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Inflammatory chemokines and their receptors in Human Visceral Leishmaniasis: Gene expression profile in peripheral blood, splenic cellular sources and their impact on trafficking of inflammatory cells

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Abstract

Chemokines play an important role in determining cellular composition at inflammatory sites, and as such, influence disease outcome. In this study, we investigated the expression profile and splenic cellular source of various inflammatory chemokines and their receptors in human visceral leishmaniasis (VL). The expression of chemokines or their receptors was measured at the gene and protein level by employing real time qPCR and a cytometric bead array assay, respectively. In addition, the cellular source of chemokines and their receptors in the spleen was identified employing gene expression analyses in sequentially selected cell subsets. We identified elevated expression of CXCL10, CXCL9, CXCL8, and decreased CCL2 from VL patients. Further, we found reduced expression of the chemokine receptors CXCR1, CXCR2, CXCR3 and CCR2, but increased expression of CCR7 on VL PBMC, compared to endemic healthy controls. Additionally, splenic monocytes were found to be the major source of CXCL10, CXCL9 and CCR2, whereas T cells were the main source of CXCR3 and CCR7. We also report a strong association between plasma IFN- γ and CXCL-10, CXCL-9 levels. Enhanced parasite burden positively correlates with increased expression of CXCL10, CXCL9, IFN- γ and IL-10. Overall our result indicates that VL patients have an elevated inflammatory chemokine milieu which correlated with disease severity. However, expression of their chemokine receptors was significantly impaired, which may have contributed to reduced frequencies of blood monocytes and neutrophils in peripheral blood. In contrast, enhanced expression of CCR7 was associated with increased numbers of activated T cells in circulation. These findings highlight the importance of chemokines for recruitment of various cell populations in VL, and the knowledge gained may help in global understandings of the complex interaction between chemokines and pathological processes, and therefore will contribute towards the design of novel chemokine based immunological therapies against VL.

Graphical Abstract

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Supporting Information

Table S1 represents Assay ID of all the TaqMan probes used to assess the gene expression of various chemokines or their receptors.

Conflict of interest

N.S. and S.S. declare no potential financial conflict of interest in relation to the content of present manuscript.

Keywords

Visceral leishmaniasis (VL); Splenic aspirates; IFN- γ ; CXCL10; CXCL9; CXCL8; CCL5; CCL2

1. Introduction

Visceral Leishmaniasis (VL) is a significant neglected tropical disease with more than 58,000 new VL cases every year (Alvar et al.). VL is still a major public health problem in the Indian subcontinent. Chemokines are chemoattractant that mediate their action by binding chemokine receptors, resulting in cell migration along the chemokine gradient, followed by cell activation and cell differentiation (Griffith et al., 2014). Altered expression of chemokines or their receptors may influence the outcome of infection (Esche et al., 2005; Rot and von Andrian, 2004; Roychoudhury and Roy, 2004). Regulation of immunity against *Leishmania donovani* depends upon the recruitment of appropriate effector cells to site of infection. Further, *Leishmania* infection induces the expression of various chemokines and chemokine receptors in the mammalian host which can subvert the host immune responses (Guerfali et al., 2008; Katzman and Fowell, 2008; Racoosin and Beverley, 1997; Ritter and Korner, 2002). In human cutaneous leishmaniasis, parasite causes the rapid modulation of the host chemokine profile to ensure its persistence, as well as recruitment of suitable host cells (Ji et al., 2003; Katzman and Fowell, 2008; Matte and Olivier, 2002; van Zandbergen et al., 2004). This suggests the potential involvement of chemokines and their receptors in disease pathogenesis.

In experimental VL, infected kupffer cells rapidly secrete CCL2, CCL3 and CXCL10 to recruit inflammatory monocytes and T cells to form a granuloma for the initiation of anti-parasitic immunity (Cotterell et al., 1999; Stanley and Engwerda, 2007). CXCL10 promotes leishmanicidal activity via stimulating the release of nitric oxide (NO) and pro-inflammatory cytokines (Gupta et al., 2009). CCL2 triggers reactive oxygen species (ROS) production to restrict parasites (Dey et al., 2005). Other inflammatory chemokines show direct antimicrobial activity on the parasite (Sobirk et al.). Skin lesions from patients with localised cutaneous leishmaniasis shows higher expression of CCL2, CXCL9, CXCL10, and lower levels of CCL3 (Ritter and Korner, 2002), but in severe diffuse cutaneous leishmaniasis,

these chemokines have lower expression, while CCL3 was found to be up-regulated (Ritter et al., 1996).

