T cell subset and cytokine profiles in human visceral leishmaniasis during active and asymptomatic or sub-clinical infection with *Leishmania donovani*[☆]

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Abstract

During an epidemiological study of visceral leishmaniasis (VL) in south-west Ethiopia, 33 VL patients, 9 treated VL patients, 14 individuals with sub-clinical infection, 34 individuals with asymptomatic infection, and 19 healthy controls were studied for T cell subsets and cytokine profiles. Negative leishmanin skin test, CD3 and CD4 lymphocytopenia, and significantly reduced numbers of memory CD4^{+} T cells were found in VL patients compared to treated VL patients or persons with self-limiting asymptomatic infection. The proportion of CD4^{+} and CD8^{+} T cells that produced IFN-γ and IL-4 after stimulation with PMA (Phorbol 12-myristate 13-acetate) and ionomycin was significantly reduced in VL patients compared to sub-clinical and asymptomatic infections or healthy controls. Plasma concentrations of IFN-γ and IL-10 were elevated in VL.
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Introduction

Leishmania parasites infect cells of the mononuclear phagocytic system in mammalian hosts and depending on the species of *Leishmania* and the immunological response of the host, infection in man leads to a spectrum of diseases [1,2]. *Leishmania donovani* causes visceral leishmaniasis which is characterised by profound suppression of cellular immunity [3] and by humoral immune responses leading to hyper-gammaglobulinemia [4] and high levels of immune complexes [5]. These abnormalities are restored after successful treatment, and generally immunity to reinfection results.

Investigations of the immunopathogenic processes of murine leishmaniasis have contributed to our knowledge about the interplay of Th1 and Th2 cytokines. T-helper response patterns determine outcome of *Leishmania* infections in that Th1 responses enhance cell-mediated immunity leading to control of infection and resistance to reinfection

whereas Th2 responses lead to a dominantly humoral response and to exacerbation of the infection [6]. Th responses are less polarised in man [7] but Th1 unresponsiveness is characteristic of active visceral leishmaniasis. Prospective studies of the progress of infection from an asymptomatic or sub-clinical stage to full blown disease and of the spectrum of disease in an endemic population are limited. Thus, Th1 and Th2 patterns during various stages of infection as well as during treatment and convalescence are understood to limited extent only.

During an on-going field study of visceral leishmaniasis in an endemic locality in Ethiopia, we studied immunological factors in patients with active visceral leishmaniasis, in persons cured after treatment, in individuals with asymptomatic and sub-clinical infection, and in endemic controls. We report on T cell subset distribution and cytokine profiles in this study population.

Materials and methods

Study area

From August 1997 to February 2000, detailed clinico-epidemiological data were collected at 6-monthly intervals in a previously described [8,9] endemic locality in south-western Ethiopia. All inhabitants of two villages Galga and Goinada with a population of 753 and 1123 respectively were studied. Previously, it had been demonstrated that the leishmanin skin test (LST, see below) was positive in 32.2% of the population and that 4.1% had a positive serological test for leishmania antibodies [9]. The prevalence of HIV antibodies in the two study villages during the study period was less than 0.1%.

Study groups of persons examined

Group 1

33 newly diagnosed patients with visceral leishmaniasis. Patients with prolonged fever, splenomegaly, weakness, wasting, and a positive Direct Agglutination Test (DAT, see below) were subjected to parasitological investigations of aspirates of lymph nodes or spleen. *Leishmania* parasites were demonstrated in smears and/or NNN (McNeal Novy Nicoll) cultures.

Group 2

9 VL patients treated 47–212 months before, and 23 patients of group 1 reexamined 3 months (10 patients), 6 months (8 patients), 9 months (8 patients), 12 months (1 patient), and 15 months (2 patients) after end of treatment; total number 32 patients.

Group 3

14 sub-clinical cases, defined as persons with positive DAT test without or with only minor constitutional symptoms.

Group 4

34 asymptomatic cases, defined as individuals with positive LST, with or without positive DAT, without signs and symptoms of disease.

Group 5

19 healthy endemic persons with negative DAT and LST, not matched for age and sex.

Investigations

In all individuals, a physical examination was done and in all the DAT and LST were performed. Fresh and preserved faecal specimens were examined for intestinal parasites.

Blood examination included determination of Packed Cell Volume (PCV), total and differential WBC count, and microscopic examination of Giemsa stained thick and thin blood slides for malaria parasites.

Treatment

VL patients were treated with intramuscular injections of sodium antimony gluconate, 20 mg/kg/day for 30 days.

Specific immunological investigations

Immunological investigations were conducted in a sample of the study population. Plasma was stored at $-20°C$ in the field and subsequently at $-70°C$ until tested. Peripheral blood mononuclear cells (PBMCs) were isolated over Ficoll–Hypaque gradients, and kept frozen first at $-70°C$ and subsequently in liquid nitrogen ($-196°C$).

Leishmanin skin test (LST)

Leishmanin (*L. infantum* ZLON49) donated by Dr. Marina Gramiccia (Istituto Superiore di Sanita, Laboratorio di Parassitologia, Rome, Italy), was administered intradermally on the volar surface of the forearm. Skin induration was measured 48–72 h later as described [10]. Induration size of 5.0 mm and above was considered positive.

Direct Agglutination Test (DAT)

The presence and titre of leishmanial antibodies were measured by the Direct Agglutination Test (DAT). DAT was performed as described [11], with serum titrations of 1/100 (numerically scaled at 0) up to 1/102400 (scaled at 10). The cut-off titre was 1/1600 (scaled at 4), as established for the area [12]. Titres were expressed in numerical scales ranging from −1 (titres < 1/100) to 11 (titres > 1/102,400).

