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T cell suppression in the bone marrow of visceral leishmaniasis patients: impact of parasite load

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Summary

Visceral leishmaniasis (VL) is a disseminated and lethal disease of reticulo-endothelial system caused by protozoan parasites *Leishmania donovani and L. infantum*, which are known to induce host T cell suppression. To understand the impact of parasite load on T cell function, the present was focused on parasite load with T cell function in bone marrow of 26 VL patients. We observed significant enrichment of forkhead box protein 3 (FoxP3)$^+$ ($P = 0{\cdot}0003$) and interleukin (IL)-10$^+$ FoxP3$^+$ regulatory T cells (T_{reg}) ($P = 0{\cdot}004$) in the bone marrow (BM) of patients with high parasite load (HPL) compared with low parasite load (LPL). Concordantly, T effector cells producing interferon (IFN)-γ ($P = 0{\cdot}005$) and IL-17A ($P = 0{\cdot}002$) were reduced in the BM of HPL. Blocking of T_{reg}-cell derived suppressive cytokines [(IL-10 and transforming growth factor (TGF)-β] rescued the effector T cells and their functions. However, it was observed that TGF-β levels were dominant, favouring T_{reg} cell differentiation. Furthermore, the low ratio of IL-6/TGF-β favours the suppressive milieu in HPL patients. Here we show the change in levels of various cytokines with the parasitic load during active VL, which could be helpful in devising newer immunotherapeutic strategies against this disease.

Keywords: bone marrow aspirate (BMA), *Leishmania donovani*, T regulatory cells (T_{reg}), visceral leishmaniasis (VL)

Introduction

Visceral leishmaniasis (VL) is a chronic infectious disease, fatal if left untreated, caused by a group of protozoan parasites of the genus *Leishmania* and transmitted by phlebotomine sandflies [1]. In the Indian subcontinent, visceral leishmaniasis is caused primarily by *L. donovani*, while *L. infantum* is the pathogen responsible for the disease in Latin America and the Mediterranean regions [2,3]. Demonstration of the amastigote form of *Leishmania* parasite in aspirates of lymph node, spleen or bone marrow is still the gold standard for diagnosis [4–6]; parasitic grading is usually used as per the World Health Organization (WHO) guidelines (0–6$^+$) of splenic aspirate [7]. The WHO grading system has also been used for bone marrow (BM) aspirate [8,9], even though the possibility of dilution by peripheral blood remains a concern. Severe parasitic infestation within the reticulo-endothelial system (RES), including visceral organs such as the liver, spleen and in the BM, is the pathological hallmark of the disease [10]. Dissemination of the disease is believed to be due to the suppressed state of immunity induced by the high parasite load (HPL) [2]. However, the role of regulatory T cells (T_{reg}) in such parasite-induced immune suppression at the disease site, i.e. bone marrow, remains unexplored.

Clearance of leishmania parasites from the infected macrophages critically requires a strong T helper type 1 (Th1)-like response with biased production of inflammatory cytokines. Such cytokines, namely interferon (IFN)-γ and interleukin (IL)-17, favour parasite clearance via macrophage activation leading to enhanced production of reactive oxygen and nitrogen species. A strong Th1-like inflammatory response has been demonstrated to be protective in both the murine model as well as in VL patients [3,11,12]. A state of immune suppression has been documented as characteristic of VL [13]. Therefore, it had been proposed and demonstrated subsequently that the suppressed state of immune response at the pathological sites facilitates parasitic growth and dissemination, leading to their infiltration in the RES of the subjects. We have shown

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previously that, in spite of a higher frequency of IFN-γ-positive T cells, the parasite remains in the BM of the VL patients [14]. We also demonstrated a higher frequency of T_{reg} cells in the patients' BM [14]. Higher levels of IL-17 and IL-22 have been proposed to be protective among endemic healthy contacts of VL patients [15]. Thus, along with several other groups, we proposed that a suppressed state of T cell response at the pathological sites of disease is critical for parasitic growth, and this may be an immune evasion strategy of the parasite. We also showed that *Mycobacterium tuberculosis* induces T_{reg} cell-mediated suppression of the immune response [16], especially at the pathological sites of miliary tuberculosis and their frequency correlates with the bacillary load of the patients [17]. We thus proposed that reciprocal levels of T_{reg} *versus* inflammatory/effector T cells (IFN-γ^+, IL-17^+) dictate the fate of parasitic survival and pathogen growth within the macrophage. We demonstrated enrichment of T_{reg} cells and IL-10 secreted by them in the BM of VL patients [14]. Here we investigated the status of T_{reg} cells, their suppressive effect on the inflammatory cytokine production relating to the parasite load of the patients [high parasitic load (HPL) *versus* low parasitic load (LPL)]. We show a higher frequency of T_{reg} cells in the BM of the HPL group as opposed to that of the LPL group. We also observed a higher frequency of T_{reg} cells producing IL-10 among HPL patients, suggesting that those enriched T_{reg} cells are the major cellular source of IL-10. These suppressive immune parameters in HPL patients correlate inversely with the lower frequencies of IFN-γ^+ and IL-17A$^+$ T cells and inflammatory cytokines. Furthermore, blocking of IL-10 and transforming growth factor (TGF)-β rescued inflammatory cytokine-producing T cells in VL. Overall, our results show the direct relationship between T_{reg} cells in the BM and the parasitic burden, suggesting that either T_{reg}-mediated suppression of the local immune response facilitates parasite load or a higher parasite burden induces T_{reg}-mediated suppression of immune response at the disease site.

We propose that T_{reg} cells and their suppressive cytokines such as IL-10, TGF-β may be indicative of the patients' parasite burden, and blocking the T_{reg} cell function may be critical for rescuing protective immunity and subsequent parasite clearance.

Material and methods

Study subject (VL patients)

BM aspirates from 26 parasitologically confirmed VL patients were included in this study after obtaining the patients' informed consent (mean age = 33·81 years, range = 10–60 years; 17 males and nine females) (Table 1). These patients were visited at the out-patient department (OPD) of Balaji Utthan Sansthan Patna, Bihar for diagnosis and treatment of their illness. The patients presented with complaints of prolonged high fever, weight loss and swelling of abdomen. Further examination and laboratory findings revealed splenomegaly, hepatomegaly, pancytopenia and hypergammaglobulinaemia, etc. BM samples (1–1·5 ml) were collected in heparinized tubes at the time of diagnosis. Microscopic L-D bodies were demonstrated as per the 2010 WHO guideline used for splenic aspirate [7]. Utmost care was taken to avoid any significant dilution of BM aspirate by bleeding, and any such specimens were excluded. The parasitic load in the BM of these patients was evaluated before recruitment into the study and the start of therapy. The study subjects were divided into HPL and LPL groups based on the parasite density in BM of the study subjects (Supporting information, Fig. S1).

