusion

Serum cytokines are part of a complex host response in kala-azar, which is not well understood. Experimental models of severe disease, systems studies, and cohort designs followed by pathological observations of the deceased patients represent the limit of ethical scientific investigation for this area. In this context, the present findings suggest that the late stage of severe kala-azar, like other severe acute infectious diseases such as sepsis, malaria, dengue, leptospirosis, and viral hemorrhagic fevers, should be interpreted as a consequence of a systemic inflammatory response.[22,52–55]

Only a limited number of proteins and pathways were studied, while many others need to be observed. As examples, it has been shown by our group that polymorphisms in the *MBL* gene alleles and the *TGF-alpha* gene –509 allele T are associated with more severe kala-azar.[35,56] The identification of parasite and host molecules that participate in the development of severe kala-azar will help to design better adjuvant therapy. Clinical experience has shown that

after patients start to bleed or if they have other complications, it is much harder to save their lives, even when using first line drugs like liposomal amphotericin B. Targeting critical molecules that are part of the host response with biopharmaceuticals may eventually save lives as it has for acute critical diseases,[57–59] or may help to assemble data for future analysis.[60] From our findings, the first and most promising pharmaceutical targets are the antagonists of IL-6.

To briefly summarize in full, in this study, we have shown that higher concentrations of IL-6, IL-8, and interferon-gamma precede death in patients with kala-azar, but not other inflammatory cytokines. Also, IL-1beta, IL-6, IL-8, interferon-gamma, and TNF-alpha are correlated with an array of clinical and laboratorial data typically found in severe kala-azar. IL-6 and interferon-gamma were strongly associated with markers of DIC. Finally, a pattern of relationships among cytokines was identified in kala-azar patients, in which IL-6 seems to play a central role in triggering symptoms and controlling other cytokines. The present data indicates that lethal kala-azar is very likely a result of an exaggerated inflammatory response and that anti-cytokine therapy, particularly anti-IL-6, should be assayed as an adjuvant therapy for the most severe forms of the disease in animal models.

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Competing interests

The authors have declared that no competing interests exist. This article is part of the DSc thesis of Dorcas L. Costa 'Fatores de prognóstico na leishmaniose visceral: Alterações clínicas e laboratoriais associadas à resposta imune, aos distúrbios da coagulação e à morte'. Graduate Program in Health Sciences: Infectious Diseases and Tropical Medicine. Belo Horizonte, MG, Federal University of Minas Gerais, August 2009.

Acknowledgements

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