Evaluation of the role of pro and anti-inflammatory cytokines in new endemic foci of *Leishmania donovani* prevalent in Sutlej river valley of Himachal Pradesh

SUSHILA DEVI NEGI[1], AFROZ ALAM[2], PRAVIN KUMAR TRIPATHI[3], MOHAMED AZHAR KHAN[1], UMAR FAROOQ [1,4,*]

[1]*Molecular and Immuno-parasitology Research Laboratory, Faculty of Applied Sciences and Biotechnology, Shoolini University, Solan, HP, India;* [2]*Faculty of Parmacy, Shoolini University, Solan, HP, India;* [3]*Department of Medical Parasitology, Postgraduate Institute of Medical Education and Research, Chandigarh, India;* [4]*Faculty of Dentistry, Taif University, Taif, KSA*

Received 25 December 2016
Accepted 16 June 2017

ABSTRACT

Objectives: Leishmaniasis is a major public health problem in tropical and subtropical countries of the world. The present study was aimed to assess the prevalence of disease as well as to know the pro and anti-inflammatory cytokine response against the infection.
Methods: 100 samples were collected from individuals suspected of leishmaniasis, from different clinics and hospitals situated in Rampur Bushair and Kinnaur, HP. Indirect ELISA was performed to detect anti-leishmanial IgG, and its isotypes in serum samples. The expression level of serum cytokines; TNF-α, INF-γ, IL-2, IL-4, IL-6, IL-10 and IL-17A was measured by cytokine bead array system (BD Biosciences) and by using Flowcytomer (BD. Cantotm II).
Results: Significantly high levels of IgG antibodies were detected in 42/100 (42%) individuals. Among IgG isotypes, the significantly high levels of IgG1 (38.1%) was observed followed by IgG3 (26.2%) and IgG4 (9.52%) respectively. However, the IgG2 subclass did not show any significant difference. The prevalence of IgG antibodies was observed high among 21-40 years of age group. The flowcytometric analysis of the cytokines IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α and IFN-γ revealed that the pro-inflammatory cytokine IL-2 was expressed in maximum levels followed by TNF-α, IFN-γ and IL-6, while IL-17A showed the minimum levels of expression. The expression of pro-inflammatory cytokine IL-2 and anti-inflammatory cytokine IL-10 was observe very high.
Conclusion: The study shown that the Sutlej river valley is a new endemic focus for *L. donovani* and the protective immune response could not develop in the native population. Cytokines analysis revealed that the parasite prevalent in this region is highly virulent and causes more pathogenic effect as this is the new endemic foci and there is no earlier exposure to the parasite.

KEY WORDS: *Leishmania donovani*, Prevalence, Cytokines, Flowcytometry

Introduction

Leishmaniasis is a vector-borne infectious disease caused by a group of protozoan parasites of the genus *Leishmania* [1]. It is one of the major public health problem in tropical and subtropical countries of the world including India. It is transmitted through the bite of infected sand flies of the genus *Phlebotomus* [2]. Visceral leishmaniasis (VL) is a serious problem in various states of India [3]. Localized cutaneous leishmaniasis (LCL) is usually due to *L. tropica* and is prevalent in the deserts of Rajasthan and Himachal Pradesh. Recently Himachal Pradesh has been a new endemic focus for VL[4]. The association of VL and human immunodeficiency virus (HIV) infection has proved that VL is an opportunistic infection due to immune suppression [5].
During early infection with *Leishmania* parasite, both resistant and susceptible hosts exhibit mixed Th1 and Th2 type responses of $CD4^+$ T cell population with IL-2, IL-4 and IL-13 production, while IL-6 is induce often together with IL-1 and TNF-α in many alarming conditions[6]. However, IL-6 has been proposed to favor the development of Th2 responses. IL-6 deficient mice led to down regulation of both Th1 (IL-12) and Th2 (IL-4, IL-10 and IL-13) associated cytokines, and in *L. major* infection, IL-6 showed to promote the development of both Th1 and Th2 responses [7].

