

RESEARCH ARTICLE

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# Cytokine profiles amongst Sudanese patients with visceral leishmaniasis and malaria co-infections

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## Abstract

**Background:** The immune system plays a critical role in the development of co-infections, promoting or preventing establishment of multiple infections and shaping the outcome of pathogen-host interactions. Its ability to mediate the interplay between visceral leishmaniasis (VL) and malaria has been suggested, but poorly documented. The present study investigated whether concomitant infection with *Leishmania donovani* complex and *Plasmodium falciparum* in naturally co-infected patients altered the immunological response elicited by the two pathogens individually.

**Results:** Circulating levels of interferon (IFN)- $\gamma$ , interleukin (IL)-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IL-17A and tumor necrosis factor (TNF) were assessed in sera of patients infected with active VL and/or malaria and healthy individuals from Gedarif State, Sudan. Comparative analysis of cytokine profiles from co- and mono-infected patients highlighted significant differences in the immune response mounted upon co-infection, confirming the ability of *L. donovani* and *P. falciparum* to mutually interact at the immunological level. Progressive polarization towards type-1 and pro-inflammatory cytokine patterns characterized the co-infected patients, whose response partly reflected the effect elicited by VL (IFN- $\gamma$ , TNF) and malaria (IL-2, IL-13), and partly resulted from a synergistic interaction of the two diseases upon each other (IL-17A). Significantly reduced levels of *P. falciparum* parasitaemia ( $P < 0.01$ ) were detected in the co-infected group as opposed to the malaria-only patients, suggesting either a protective or a non-detrimental effect of the co-infection against *P. falciparum* infection.

**Conclusions:** These findings suggest that a new immunological scenario may occur when *L. donovani* and *P. falciparum* co-infect the same patient, with potential implications on the course and resolution of these diseases.

**Keywords:** Visceral leishmaniasis, Malaria, Co-infection, Cytokines, Sudan

## Background

Co-infection of a host by multiple parasite species is a commonly observed condition, where individual susceptibility and infectiousness are affected at various levels [1-3]. Inter-microbial competition along with micro-environmental and immunological conditioning govern the magnitude and type of interactions across poly-parasitism, promoting or hampering establishment of multiple parasite infections and their ability to persist and spread to new patients [4]. At the immunological level, cross-regulation of pathogen-associated pathways is achieved through cytokine signaling; an integrated network responsible for the controlled polarization and amplification of immune responses [5,6].

As a result, cytokines secreted in response to one parasite species may act synergistically or antagonistically with those elicited by another species, enhancing (cross-immunity) or impairing (immune-suppression) simultaneous control of infections. Cytokine ability to shape the immune system into efficacious responses arises from their downstream actions on the effector mechanisms, with great variation across different host-parasite systems [6,7]. Conversely, upstream cytokine functions are more stereotypical, while remaining predictive of effector efficacy. Hence, their characterization in poly-parasitized models provide a valuable and convenient framework for understanding co-infection dynamics [6,8].

Visceral leishmaniasis (VL) and malaria are two major parasitic diseases which overlap geographically and may co-exist in the same patients [9,10]. Partially sharing the same host tissue niches, the two infections have the

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ability of evading and subverting immune surveillance, with clinical outcomes largely dependent on the immunological status of the host. Obligate intracellular parasites of the visceralizing *Leishmania donovani* complex successfully colonize macrophages and other reticulo-endothelial cells of the lymphoid system, by altering signaling pathways associated with parasite killing and adaptive immunity engagement [11,12]. As a result, phagocytes harboring *Leishmania* parasites are incapacitated to function as cytolytic and T-cell priming effectors, causing immune dysfunction and tissue injury. Resistance to infection is conferred by development of effective T helper cell 1-type (Th1) responses, mounted upon release of a pleiotropic interleukin (IL)-12 and interferon (IFN)- $\gamma$  cytokine network, and boosted by pro-inflammatory (tumor necrosis factor (TNF), IL-23, IL-17A) and Th2-promoting (IL-4) mediators [13-17]. Thus, in contrast to the classical Th1-Th2 paradigm suiting predictions of resistance/susceptibility to cutaneous leishmaniasis [12], clearance of *L. donovani* appears to be blunted by induction of the regulatory T cell subset Tr1, rather than Th2 or Th3 clusters, through an IL-10 mediated mechanism [18-20]. Anergic IL-10 producing T cells have also been detected in response to *Plasmodium falciparum* infections [21-25], which account for the largest proportion of malaria disease. Complex, stage-specific networks of antibody-dependent and cell-mediated interactions provide immunity against *Plasmodium* spp., with clinical implications depending on the type and timing of cytokine release. Early type-1 responses, dominated by IFN- $\gamma$ , IL-2 and TNF, have been reportedly associated with inhibition of liver stage development [26-31], resolution of acute malaria parasitaemias [32-34] and delay of re-infection [35], as confirmed by the absolute requirement of IFN- $\gamma$  in the effector mechanism of sporozoite-induced protective immunity [35-38]. Release of these cytokines, initiated by the innate immune system (Natural killer (NK) cells,  $\gamma\delta$ T- and  $\alpha\beta$ T-cells) [39-41] and sustained by *Plasmodium*-specific CD8<sup>+</sup> and CD4<sup>+</sup> cells [32,37,39], requires to be timely counterbalanced by a switch to type-2 responses which propagate humoral immunity against the erythrocytic stage, and limit the pathogenicity of pro-inflammatory cytokines [42,43]. Similar symptom-suppressing activities appear to be mediated by IL-10 and transforming growth factor (TGF)- $\beta$ , which in the attempt to reduce immunopathology, can interfere with the protective effects of IFN- $\gamma$  and TNF and allow the parasite to grow uncontrollably [21,44,45].