Plasma levels of cytokines

Plasma levels of interferon (IFN)-γ, interleukin (IL)-4, and IL-10 were measured in a solid phase sandwich ELISA.

Plates were supplied pre-coated with mouse monoclonal antibodies to human IFN-γ, IL-4, or IL-10. Kits for IFN-γ, interleukin (IL)-4, and IL-10 were from Diaclone Research (BesanÇon, Cedex, France) with detection limit less than 5.0 pg/ml. Test procedures were performed as described by the manufacturers.

FACS scan analysis

Frozen PBMC were thawed and resuspended in Iscoves IMDM medium supplemented with 10% Foetal Calf Serum (FCS) containing penicillin and streptomycin. Cells were counted by Coulter cell counter (Coulter Multisizer II) and adjusted to a density of 1×10^7/ml. Microtitre plates containing 1×10^6 cells per well were incubated with saturating amounts of flourescein labelled MoAbs to CD4, CD8, CD45RO, CD27, and the corresponding isotype controls for 30 min at 4°C in the dark. Allophycocyanin (APC)-conjugated CD4 and Peridinin Chlorophyll Protein (PerCP)-conjugated CD8 were purchased from Becton Dickinson (San Jose, CA). Fluorescein isothiocyanate (FITC)-conjugated CD27 and phycoerythrin (PE)-conjugated CD45RO were from Sanquin Reagents (Amsterdam). Cells were finally washed twice and resuspended in 150 μl fixation buffer (4% paraformaldehyde), after which data acquisition and analysis were performed by 4-color flow cytometry (FACS Calibur; Becton Dickinson, San Jose, CA), and using Cell Quest software (Becton Dickinson, San Jose, CA). In all circumstances, a minimum of 50,000 events in a lymphocyte gate was analysed. $CD4^+$ and $CD8^+$ T cells were sub-divided into naïve ($CD45RO^-CD27^+$), memory ($CD45RO^+CD27^+$), effector ($CD45RO^-CD27^-$), and memory–effector ($CD45RO^+CD27^-$) subsets. Phenotype characterisation of $CD4^+$ and $CD8^+$ T cells was performed according to the procedures described by Hamann et al. [13] except that we have used CD45RO marker instead of CD45RA. Total and differential counts of leucocytes were coupled with flow cytometric data to determine counts of CD3, CD4, and CD8 T cells including counts of T cell subsets classified as naïve, memory, and effector cell sub-populations. A sequential collection of PBMC available for VL patients before and after treatment, and sub-clinical cases were analysed.

Intracellular staining for IFN-γ and IL-4

The procedures for intracellular staining of IFN-γ and IL-4 were adapted from Jung et al. [14] and Waldrop et al. [15] and modified as follows. Frozen PBMC were thawed and resuspended in Iscoves IMDM medium supplemented with 10% Foetal Calf Serum (FCS) containing penicillin and streptomycin; washed twice with Iscoves IMDM and resuspended in IMDM containing 10 ng/ml PMA (Phorbol 12-myristate 13-acetate, Sigma P-8139), 2 μg/ml ionomycin (Sigma, I-0634), and 3 μM monensin (M-5273) or in medium control (complete medium containing only 3 μM

monensin). Cells were aliquoted into 24-well plates (2×10^6 cells per well) and incubated 4 h at 37°C under 5% CO_2. Cells were then harvested and washed with 0.5% BSA/PBS and stained with CD4-APC and CD8-PerCP MOAbs for 30 min in the dark at 4°C. After further washing, cells were first fixed in ice with 4% PFA (paraformaldehyde) for 5 min and subsequently treated with FACS permeabilising solution at room temperature for 10 min. Cells were then washed and subjected to intracellular staining using saturated concentrations of IL4-PE and IFN-γ-FITC (both Becton Dickinson, San Jose, CA), for 30 min at 4°C in the dark. Cells were finally washed twice and resuspended in 150 μl of fixation buffer (4% PFA), after which data acquisition and analysis were performed as described above. A minimum of 10,000 events in a lymphocyte gate was analysed.

Data analysis

Data on cell counts and plasma levels of cytokines were analysed first by one-way ANOVA and by group-wise comparison using the non-parametric statistic, Kruskal–Wallis [Wilcoxon two-sample] test. SPSS for Windows Standard Version (release 7.5, 1996; SPSS Inc.) and EPINFO version 6.03 (CDC/WHO) were used.

Data on intracellular production of IFN-γ and IL-4 by $CD4^+$ and $CD8^+$ T cell subsets were transformed into a standardised relative index (SRI), computed as the difference in percentages of cytokine producing cells between stimulated and unstimulated cell cultures further divided by the percentage of cytokine producing cells of the respective unstimulated cultures. The SRI enabled us to make a uniform comparison between different sets of experiments, and to show how many times higher the excess cytokine production was attributed to stimulation in comparison with unstimulated cells. It also helped to standardise the differences observed by unstimulated cells of different patient groups.

Results

Characteristics of study subjects and clinical findings

The duration of illness of VL patients was 6.9 (mean) ± 4.5 months (SD). Anemia was common in all groups (Table 1). None of the VL patients presented with malaria, pulmonary tuberculosis, pneumonia, or other intercurrent infections and diarrhea was infrequent. Concomitant infestation by helminths was more common in VL patients (Table 1). *Giardia lamblia* and *Entamoeba histolytica* were the commonest protozoa.