Correspondence to: Dr. Umar Farooq
Email: ufarooq8@gmail.com

Whereas, IL-2 produced by Th-1 cells, is a potent inflammatory cytokine mediating multiple immune responses on activated B cells, monocytes, and natural killer (NK) cells [8,9]. IL-4 is considered to be the signature cytokine of Th2 response. IL-4 is involved in the down- regulation of the Th1 type of response in human leishmaniasis[10,11]. But it

also does not suppress the production of IFN- γ in cure of leishmaniasis. IL-10 is an important immune-modulatory cytokine that can be produced by monocytes, macrophages, mast cells, natural killer cells, B cells, $CD4^+$, $CD8^+$ T cells, T reg, Th1, Th2 and Th-17 cell subset [12,13]. It is able to block the production of IL-1, IL-6 and TNF-α and other cytokines[14]. Some studies show that IL-10 blockade can enhance VL, PBMC proliferation, IFN-γ responses and inhibit VL serum promoted parasite replication in macrophages[15.16.17]. In addition, IL-10 was also shown to induce the proliferation of B-lymphocytes and most notably their differentiation into plasma cells secreting immunoglobulins at high rate [18]. IL-10 inhibits the microbicidal activity of interferon gamma (IFN-γ) treated macrophages, against intracellular parasites[19]. IL-10 and IL-4 inhibit intracellular killing of *L. infantum* and *L. major* by human macrophages by falling nitric oxide production. IL-17 is a pro-inflammatory cytokine that is secreted primarily by activated T cells (CD4, CD8 cells). It stimulates a variety of cells (e.g. fibroblasts, macrophages, endothelial cells) to produce inflammatory mediators including IL-1, TNF-α and chemokines. TNF-α is a cytokine involved in systemic inflammation and is a member of cytokines that stimulate the acute phase reactions [20]. The primary sources of TNF-α are monocytes/macrophages activated by various parasite products. TNF-α is not just a pro-inflammatory cytokine, it has also been proposed to be an immune-regulatory molecule that can alter the balance of T regulatory cells [21]. Studies have shown that large CL lesions correlate with a higher frequency of lymphocytes producing *Leishmania* soluble antigen specific inflammatory cytokines (IFN-*γ* or TNF-*α*)[22]. Infection of *Leishmania* in human is detected by the appearance of anti-leishmanial antibodies in the sera of the patients. During CL, they are present at low levels during active phase of the disease [23]. In contrast, strong anti-leishmanial antibody titers are well known in VL [24.25]. Critical analysis of *Leishmania* antigen specific immunoglobulin isotopes have shown raised levels of IgG, IgM, IgE and IgG subclasses during disease [26,27,28,29]. IgG not only fails to deliver protection against this intracellular parasite, but it actually contributes to disease progression [30]. The present study was aimed to determine the prevalence of Visceral Leishmaniasis and role of cytokines in regression and progression of pathogenicity and immunity in *Leishmania* infected individuals in the Sutlej river valley of Himachal Pradesh.

Materials and Methods

Study area: Himachal Pradesh

The study area comprised different clinics and Hospitals situated at Rampur Bushair and Kinnaur district, situated in Sutlej river valley of Himachal Pradesh, India. These areas are the most active endemic pockets and previously demarcated as endemic area for human leishmaniasis.

Ethical approval

Ethics committee of University approved the project.

Sample collection

Samples were collected from leishmaniasis individuals attending out patients department of regional hospitals situated in Rampur Bushier and Kinnaur District, Himachal Pradesh or clinics/hospitals situated at other areas of Sutlej river valley. This area has been identified as a new endemic focus for *L. donovani.* Overall, 100 samples were collected during July 2010 to March 2013 from visceral leishmaniasis patients. Five millilitres of blood was obtained from each individual before starting the anti-leishmanial therapy. Venous blood samples were collected in sterile vacutainers for serum collection and in NNN Medium for *Leishmania* parasite cultivation. The skin scraping was taken from cutaneous leishmaniasis patients and inoculated in NNN medium. The written informed consent was taken from each individual before obtaining the sample. The serum samples were kept in -20^0 C till use.

Preparation of *Leishmania* Antigen

Antigen for ELISA was prepared from promastigotes cultured in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (Sigma-Aldrich). The continuous culture of *Leishmania* strains isolated from clinical samples was maintained in RPMI-1640 medium (Hi-media) with 10% FBS. Initially, the isolate was grown in NNN media with 10% rabbit blood and then the promastigotes were shifted to RPMI-1640 with 10% FBS streptomycin (150 µg/ml), penicillin G (100 µg/ml) and gentamycin (150 µg/ml) at pH 7.2. The parasites were harvested in late log phase and centrifuged at 1000-1500 rpm for 10 minute. After that the supernatant was taken and centrifuged at 3000 rpm for 10 minutes. The pellet was washed 3-4 times with PBS and re-suspended in ~1ml sterile distilled water and kept at 4ºC overnight. Then sonicated at 20 kilocycles: 3

cycles for 1 min. each with 1 min intermittent gap. Then centrifuged at 10,000g for 10 min at 4ºC and the protein of supernatant was estimated by Lowry's method and stored in aliquots at -20ºC [31].