Despite VL and malaria co-infection cases being encountered across co-endemic areas, little work has been done so far to examine the dynamics of this co-infection and its effect on host immunity. Studies performed in co-infection murine models of *P. chabaudi chabaudi* and *L. infantum* [46], and of *P. yoelii* and *L. mexicana amazonensis* [47,48] have highlighted an exacerbating

effect of the two diseases upon each other, particularly for leishmaniasis, whose enhanced parasite load was attributed to the *Plasmodium*-triggered release of splenic IL-4, as assessed by gene expression [46]. Conversely in golden hamsters, pre-inoculation with different *L. infantum* strains resulted in a reduced proliferation of *P. berghei*, with no aggravation of the *Leishmania* infection [49]. Whilst these discrepancies reflect the difficulty in extrapolating animal model data, particularly when dealing with multiple infections, they agree on recognizing the immune system as a major determinant of *Leishmania* and *Plasmodium* spp. interactions upon co-infection.

In the present study, the cytokine profiles of naturally co-infected patients were examined. Blood samples from patients actively infected with VL and/or malaria and from healthy individuals were collected during an exploratory survey conducted in Gedarif State, Sudan, and the level of nine different cytokines selected from across the four major response arms of the immune system were assessed simultaneously. The comparative analysis between co- and mono-infected groups highlighted substantial differences in the cytokine profile of these patients and their levels of *P. falciparum* parasitaemia, emphasizing the importance of immune-mediated interactions in poly-parasitism.

## Methods

### Study site, study cases and ethical considerations

The sample collection was performed in February 2011 in the village of Tabarak Allah, an endemic area of *L. donovani*, located in Gedarif State, Sudan. Patients were recruited at Tabarak Allah Hospital, which hosts a VL treatment center managed by Médecins sans Frontières since January 2010. Seasonal and unstable malaria prevails in the area, where co-infection rates of 18% to 45% were recorded amongst Tabarak Allah VL in-patients (2005-2010) [10].

All individuals included in the study originated from Gedarif State and aged six years or above. Eligibility for the study was precluded to children up to age six, due to immature status of their immune system [50,51]. Individuals with previous history of VL were also excluded to ensure relapse cases were not enrolled in the study. Included patients reported no history of immune-related disorders, or of ongoing infectious diseases (other than VL and malaria). Clinical and laboratory examinations were performed, including assessment of hemoglobin levels (by HemoCue) and white blood cell (WBC) counts (by microscopy), and their outcomes recorded on anonymized case record forms. Plasma and serum were obtained from peripheral blood and stored at -70°C until tested. None of the subjects received anti-leishmanial chemotherapy before collection of blood samples, while a minimum of two-week lapse from previous treatment

was observed for anti-malarial drugs. Written informed consent was obtained from each study participant above 18 years of age or guardian who consented on their behalf, after providing information on the study aim and procedures in the local language. The survey was conducted with the approval of the Sudanese Minister of Health (National Research Ethics Review Committee), who granted National Ethical Clearance (Nr. 140-10-11).

### Diagnostic algorithm

For categorization of study subjects, the following diagnostic algorithm was implemented. All patients presenting at the study hospital with symptoms of VL and/or malaria, including fever, weight loss, hepatosplenomegaly and anemia, were given physical examination. Finger-prick blood was assessed by microscopy for diagnosis of malaria and by direct agglutination test (DAT) for diagnosis of VL. Assessment of *P. falciparum* parasitaemia was performed by microscopy, counting the total number of parasites per 200 WBCs, as previously described [52]. Artemisinin-based combination therapies were administered to patients positively diagnosed for malaria. The DAT was performed on filter paper-spotted blood, using freeze-dried antigen and control sera from the Royal Tropical Institute (Amsterdam, the Netherlands). A cut-off titer of 3,200 was used, as previously established for the area [53]. Accordingly, patients meeting the WHO clinical definition for VL (fever for >2 weeks with either anemia or splenomegaly) [54], and having a DAT titer >3,200, but no history of VL were diagnosed with primary active VL and received a 30-day course of parenteral sodium stibogluconate, conforming to the national policy, along with the anti-malarial regimen, if required. Patients who tested negative for both VL and malaria were excluded from the study and referred to the hospital medical staff for alternative diagnoses. In total, 102 participants (77 VL and/or malaria confirmed patients and 25 healthy controls) were included at study entry. Prior to initiation of specific chemotherapies, peripheral blood was collected from enrolled participants and processed to obtain serum and plasma samples. A second subsequent evaluation of all specimens to confirm (or exclude in case of healthy controls) diagnosis of VL and/or malaria was independently performed at the Royal Tropical Institute, the Netherlands. Specific antibodies to *Leishmania* were measured in sera or, when unavailable, filter paper-spotted blood using the DAT and two commercially available rk39 tests, the DiaMed IT-Leish<sup>®</sup> (Diamed AG, Cressier sur Morat, Switzerland) and the Kalazar Detect<sup>™</sup> (InBios International, Inc., Washington, USA). The following conditions were considered indicative of VL infection: a) DAT titers  $\geq 3,200$ , with or without positive rk39 test