Distribution of T cell subsets in healthy controls

The data on T cell subset distribution in the healthy controls are consistent with earlier reports from Ethiopia and

Table 1
Demographic, clinical, and laboratory characteristics of study subjects

Characteristics	Groups of study subjects				
	1	2	3	4	5
Number of subject (M, F)	$N = 33 (24, 9)^5$	$N = 32 (24, 8)^5$	$N = 14 (8, 6)$	$N = 34 (23, 11)$	$N = 19 (8, 11)^{1,2}$
Age (median, IQ range)	$14 (12, 16)^4$	$14.4 (11.1, 18.7)^4$	17 (12.8, 47)	$22.7 (13.8, 40.5)^{1,2,5}$	$13 (7, 17)^4$
BMI (median, IQ range)	$14.4 (13.3, 17.4)^{2,4}$	$15.4 (14.6, 19.3)^1$	16.2 (14.7, 19.2)	$19.2 (15.4, 20.5)^1$	15.5 (14.4, 18.3)
PCV (%, median, IQ range)	$25 (23, 30)^{2,3,4,5}$	$36 (31, 40)^1$	$38 (34, 44)^1$	$36.5 (33, 38)^1$	$34 (29, 37)^1$
WBC count/mm3 (median, IQ range)	$4150 (3550, 5000)^{2,4,5}$	$5100 (4750, 6050)^1$	5175 (3600, 6250)	$6050 (4650, 7650)^1$	$5650 (5100, 8900)^1$
% of patients with pos. DAT	100	95.8	100	50	0.0
% of patients with pos. LST	0.0	31.6	0.0	100	0.0
% of patients with helminths	30.3	18.8	7.1	2.9	0.0
% of patients with protozoa	18.2	21.9	16.7	15.4	0.0

DAT = Direct Agglutination Test; LST = leishmanin skin test; PCV = Packed Cell Volume; WBC = white blood cell count; M = males; F = females. Groups 1 to 5 are: 1 = active visceral leishmaniasis patients, 2 = treated visceral leishmaniasis patients, 3 = persons with sub-clinical infection, 4 = persons with asymptomatic infection, and 5 = healthy controls. Statistically significant differences (SSDs) are based on P values < 0.05. Statistical note: superscripts ([1,2,3,4,5]) are groups with which statistically significant differences (SSDs) were observed. (a) Age: SSDs between group 4 versus groups 1, 2, and 5; (b) BMI: SSDs between group 1 versus groups 2 and 4; (c) PCV: SSDs between group 1 versus all other groups; (d) Total WBC count: SSDs between group 1 versus groups 2, 4, and 5.

differ from those in European populations with reduced $CD4^+$ and increased $CD8^+$ T cells (Figs. 1 and 2) [16–20]. Characteristics and significance of these findings have been discussed elsewhere [21,22]. The numbers of $CD3^+$, $CD4^+$, and $CD8^+$ cells in males and females were not significantly different but the mean and median CD4:CD8 ratio in females were higher (1.45 and 1.31) than in males (0.90 and 0.83) (data not shown) as reported by Tsegaye et al. [16] and
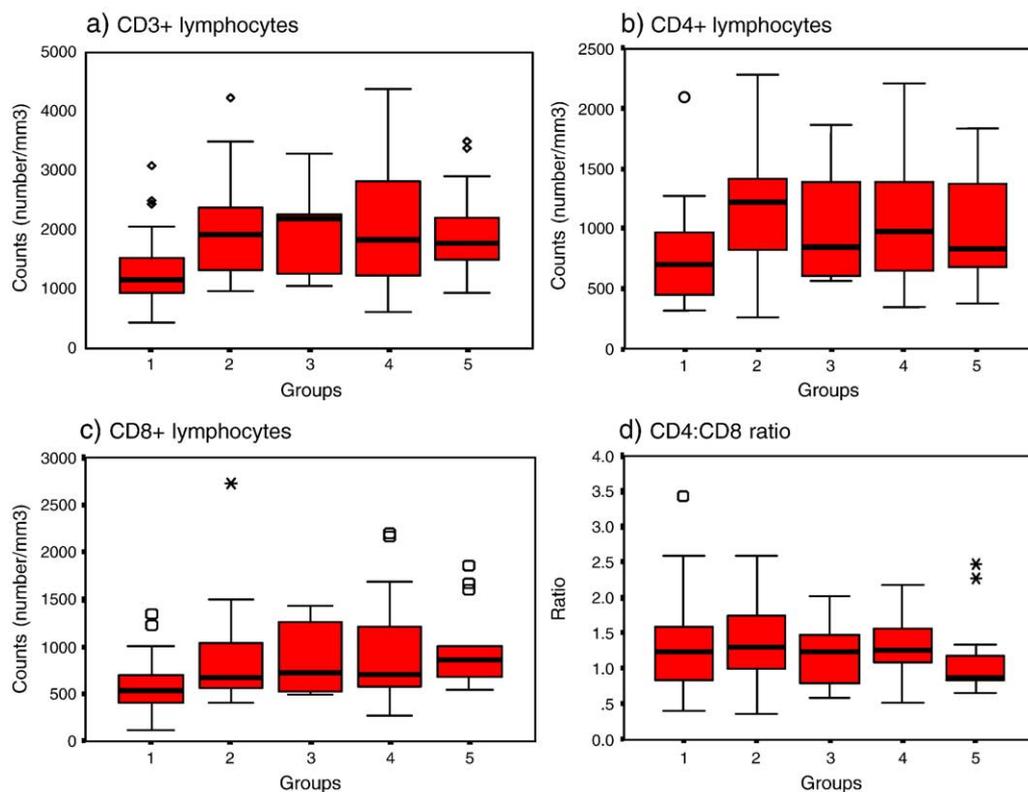