Reagents

Isolated BM mononuclear cells (MNCs) were cultured in RPMI medium, supplemented with L-glutamine (G-5763; Sigma Chemicals Co., St Louis, MO, USA), antibiotics (Pen-Strep-Ampho Sol; Biological Industries, Kibbutz Beit Haemek, Israel) and 10% fetal calf serum (FCS) (Biological Industries); 0·5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) was used as the staining buffer for surface staining, while 2% formaldehyde in PBS was used for cell fixation. Cell permeabilization for intracellular antigen detection was performed with 0·5% saponin (S-7900; Sigma). Monoclonal antibodies were used for surface and intracellular antigen detection. Fluorescein isothiocyanate (FITC)-conjugated IL-17A, forkhead box protein 3 (FoxP3) (BD Pharmingen, San Diego, CA, USA), phycoerythrin (PE)-conjugated CD25, IFN-γ (BD Pharmingen), peridinin chlorophyll/cyanin (PerCP/Cy5) PE-conjugated CD4 (BD Pharmingen) and FoxP3 staining kits were purchased from e-Biosciences (Cat. no. 71–5776-40; e-Biosciences, San Diego, CA, USA).

Demonstration of parasite in BM aspirate

Clean labelled slides were used for thin BM smear preparation. After air-drying, the slides were stained with Giemsa stain (GS500-500ML; Sigma-Aldrich), according to the manufacturer's protocol. Following identification of parasitic density on the slides using eyepiece (×10) and objective (×100), the parasite density was scored by means of a logarithmic scale ranging from 0 (no parasite per 1000 oil immersion fields) to +6 (> 100 parasites per field) based on the WHO diagnosis criteria (WHO, March 2010).

Preparation of whole cell lysate antigen from *L. donovani*

Parasites from late log phase growth were collected by centrifugation (6500 ***g***) for 10 min at 4°C. The pellet of LD was washed twice with sterile PBS and finally resuspended in PBS and then sonicated. Protein concentration was assessed with an ND-1000 spectrophotometer (NanoDrop

Technologies, Wilmington, DE, USA). Ten μg of sonicated soluble protein (titrated dose) was used to stimulate 1–2 million cells in 1-ml culture.

Isolation of MNCs from BM aspirate

Briefly, 1–1·5 ml of BM samples was collected in heparinized tubes at the time of diagnosis (microscopic parasitic demonstration). Collected BM samples were centrifuged at 845 g for 5 min in a micro-centrifuge. The pellets containing cells were separated from the supernatant. Supernatant was collected and stored at −80°C and used for the detection of soluble cytokines in BM aspirate (IL-17A, IL-6 and TGF-β). BM MNCs were isolated from pellets of BM aspirate through Ficoll Hypaque gradient centrifugation; isolated cells were washed thrice with incomplete RPMI (without FBS) and finally suspended in complete RPMI-1640 supplemented with 10% FBS (Caisson Laboratories, Logan, UT, USA). The cell viability was estimated by trypan blue dye exclusion test. Samples with more than 95% viability were used for *in-vitro* culture.

In-vitro cell culture

Isolated BM MNCs were cultured (2×10^6 cells/ml) in 96-well *U*-bottomed microtitre plates (BD Falcon/BD Biosciences). Plates were coated with purified α-CD28 and α-CD49d antibody, 5 μg/ml in carbonate buffer, pH 9·6 (AbdSerotec, Oxford, UK; LE/AF, Cat. no. MCA70EL and MCA923EL, respectively); 0·5 million cells were seeded per well and stimulated with LD antigen (whole cell lysate, 10 μg/ml) for 48 h at 5% CO_2 at 37°C. For blocking studies, purified monoclonal antibodies of IL-10 (10 μg/ml, NA/LE; BD Pharmingen) and TGF-β (10 μg/ml; BD Pharmingen) was mixed individually or in combination with BM MNCs (2 million/ml). Monensin (Golgi transport inhibitor; 1 mM; Sigma-Aldrich) was added in the last 6 h of culture.

Surface and intracellular staining

Ex-vivo and *in-vitro* isolated and cultured cells were incubated directly with fluorescence-labelled monoclonal antibodies in staining buffer (5% BSA in PBS) for 15 min on ice. After washing, fixation was performed in 2% formaldehyde in PBS for 15 min at room temperature. Cells were then permeabilized by using permeabilization buffer (0·5% saponin) for 15 min at room temperature. After washing, the cells were incubated at room temperature for 30 min with a titrated dose of intracellular monoclonal antibodies (IL-10, IL-17A and IFN-γ, etc.) in permeabilization buffer. Finally, after washing twice with staining buffer, cells were transferred to a fluorescence activated cell sorter (FACS) tube (BD Falcon; BD Biosciences) for data acquisition by three/four-colour flow cytometer (FACSCalibur; BD Biosciences).

Cells staining for FoxP3 antigen

Briefly, 1×10^6 MNCs were first surface-stained for CD4 and CD25 and were further stained intracellularly for FoxP3 antigen using a FoxP3 staining (FITC/PE) kit (Cat. no. 00–5123-43; eBiosciences). To ensure the specificity of CD4, CD25 and FoxP3 staining, we performed fluorescence minus one (FMO) and isotype staining of the same specimens. To enumerate the IL-10-producing FoxP3$^+$ cells, antigen-stimulated cells were stained for IL-10 and FoxP3 using the same protocol. To confirm the staining specificity, we performed FMO for IL-10. For enumeration of FoxP3$^+$ T_{reg} cells, 2×10^5 events were acquired to obtain the analysable number of FoxP3$^+$ cells.

Estimation of IL-6, TGF-β, IL-10 and IL-17A in BM aspirates

To evaluate the soluble levels of various cytokines in the BM aspirate, sandwich enzyme-linked immunosorbent assay (ELISA) was performed using kits specific for IL-6 (eBioscience; cat no. 88–7066-22), TGF-β (eBioscience; cat no.88–8350-88), IL-17A (eBioscience; cat no.88–7176-88) and IL-10 (eBioscience; cat no.88–7106-88), following the manufacturer's protocol.

Ethics declaration

The research project was approved by the Institutional Ethics Committee of AIIMS, New Delhi (Ref. no. IEC/NP-331/2010; 28 December 2010). Written informed consent was obtained from all study subjects. For minor children and for patients who could not read and write, written informed consent was provided by their legal guardian.

Statistical analysis

GraphPad Prism-5 was used for statistical analysis. Median and standard deviation (s.d.) were used for scatter analysis. The significance between two groups was calculated by unpaired two-tailed and Mann–Whitney *U*-tests. When more than two groups were compared simultaneously, significance was determined by analysis of variance (ANOVA) using a Bonferroni correction for multiple comparisons. A P-value $< 0{\cdot}05$ was considered significant.