outcomes; b) DAT titers =1,600 with at least one confirmatory rk39 test; c) DAT titers <1,600 with positive result in the field (>3,200) and a positive rk39 test. Samples that did not fulfill these criteria were excluded from the study ( $n = 8$ ) or re-categorized ( $n = 3$ ), if tested positive for malaria only. Thin and thick blood smears of all study participants were microscopically re-assessed to confirm or exclude presence of *P. falciparum* parasites. When slide re-examination resulted in discordant outcomes, a rapid test (SD Bioline, Standard Diagnostics, Inc., Korea) for detection of *P. falciparum* and *Plasmodium* spp. was performed on the corresponding serum sample. Positive results obtained with the serological test were considered confirmatory of malaria cases ( $n = 4$ ), while specimens which tested negative were excluded from the analysis ( $n = 2$ ). In addition, five other cases (3 healthy controls, 1 co-infected patient and 1 malaria patient) were excluded from the study, due to poorly reliable test outcomes, missing samples or diagnosis of non-*P. falciparum* malaria. From the 102 participants included at study entry, 15 were excluded because they did not match the diagnostic criteria, narrowing the sample size to 87 cases.

### Clinical groups

#### Group 1

Primary VL cases ( $n = 29$ ), defined as VL-seropositive individuals who fulfilled the clinical case definition of VL and tested negative for malaria.

#### Group 2

Clinical malaria patients ( $n = 21$ ). This group included parasitologically-confirmed cases of *P. falciparum* malaria who presented at hospital with clinical symptoms, such as fever, hepato-splenomegaly and anemia, and lacked *Leishmania*-specific antibodies.

#### Group 3

VL and malaria co-infected patients ( $n = 15$ ), defined as VL-seropositive individuals diagnosed with a *P. falciparum* malaria infection.

#### Group 4

Healthy endemic controls ( $n = 22$ ) with a VL-seronegative profile and no microscopically detectable malaria in peripheral blood.

### Cytokine measurement

Cytokine levels in patients' samples were determined using a 9-multiplex magnetic bead-based immunoassay (HCYTOMAG-60 K, Millipore BV, Amsterdam, the Netherlands), performed according to manufacturer's instructions. Briefly, 25  $\mu$ L of magnetic beads internally labeled with multiple fluorophores and coated with specific capture antibodies against one of the nine cytokines (TNF, IL-2, IL-4, IL-6, IL-

10, IL-12p70, IL-13, IL-17A and IFN- $\gamma$ ) was added to 25  $\mu$ L of patient sample and an equal amount of assay buffer. Standards and quality controls for each cytokine were mixed likewise. After an overnight incubation followed by extensive wash to remove unbound proteins, 25  $\mu$ L of biotinylated detection antibodies was added and the fluorescence of the streptavidin-phycoerythrin complex measured by a MAGPIX<sup>®</sup> (Luminex, Austin, USA). A minimum of 50 beads per cytokine was measured. Interpolation of sample concentrations using a five-parameter logistic standard curve was performed with the MILLIPLEX<sup>®</sup> Analyst 5.1 software (Merck Millipore, Billerica, USA). The lower detection limits of the assay were: 0.53 pg/mL for TNF, 0.54 pg/mL for IL-2, 0.34 pg/mL for IL-4, 0.97 pg/mL for IL-6, 0.59 pg/mL for IL-10, 0.74 pg/mL for IL-12p70, 0.57 pg/mL for IL-13, 0.22 pg/mL for IL-17A and 0.17 pg/mL for IFN- $\gamma$ . Comparative analysis of cytokine profiles included both serum ( $n = 83$ ) and, when unavailable, plasma samples ( $n = 5$ ), since exclusion of plasma assessments had no effect on the outcome of the analysis.

#### Statistical analysis

Group-wise comparison of cytokine values was performed using nonparametric statistics. Mann-Whitney U test and Kruskal-Wallis test were used to examine whether continuous variables from two or multiple groups, respectively, originated from the same distribution, whilst comparison of categorical variables was performed using the Chi-square test.  $P$  values  $<0.05$  were considered indicative of statistical significance. Spearman's ( $r_s$ ) rank correlations were computed to assess statistical dependence between cytokine levels and the corresponding patient demographic/clinical

characteristics and between each cytokine pair. Data analysis was conducted with STATA software (College Station, TX, USA).