Enzyme linked Immunosorbent Assay (ELISA)

ELISA was performed as described by Afrin *et al* [32], and Crewther [33]. The optimum dilutions were determined by checker board titrations. Purified antigen was individually diluted to the optimized concentration of 2.5µg per well in carbonate bicarbonate buffer (pH-9.6) and 100µl of antigen was added to each well of micro-titer plates. The plates were kept at 4^0 C overnight. The antigen-coated plates were washed thrice with phosphate buffer saline with 0.1 % Tween 20 (PBST) pH 7.2. One hundred µl of 2% bovine serum albumin (BSA) was then added to each well to block the remaining unbound sites on the plate. Plate was incubated at 37^0 C for one hour and washed thrice with PBST. One hundred microlitre of optimally diluted test and control sera were added in duplicate. After one hour incubation at 37°C, washed three times with PBST. Horseradish peroxidase (HRP) enzyme labeled antihuman IgG (Sigma Aldrich),and IgG1, IgG2, IgG3 and IgG4 HRP conjugates (Invitrogen) were added as secondary antibodies. After one hour incubation at 37°C, plate again washed with PBST and 100 µl of chromogenic substrate i.e. hydrogen peroxide (H_2O_2) and orthophenylenediamine dihydrochloride (OPD, Hi-Media) was added to the plate wells and kept at room-temperature for 15 minutes in dark. The reaction was stopped by adding 100 µl of 6% H_2S0_4 and the absorbance was read on ELISA reader (Biotek, ELx800MS, US) at 490nm.

Analyses of cytokines profiles by Flowcytometric Analysis

The level of serum cytokine was performed by cytokine bead array (BD Biosciences), a system which is a bead-based on flowcytometric assay that permits the simultaneous analysis of different cytokines in a single micro plate well. Serum concentrations of cytokines viz. TNF-α, INF-γ, IL-2, IL-10 and IL-17A were measured by using Cytometric Bead Array (CBA Human soluble protein master buffer kit, B.D. Biosciences) according to manufacturer's instruction and the data was analyzed by Flowcytome BD FACS Canto^{tm} II).

Statistical Analysis

Results were expressed as Mean ± Standard Error (S.E.M.). Different between means were evaluated using t test and $p \leq 0.05$ difference was set as statistically significant.

Results

A total of 100 samples from visceral leishmaniasis patients were analyzed by ELISA. Of these 42 were of males and58 of females. Anti-leishmanial IgG antibody response was found significantly high in 42% serum samples. Of these IgG positive samples, 59.9% were from female patients and 40.5% from males. The highest prevalence of *Leishmania-sis* was found in the age group of 21-40 years, both in males and females (Table 1).

Table 1. Sero-prevalence of total IgG in leishmaniasis in Rampur Bushier and Kinnour District of Himachal Pradesh (n=100).

Sex(n=100)	Age group (Years)	Total	No of Specimens Negative	No of Specimens Positive
Male (n=42)	0-20	8	5	3 (7.1%)
	21-40	29	18	11 (26%)
	41-60	5	2	3 (7.1%)
	61-80	Nil	Nil	Nil
Female (n=58)	0-20	11	8	3 (5.2%)
	21-40	32	18	14 (24%)
	41-60	14	7	7 (12%)
	61-80	1	0	1 (1.7%)
Total		100	58	42

Table 2. Frequency of IgG subclasses (IgG1, IgG3 & IgG4) of leishmaniasis in Rampur Bushier and kinnour District of Himachal Pradesh (n=42).

Whole IgG=42	IgG1=16 (38.1%)	IgG3=11 ((26.1%)	IgG4=4 (.52%)
	Male=7 (43.75%) Female=9 (56.25%)	Male=5 (45.45%), Female=6 (54.54%)	Male=1 (25%), Female=3 (75%)

The subclasses of IgG antibodies were further analyzed in all the 42 IgG positive samples, out of which 17(40%) were from males and 25(60%) from females. In present, study

the frequency of IgG1 subclass was observed to be the highest i.e. 38.1% followed by IgG3 (26.2%) and IgG4 (9.52%) respectively (Table 2 and 3). IgG2 antibodies did not show any significant difference in our study, showing that this antibody has minimum role in leishmaniasis.