Results

Enrichment of T_{reg} cells in BM of VL patients correlates with parasite load

To reveal the status of T_{reg} cells and suppressive cytokines (IL-10 and TGF-β) in patients with HPL in BM, we enumerated the frequency of activated T (CD4$^+$CD25$^+$) cells and T_{reg} (CD4$^+$CD25^{br} FoxP3$^+$) cells in BM. We observed a significantly higher percentage frequency of activated T cells in HPL patients ($n = 12$) compared with LPL patients ($n = 14$)

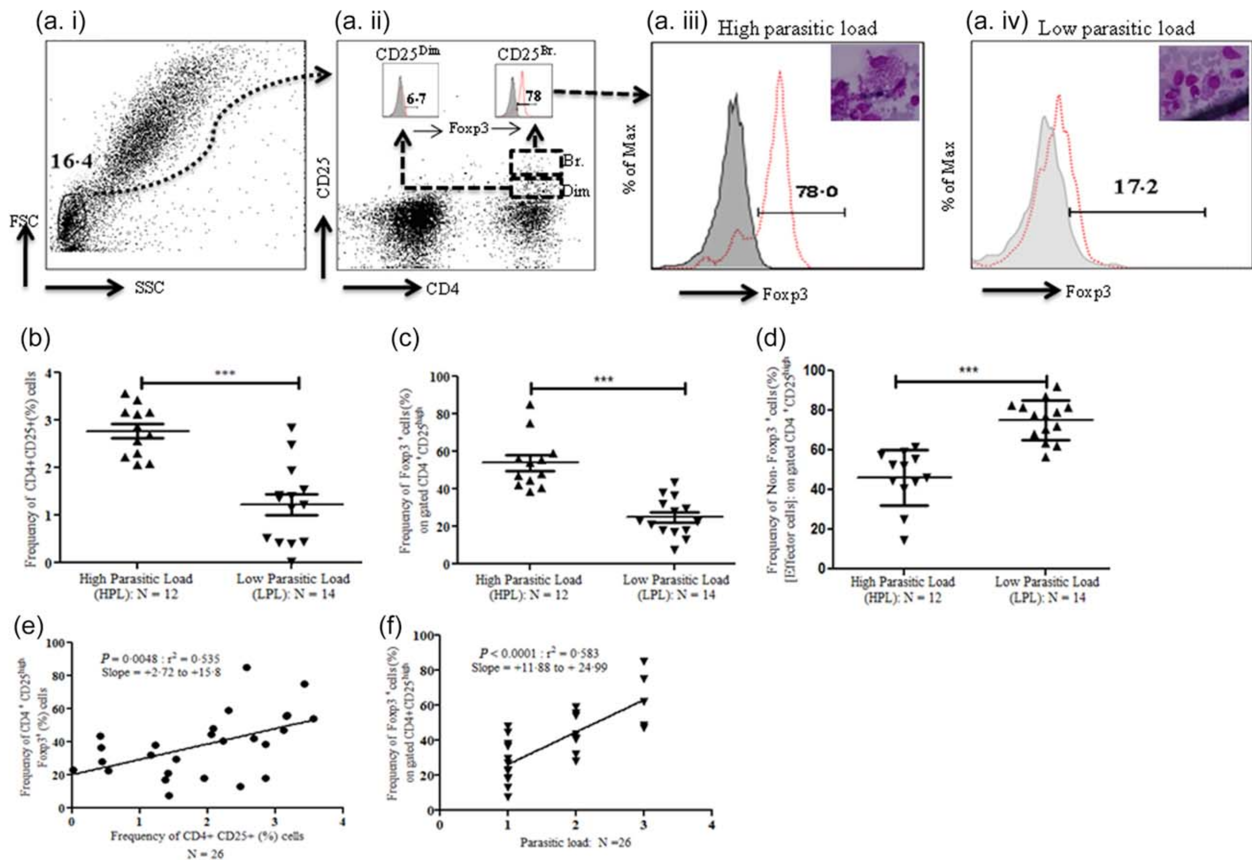

Fig. 1. Enrichment of regulatory T cells (T_{reg}) cells in bone marrow of visceral leishmaniasis (VL) patients correlates with parasite load. (a) Enumeration of T_{reg} [forkhead box protein 3 ($FoxP3^+$)] frequency: bone marrow mononuclear cells (BM MNCs) from VL patients were used for immunophenotyping of T_{reg} cells. $CD4^+CD25^+$ population of T cells was gated on pregated lymphocytes (based on forward- *versus* side-scatter); further, the dual population of $CD4^+CD25^+$ T cells was divided into $CD4^+CD25^{dim}$ and $CD4^+CD25^{br}$ (Fig. 1a, ii: inset). Intranuclear expression of FoxP3 was observed in these two populations. Histogram plot of FoxP3 expressing $CD4^+CD25^{dim}$ or $CD4^+CD25^{br}$ (thin red dotted line) was overlaid on FoxP3 fluorescence minus one (FMO) (solid dark field). $CD4^+CD25^{br}$ were found to be the major population of activated T cells expressing FoxP3 (br = bright). (b) Increased percentage frequency of activated ($CD4^+CD25^+$) T cells in BM of VL patients with high parasitic load (HPL). The percentage frequency of activated T cells ($CD4^+CD25^+$) was analysed from BM-derived MNCs of VL patients ($n = 26$). We observed a significantly higher percentage frequency of $CD4^+CD25^+$T cells in VL patients with HPL [($2^+$and 3^+: $n = 12$), mean ± standard deviation (s.d.): 2·76 ± 0·15%] compared with low parasitic load [(1^+: $n = 14$) mean ± s.d.: 1·23 ± 0·21%; $P = 0{\cdot}002$, unpaired *t*-test]. Horizontal lines in scatter-plot depicted as the mean value (br = bright). (c) Increased T_{reg} frequency ($CD4^+CD25^+FoxP3^+$) in BM of VL patients with HPL: significantly increased percentage frequency of $FoxP3^+$ T cells on pregated $CD4^+CD25^+$ were observed in BM MNCs of VL patients with HPL ($n = 12$: mean ± s.d.: 53·84 ± 4·06%) compared to the patients with low parasitic load (LPL) ($n = 14$: mean ± s.d.: 24·89 ± 2·70%) ($P = 0{\cdot}0001$: unpaired *t*-test). Horizontal lines in scatter-plot depicted as mean value. (d) Significantly decreased frequency of T effector cells ($CD4^+CD25^+FoxP3^–$) in bone marrow of VL patients with HPL: significantly decreased percentage frequency of T effector cells [($CD4^+CD25^+FoxP3^–$): i.e. 100%: % frequency of $CD4^+CD25^+FoxP3^+$] in BM MNCs of VL patients with HPL ($n = 12$: mean ± s.d.: 46·16 ± 4·06%) compared to the patients with LPL ($n = 14$: mean ± s.d.: 75·12 ± 2·70%) ($P = 0{\cdot}0001$: unpaired *t*-test). Horizontal lines in scatter-plot depicted as mean value. (e) Increased frequency of T_{reg} ($CD4^+CD25^+FoxP3^+$) cells in BM of VL patients was correlated positively with higher percentage frequency of activated T cells ($CD4^+CD25^+$): further, we observed a significant positive correlation between percentage frequency of T_{reg} ($CD4^+CD25^+FoxP3^+$) cells with percentage frequency of activated cells ($CD4^+CD25^+$) in VL patients ($P = 0{\cdot}004$: Pearson's correlation coefficient $r^2 = 0{\cdot}535$). (f) Increased frequency of T_{reg} ($CD4^+CD25^+FoxP3^+$) cells in BM of VL patients was correlated positively with parasitic load: we observed significant positive correlation between percentage of T_{reg} frequency ($CD4^+CD25^+FoxP3^+$) with parasitic load in VL patients ($P < 0{\cdot}0001$: Pearson's correlation coefficient $r^2 = 0{\cdot}583$). [Colour figure can be viewed at wileyonlinelibrary.com]