Fig. 1. Groups 1 to 5 are: 1 = active visceral leishmaniasis patients, 2 = treated visceral leishmaniasis patients, 3 = persons with sub-clinical infection, 4 = persons with asymptomatic infection, and 5 = healthy controls. Statistically significant differences (SSDs) are based on P values < 0.05. Box plot shows median (middle thick line across the box), inter-quartile [IQ] range (vertical ends of the box), extremes (*), outliers (○), and whiskers [⊥] (lines extending from the box to the highest and lowest values excluding the outliers and extremes). Extremes are cases with more than 3.0 box lengths from the upper or lower edge of the box; and outliers are cases with 1.5–3.0 box lengths from the edges of the box. Extremes and outliers have been included in statistical computations. Note: $CD4^+$ lymphocyte count and CD4:CD8 ratio are lower than in European populations whilst $CD8^+$ lymphocyte count is higher [16–20]. Statistical note: (a) CD3 counts: SSDs between group 1 versus groups 2, 4, and 5; (b) CD4 counts: SSDs between group 1 versus groups 2 and 4; (c) CD8 counts: SSDs between group 1 versus groups 2, 4, and 5; (d) CD4/CD8 ratio: SSD between groups 4 and 5.
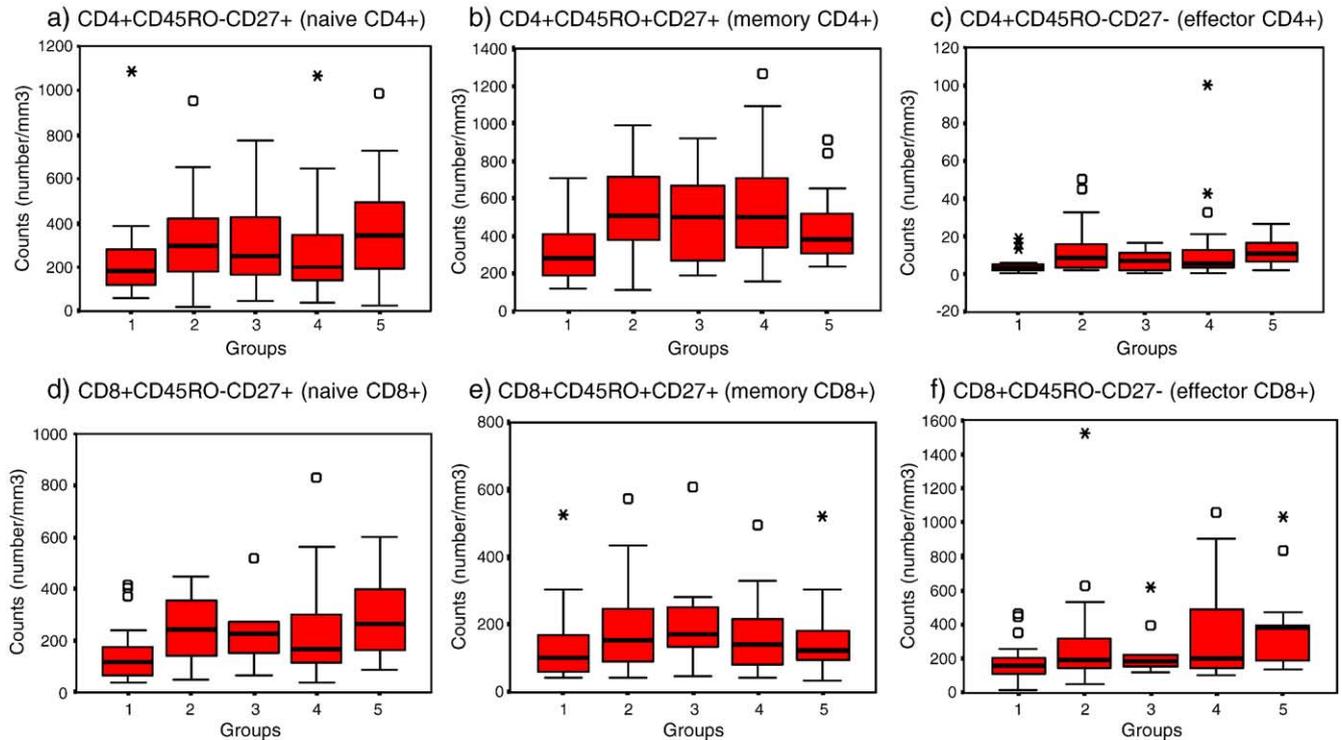
Fig. 2. Groups 1 to 5 are: 1 = active visceral leishmaniasis patients, 2 = treated visceral leishmaniasis patients, 3 = persons with sub-clinical infection, 4 = persons with asymptomatic infection, and 5 = healthy controls. Statistically significant differences (SSDs) are based on P values < 0.05. Box plot shows median (middle thick line across the box), inter-quartile [IQ] range (vertical ends of the box), extremes (*), outliers (○), and whiskers [⊥] (lines extending from the box to the highest and lowest values excluding the outliers and extremes). Extremes are cases with more than 3.0 box lengths from the upper or lower edge of the box; and outliers are cases with 1.5–3.0 box lengths from the edges of the box. Extremes and outliers have been included in statistical computations. Note: naïve CD4$^+$ and CD8$^+$ counts and memory CD4$^+$ counts are lower than in European populations whilst effector CD8$^+$ counts are higher [16–20]. Statistical note: (a) naïve CD4 counts: SSDs were not observed; (b) memory CD4 counts: SSDs between groups 1 versus 2, 4, and 5; (c) effector CD4 counts: SSDs between groups 1 versus 2 and 5; (d) naïve CD8 counts: SSDs between groups 1 versus 2 and 5; (e) memory CD8 counts: SSDs were not observed; (f) effector CD8 counts: SSDs between groups 1 versus 4 and 5.