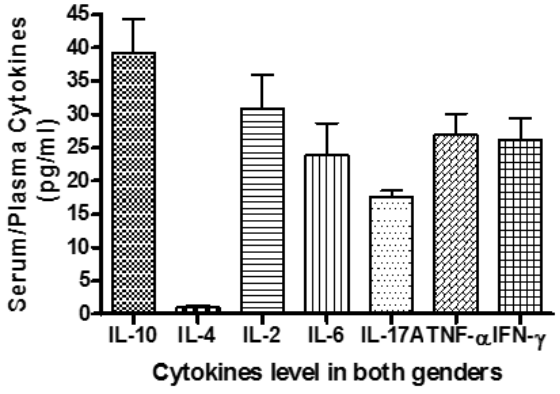
Cytokines analysis

The expression level of pro-inflammatory cytokines viz., IL-2, IL-6, IL-17A, TNF-α and IFN-γ was determined in all the 100 serum samples. IL-2 has showed the significantly high level of expression followed by TNF-α, IFN-γ, IL-6 and IL-17A respectively (Figure 1). On gender basis, significantly high expression of pro-inflammatory cytokines IL-6 was observed in males followed by IFN-γ, TNF-α and IL-2, while, expression level of IL-17A was not significant (Figure 2).

Table 3. Sero-prevalence of IgG subclasses (IgG1, IgG2, IgG3 &IgG4) in leishmaniasis in Rampur Bushier and Kinnour District of Himachal Pradesh in different age groups (n=42).

Age group	IgG1=16		IgG3	=11	IgG4=4	
0-20	Male	1 (6.25%)	Male	1 (9.1%)	Male	Nil
	Female	1 (6.25%)	Female	1(9.1%)	Female	Nil
21-40	Male	5 (31.25%)	Male	3 (27.3%)	Male	1(25%)
	Female	6 (37.5)	Female	4 (36.4)	Female	2 (50%)
41-60	Male	1 (6.25%)	Male	1(9.1%)	Male	Nil
	Female	2 (12.5%)	Female	6 (54.5%)	Female	1 (25%)
61-80	Male	Nil	Male	Nil	Male	Nil
	Female	Nil	Female	1(9.1%)	Female	Nil

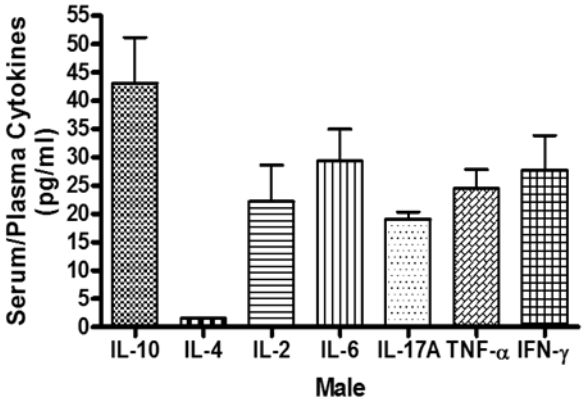
Figure 1. Expression level of pro and anti-inflammatory cytokines in both genders.



However, IFN-γ showed significant expression among females, followed by TNF-α, IL-17A, IL-6 and IL-2 respectively (Figure 3). The pro and anti-inflammatory cytokines were also studied in both males and females (Figure 4).

The expression levels of anti-inflammatory cytokines IL-4 and IL-10 were also determined.

Figure 2. Expression level of pro and anti-inflammatory cytokines in males**.**



IL-4 did not show any significant difference while the levels of IL-10 were significantly high in both the sexes, males and females. Thus, the flowcytometric analysis of pro and anti-inflammatory cytokines among the leishmaniasis patients revealed that the parasite prevalent in this region is highly virulent and may have more pathogenic effect to the patients.

Figure 3. Expression level of pro and anti-inflammatory cytokines in females.