(Fig. 1b) ($P = 0.0002$; unpaired two-tailed *t*-test). Furthermore, we observed a significantly higher percentage frequency of $FoxP3^+$ T_{reg} cells ($CD4^+$ $CD25^{br}FoxP3^+$) in patients with HPL compared with low LPL (Fig. 1c) ($P = 0{\cdot}0001$; unpaired two-tailed *t*-test). Moreover, we observed a significantly lower percentage frequency of T effector cells ($CD4^+CD25^{br}$ $FoxP3^–$) in HPL patients ($n = 12$: 46·2 ± 4·06) compared with LPL patients ($n = 14$: 75·1 ± 2·71) (Fig. 1d) ($P = 0{\cdot}0001$; unpaired two-tailed *t*-test). The percentage frequencies of activated T ($CD4^+CD25^+$) cells also correlated significantly

with increased frequency of T_{reg} ($FoxP3^+$) cells (Fig. 1e) ($P = 0{\cdot}004$; Pearson's correlation coefficient $r^2 = 0{\cdot}535$). Moreover, a higher T_{reg} frequency in BM was correlated positively with parasitic load in the BM of VL patients (Fig. 1f) ($P < 0{\cdot}0001$; Pearson's correlation coefficient $r^2 = 0{\cdot}583$). These results established a direct correlation between parasitic burden in BM with T cell activation ($CD4^+CD25^+$), increased T_{reg} cells ($FoxP3^+$) and decreased T effector cell ($FoxP3^-$) frequency.

Enrichment of IL-10-producing T_{reg} cells in VL patients with HPL

We further characterized the source of IL-10 producing T cell, whether it is $CD4^+CD25^{br.}$ $Foxp3^+$ or $CD4^+CD25^{dim}$ $Foxp3^+$ T cells. We observed that $CD4^+CD25^{br}FoxP3^+$ T cells (Fig. 2a, iii, iv) (red dotted line of overlay histogram) are the major producers of IL-, rather than $CD4^+CD25^{dim}FoxP3^+$ cells. Interestingly, a significantly higher proportion of T_{reg} cells were IL-10 producers in HPL patients ($n = 10$) compared to that of LPL patients ($n = 12$) (Fig. 2b) ($P = 0{\cdot}004$; unpaired two-tailed *t*-test). When we compared the ELISA-based soluble level of IL-10 among these groups, we observed that the level of IL-10 was significantly higher in the HPL group ($n = 11$) (Fig. 2c) ($P = 000{\cdot}1$: $208{\cdot}4 \pm 16{\cdot}09$) than that compared with the LPL group ($n = 13$) ($106{\cdot}3 \pm 3{\cdot}44$). This indicates that the parasitic load may influence IL-10 production by T_{reg} cells in the BM of visceral leishmaniasis patients. Conversely, the higher