## Results

### Study population

The sex, age, hemoglobin level, WBC count and *P. falciparum* infection intensity of patients diagnosed with VL and/or malaria are summarized in Table 1. With the exception of malaria parasitaemia, which appeared significantly reduced amongst co-infected patients ( $P <0.01$ ), no major differences in the baseline distribution of these variables were observed, with patients sharing most of their demographic and clinical characteristics. Mild anemia with normal leukocyte counts characterized most of the actively infected population, who largely consisted of young boys. Co-infected patients displayed some intermediate features between the VL and the malaria groups, including lower male to female ratio, younger age and milder anemia as compared with the VL patients.

### Circulating cytokine profiles in VL and malaria mono-infected patients

Significantly increased levels of pro-inflammatory cytokines characterized the VL group compared with the healthy individuals (circulating cytokine levels of all study participants are reported in Additional file 1). Tumor necrosis factor, a marker of local and systemic inflammation, and the type-1-inducing cytokine IFN- $\gamma$  were both strongly up-regulated amongst VL patients, as compared with healthy controls ( $P <0.0001$ ) (Figure 1A and B). Circulating IL-12p70 was also enhanced by the

**Table 1 Baseline characteristics of patients with VL and/or malaria recruited at Tabarak Allah Hospital, Sudan**

Characteristics	Patient group				P value
	1	2	3	Total	
Subjects ( $n$ )	29	21	15	65	
Male/Female ( $n$ )	20/8 <sup>a</sup>	11/9 <sup>b</sup>	9/6	40/23 <sup>c</sup>	0.5
Age (years)	19 (9-29) <sup>d</sup>	8 (7-26)	16 (9-21)	15 (8-24) <sup>e</sup>	0.2
Hb level (g/dL)	9.6 (7.1-12.4)	10.3 (9.1-11.2)	9.9 (7.5-11.5)	10.0 (8.4-11.4)	0.8
WBC count/mm <sup>3</sup>	5650 (4925-6900) <sup>f</sup>	6800 (5900-8000) <sup>g</sup>	5400 (4250-7150) <sup>h</sup>	6050 (4950-7400) <sup>i</sup>	0.2
DAT titer	25600 (6400-102400)	NA	12800 (6400-102400)	19200 (6400-102400)	0.9 <sup>†</sup>
<i>P.f. parasitaemia</i> (parasites/ $\mu$ L)	NA	825 (355-2325) <sup>j</sup>	53 (38-585) <sup>k</sup>	370 (51-1113) <sup>l</sup>	0.005 <sup>‡</sup>

When not otherwise indicated, data shown represent median and (interquartile range).

Hb = hemoglobin; WBC = white blood cell; DAT = direct agglutination test; *P.f.* = *Plasmodium falciparum*; NA = not applicable.

Groups 1 to 3 are: 1 = visceral leishmaniasis patients, 2 = malaria patients, 3 = visceral leishmaniasis-malaria co-infected patients.

The Kruskal-Wallis test was used to calculate the  $P$  values, except for the variable sex for which the Chi-Square test was used, and for the variables DAT titer and *P.f. parasitaemia*, for which the Mann-Whitney test was used.

<sup>†</sup> $P$  value refers to differences between groups 1 and 3 only.

<sup>‡</sup> $P$  value refers to differences between groups 2 and 3 only.

Sex data based on  $a$  28 patients  $b$  20 patients  $c$  63 patients. Age data based on  $d$  27 patients and  $e$  63 patients. WBC count data based on  $f$  18 patients,  $g$  11 patients,  $h$  13 patients and  $i$  42 patients. *P. falciparum* parasitaemia data based on  $j$  12 patients,  $k$  10 patients and  $l$  22 patients. Two co-infected patients received artemether i.m. three weeks prior to diagnosis and their data on *P. falciparum* parasitaemia were excluded to reduce possible bias.