Kassu et al. [17]. The relevance of this gender related difference as a possible risk factor for the increased incidence of VL in males needs further investigation.

Distribution of T cell subsets in patient groups

For comparison of T cell subset counts, children under 6 years of age were excluded, as recommended [23]. Profiles of CD3$^+$, CD4$^+$, and CD8$^+$ T cell subset counts and CD4:CD8

ratios in the different groups are presented in Fig. 1. Significant increases (P < 0.05) in CD4$^+$ and CD8$^+$ T cells (and CD4: CD8 ratio) were associated with cure after treatment.

CD45RO and CD27 expression in CD4$^+$ and CD8$^+$ T cell subsets

There was a significantly lower number of naïve (CD45RO$^-$CD27$^+$) CD8$^+$ T cells in the active visceral leishmaniasis patients compared to treated patients and healthy

Fig. 3. The x-axis shows time ranges in months after treatment of visceral leishmaniasis patients, i.e., 0 = immediately before treatment, 3–6 months and 9–15 months in comparison with HC = Healthy Controls. Statistically significant differences (SSDs) are based on P values < 0.05. Box plot shows median (middle thick line across the box), inter-quartile [IQ] range (vertical ends of the box), extremes (*), outliers (○), and whiskers [⊥] (lines extending from the box to the highest and lowest values excluding the outliers and extremes). Extremes are cases with more than 3.0 box lengths from the upper or lower edge of the box; and outliers are cases with 1.5–3.0 box lengths from the edges of the box. Extremes and outliers have been included in statistical computations. Statistical note: (a) CD3$^+$: SSDs observed between visceral leishmaniasis patients before treatment and cured visceral leishmaniasis patients 9–15 months after treatment and healthy controls; (b) CD4$^+$: SSDs observed between visceral leishmaniasis patients before treatment and cured visceral leishmaniasis patients 9–15 months after treatment; (c) CD8$^+$: SSDs observed between visceral leishmaniasis patients before treatment and healthy controls; (d) CD4:CD8 ratio: SSDs observed between treated visceral leishmaniasis patients and healthy controls, the ratio being increased in visceral leishmaniasis before and after treatment cf. healthy controls; (e) CD4$^+$CD45RO$^-$CD27$^+$ (naïve CD4$^+$): SSDs observed between untreated visceral leishmaniasis patients and cured visceral leishmaniasis patients 9–15 months after treatment; (f) CD4$^+$CD45RO$^+$CD27$^+$ (memory CD4$^+$): SSDs observed between visceral leishmaniasis patients before treatment (lowest counts) and healthy controls/and cured visceral leishmaniasis patients (highest counts); (g) CD4$^+$CD45RO$^-$CD27$^-$ (Effector CD4$^+$): no SSDs observed; and (h) CD8$^+$CD45RO$^-$CD27$^+$ (naïve CD8$^+$): SSDs observed between visceral leishmanaisis patients before treatment (lowest counts) and cured visceral leishmaniasis patients 9–15 months after treatment (highest counts)/healthy controls, and within groups of cured visceral leishmaniasis patients evaluated 3–6 and 9–15 months after treatment; (i) CD8$^+$CD45RO$^+$CD27$^+$ (memory CD8$^+$): no SSDs observed; and (j) CD8$^+$CD45RO$^-$CD27$^-$ (Effector CD8$^+$): SSDs observed between visceral leishmaniasis patients before treatment and healthy controls.