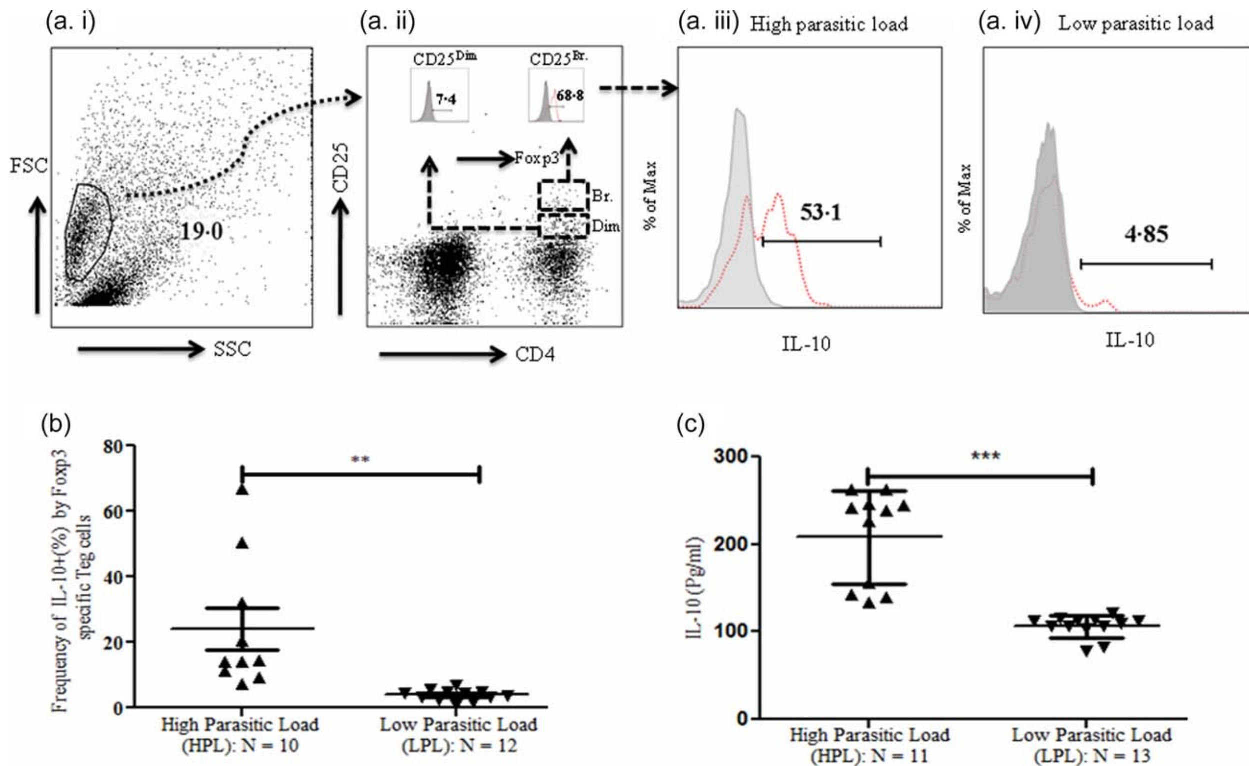

Fig. 2. Enrichment of interleukin (IL)-10 producing regulatory T cells (T_{reg}) cells in visceral leishmaniasis (VL) patients at disease site. (a) Enumeration of IL-10-producing T_{reg} ($CD4^+FoxP3^+IL\text{-}10^+$) cells in bone marrow mononuclear cells (BM MNCs): isolated MNCs from bone marrow of VL patients were cultured for the assessment of IL-10 production by antigen-specific T_{reg} cells in response to *Leishmania donovani* antigen (whole cell lysate; 10 μg/ml). Cells were cultured for 72 h in animal cell culture conditions (incubation at 37°C with 5% CO_2). Monensin (Golgi transport inhibitor; 1 mM) was added in the last 6 h of culture. On forward- *versus* side-scatter gated lymphocytes, the dual population of $CD4^+CD25^+$ was defined and divided further into $CD4^+CD25^{dim}$ and $CD4^+CD25^{br}$ T cells. Histogram plot of FoxP3 expressing $CD4^+CD25^{dim}$ or $CD4^+CD25^{br}$ (thin red dotted line) was overlaid on FoxP3 fluorescence minus one (FMO) (Fig. 2a, ii: inset). IL-10 expression was then assessed on $CD4^+CD25^{br}$ $FoxP3^+$ T cells. For this, IL-10 expressing $CD4^+CD25^{br}$ $FoxP3^+$ T cells were overlaid on IL-10 FMO (Fig. 2, iii and iv). (b) Increased frequency of IL-10-producing T_{reg} cells at disease site in VL patients with high parasitic load (HPL): $CD4^+FoxP3^+$ pregated lymphocytes were used for illustration of the IL-10-producing T_{reg} population (histogram). T_{reg}-specific IL-10 population was increased significantly in VL patients with HPL in response to whole cell lysate of *L. donovani* (10 μg/ml) [$n = 10$: mean ± standard deviation (s.d.): $24{\cdot}03 \pm 6{\cdot}31$%] compared to the patients with low parasitic load (LPL) ($n = 12$: mean ± s.d.: $3{\cdot}87 \pm 0{\cdot}49$%) ($P = 0{\cdot}002$: unpaired *t*-test). Horizontal lines in scatter-plot depicted as mean value. (c) Significantly increased soluble level of IL-10 in VL patients with HPL: enzyme-linked immunosorbent assay (ELISA)-based soluble level of IL-10 in BM aspirate of HPL ($n = 11$: mean ± s.d.: $208{\cdot}5 \pm 16{\cdot}09$ pg/ml) patients was significantly higher compared with LPL ($n = 13$: mean ± s.d.: $106{\cdot}3 \pm 3{\cdot}44$ pg/ml) patients ($P = 0{\cdot}0001$: unpaired *t*-test). Horizontal lines in scatter-plot depicted as mean value. [Colour figure can be viewed at wileyonlinelibrary.com]

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frequency of IL-10-producing T_{reg} cells may facilitate parasite growth by suppressing the host effector immune response.

Decreased frequency of IFN-γ- and IL-17A-producing cells in VL patients with HPL

To evaluate the status of effector T cells in cytokine production, we enumerated the frequency of IFN-γ- and IL-17A-producing cells. The percentage frequency of both IFN-γ- and IL-17A-producing $CD4^+$ T cells (Fig. 3a, b) was significantly lower in HPL patients ($n = 11$) compared to that of LPL patients ($n = 13$) ($P = 0{\cdot}005$ and $0{\cdot}002$, respectively; unpaired two- tailed *t*-test). Furthermore, we correlated the percentage frequency of T_{reg} cells with CD4-specific IFN-γ- and IL-17A-producing cells in the HPL group. We observed a negative correlation for both IFN-γ and IL-17A with T_{reg} cells. Moreover, the correlation was highly significant for IFN-γ than IL-17A ($n = 12$) (Fig. 3c). These findings strongly suggest a parasite load role in dampening the effector T cells producing proinflammatory cytokines (IFN-γ and IL-17A), most probably by augmenting the suppressive T_{reg} cells and their function.

Increased soluble level of IL-6 and TGF-β in VL patients with HPL

In vitro, naive T cells can be differentiated into Th17 cells in the presence of IL-6 and TGF-β [18,19]. Conversely, soluble TGF-β promotes T_{reg} cells by enhancing the expression of FoxP3 in $CD4^+$ T cells. It has also been observed that IL-6 inhibits TGF-β-induced T_{reg} differentiation [18]. To evaluate the status of soluble levels of IL-6 and TGF-β in the study group, we performed ELISA and observed that