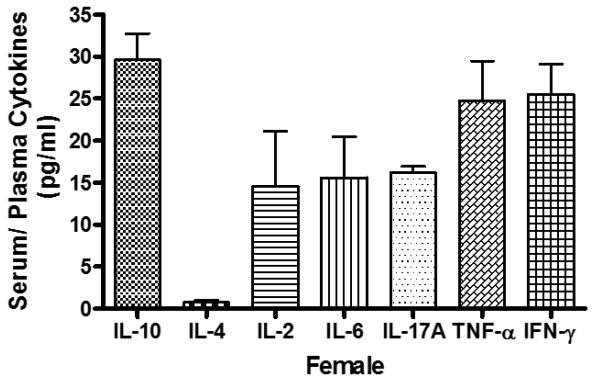
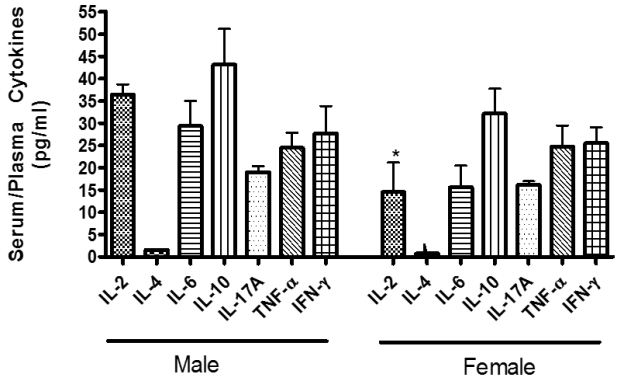


Figure 4. Significant level of pro and anti-inflammatory cytokines in both males and females.



Discussion

Leishmaniasis is characterized by high levels of *Leishmania* specific antibodies [24]. In visceral leishmaniasis patients, significantly high levels of anti-leishmanial antibodies in serum samples have been reported when compared to cutaneous leishmaniasis [33,34]. In the present study, the anti-leishmanial IgG antibodies and its subclasses i.e. IgG1, IgG2, IgG3 and IgG4 antibodies were assessed by ELISA, which like IFAT, is a sensitive technique for the diagnosis of leishmaniasis [35,36].

In our study, the IgG antibody was detected in 42% samples against leishmanial promastigote antigen. This is in agreement with other studies which have reported the predominance of the anti-leishmanial IgG antibodies in visceral leishmaniasis patients from different parts of the world [26,37], as well as from Indian kalaazar patients [34.38,39]. In the present study significant difference was found in between IgG1 and IgG3 subclass while IgG4 has shown moderate difference with IgG1 and IgG3. However, the prevalence of IgG2 was observed very low among the subject. This may be due to small sample size or the isolates prevalent in this region is not able to develop IgG2 response. Similarly, the earlier studies reported the absence or very low prevalence of IgG2 antibodies in the population of leishmaniasis endemic regions [26,28]. The moderate of low prevalence of IgG4 antibody among the population of present study has been supported by previous study of Zwingenberger et al [40] reported low prevalence of IgG4 antibodies among the study population residing in de Sao Paulo, Brazil. Diverse prevalence of IgG antibodies among the population may be due to the new endemic foci of *L. donovani*. The study area (Sutlej River Valley of Himachal Pradesh) is an endemic region for cutaneous leishmaniasis. Indian VL patients also showed a predominance of IgG1 subclass antibody response, but the levels of the IgG3, IgG4, and IgG2 antibody level were also significant. The subclasses of human IgG antibodies are equipped with unique functional and biological properties, including their response to various types of parasitic antigens [41]. The elicitation of IgG1, IgG3, and IgG4 antibodies in VL patients may be due to the presence of protein antigens, and the elicitation of IgG2 antibodies in VL patients might be due to the presence of carbohydrate antigens, as reported for viral, bacterial, and parasitic infections [42].

ELISA analysis indicates that the serum IgG appears to be maximally related to the IgG1 isotype, although significant reaction with IgG3 followed by IgG4 and IgG2 was not observed in any of the serum samples. The study reveals that the strain prevalent in Sutlej river valley elicits IgG1 specific immune response in individual infected with *Leishmania spp* and predominating over other subclasses. However, IgG2 was not detected in any of the individual. Further, the prevalence of IgG1 pre-dominatingly was observed among the native population of Sutlej river valley while all three isotypes i.e. IgG1, IgG3 and IgG4 were observed in the population, migrated from VL endemic region to Sutlej river valley for the development of hydroelectric and other projects. The discrimination of IgG subtype prevalence between these two populations may be due to the new endemic foci for *L. donovani*. Further the study population was divided into different age group, the young age group (21-40-year age) showed the maximum frequency i.e. this age group is more prone to infections. We have observed that females are more susceptible to infection of *Leishmania* species as the frequency of detection of anti-leshmanial IgG antibody was higher amongst females in comparison to

those of males.