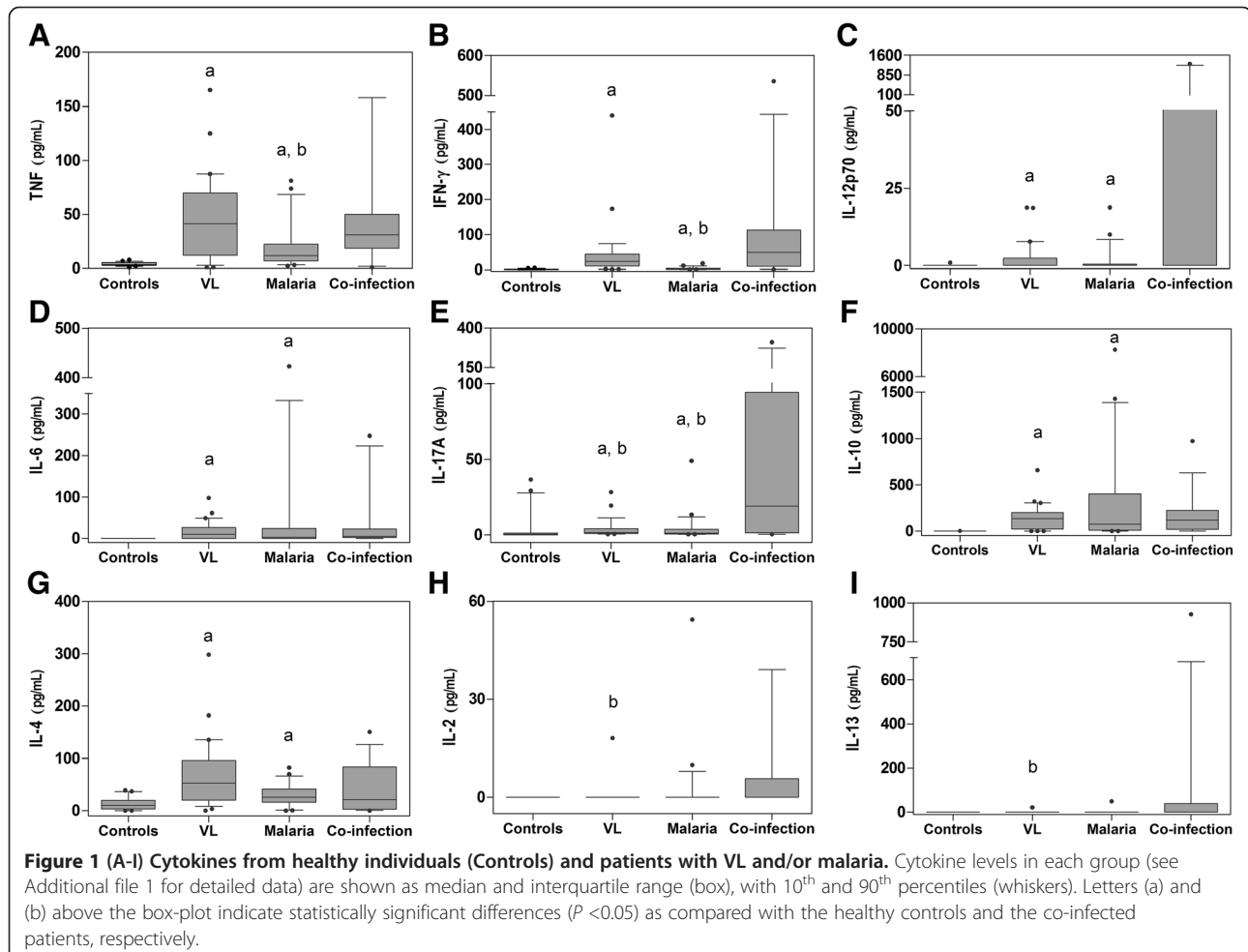
*Leishmania* infection ( $P < 0.001$ ): undetectable in all, but one control, this interleukin reached or exceeded the detection limit (0.74 pg/mL) in nearly half of the VL patients (Figure 1C). Similarly, the serum level of IL-6 was negligible in all healthy individuals ( $< 0.97$  pg/mL), but raised to a 10-fold higher value in the VL group ( $P < 0.0001$ ) (Figure 1D). Systemic inflammation amongst VL patients was confirmed by IL-17A, whose circulating levels appeared to be significantly induced ( $P < 0.001$ ) (Figure 1E). Visceral leishmaniasis cases also exhibited an increase in their anti-inflammatory and regulatory cytokine patterns, as shown by the higher amounts of IL-4, and particularly IL-10 ( $P < 0.0001$ ) (Figure 1F and G). No difference in the circulating level of IL-2 and IL-13, on the other hand, distinguished VL-affected and healthy individuals, for whom the level of these two cytokines resulted mostly under the assay detection limits (0.54 pg/mL and 0.57 pg/mL, respectively) (Figure 1H and I).

Clinical malaria cases exhibited an immunological profile qualitatively similar to that of the VL patients, with

increased concentrations of TNF, IL-6 and IL-10 ( $P < 0.0001$ ) and, to a less extent, IFN- $\gamma$  and IL-17A ( $P < 0.01$ ), IL-4 and IL-12p70 ( $P < 0.05$ ) (Figure 1A-G). Interleukin-2 and IL-13 persisted at negligible levels (Figure 1H and I).

#### Circulating cytokine profiles in VL and malaria co-infected patients

Co-occurrence of malaria and VL in the same patients deeply altered their immunological response as compared with the single infections. Overall, co-infection of *Leishmania* and *Plasmodium* resulted in a marked elevation of type-1 and pro-inflammatory cytokine patterns, presumably triggered by the *Leishmania* disease. Whereas comparable amounts of IFN- $\gamma$  and TNF were detected in the sera of VL mono- and co-infected patients, the level of these two cytokines significantly raised when malaria patients were co-diagnosed with VL ( $P < 0.001$  and  $P < 0.01$ , respectively) (Figure 1A and B). Up-regulation of pro-inflammatory IL-17A distinguished the co-infected patients from both VL and malaria mono-infection cases ( $P < 0.05$ ) (Figure 1E),



indicating that synergistic interactions of the two diseases upon each other concurred to its release. Strikingly different IL-17A concentrations were measured amongst the co-infected patients, whose demographic and clinical characteristics, however, did not display any correlation with the cytokine level. In the co-infected cohort, IL-2 and IL-13 concentrations exceeded the detection limit with a higher frequency (4 out of 15 samples) than amongst VL patients (1 out of 29 samples), resulting in significant differences ( $P < 0.05$ ) between the two groups (Figure 1H and I).