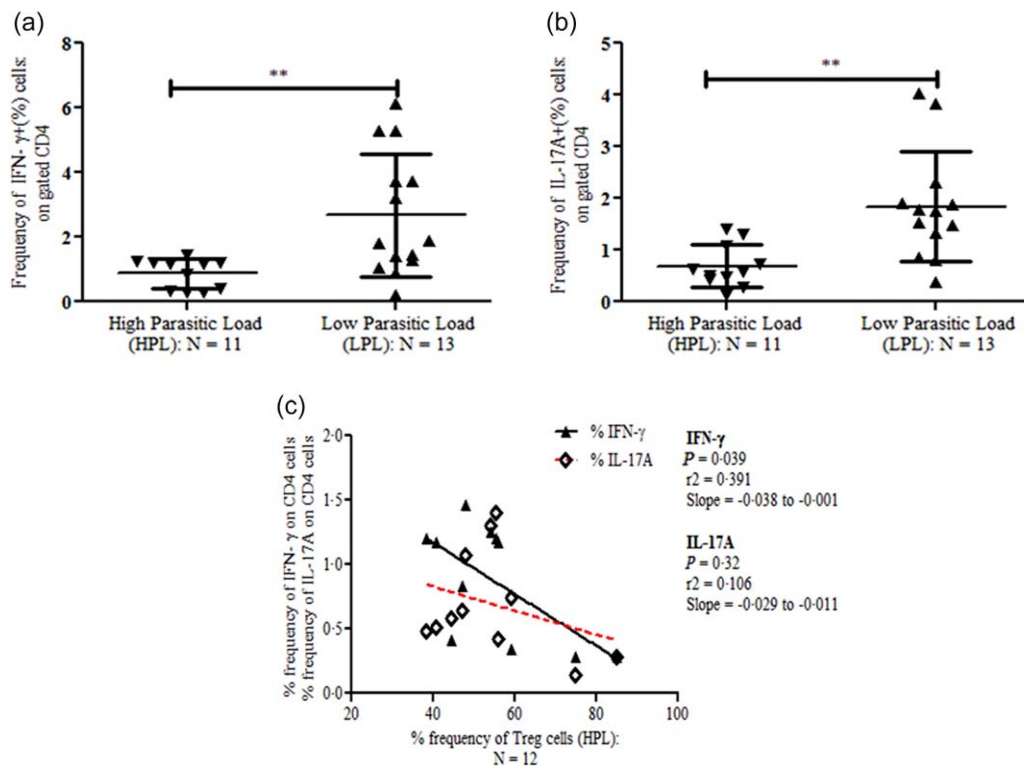

Fig. 3. Decreased frequency of interferon (IFN)-γ- and interleukin (IL)-17A-producing cells in visceral leishmaniasis (VL) patients with high parasitic load (HPL). Enumeration of IFN-γ- and IL-17A-producing cells in bone marrow mononuclear cells (BM MNCs): isolated MNCs ($n = 24$) from bone marrow of VL patients were cultured for IFN-γ and IL-17A production: BM -MNCs were cultured for 24 h. At the 0 h time-point, whole cell lysate of *Leishmania donovani* antigen (10 µg/ml) and at the 18 h time-point, monensin was supplemented in culture. Finally, at the end of 24 h of culture, cells were harvested and used for intracellular staining of IFN-γ- and IL-17A-producing cells using anti-human IFN-γ and IL-17A monoclonal antibodies along with anti-human CD4 antibodies. (a) Scatter-plot of IFN-γ-producing cells on gated $CD4^+$ T cells: significantly decreased percentage frequency of IFN-γ-producing cells in patients with HPL ($n = 11$: mean ± s.d.: $0{\cdot}87 \pm 0{\cdot}13\%$) in comparison with patients with LPL ($n = 13$: mean ± s.d.: $2{\cdot}72 \pm 0{\cdot}54\%$) ($P = 0{\cdot}005$). (b) Scatter-plot of IL-17A-producing cells on gated $CD4^+$ T cells: as of IFN-γ; the percentage frequency of IL-17A also follows the same trend between HPL ($n = 11$: mean ± s.d.: $0{\cdot}68 \pm 0{\cdot}12\%$) and low parasitic load (LPL) ($n = 13$: mean ± s.d.: $1{\cdot}83 \pm 0{\cdot}29\%$) ($P < 0{\cdot}002$). (c) Increased percentage frequency of regulatory T cells (T_{reg}) ($CD4^+CD25^+FoxP3^+$) cells in BM of HPL patients was correlated negatively with proinflammatory cytokine- (IFN-γ and IL-17A)-producing cells. We observed a significant negative correlation between percentage frequency of T_{reg} ($CD4^+CD25^+FoxP3^+$) cells with a percentage frequency of IFN-γ ($P = 0{\cdot}03$: $r^2 = 0{\cdot}39$ with slope $= -0{\cdot}038$ to $-0{\cdot}001$) and IL-17A ($P = 0{\cdot}32$: $r^2 = 0{\cdot}10$ with slope $= -0{\cdot}029$ to $-0{\cdot}011$)-producing cells on gated $CD4^+$ T cells. [Colour figure can be viewed at wileyonlinelibrary.com]

IL-6 was significantly higher in the HPL ($n = 12$) (Fig. 4a) ($P = 0{\cdot}053$: $187 \pm 16{\cdot}8$) compared with the LPL ($n = 11$) ($144{\cdot}4 \pm 12{\cdot}5$) groups. Similarly, the soluble level of TGF-β was significantly higher in HPL patients ($n = 11$) (Fig. 4b) ($1123 \pm 155{\cdot}4$) compared with LPL patients ($n = 13$) ($533{\cdot}0 \pm 101{\cdot}4$). Further, we observed that the soluble level of IL-17A was relatively low in the HPL group ($n = 12$) (Fig. 4c) ($57{\cdot}99 \pm 5{\cdot}2$) compared with the LPL group ($n = 14$) ($72{\cdot}21 \pm 4{\cdot}19$). Why, in spite of high levels of both IL-6 and TGF-β, we observed lower numbers of Th17 cells and IL-17A levels in VL HPL patients was surprising. To understand and explain this apparent discrepancy, we calculated the ratio of IL-6 over TGF-β, which shows the relative dominance of one cytokine over the other. We observed a significantly lower IL-6/TGF-β ratio among HPL patients ($n = 11$) ($P = 0{\cdot}011$) relative to that of LPL patients ($n = 11$) (Fig. 4d). This indicates TGF-β dominance in the BM of HPL patients favouring T_{reg} cell differentiation, resulting in the suppression of Th17 function or differentiation. Therefore, we conclude that HPL somehow triggers a dominant TGF-β environment in BM, and probably that inhibits the protective Th17 cells.

Rescue of IFN-γ- and IL-17A-producing cells upon blocking TGF-β and IL-10

To delineate the suppression of effector T cells by T_{reg} cells in *in-vitro* culture, BM MNCs ($n = 8$) VL patients were stimulated with leishmanial antigen in the presence or absence of neutralization/blocking monoclonal antibodies against IL-10 and TGF-β (10 μg/ml each). Using one-way ANOVA, we observed the significant rescue of both IFN-γ- ($P = 0{\cdot}0001$; Fig. 5a) and IL-17A- ($P = 0{\cdot}0001$; Fig. 5b) producing $CD4^+$ T cells upon blocking or neutralization of these suppressive cytokines. When we analysed the data further for Bonferroni correction for multiple comparisons, significant rescue of IFN-γ was observed among antigen stimulation and blocking of both suppressive