In present study, the serological data demonstrate the potentiality of IgG1isotype as a marker for the detection of individuals with leishmaniasis. Furthermore, the serological analysis is also suggesting that the females are more prone to the infection than the males.

We have also measured the expression level of pro and anti-inflammatory cytokines which helps in protection and progression of the disease by using different cytokines viz. IL-2, IL-4, IL-6, 1L-10, 1L-17A, TNF-α and IFN-γ, to know whether these cytokines play role in protection or pathology. The expression level of pro-inflammatory cytokines i.e. IL-2, IL-6, 1L-17A, TNF-α and IFN-γ was analyzed. Among all pro-inflammatory cytokines, IL-2 cytokine showed highest level of expression followed by TNF-α, IFN-γ, IL-6 and IL-17A has shown minimum level of expression. The expression level of IL-2 was significantly very high in males comparison to those of females. Ganguly and co- worker [43] have demonstrated the elevated level of IL-2 in VL and PKDL patients from Kolkata (India) and also observed that the helper T cells in treated individuals with anti-leishmanial drugs failed to synthesize IL-2 in PKDL patients. The detection of high level of IL-2 cytokines in our subject might be due the low exposure of disease.

Regarding to gender, we have observed significantly high expression of IL-6 in males whereas expression level in females was less. In concurrence of our findings the levels of IL-6, was high in patients with self-healing lesions, but was not detectable or very low in DCL lesions [44,45,46], whereas, IL-17A cytokines showed no significant change in both genders. Interestingly this cytokine expression was found to be lowest among all the cytokines detected. Recent finding has also shown a very low expression of IL-17A in the splenic biopsies of both pre-and post-treatment VL patients [47].

The expression of IFN-γ cytokine was observed maximum in females, followed by TNF-α, IL-17A, IL-6 while IL-2 was detected in low level. Similarly, the high expression levels of IFN- γ had been reported from Kolkata in VL patients [48]. Our findings and the earlier studies both suggest that IFN-γ helps in the progression of disease and is unable to control that.

We have studied the expression level of anti-inflammatory cytokines i.e. IL-4 and IL-10. Among the anti-inflammatory cytokines, IL-10 showed the maximum expression. The frequency of detection of IL-10 was significantly high in the females. In support of our findings the high expression levels of IL-10 has been reported from Kolkata, the authors observed the expression of IL-10 was significantly high among VL patients, before and after treatment [48].

Our study revealed that the high-level expression of IL-10 in males amongst the study population, is able to protect from parasitic infection, rendering them more resistant in comparison to those of females. The high expression of IL-10 in absence of significant IL-4, also indicates towards the high virulence of parasite prevalent in this study region.

Conflict of Interest

We declare that we have no conflict of interest.

Acknowledgements

Authors express their gratitude to Dr. Ajeet Negi, Senior Medical Officer In-charge, MGMSC (Govt. of India) Khaneri, Rampur, Distt. Shimla H.P.) for providing few samples from Leishmaniasis patients.

References

1 Van Griensven J, Zijlstra EE, Hailu A. Visceral Leishmaniasis and HIV Co-infection: Time for Concerted Action. PLOS Negl Trop Dis. 2014; 8(8): e3023.

2 Dujardin JC, Campino L, Cañavate C, Dedet JP, Gradoni L, Soteriadou K, et al. Spread of vector-borne diseases and neglect of Leishmaniasis in Europe. Emerg Infect Dis. 2008;14 (7): 1013–1018.

3 Perasons RD, De Queiroz Sousa A. Clinical spectrum of leishmaniasis. Clin Infect Dis. 1996;22:1-13.

4 Sharma NL, Mahajan VK, KangaA, SoodA, Katoch VM, Mauricio I, et al. Localized cutaneousleishmaniasis due to Leishmaniadonovani and Leishmaniatropica: preliminary findings of the study of 161 new cases from a new endemic focus in himachalpradesh, India. Am J Trop Med Hyg. 2005;72(6): 819-24.

5 Alvar J, Aparicio P, Aseffa A, Den BoerM,CañavateC, DedetJP, et al. The re-lationship between leishmaniasis and AIDS: the second 10 years. Clin Microbiol Rev. 2008; 21: 334-59.

6 Heinzel FP, Schoenhaut DS, RerkoRM, RosserLE,Gately MK. Recombinant Interleukin 12 cures mice infected with Leishmania major. J Exp Med. 1993;177(5): 1505-9.