Although the roles and contributions of various chemokines and their receptors in immune responses have been extensively investigated (Costa et al., 2013; Hailu et al., 2004; Kurkjian et al., 2006), but our current knowledge on the role of these molecules during Leishmaniasis is largely derived from experimental models (Menezes-Souza et al.). Indeed there are only few human VL studies which focused on changes in chemokines expression and their possible contribution to disease outcome (Gupta et al., 2009; Hailu et al., 2004).

Inflammatory chemokines are obvious candidates for study as they selectively recruit monocytes, neutrophils and T cell subsets. We hypothesized that impaired expression of various chemokines or their receptors may contribute to disease by causing altered cell migration. Considering the potential importance of chemokines in cellular recruitment and disease outcome, we assessed expression of different inflammatory chemokines and chemokine receptors, their cellular splenic source and the association between chemokine expression and disease severity in VL. Chemokine mRNA levels were corroborated with protein level expression. Our study revealed increased expression of chemokines by monocytes and neutrophils, as well as chemokine receptors by activated T cells, thereby supporting the idea of this family of molecules as potential targets to improve treatments for chronic VL (Bhattacharyya et al., 2002).

2. Materials and Methods

2.1 Study Subjects

All patients included in this study presented with symptoms of VL at the Kala-azar Medical Research Center (KAMRC), Muzaffarpur, Bihar, India. In total, 71 individuals were recruited for the study, comprising 32 untreated VL cases, 10 individuals following six months of therapeutic cure and 19 healthy volunteers from the disease endemic area of Bihar. In addition, splenic aspirates from nine separate, untreated VL cases were used. VL Patients were identified as Day (D)-0 before initiation of drug treatment and as Day (D)-dis for the same patient after 15 doses of Amphotericin B treatment. For each patient, disease was clinically confirmed by a fever >2 weeks, splenomegaly, positive serology for recombinant antigen (r)-K39 and/or by demonstration of amastigotes in splenic aspirate smears. All patients were treated intravenously with 0.75mg/kg Amphotericin B on alternate days for a month. Patients with previous cases of VL or infected with HIV, hepatitis, tuberculosis or children <14 years were excluded from the study. All patients underwent clinical follow up initially at an interval of seven days until their drug treatment was completed, and later at post-six months of drug treatment. Patients were evaluated for cure in response to treatment by haematological and clinical profile. Healthy volunteers (EC) living in endemic area were included in the study as experimental controls. Standardised questionnaire forms were used to record clinical features, laboratory results and treatment outcome of all cases included in the study. Patients' data were entered onto a data sheet and data were retrieved from the hospital electronic record system. The flow representation of various immunological assays performed on study subjects is shown in Figure 1, and the prevalence of clinical symptoms observed at the time of enrolment of VL patients are

presented in Figure 2A. Aggregate clinical features of all the subjects recruited in the study are presented in Table 1.

2.2 Ethics Statements

The enrolment of human subjects complies with the principles laid down in the Helsinki declaration. Institutional ethical approval (Dean/2008–09/314, Dean/2012–2013/89) was obtained from the ethical review board of Banaras Hindu University (BHU), Varanasi, India. Informed written consent was obtained at the time of enrolment of each case from participants or their legal guardian, in the case of minors. Only patients who were not treated before and agreed to participate in the study were enrolled. All clinical treatment and follow-up records were maintained using standardised case report forms on an electronic server.

2.3 Splenic aspirate and whole blood collection

Splenic aspirates were routinely collected for diagnostic purposes before initiation of VL treatment. Approximately 150ul of splenic biopsy was obtained, following preparation of smears, left over splenic tissue was placed directly into 1ml of RPMI, supplemented with 10% (v/v) heat inactivated fetal calf serum (Gibco, USA), 25mM HEPES (Gibco, USA), 50Units/ml Penicillin and 50µg/ml Streptomycin (PenStrep, Gibco, USA) and 5IU/ml heparin. Six ml of heparinised peripheral venous whole blood was collected from each patient before and after Amphotericin B treatment, as well as from endemic healthy controls, while 2 ml of blood was obtained from individuals following six months of drug treatment.