To exclude that the cytokine profiles observed for VL and/or malaria infected patients may have been biased by differences in their demographic and clinical features, the study population was stratified by sex, age or *P. falciparum* parasitaemia (Table 2). For age, patients were matched by means of two groups (6-15 years, >15 years) selected to yield similar group-wise compositions, while for *P. falciparum* parasitaemia, an infection intensity >100 parasites/ $\mu$ L of blood was chosen (corresponding to >0.002%, the level above which patients may become symptomatic). None of the three variables displayed a systematic confounding effect on the cytokines examined here, whose variations amongst the three study groups remained overall stable (Figure 2). Increased concentrations of IFN- $\gamma$  distinguished the co-infected from the malaria mono-infected patients in all of the matched groups, whereas significant differences in TNF levels were observed for men only and for patients above 15 years of age. Importantly, for TNF and IFN- $\gamma$  the lower *P. falciparum* infection intensity recorded amongst the co-infected cohort did not flaw the comparison with the malaria patients, as statistically significant differences still distinguished the two groups after parasitaemia stratification. Increase in co-infected patient IL-17A level appeared to be mainly triggered by malaria, as the difference with this group reduced after age and parasitaemia matching, but persisted towards VL patients in women only and in subjects older than 15 years. No significant differences were observed for the remaining six cytokines,

including IL-2 and IL-13, for which most patients displayed negligible levels (data not shown).

#### Correlation between circulating cytokines in VL and malaria co-infected patients

Specific mechanisms cross-regulate the production of cytokines, with positive and negative feed-back loops to control secretion of signaling molecules. To understand the link interconnecting each of the cytokines examined here, their correlation was investigated using the Spearman's correlation rank test. As shown in Table 3, multiple positive correlations were identified between pro-inflammatory (TNF vs. IL-6) and type-1 cytokines (IFN- $\gamma$  vs. IL-12p70), as well as for type-1 vs. pro-inflammatory (IFN- $\gamma$  & IL-12p70 vs. TNF, IL-6 & IL-17A), and type-2 vs. pro-inflammatory (IL-13 vs. TNF, IL-6 & IL-17A) and type-1 cytokines (IL-13 vs. IL-12p70 & IFN- $\gamma$ ). Conversely, no correlation could be identified between the examined cytokines and the DAT titers as well as the *P. falciparum* parasitaemia of co-infected patients (data not shown).

#### Discussion

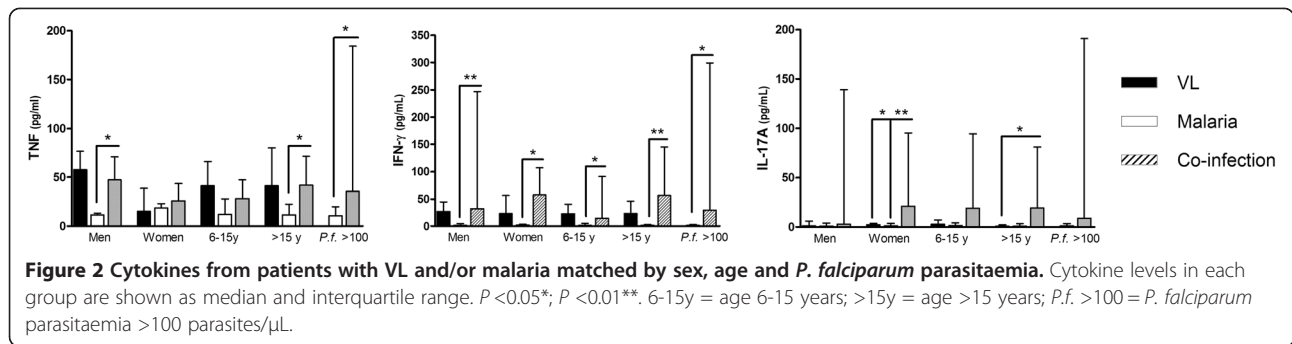
The ability of *Leishmania* and *Plasmodium* parasites to manipulate host immunity and co-inhabit part of the same lymphoid tissues suggests the possibility that the two diseases may interact with each other, when co-occurring in the same host. This is demonstrated for the first time in naturally co-infected patients by the pilot study presented here. Comparative analysis of cytokine profiles from co- and mono-infected patients highlighted substantial variations in the immune response mounted upon co-infection, confirming the ability of *L. donovani* and *P. falciparum* to mutually interact at the immunological level. Patients harboring both leishmanial and malarial parasites responded with an overall increase in type-1 and pro-inflammatory cytokine release, which partly reflected the effect elicited by VL (TNF, IFN- $\gamma$ ) and malaria (IL-2), and partly resulted from a synergistic interaction of the two diseases upon each other (IL-17A).

**Table 2 Characteristics of patients with VL and/or malaria matched by sex, age or *P. falciparum* parasitaemia**

Characteristics	Patient group			Total
	1	2	3	
Subjects (n)	29	21	15	65
Male/Female (n)	20/8	11/9	9/6	40/23
Age 6-15 years (n)	13	14	7	34
Median (Interquartile range)	9 (7-13)	8 (7-9)	9 (7-10)	
Age >15 years (n)	14	7	8	29
Median (Interquartile Range)	26 (20-35)	27 (25-30)	19 (17-27)	
<i>P.f.</i> parasitaemia >100 parasites/ $\mu$ L (n)	NA	12	4	16
Median (Interquartile range)	NA	825 (355-2325)	640 (290-1088)	

*P.f.* = *Plasmodium falciparum*; NA = not applicable.