a) CD3 counts
b) CD4 counts
c) CD8 counts
d) CD4:CD8 ratio
e) CD4+CD45RO-CD27+ (naive CD4+)
f) CD4+CD45RO+CD27+ (memory CD4+)
g) CD4+CD45RO-CD27- (effector CD4+)
h) CD8+CD45RO-CD27+ (naive CD8+)
i) CD8+CD45RO+CD27+ (memory CD8+)
j) CD8+CD45RO-CD27- (effector CD8+)
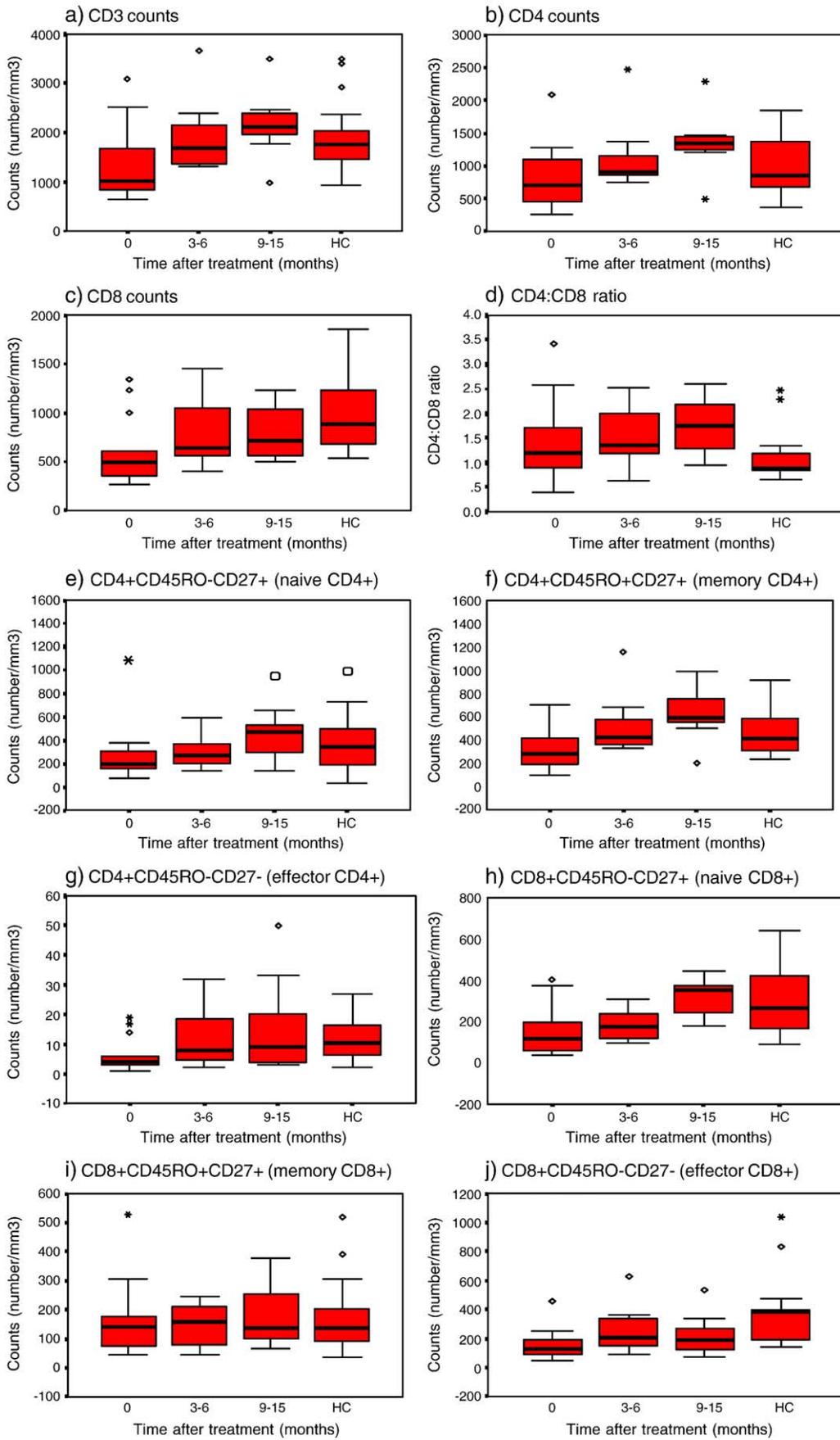
controls (Fig. 2d); but not for naïve CD4$^+$ T cells (CD45RO$^-$CD27$^+$). Memory CD4$^+$ T cells (CD45RO$^+$CD27$^+$) were lowest in active VL patients (Fig. 2b, $P < 0.05$); and memory CD8$^+$ T cells (CD45RO$^+$CD27$^+$) were not different between the groups (Fig. 2e).

Pre- and post-treatment T cell subset distribution

Sequential pre- and post-treatment data of VL patients show that the numbers of CD3$^+$, CD4$^+$, naïve CD4$^+$, naïve CD8$^+$, and memory CD4$^+$ T cells increased and reached the highest numbers around 12 months after treatment (Fig. 3). In particular, the numbers of memory CD4$^+$ T cells increased significantly ($P < 0.05$) after cure (Fig. 3). These observations indicate the restoration of immune homeostasis as well as a build-up of memory.

Intracellular detection of IFN-γ and IL-4

The numbers of CD4$^+$ and CD8$^+$ T cells producing IFN-γ and IL-4 after a 4-h stimulation with PMA and ionomycin in the presence of monensin were enumerated by 4-color staining and flow cytometry. Results expressed as standardised relative index (SRI), show that the median SRI of CD8$^+$ T cells assessed for IL-4 production was less than 3.0 in all groups of patients (Fig. 4). IL-4 and IFN-γ production by both CD4$^+$ and CD8$^+$ cells was lowest in active VL patients ($P < 0.05$), and highest in the control group. The median SRI values of asymptomatic subjects (group 4) were not different from those of healthy controls ($P = 0.16–0.63$), suggesting that T cells from asymptomatic individuals have the same capacity to produce IFN-γ and IL-4 as healthy controls.