2.4 Complete blood count, PBMC isolation and enrichment of cells from splenic aspirates

One ml of whole blood from VL patients was used for complete blood counts (CBC) to measure haematological parameters, while another one ml was centrifuged at 1800 rpm for 10 min at 20°C to collect the plasma for estimation of circulating antibodies, cytokines and chemokines. The remaining four ml of blood was used for isolation of PBMC. PBMC were isolated using a ficoll-hypaque™(GE Healthcare) gradient, according to the manufacturer's instruction. Following the second wash with 1X PBS, the PBMC pellet was stored in RLT buffer at –80°C for gene expression studies.

For sequential, selective enrichment of CD14⁺ (monocytes), CD3⁺ (T cells) and CD19⁺ (B cells) from splenic biopsies, MACS Micro Beads (Miltenyi, Germany) were used as per manufacturer's instructions and described elsewhere (Ansari et al., 2011; Nylen et al., 2007). Briefly, 20µL of CD14 Micro Beads were added per 10⁷ PBMC and after 15 minutes of incubation at 4°C, cells were washed using PBS supplemented with 0.5% BSA and 2mM EDTA. This was followed with magnetic separation of CD14⁺ cell subsets using MS columns (Miltenyi Biotec). Cells remaining after depletion of CD14⁺ fraction are further used for enrichment of CD3⁺ and CD19⁺ subsets respectively, by repeating the same process as described above. Purity of positively enriched cell populations was further confirmed by flow cytometry. Positively selected CD14⁺, CD3⁺, CD19⁺ cells were mixed with 350µl of RLT buffer and stored at –80°C until used for RNA isolation and gene expression studies.

2.5 Serological measurement of rK39 titre by ELISA

Serological detection of rK39 was performed by ELISA, as described elsewhere (10). Briefly, 96-well, flat bottom microtitre plates were coated with 25ng/well of rK39 antigen by overnight incubation at 4°C. After washing with PBS/0.1% (v/v) Tween 20, 150µl/well of 1% (w/v) BSA in PBS was used to reduce non-specific binding by incubating for two hours at room temperature. After washing, 100ul of 1:400 diluted plasma samples were added in duplicate wells and incubated for 1hr. This was followed by another washing cycle and addition of 100ul of HRP (1:16000) and incubation for 30 minutes. 100ul of TMB was added after six washes and left for 15 minutes in the dark. To stop the reaction, 100ul of stop solution was added and the optical density was recorded at 450nm in an ELISA reader. Positive (1:400 diluted plasma pool of confirmed VL cases) and negative (1:400 dilution of plasma pool from non-endemic healthy) controls were run in each plate to standardize the readings and plate variations. The cut-offpoint between negative and positive readings was calculated by the mean of the negative controls plus 3 standard deviations. The positive control was used to calculate relative percentage positivity (PP) for each test sample. Cut off and PP values were determined as previously described (10).

2.6 Cytokine measurement

IL-10 and IFN- γ concentrations were measured in plasma using ELISA Max Deluxe Set Human IL-10 (Cat No. 430605) and IFN- γ (Cat No. 430105) Biolegend's commercial kit(US), as per manufacturer's protocol.

2.7 Chemokine measurement

Plasma level of chemokines was measured using BD Biosciences Cytometric Bead Array (CBA) Human Chemokine Commercial Kit (Cat No. 552990), as per manufacturer's instruction. Briefly, 50 µL of mixed capture Beads, 50 µL of plasma test sample and 50 µL of Human chemokine PE detection antibody (all reagents from BD Biosciences Chemokine kit, US) were added to each test assay tube and incubated for 3 hours at room temperature in dark. Following incubation and washing, cells were suspended in 300 µL of Wash Buffer (BD Chemokine kit), for acquisition on a Flow Cytometer.