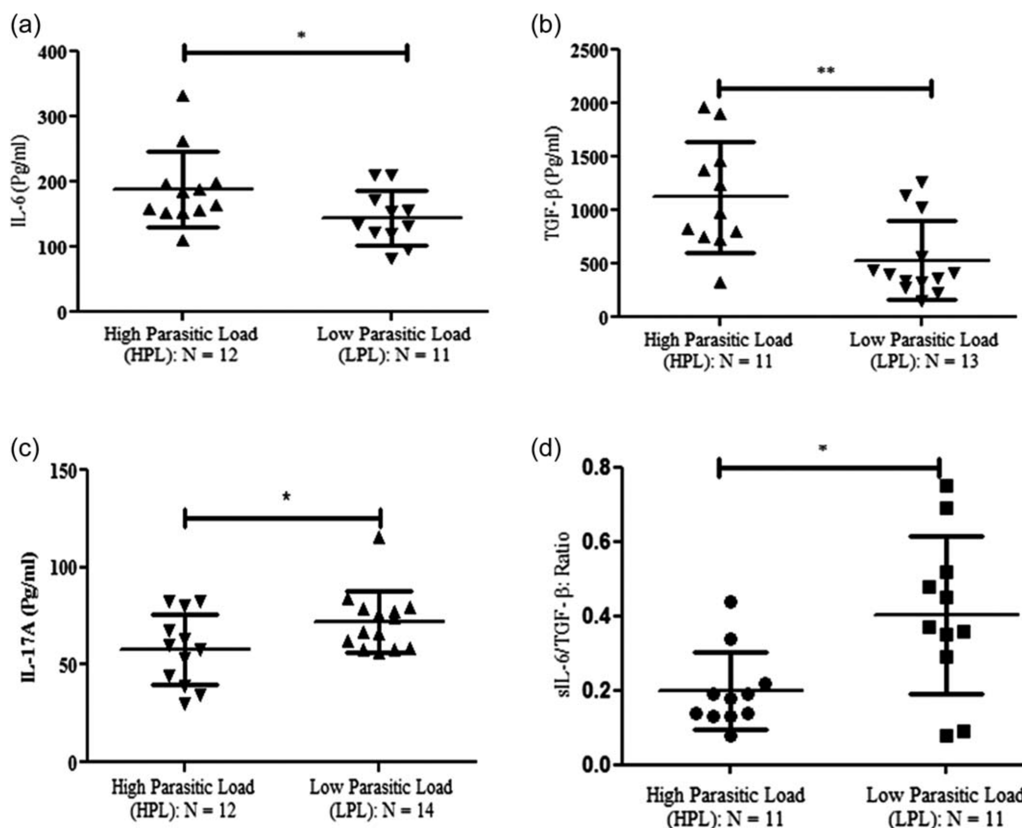

Fig. 4. Soluble level of interleukin (IL)-6, transforming growth factor (TGF)-β and IL-17 in visceral leishmaniasis (VL) patients with parasitic load: sandwich enzyme-linked immunosorbent assay (ELISA)-based assay was performed to measure the soluble levels of IL-6, TGF-β and IL-17 in bone marrow (BM) aspirates of VL patients. Cytokine (IL-6, TGF-β and IL-17) levels were evaluated based on company protocols. (a) Significantly increased level of IL-6 in BM aspirate of high parasitic load (HPL) [$n = 12$: mean ± standard deviation (s.d.): $187{\cdot}9 \pm 16{\cdot}84$ pg/ml] patients compared with low parasitic load (LPL) ($n = 11$: mean ± s.d.: $144{\cdot}4 \pm 12{\cdot}5$ pg/ml) ($P = 0{\cdot}05$: unpaired *t*-test). Horizontal lines in scatter-plot depicted as mean value. Similarly, for (b) TGF-β in BMA of HPL ($n = 11$) compared with LPL ($n = 13$) ($P = 0{\cdot}003$: Mann–Whitney *U*-test) and (c) IL-17A: significantly decreased soluble level of IL-17A was observed in patients with HPL ($n = 12$: mean ± s.d.: $57{\cdot}9 \pm 5{\cdot}27$ pg/ml) compared with LPL ($n = 14$: mean ± s.d.: $72{\cdot}21 \pm 4{\cdot}19$ pg/ml) ($P = 0{\cdot}04$: unpaired *t*-test). (d) sIL-6/TGF-β: significantly decreased ratio of sIL-6 *versus* TGF-β was observed in HPL patients ($n = 11$) compared with LPL ($n = 11$) patients ($P = 0{\cdot}02$: Mann–Whitney *U*-test, unpaired *t*-test).

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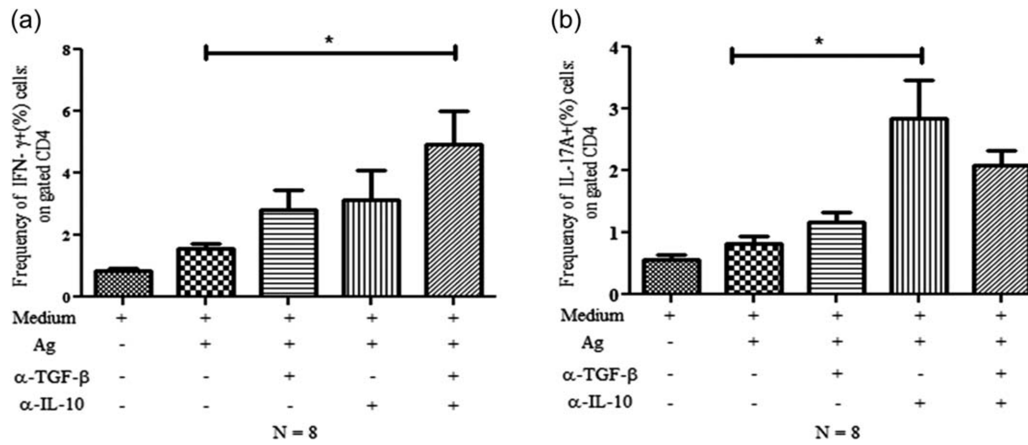

Fig. 5. Rescue of both interferon (IFN)-γ- and interleukin (IL)-17A-producing cells upon blocking of suppressive cytokines [transforming growth factor (TGF)-β and IL-10]. Mononuclear cells (MNCs) from bone marrow of visceral leishmaniasis (VL) patients were cultured for 48 h with/without *Leishmania donovani* antigen (10 μg/ml, (whole cell lysate) along with neutralizing antibodies for IL-10 and TGF-β (10 μg/ml) alone or in combination. Monensin was supplemented in the last 6 h of culture. Rescue of IFN-γ and IL-17A on $CD4^+$ T cells was assessed by flow cytometry. (a) Column graph showing the one-way analysis of variance (ANOVA) significant rescue of IFN-γ^+ cells on $CD4^+$ T cells. Data are average of eight independent experiments. Statistical Bonferroni correction for multiple alignment revealed significant rescue of IFN-γ by blocking of both suppressive cytokines (TGF-β and IL-10) (Fig. 5a); (b) Similar to IFN-γ; column graph of IL-17A showing the one-way ANOVA significant rescue of IL-17A$^+$ cells on gated $CD4^+$ T cells. Data are average of eight independent experiments. Statistical Bonferroni correction for multiple alignments revealed the significant rescue of IL-17A upon blocking of IL-10 alone (Fig. 5b).

cytokines (α-IL-10 and α-TGF-β). In the case of IL-17A, a significant rescue was observed among antigen stimulation and blocking of IL-10 (α-IL-10) only. Therefore, our findings indicate the suppression of IFN-γ- and IL-17A-producing effector T cells in the BM of HPL by suppressive cytokines such IL-10 and TGF-β derived from T_{reg} cells present in the BM. This is substantiated further by higher expression of IL-10RA receptor (CDw210A) on both peripheral and BM-derived T cells of HPL patients (data not shown).

Discussion

The gold standard of leishmaniasis diagnosis (VL) is the microscopic demonstration of the amastigote form of parasite (LD bodies) in splenic and BM aspirates [4,20]. The visceral organs are well-known homing organs of parasites [3]. Therefore, understanding the suppressed state of the host immune response against the parasites necessitates the study of immune cells or parameters of BM. Suppression of the T cell response in VL is well demonstrated, and contributes to the dissemination of parasites in various visceral organs of reticulo-endothelial organs. This appears to be one of the major immune evasion strategies of *L. donovani*. However, the precise relation between parasite burden and T cell response in these affected organs remains inconclusive. Some critical and unresolved issues are: (i) which immune cells are involved in local suppression of T cells, (ii) how do they influence the parasite burden or (iii) how does the parasite load influence the host immune state at the disease site? To address some of these issues, we focused our attention on the T effector and T_{reg} cells in the BM of VL patients with HPL as opposed to LPL patients. Few studies from this viewpoint have been carried out using peripheral blood [21]. However, a correlation between immune parameters with parasitic load at the BM remains to be resolved.