7 Roger KA, Derkry GK, Mbow ML, Gillespie RD, Brodskyn CL, Titus RG. Type 1 and type 2 response

to Leishmania major. FEMS Microbial Lett. 2002; 209: 1-7.

8 Beadling C, Johnson KA. Isolation of interlukin2-induced immediate early genes. Proc Natl Acad Sci USA. 1993; 90(7): 2719-23.

9 Safro BY, WilsonNO, Bond VC, Stiles JK. Plasmodium berghei ANKA infection increases Foxp3, IL-10 and IL-2 in CXCL-10 deficient C57BL/6mice. Malaria J. 2011; 10:69.

10 Alexander J, Carter KC, Al-Fasi N, SatoskarA, Brombacher F. Endogenous IL-4 is necessary for effective drug therapy against visceral leishmaniasis. Eur J Immunol. 2000;30:2935-43.

11 Wilson AJ, Coltman DW, Pemberton JM, Overall ADJ, ByrneKA,KruukLEB. Maternal genetic effects set the potential for evolution in a free-living vertebrate population. J Evol Biol. 2005; 18:405-14.

12 Moore ID, Norton TW, Williams JE.Modelling environmental heterogeneity in forested landscapes. J Hydrol. 1993; 150 : 717-747.

13 Kubo M, Yasutaka M. Transcriptional regulation of the anti-inflammatory cytokine IL-10 in acquired immune cells. Fronteirs Immune. 2012; 3:275.

14 De Waal Malefyt R, Abrams J, Bennett B, Figdor CG, DeVries JE. Interleukin 10(IL10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med. 1991; 174: 1209-1220.

15 Nylen S, SacksD. Interleukin-10 and the pathogenesis of human visceral leishmaniasis.Trends Immunol. 2007;28.: 378–384.

16 Carvalho EM, Bacellar O, Brownell C, Regis T, Coffman RL. Restoration of IFN-gamma production and lymphocyte proliferation in visceral leishmaniasis. J Immunol. 1994;152: 5949-56.

17 Ghalib HW, Piuvenzam MR, Skeily YA, Sidding V, Hashim FA, El-Hassan AM, et al. Interleukin 10 production correlates with pathology in human Leishmania donovani infection. J Clin Invest. 1993; 92(1): 324–329.

18 Russell DG, Xu S, Chakraborty P. Intra cellular trafficking and the parasitophorous vacuole of *Leishmania Mexicana* infected macrophages. J Cell Sci. 1992;103*:*1193-1210.

19 Bogdan C, MollH, SolbachW,Rollinghoff M.Tumour necrosis factor-alpha in combination with interferon-gamma, but not with interleukin 4 activates murine macrophages for elimination of Leishmania majoramastigotes. Eur J Immunol. 1990;20: 1131–1135.

20 Depinay N, FranetichJF,GrunerAC, Mauduit M, Chavatte JM, Lutty AJ, et al. Inhibitory effect of TNF-α on malaria pre-erythrocytic stage development: influence of host hepatocyte/parasite combination. Plos One. 2011; 6 (3). http://dx.doi.org/10.1371/journal.pone.0017464.

21 Wu AJ, Hua H, Munson SH, McDevitt HO. Tumor necrosis factor-*α* regulation of CD4+C25+ T cell levels in NOD mice. ProcNatlAcadSci U S A. 2002;99(19): 12287–12292.

22 Antonelli LR, Dutra WO, Almeida RP, Bacellar O, Carvalho EM, GollobKJ. Activated inflammatory T cells correlate with lesion size in human cutaneous leishmaniasis. Immunol Lett. 2005;101: 226–230.

23 Behin R, Jacques L**,**Immune response to Leishmania. Crit Rev Trop Med. 1984; 2.

24 Bray RS. Immunodiagnosis of leishmaniasis.In Cohen S, and Sadun EH (ed.), Immunology of parasitic infections. Blackwell Scientific Publications, Oxford, England. 1976:65-76.

25 Neogy AB, Nandy A, Dastidar BG, ChowdhuryAB.Antibody kinetics in kala-azar in response to treatment. Ann Trop Med Parasitol. 1987;81:727.

26 Anam K, Afrin F, Banerjee D, Pramanik N, Guha SK, Goswami RP, et al. Immunoglobulin subclass distribution and diagnostic value of Leishmaniadonovani antigen-specific immunoglobulin G3 in Indian kala-azar patients. Clin Diagn Lab Immunol. 1999; 6: 231-5.