2.8 RNA isolation, cDNA synthesis and Real time PCR

RNA was isolated from enriched splenic cell subsets and whole PBMC stored in RLT buffer using the RNAeasy mini-kit (Qiagen, Germany), as per manufacturer's instructions. cDNA synthesis was performed on RNA samples using High Capacity cDNA Reverse transcription kits (Applied Biosystems, USA), as per the manufacturer's instructions in a 20µl reaction volume. Real time PCR was performed using an ABI-Prism 7500 machine (Applied Biosystems, USA). cDNA specific for FAM-MGB-labelled probes for CCR2, CCR6, CCR7, CXCR1, CXCR2, CXCR3, CCL5, CXCL8, CXCL9, CXCL10 and VIC-MGB labelled 18S probewere used to determine normalised expression of the gene of interest relative to 18S mRNA (all reagents from Applied Biosystems).

2.9 Statistical Analyses.

FCAP Array software was used to analyse BD Cytometric Bead Array (CBA) data. Statistical significance was determined using PRISM5 (GraphPad Software, La Jolla, CA, USA). P values < 0.05 were considered as statistically significant and outliers were removed from analyses using Grubbs' test. Statistical data was analysed by both paired (paired cases; D-0 and D-Dis) and unpaired t tests (unpaired cases; D-0 and 6M, D-0 and EC).

3. Results

3.1 Characteristics of the study groups

Subjects involved in the study were selected, based on serological analysis performed by detection of antibodies against recombinant (r)-K39 antigen in plasma. Figure 2B shows a comparison of rK39 titre from active VL, cured VL, six months post-treated (6M) cases and endemic control (EC) individuals included in the study. As expected, VL patients showed relatively high titres for rK39, before and after treatment, when compared with 6M and EC groups (*P<0.05 and ***P<0.001 respectively).

3.2 Ex vivo mRNA expression of chemokines and chemokine receptors on peripheral blood mononuclear cells obtained from VL patients and EC

First we investigated whether altered expression of chemokines or their receptors on PBMC was a feature in VL. We measured the gene expression profile of typical inflammatory chemokines and their receptors, related to mobilisation of major effector cells such as T cells, monocytes and neutrophils. Gene expression analyses showed significant up-regulation in the expression of chemokines CXCL10 (*P<0.05), CXCL9 (**P<0.01), CXCL8 (*P<0.05) in VL PBMCs, while no changes in CCL5 expression were found, as compared to EC. Chemokine receptor CCR7 (*P<0.05) was found to be upregulated, whereas expression of CXCR1 (*P<0.05), CXCR2 (*P<0.05), CXCR3 (**P<0.01) and CCR2 (*P<0.05) were down-regulated at D-0, compared with EC (Figure 3). No significant changes in CCR6 expression were found.

3.3 Changes in plasma chemokine levels in VL.

Analysis of plasma obtained from Pre- and Post-VL pairs, 6M and EC by CBA showed increased circulating levels of the inflammatory chemokines CXCL10 (**P<0.01 and ***P<0.001), CXCL9 (**P<0.01 and ***P<0.001), CXCL8 (P=ns and **P<0.01), and reduced levels of CCL2 (P=ns and *P<0.05), whereas no changes in CCL5 production between D-0 patients and 6M post-treated or EC, respectively, were found (Figure 4). These changes in chemokine protein levels were in line with their mRNA levels of expression (Figure 3).

3.4 Splenic cellular sources of chemokines and their receptors in VL.

To identify the cellular source of inflammatory chemokines and their receptors in a target organ of VL, we assessed *ex-vivo* mRNA expression in positively enriched splenic cell subsets. Gene expression analyses showed the highest levels of CXCL10, CXCL9, CXCR1, CXCR2 and CCR2 expression by CD14⁺ cells, while CD3⁺ T cells showed the highest

levels of CXCR3 and CCR7 expression. Thus, monocytes are the main source of CXCL10 and CXCL9 and express CCR2, while T cells express CXCR3 and CCR7 receptors in the spleens of VL patients (Figure 5). The highest level of CCR6 expression was in T cells, closely followed by CD14⁺ cells. Unfortunately, our cellular analysis was restricted to monocytes, T cells and B cells due to the limited availability of splenic tissue, and as a result, no other cell subsets were investigated.