A sequential analysis of SRI profiles of 5 sub-clinical patients, with suspected incubating VL (i.e., individuals with high titres of antibodies and with or without minor constitutional symptoms), was performed for a period of 3 to 12 months. Two of these converted to active VL in this period. The SRI values of these two patients were indistinguishable from those of active VL patients throughout the period of investigation. On the other hand, a 10–20-fold increase of SRI values was noted in two of the three who did not convert to active VL (data not shown). SRI
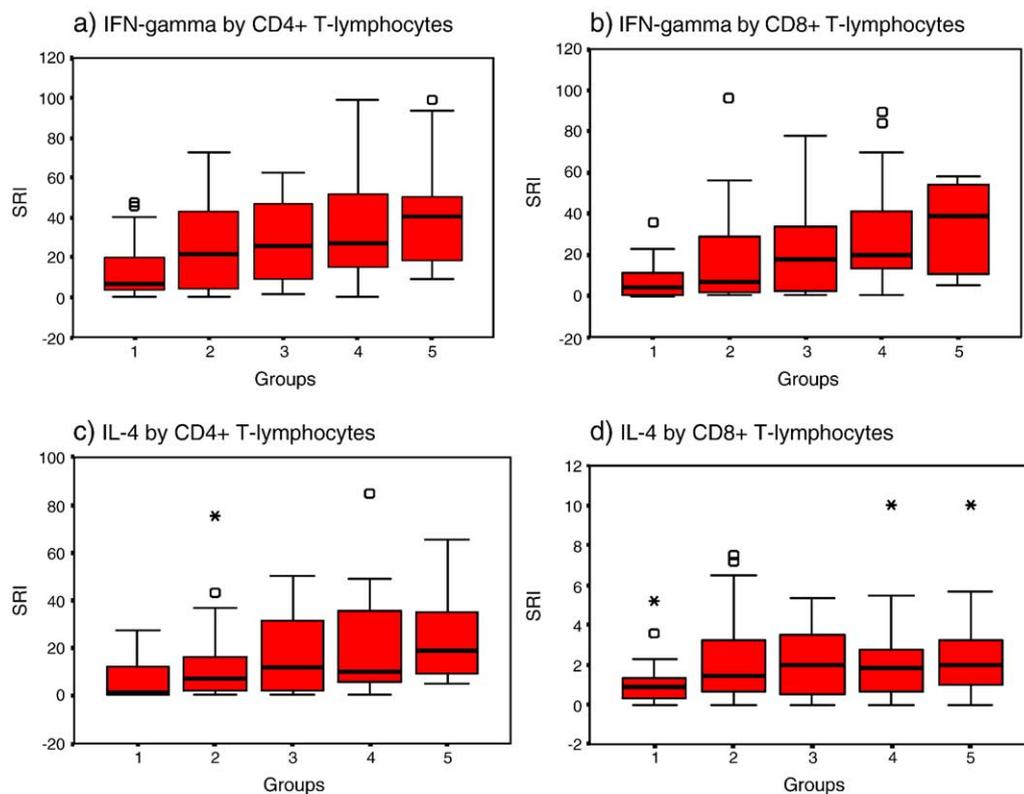

Fig. 4. Groups 1 to 5 are: 1 = active visceral leishmaniasis patients, 2 = treated visceral leishmaniasis patients, 3 = persons with sub-clinical infection, 4 = persons with asymptomatic infection, and 5 = healthy controls. Statistically significant differences (SSDs) are based on P values < 0.05. Box plot shows median (middle thick line across the box), inter-quartile [IQ] range (vertical ends of the box), extremes (*), outliers (○), and whiskers [⊥] (lines extending from the box to the highest and lowest values excluding the outliers and extremes). Extremes are cases with more than 3.0 box lengths from the upper or lower edge of the box; and outliers are cases with 1.5–3.0 box lengths from the edges of the box. Extremes and outliers have been included in statistical computations. Data on intracellular production of IFN-γ and IL-4 by CD4$^+$ and CD8$^+$ T-cell subsets were transformed into a standardised relative index (SRI), by computing the difference in percentages of cytokine producing cells between stimulated and unstimulated cell cultures and further dividing it by the percentage of cytokine producing cells in unstimulated culture of the respective patient or individual. Statistical note: (a) IFN-γ by CD4$^+$ T cells: SSDs observed between group 1 versus groups 4 and 5; (b) IFN-γ by CD8$^+$ T cells: SSDs observed between group 1 versus groups 3, 4, and 5; and group 2 versus 4 and 5; (3) IL-4 by CD4$^+$ T cells: SSDs observed between group 1 versus groups 3, 4, and 5; and group 2 versus 5; (d) IL-4 by CD8$^+$ T cells: SSDs were observed between groups 1 and 5.

a) IFN-gamma in plasma
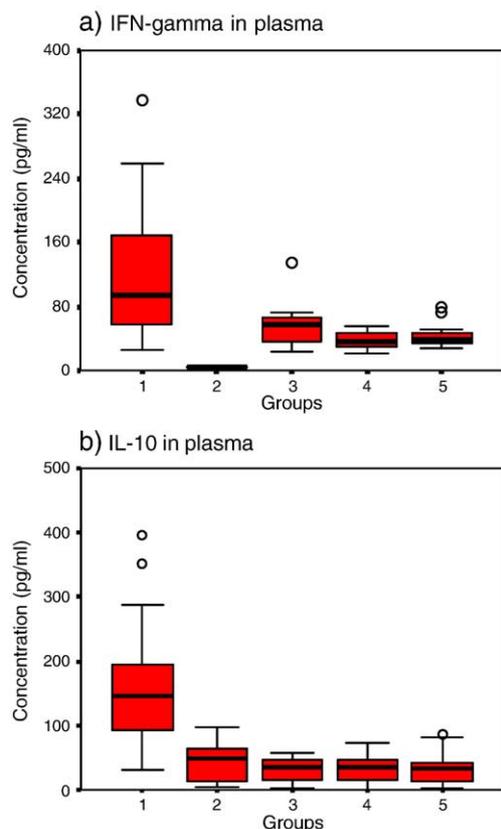

b) IL-10 in plasma

Fig. 5. Groups 1 to 5 are: 1 = active visceral leishmaniasis patients, 2 = treated visceral leishmaniasis patients, 3 = persons with sub-clinical infection, 4 = person with asymptomatic infection, and 5 = healthy controls. Statistically significant differences (SSDs) are based on P values < 0.05. Box plot shows median (middle thick line across the box), inter-quartile [IQ] range (vertical ends of the box), extremes, outliers (○), and whiskers [⊥] (lines extending from the box to the highest and lowest values excluding the outliers and extremes). Extremes are cases with more than 3.0 box lengths from the upper or lower edge of the box; and outliers are cases with 1.5–3.0 box lengths from the edges of the box. Extremes and outliers have been included in statistical computations. Statistical note: (a) IFN-γ in plasma: SSDs observed between group 1 versus groups 2, 3, 4, and 5; (b) IL-10 in plasma: SSDs observed between group 1 versus groups 2, 3, 4 and 5.

values in the third patient were high at the beginning and remained high during 5 periods of half-yearly sampling.