Groups 1 to 3 are: 1 = visceral leishmaniasis patients, 2 = malaria patients, 3 = visceral leishmaniasis-malaria co-infected patients.



Secretion of IL-13 in co-infected patients significantly exceeded the amounts found in VL patients and displayed positive correlations with most of the examined cytokines. Although this trend can be seen as an attempt of the immune system to contain the effects elicited by type-1 and pro-inflammatory cytokines (these patients exhibited some of the highest concentrations of IFN- $\gamma$ , TNF and IL-17A), the finding remains poorly representative, reflecting the response of 4 patients only, with the remaining co-infected patients ( $n = 11$ ) displaying negligible levels of IL-13 just as most of VL and malaria patients.

Measurement of IL-17A levels allowed to distinguish the co-infected patients from both VL and malaria mono-infected counterparts, indicating that both diseases synergistically concurred to its up-regulation. Better known for its pro-inflammatory effects in allergic and autoimmune conditions, IL-17 has been recently implicated in the protective immunity towards bacterial, fungal and protozoan infections [55], where it is thought to mediate recruitment of neutrophils to the epithelial and mucosal surfaces and induce production of antimicrobial peptides [55,56]. Its release by CD4<sup>+</sup> Th17 cells has been associated with resistance to human VL [57] and positive resolution of murine *L. donovani* infections [58], suggesting that Th17 and Th1 cytokines may play complementary roles in parasite clearance. Hence,

the increased concentrations of IL-17A found in the co-infected vs. the VL mono-infected cohort, besides the already elevated IFN- $\gamma$  and IL-12p70, may be indicative of a favorable, possibly improved, prognosis for VL, though the present data do not allow to draw conclusions in this respect. In support of this speculation is the finding of a recent study conducted in Barbar el Fugarra, a Sudanese village situated only a few tens of kilometers away from Tabarak Allah Hospital (where patients in this study were recruited), in which peripheral blood mononuclear cells (PBMCs) isolated from VL-seropositive individuals who did not develop disease at any time during the 6-year survey, secreted significantly higher IL-17 levels when challenged with *L. donovani* in comparison with VL-seropositive individuals who became symptomatic within 6 months from the evaluation [57]. Interestingly, malaria appeared as the major trigger of this IL-17A up-regulation in co-infected patients, given that no relationship between patients' demographic and clinical variables and the corresponding IL-17A serum level could be identified in this group (nor in any other group). Expansion of IL-17-producing cells (either CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells or macrophages) and related cytokines (IL-17, IL-22 and IL-23) has been observed in *P. vivax* natural infections [59] as well as *P. berghei*, *P. chabaudi* and *P. fragile* animal models [60-62], where these interleukins have been shown to

**Table 3 Correlation coefficients between cytokine levels from VL-malaria co-infected patients recruited at Tabarak Allah Hospital, Sudan**

Coefficient $r_s^{\dagger}$	Cytokine correlation								
	TNF	IL-6	IL-17A	IFN- $\gamma$	IL-12p70	IL-13	IL-10	IL-4	IL-2
TNF		0.80 <sup>***</sup>	0.63 <sup>*</sup>	0.69 <sup>**</sup>	0.66 <sup>**</sup>	0.75 <sup>**</sup>	0.59 <sup>*</sup>	0.40	0.20
IL-6			0.55 <sup>*</sup>	0.68 <sup>**</sup>	0.82 <sup>***</sup>	0.68 <sup>**</sup>	0.61 <sup>*</sup>	0.27	0.13
IL-17A				0.64 <sup>**</sup>	0.68 <sup>**</sup>	0.70 <sup>**</sup>	0.14	0.30	0.20
IFN- $\gamma$					0.75 <sup>**</sup>	0.78 <sup>**</sup>	0.46	0.59 <sup>*</sup>	0.39
IL-12p70						0.85 <sup>***</sup>	0.34	0.19	0.34
IL-13							0.28	0.32	0.53 <sup>*</sup>
IL-10								0.14	0.12
IL-4									-0.15

<sup>†</sup>Spearman's ( $r_s$ ) rank correlations were computed and statistical significance was considered when  $P < 0.001^{***}$ ,  $P < 0.01^{**}$  or  $P < 0.05^*$ .