A significant correlation was noted between the distinct function of certain T cell subsets and parasite load in the BM of the patients. Taking cues from our previous study, where we noted a significant enrichment of T_{reg} cells in the BM of VL patients (mean 48·7%) compared to the BM of healthy subjects (mean 10·1%) [14], we enumerated T_{reg} cells in the BM of VL patients and their correlation with the parasite load. We observed a higher frequency of T_{reg} cells ($CD4^+CD25^{br}FoxP3^+$) than that of T effector cells ($CD4^+CD25^{br}FoxP3^-$) in the BM of HPL patients and the reverse was true for the LPL patients. This indicates a strong T_{reg}-mediated suppression of effector T cells in the BM of VL. The frequency of IL-10-producing T_{reg} cells was also higher in the BM of HPL VL patients. Moreover, a higher frequency of suppressive (IL-10$^+$) T_{reg} cells in the BM correlated negatively with the frequencies of IFN-γ^+ and IL-17A$^+$ T effector cells among HPL patients. We also observed a reverse pattern in LPL patients. Therefore, our results indicate that HPL correlates closely with the suppressive state of the T cell response in the BM. IL-10 may also be produced by recently activated Tr1 cells, which are essentially $FoxP3^-$. However, we did not observe any significant presence of $FoxP3^-$ IL-10-producing Tr1 cells in

Table 1. Demographic and haematological investigation table: showing the details of visceral leishmaniasis patient included in this study

Number of patients	$n = 26$	
Demographic characteristic		
Age (mean ± s.d.)	33·81 ± 11·35	
Sex (M/F)	17/09	
Ethnicity	Indian	
Diagnosis		
*Parasitic demonstration	No. of patients	Parasitic load
In bone marrow	14	+1
	07	+2
	05	+3
Haematological investigations		
†WBC count (mean ± s.d. × 10^3)	3·72 ± 2·18	
†Platelets count (mean ± s.d. × 10^3)	100·9 ± 43·59	
‡Hb (mean ± s.d.)	8·98 ± 2·08	

*Parasitic load in bone marrow was calculated according to standard criteria of WHO (2010). †White blood cell count (WBC) and platelets count in thousands. ‡Haemoglobin (Hb) in mg/dl; s.d. = standard deviation.

the BM of the VL patients that we studied. We believe that a causal link exists between the parasite load and functional status of T cell responses in the BM of HPL VL patients. However, which one is the cause or effect is not evident in our study between the immune suppression and parasite load. Further mechanistic study is needed to decipher the issue conclusively. Either HPL induces T_{reg} cells and suppresses the protective T cell response or the dominance of T_{reg} cells abrogates the immune containment of parasites, thus failing to control the parasite multiplication as well as the dissemination observed in VL patients. Delineating a precise causal link between parasite load and the T_{reg}-mediated suppression requires further detailed studies, including experiments in animal models.

As expected from our positive correlation between the parasite load and T_{reg} cell frequency, we observed significantly higher levels of T_{reg} cell-associated cytokines IL-10 and TGF-β in HPL patients. However, we also noted a simultaneous and significant rise in IL-6 levels in the BM of HPL patients compared to those of the LPL group. In our HPL group, therefore, there were higher levels of both IL-6 and TGF-β. However, the frequency of IL-17A$^+$ T cells (Th17) was significantly low. This appears paradoxical, as a combined elevation of these two cytokines is likely to facilitate Th17 cells, conferring an immune checkpoint against parasite multiplication [22]. To address this paradox, we evaluated the ratio of TGF-β to IL-6 present in the BM of patients. We found a significantly lower IL-6 : TGF-β ratio among HPL and the reverse in LPL. This indicates a direct correlation between higher levels of TGF-β and the increased parasitic burden of either one leading to the other, causing the dominance of T_{reg} cell differentiation suppressing the differentiation of protective Th17 cells. Our results indicate the possibility of a parasite load-dependent preferential rise in TGF-β (along with IL-6), thereby creating a cytokine milieu conducive for the generation of suppressive T_{reg} cells compared to that of protective Th17 cells, albeit in the presence of IL-6. This vicious cycle may result in unchecked parasite multiplication. Alternatively, HPL may also drive such a preferential production of TGF-β. Therefore, our result does not rule out either of these two possibilities. A longitudinal immune profiling study will be required to resolve the causality relationship of our observational findings. The cellular source of parasite-induced elevation of TGF-β may be infected macrophages [22,23]. We envisage that preferential secretion of TGF-β in the BM of VL patients may represent a critical immune evasion strategy of leishmanial parasites. Once such TGF-β bias is achieved, T_{reg} cells differentiate to increase TGF-β further to suppress the effector T cells actively, resulting in uncontrolled parasite multiplication. This may then be apparent in a positive correlation between HPL and the suppressive immune parameters, as we observed that high T_{reg} cells elevated suppressive cytokines TGF-β/IL-10 and low effector cells and cytokines IFN-γ^+ and IL-17A$^+$ T cells. Our findings of rescuing both IFN-γ^+ and IL-17A$^+$ T cells upon blocking of TGF-β/IL-10, either alone or in combination, substantiate that TGF-β/IL-10 present in HPL BM indeed suppress the effector T cells, leading to failure in the immune containment of parasites. This, in turn, manifests as a close correlation between the parasite load and suppressive immune parameters in the BM. However, this effect may be due to either a direct effect of these cytokines on the effector T cells or an indirect effect on the antigen-presenting cells. Further study will be required for resolving this issue.

Overall, our findings reiterate certain critical issues of immune suppression observed in VL. In this study, we demonstrate (i) a close relationship between parasite burden and T cell suppression mediated by T_{reg} cells in BM and (ii) a TGF-β (and also IL-10)-dominant local milieu facilitates T_{reg} cell differentiation, resulting in the suppression of protective T cells. Major cellular sources of TGF-β may be infected macrophages [22] and T_{reg} cells, as we have shown previously [16,17]. Whatever the initial source of this cytokine may be, once the infection sets the milieu suitable to drive T_{reg} cell generation, a perpetual state of T cell suppression ensues in the BM. This leads eventually to uncontrolled parasite multiplication and dissemination. However, the contribution of a similar pathway may not be inferred for other pathological sites of visceral leishmaniasis due to differences in the architectural and immune milieu in spleen, liver and lymph nodes. Suppression of the host T cell response has also been well documented in *L. infantum* infection [2,3,24]. Considering the similarity of the immune response, we envisage that the parasite burden may also suppress the T cell response during *L. infantum* infection. We propose that blocking suppressive elements such as T_{reg} cell-favouring cytokines may rescue

the protective immune response and immune containment of the leishmania parasites. Conversely, evaluating these parameters may prove critical, as biomarkers for disease severity pave the ways to immune rescue therapy and vaccination.

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Disclosure

The authors declare no financial or commercial conflicts of interest.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Microphotograph showing the intracellular *Leishmania donovani* bodies (LD bodies) in bone marrow aspirate of visceral leishmaniasis study subject. Thin microscopic smear slides were stained with Giemsa stain and examined microscopically under 10×100 magnifications. Presence of LD bodies was graded on a scale from 1^+ to 6^+. Amastigote (LD bodies) 1–10 in 10, 100 or 1000 fields were graded as 3^+, 2^+ and 1^+. (a) Representative bone marrow LD bodies demonstration in the low parasitic load (LPL) (1+) study group. (b) Bone marrow LD bodies demonstration in the high parasitic load group (HPL) (2+) and (c) bone marrow LD bodies demonstration in the HPL group (HPL) (3+).