3.5 Inflammatory chemokine levels positively correlate with parasite burden

To investigate any association between disease severity, measured by parasite burden, and plasma level of chemokines (Figure 4), we performed a correlation analyses. We used splenic score as a measure of parasite load (Sudarshan et al., 2015), and our results indicate a positive correlation between plasma CXCL9 ($r^2=0.2769$, $*P=0.0533$), CXCL10 ($r^2=0.4498$, $**P=0.0087$) and splenic score. In addition, higher levels of the cytokines IFN- γ ($***P<0.001$ and $***P<0.001$) and IL-10 ($***P<0.001$ and $***P<0.001$) were observed in VL patients at D-0, when compared with D-dis or EC (Figure 6a), and a similar significant positive correlation between plasma level of IFN- γ ($r^2=0.3439$ and $*P=0.0275$), IL-10 ($r^2=0.3020$ and $*P=0.05$) and splenic score was found. Higher concentrations of IFN- γ were also positively correlated with elevated expression of CXCL10 ($r^2=0.7026$ and $***P<0.0001$) and CXCL9 in plasma ($r^2=0.5506$ and $****P=0.0004$) (Figure 6).

3.6 Changes in immune cell population in the VL blood

To assess the relationship between clinical cure of VL and cellular profile in the peripheral blood, we investigated the frequencies of various cell types (WBC, lymphocytes, monocytes and neutrophils) using CBC during the course of drug treatment (n=18). We found a significant reduction in total WBC count ($***P<0.001$) at D-0, when compared with D-Dis or 6M (Figure 7). Similarly, frequencies of monocytes and neutrophils ($***P<0.001$) were significantly decreased at D-0, when compared with D-Dis or 6M. In contrast, significantly elevated frequency of lymphocytes ($***P<0.001$) were reported at D-0, which decreased with treatment. Haematological parameters started to improve within one week of drug administration, yet we observed differential trafficking of immune effector cells over the course of drug treatment.

Discussion

Successful immunity against *Leishmania* depends upon the recruitment of appropriate effector cells at the site of parasite entry and residence, inflammatory chemokines and their receptors play a vital role in this process. We assessed expression and source of inflammatory chemokines or their receptors and their association with frequencies of immune effector cells in peripheral blood of VL. We demonstrated significantly enhanced expression of CXCL8, CXCL9 and CXCL10 at both mRNA and protein levels in D-0 patients. Additionally our data corroborates elevated production of IFN- γ inducible chemokines during VL (Hailu et al., 2004). However, we observed a significant reduction in the expression of their receptors, including CXCR1, CXCR2 and CXCR3, at the mRNA level in D-0 PBMC, as compared to D-Dis and EC. This suggests that VL may result in modulation of expression of chemokines or their receptors. No significant changes in the

expression of CCL5 and its receptor CCR5 were found, but significantly reduced expression of CCL2 and its receptor CCR2 was identified. One possibility is that production of CXCL10, CXCL9 and CXCL8 might have been increased in VL as a compensatory mechanism for the decreased expression of their receptors CXCR1, CXCR2, CXCR3.

We further employed gene expression analyses in sequential selectively enriched splenic cell subsets to establish the sources of inflammatory chemokines and their receptors in spleen of pre-treated VL patients. The highest expression of CXCL10, CXCL9 and receptors CXCR1, CXCR2 and CCR2 was found in CD14⁺ cells, whereas CD3⁺ cells mainly expressed CXCR3, CCR6 and CCR7 in the spleen of VL patients. Neutrophils have been known to express CD14 receptors (Antal-Szalmás et al., 1997; Haziot et al., 1993) therefore it's possible that high expression of CXCR1 and CXCR2 receptors by CD14⁺ cells in our samples may include and indicate neutrophils as a potential source, in line with previous reports (Gordon et al., 2005; Li et al., 2002).

Moreover, we have previously showed a direct positive correlation between peripheral blood parasitemia and splenic score in VL patients (Sudarshan et al., 2015), so in present study we employed splenic scores available from routine diagnostic procedure as a measure of disease severity in patients. A positive correlation between splenic score and plasma CXCL9, CXCL10, IFN- γ and IL-10 levels signify association between high levels of immune activation during VL with enhanced parasite burden. This indicates a host strategy to control parasitemia during ongoing VL by increasing expression of pro-inflammatory and regulatory molecules together. CXCL10 can promote a protective immune response by up-regulating pro-inflammatory mediators, Th1 cell cytokines and NO in an *in-vivo* model of VL (Gupta et al., 2009). VL patients have high levels of IFN- γ and IL-10, which is in agreement with previous studies, and it has been reported that IL-10 plays role in the survival of amastigotes present in the splenic aspirate cells (Gautam et al.; Murray et al., 2002). IFN- γ also modulates chemokine production and cellular immune responses, and induces expression of CXCL10 and CXCL9 by monocytes (Dufour et al., 2002; Hailu et al., 2004). Responses similar to VL patients in some endemic individuals indicate that recall proliferative and cytokine responses are primed.