reduce parasite intensity and protect against fatal outcomes [59-61]. Conversely, a clear role of IL-17 immunity in *P. falciparum* infections is yet to be demonstrated. Transcriptional profiling of PBMCs isolated from *P. falciparum*-infected patients has recently highlighted a Th17/Th $\alpha$  $\beta$  driven bias in the immune response mounted against malaria, with up-regulation of several Th17- and neutrophil-related genes [63,64] and induction of a NK cell-mediated humoral response *via* interferon  $\alpha$  and  $\beta$  [63]. Triggering of this Th $\alpha$  $\beta$  immunity, in particular, was shown to inhibit the IL-12 driven Th1 response [63], necessary for boosting clearance of malaria parasites [29,65-67], particularly during the pre-erythrocytic stage, when cell-mediated immunity is essential for control of infection. If induction of a Th17 response may, therefore, indirectly impair host ability to contain malaria through suppression of macrophage activities, the IFN- $\gamma$  dominant response elicited by VL may partially compensate for this deficiency and act as a pre-priming stimulus upon *Plasmodium* infection, for the development of malaria adaptive immunity (*via* NKT cells, e.g.) and the nitric oxygen-dependent suppression of intra-hepatocytic forms. The above-shown data confirm the leading presence of IFN- $\gamma$  ( $P < 0.0001$ ), followed by TNF ( $P < 0.05$ ) and IL-4 ( $P < 0.05$ ), in the sera of VL patients as compared with the malaria ones, and clearly identify a shift towards type-1/pro-inflammatory polarization when malaria co-occurred with VL. In addition, a significantly reduced *P. falciparum* infection intensity was observed among co-infected patients, suggesting improved tolerance of these individuals to the malaria disease. Whether this reduced susceptibility resulted from the VL-driven pre-immune response remains to be demonstrated. The pioneer work of Adler *et al.* on co-infected hamsters [49] highlighted a reduced proliferation of *P. berghei* for effect of the *Leishmania* infection, supporting the idea of a VL-triggered cross-immunity against malaria, whereas the more recent mouse model data [46-48] seem to suggest the opposite conclusion. It is worthy to note that animals were challenged with blood-stage parasites rather than with sporozoites, bypassing the naturally occurring liver phase against which cellular immunity is most effective and most likely to be developed in response to VL (*Leishmania* parasites visceralize in the liver, too). Moreover, mice and hamsters are not equally representative models of the VL disease, whose clinicopathological features in humans are better reproduced by the golden hamster model [68].

The exploratory nature of this survey implies its design and findings are limited by the small sample sizes and the lack of subject matching between groups, although no significant difference in the distribution of patients' demographic and clinical features was observed. Diagnosis of VL in clinical suspects was confirmed by serology, according to the national policy, precluding any analysis on parasite loads and their link with cytokine profiles. Assessment of

malaria parasitaemia, on the contrary, was performed on peripheral blood films, but the low sensitivity of microscopy observation inevitably limits its reliability as a quantitative assay. Moreover, in the absence of a molecular screening of the recruited individuals, the risk of sub-microscopy malaria infections being carried by the VL patients and/or apparently healthy controls cannot be excluded. Malaria mono- and co-infected patients exhibited different *P. falciparum* blood parasitaemias. Whether these differences are linked to their particular diagnosis, however, is unknown, as patients were recruited sequentially and discernment between clinical and sub-clinical co-infection cases is not possible if one of the two diseases manifests with symptoms. Therefore, recruitment of asymptomatic, but parasitaemic individuals for each of the two infections may be useful to control for non-homogeneous group-wise comparisons. Absence of pre-existent disorders was based on patient reporting only, with no diagnostic procedure performed, other than those ones aimed to confirm VL or malaria. Finally, longitudinal rather than cross-sectional assessments should be endorsed, as they could help identifying those fundamental associations amongst parasite load, cytokine response and clinical picture which are keys to the interpretation of present data. Similar studies may not only clarify the exact role of the VL-malaria co-infection on *P. falciparum* proliferation, but they would be pivotal for understanding the clinical implications that arise from the different cytokine profiles.

## Conclusions

Immune-mediated interactions between *L. donovani* complex and *P. falciparum* appear to shape the immunological response taking place in the co-infected host and possibly the intensity of infections that follow. Similar scenarios have been depicted with other malaria co-infections, indicating that the potential implications arising from multiple pathogen-host relations should be addressed when designing malaria vaccine trials. Careful consideration of parasite interplays should be taken when defining the best strategy for clinical management of VL-malaria co-infections, to ensure that immune homeostasis may be restored without harming patient's clinical course.

## Additional file

**Additional file 1: Cytokine levels (pg/mL) as measured in all patient samples.**

## Abbreviations

VL: Visceral leishmaniasis; IFN- $\gamma$ : Interferon-gamma; IL: Interleukin; TNF: Tumor necrosis factor; Th: T helper; NK: Natural killer; TGF- $\beta$ : Transforming growth factor-beta; WBC: White blood cell; WHO: World Health Organization; DAT: Direct agglutination test; M $\Phi$ : Macrophage; Hb: Hemoglobin; *P.f.* *Plasmodium falciparum*; PBMC: Peripheral blood mononuclear cell.



### Competing interests

The authors declare that they have no competing interests.

### Authors' contribution

EvdB designed the study, carried out the cytokine measurement and drafted the manuscript. AT recruited the study participants, performed collection of data and samples and participated to the sample analysis. MS performed the statistical analysis of the data. PM and EM conceived of the study, participated in its design, contributed to the sample analysis and helped to draft the manuscript. MG revised the critical content of the manuscript. BN participated to the study design and coordinated the work in the field. HS conceived of the study, participated in its design and coordination, contributed to the sample analysis and helped to draft the manuscript. All authors read and approved the final manuscript.

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