Plasma concentrations of IFN-γ, IL-4, and IL-10

Plasma concentrations of both IFN-γ and IL-10 were significantly higher in patients with active VL (Fig. 5), and above the 75%ile concentration of controls in 77.7% and 92.3% of the active VL patients, respectively (data not shown). IL-4 was not detectable in plasma of any patient or control.

Discussion

Protective immunity in *Leishmania* infections is generally attributed to an immune response that generates IFN-γ producing CD4+ cells [24]. Thus, many studies of *Leish-*

mania vaccines have focused on antigens that generate CD4+ Th1 cells [25]. Changes in CD4+ but not CD8+ T cell subsets were found to be associated with active VL implying that impaired cell-mediated immune responses are related to suppression of the CD4+ T cell subset [26]. On the other hand, the role of CD8+ T cells during cell-mediated responses to *Leishmania* has been equivocal. In experiments involving CD8-deficient mice, control of *L. major* infection did not depend on CD8+ T cell subsets [27]. Other studies have shown that CD8+ T cell subsets contribute significantly to the production of IFN-γ [28 29]. In this study, we observed that CD8+ T cells in all groups of individuals contribute little to the production of IL-4 and that both CD4+ and CD8+ T cells of VL patients could not be stimulated to produce either IFN-γ or IL-4 suggesting a profound impairment of cell-mediated immune responses. After cure, both cell groups regained the capacity to produce IFN-γ and IL-4. In contrast, we found elevated or high plasma levels of IFN-γ and IL-10 during active VL (Fig. 5), as others did before [30–32]. IL-10, IL-4, and IFN-γ have been detected in PKDL lesions [33,34], bone marrow [35], spleen [36], and lymph nodes of VL patients [33]. The observed disparity between high plasma concentrations of IFN-γ and the decreased capacity of T cells to produce it imply that IFN-γ may be of different origin, presumably NK cells. It is well known that NK cells when stimulated with IL-12 and IL-15 produce increased amounts of IFN-γ; and in VL, where plasma concentrations of IFN-γ inducing cytokines are increased [37], the proposition that NK cells could be the source of IFN-γ is convincing. Why T cell responses are depressed in VL amidst high plasma concentrations of IFN-γ remains to be answered. The chronic stimulation of T cells in active VL due to high antigenemia could lead to apoptotic elimination of Fas and Fas-L expressing T cells.

IL-10 plays a key role in the pathogenesis of leishmaniasis especially in the down-regulation of Th1 responses [32,33,38,39]. This is supported by observations of macrophage deactivation and inhibition of leishmanicidal functions [40–42], suppression of IFN-γ production by T and NK cells [43], and down-regulation of IL-12 signals of macrophage activation [44]. The possible role of other mechanisms that may interfere with ligand binding and signalling events of IFN-γ and its receptors like secretion of soluble IFN-γ receptors or down-regulation of its expression on mononuclear phagocytic cells has not been ruled out.

The parasite replication and strong Th1 response that were concurrent phenomena in a hamster model of VL [45], are reminiscent of the high plasma levels of IFN-γ in our VL patients, which imply that a negative regulation of intact Th1 responses exists during active VL. The view that Th1/Th2 polarity determines the direction and nature of cell-mediated immune responses in leishmania infections, so clearly described in animal models, is not so clearly delineated in human VL.

Our data, although limited by sample size, also indicate that the impairment of T cell function may be an early event. This is in line with the general observation that lympho-proliferative responses [33] and in vitro production of IFN-γ, IL-4, and IL-10 [26,38–40,46] by PBMCs of VL patients are suppressed in response to specific antigen or polyclonal stimulation in vitro.

Previously, reduced levels of memory T cells (CD3$^+$ CD45RO$^+$) and of CD2$^+$ and CD4$^+$ cells have been reported [30,47]. We have now observed that the numbers of memory CD4$^+$ T cells (Fig. 2b) were also markedly increased in persons with asymptomatic infection with positive leishmanin skin test. The significance of the increase of memory CD4$^+$ T cells in this group and the relationship with a positive leishmanin test and acquired immunity needs further explanation. It will be of interest to evaluate other indicators of T cell memory like CCR7 (reviewed by Jenkins; in Ref. [48]) in patients with VL.

A major hurdle in assessing the type of Th responses during infections has been the lack of definite markers for Th1 and Th2 phenotypes. In experimental vaccine studies of leishmaniasis, such criteria as the induction of IL-12 production and proliferation of IFN-γ producing CD4$^+$ T cells are relied upon in assessing the effectiveness of vaccines. However, several vaccines that induced a Th1-type response did not lead to protection of challenge infection in animal models [25,49,50], undermining the equation between protective immune responses of *Leishmania* infection and Th1 responses. The inclusion of a procedure that evaluates the cellular immune phenotype in terms of CD4$^+$ memory might prove a useful addition in assessing the immunopro-phylactic potentials of immunogens and vaccines. Thus, the inclusion of CD45RO and CD27 markers or other markers such as CCR7 as phenotypes of a sustained acquired or vaccine-induced immunity needs further evaluation.

In this study, we observed that helminthic infections were more prevalent in VL patients compared to other groups (Table 1). In view of the fact that helminths induce production of type 2 cytokines, it would be of interest to examine the role of helminthiases, if any, in the develop-ment of active VL in endemic areas.

Acknowledgments

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