Enhanced expression of inflammatory CXC chemokines detected during VL can modulate trafficking of effector cells in circulation. Considering the importance of inflammatory cells in infection outcome, we explored frequency of cell subsets in peripheral blood. Despite higher expression of CXCL8, comparatively lower frequencies of neutrophils were observed in circulation, which implicates the effect of reduced expression of CXCR1 and CXCR2 receptors in D-0 VL. Neutrophils have been shown to play a role in determining VL outcome through CXCR2 (Mehrotra et al.). Similarly, reduced frequencies of monocytes in circulation can be attributed to reduced expression of CCL2 and CCR2. CXCL10 and CXCL9 mobilizes Th1 cells and recruitment of IFN- γ producing cells promotes macrophage activation and parasite clearance. However, enhanced CXCL10 and CXCL9 production positively correlates with disease severity in VL, and their excessive production can lead to tissue destruction by uncontrolled inflammation (Amato et al., 2003; Ritter and Korner, 2002). Higher expression of the CCR7 receptor might be connected to enhanced

recruitment of T cells in circulation (Nylen et al., 2007). CXCR3 is mainly expressed by activated IFN- γ producing Th1 cells (Bonecchi et al., 1998) and significantly reduced expression of CXCR3 might be responsible for impaired IFN- γ responses in VL. IFN- γ produced by *Leishmania*-specific CD4 T cells is required for parasite killing (Kumar et al.).

Since VL show organ specific immunity (Stanley and Engwerda, 2007) so, one of the crucial approach to better understand the underlying immune mechanisms can be to compare immune activation profile in peripheral blood and spleen. But unfortunately we have limited access of splenic biopsy from VL patients, so we were unable to perform any comparative study between spleen and peripheral blood. Furthermore, in present study six-month post treated individuals lies in narrow age range as compared to VL and EC groups. And considering age as possible confounding factor it remains the outstanding question to understand the impact of age on plasma level of chemokine and cytokine expression. Further study is necessary to understand the impact of age variation on plasma level expression of inflammatory cytokines and chemokines.

5. Conclusions

In summary, the recruitment of suitable effector cells to sites of inflammation by chemokines is a prerequisite to generate protective immune responses. Elevated expression of CXC chemokines in VL patients indicates these chemokines might play an important role in the production of multiple inflammatory mediators by effector cells which may contribute to disease severity. Overall, our results suggest that, VL patients have a sustained inflammatory milieu with impaired expression of inflammatory chemokine receptors and reduced recruitment of monocytes and neutrophils, but enhanced trafficking of activated T cells in the blood. This connection between chemokine profile and disease outcome suggests a potential role for chemokine-based therapies in VL. Our data provide new insights into VL pathogenesis and contributes to understandings of host responses to parasites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

VL Visceral leishmaniasis

CXCL10	C-X-C Motif Chemokine Ligand 10
CXCL9	C-X-C Motif Chemokine Ligand9
CXCL8	C-X-C Motif Chemokine Ligand 8
CCL5	Chemokine (C-C motif) Ligand 5
CCL2	Chemokine (C-C motif) Ligand 2
CX3CR1	CX3C Chemokine receptor 1
CCR2	C-C Chemokine receptor type 2
IFN-γ	Interferon- γ
IL-10	interleukin-10
NO	Nitric oxide
PBMC	Peripheral blood mononuclear cell
ROS	Reactive oxygen species
CBC	Complete blood count
EC	Endemic control

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Figure 1. Schematic representation of various assays performed on VL and EC subjects.

Figure 2. Characteristic of the study groups

A.Prevalence of various clinical manifestations in VL patients at the time of disease presentation. Each vertical column bar in the graph represents the frequency of a clinical parameter at D-0 **B. rK39 titer in blood plasma of study subjects.** Plasma samples were analyzed for rK39 titer by performing ELISA in pre (n=18) and post (n=18) VL pairs, 6-month post treated VL (n=10) and in endemic healthy controls (n=11). Statistical analysis was done by Mann-Whitney test in unpaired samples (VL and 6M/EC). Statistical significance is indicated with P value and each dot represents a sample in the vertical scatter plot.

Figure 3. Representative ex-vivo mRNA expression of chemokines and their receptors in VL and EC PBMC

Graphs show normalized expression of chemokines CXCL10, CXCL9, CXCL8, CCL5 and their receptors CXCR1, CXCR2, CXCR3, CCR2, CCR6, CCR7 over 18S by qPCR in PBMC pairs from VL patients before (n=14) and after therapeutic cure (n=14) and in Endemic controls (n=8). Statistical significance is indicated by a P value, *p 0.05, **p 0.01, ***p 0.001. Paired samples (D-0 & D-Dis) were analyzed using Wilcoxon signed rank test whereas unpaired samples (D-0 & EC) were analyzed using Mann-Whitney test. Each dot represents a sample on vertical scatter plot, black dot denotes sample at D-0, grey dot shows sample at D-dis whereas clear dot represents EC.

Figure 4. Plasma level expression of chemokines

Graphs show concentration of CXCL10, CXCL9, CXCL8, CCL5, CCL2 in pg/ml at Day (D)-0, Day (D)-dis, Six months (6M)-post therapy and in Endemic controls (EC). Results are presented on a vertical scatter dot plot (Mean and SEM). A non-parametric Wilcoxon matched paired t-test was applied to compare D-0 patients with same patient at D-dis, while Mann Whitney test is applied between D-0 and 6M/EC. P values *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$). Outliers were removed using a Grubbs' outlier test.

Figure 5. Source of chemokines and their receptors in VL spleen

mRNA expression analyses of chemokines CXCL10, CXCL9 and chemokine receptors CCR2, CXCR1, CXCR2, CXCR3, CCR6 and CCR7 over 18S was performed in sequentially enriched splenic CD14⁺, CD3⁺, CD19⁺ cells from untreated VL patients. Statistical analyses were executed by a non-parametric Wilcoxon matched paired t-test and statistical significance was represented using a P value. Black dot represents monocytes, grey denotes T cells and clear dots show B cells. All these cell subsets are positively selected from splenic biopsy of VL patient (n=9).

Figure 6. Correlation of splenic score with plasma level expression of chemokines and cytokines
 Untreated VL shows significantly higher production of IFN-g and IL-10 at plasma level and enhanced levels of IFN-g and IL-10 positively correlates with splenic score. A direct correlation between plasma level of CXCL10 and CXCL9 with splenic score and between IFN-g and CXCL10, CXCL9 levels in plasma has been demonstrated. Statistical significances are shown with P value and each dot represents a sample.

Figure 7. Cellular profile in peripheral blood of VL patients at different stages of drug treatment
 Vertical bar graphs show Total white blood cells (WBC) Count/ μ l whole blood and Percentage of Lymphocytes, Monocytes and Neutrophils in peripheral blood of untreated VL (0), during course of drug treatment at day 7, 14, 21, after drug treatment at day of discharge (30) and post 6 months of treatment. Statistical analyses were performed using Wilcoxon signed rank test and statistical significances are shown with a P value.

Table 1:

Aggregate clinical features of the subjects enrolled in study

Variables	VL (n=41)	6M (n=10)	EC (n=19)
Age (years)	30.12±14.96 (28.0) ^a	14.52±12.76 (11)	34.89±11.70 (34)
Sex (M/F)	28/13	3/7	10/9
WBCcounts (mm ³), Pre treatment	3548.72±1428.99 (3400.00)	ND	ND
Splenic score ^{b, #}	2.34±1.03 (2.0)	NA	NA
Splenic enlargement (cm), (Pre-treatment)	3.56±2.31 (3.0)	NA	NA
Duration of illness (days)	34.29±32.77 (22.0)	NA	NA

^aMedian value are given within brackets.^bParasite scoring is on logarithmic scale from 0 to 6, where 0 means no parasite in 1000 microscopic fields (1000X), 1 is 1–10 parasites per 1000 fields and 6 is >100 parasites per field.[#]Splenic scores presented are based on patients underwent biopsy (some patients are treated based on rk39 strip test only)

ND= not done; NA= not applicable