27 Ghosh MK, Nandy A, Addy M, Maitra TK, Ghose AC. Subpopulations of T lymphocytes in the peripheral blood, dermal lesions and lymph nodes of post kala-azar dermal leishmaniasis patients. Scand J Immunol. 1995;41:11-17.

28 Da Matta VL, Hoshino-Shimizu S, Dietze R, Corbett CE. Detection of specific antibody isotypes and subtypes before and after treatment of American visceral leishmaniasis. J Clin Lab Anal. 2000;14:5-12.

29 Ryan JR, Smithyman AM, Rajasekariah GH, Hochberg L, Stiteler JM, MartinSK. Enzyme-linked immunosorbent assay based on soluble promastigote antigen detects immunoglobulin M (IgM) and IgG antibodies in sera from cases of visceral and cutaneous leishmaniasis. J Clin Microbiol. 2002; 40:1037-43.

30 Miles SA, Conrad SM, Alves RG, Jeronimo SM, Mosser DM. A role for IgG immune complexes during infection with the intracellular pathogen Leishmania. J Exp Med. 2005;201:747-54.

31 Lowry OH, Rosenbrough NJ, Farr AL,RandallRJ.Protein measurement with the Folin phenol reagent. J Biol Chem. 1951; 193:265-275.

32 Afrin F, Ali N. Protection of mice against visceral leishmaniasis by immunization with promastigote antigen incorporated in liposomes. J Parasitol. 1997; 83:7-75.

33 Crowther JR. The ELISA guidebook. Methods in molecular biology. 2001; 149.

34 Badaro R, Duarte MISL.Leishmaniose visceral (kalazar). *Tratado de Infectologia*, Atheneu, São Paulo. 1997; 234-1259.

35 Choudhry A, Guru PY, Saxena RP, Tandon A, Saxena KC.Enzyme- Linked immunosorbent assay in the diagnosis of kala-azar in Bhadohi (Varanasi), India. Trans R Soc Trop Med Hyg. 1990; 84:363-36.

36 Hommel M, Peters W, Ranque J, Quilici M, LanotteG. The micro-ELISA technique in the serodiagnosis of visceral leishmaniasis. Ann Trop Med Parasitol. 1978;72:213-218.

37 Atta AM, Oliveira AD, Correa J, Atta ML, Almeida RP,Carvalho EM. Anti-leishmanial IgE antibodies: a marker of active disease in visceral leishmaniasis. Am J Trop Med Hyg. 1998;59:426-30.

38 Ghose AC, Haldar JP, Pal SC, Mishra BP, Mishra KK. Serological investigations on Indian kala-azar. Clin Exp Immunol. 1980;40:318-26.

39 Haldar JP, Ghose S, SahaKC,Ghose AC. Cell-mediated immune response in Indian kala-azar and post-kala-azar dermal leishmaniasis. Infect Immun. 1983; 42:702-7.

40 Zwingenberger K, Harms G, Pedrosa C, Omena S, Sandkamp B, Neifer SK. Determinants of the immune response in visceral leishmaniasis: evidence for predominance of endogenous interleukin 4 over interferon-γ production. Clin Immunol Immunopathol.1990;57:242-249.

41 Shakib F. Is IgE mediated hypersensitivity an autoimmune disease? Allergy. 1990; 45(1):1-9

42 Hussain R, Kifayet A, Chiang TJ. Immunoglobulin G1 (IgG1) and IgG3 antibodies are markers of progressive disease in leprosy. Infect Immun. 1995; 63(2):410-415.

43 Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol.1989; 7:145-173.

44 Convit J, Ulrich M, Fernández CT, Tapia FJ, Cáceres-Dittmar V, Castés M, RondónAJ.The clinical and immunological spectrum of American cutaneous leishmaniasis. Trans R Soc Trop Med Hyg. 1993;87:444-8.

45 Ritter U, MollH, Laskay T, Brocker V, VelazcoO, Becker I,Gillitzer R. Differential expression of chemokines in patients with localized and diffuse cutaneous American leishmaniasis. J Infect Dis. 1996; 173:699-709.

46 Nylen S, and Sacks D. Interleukin-10 and the pathogenesis of human visceral leishmaniasis. Trends Immunol. 2007;28: 378-84.

47 Ansari NA, Kumar R, Gautam S, Nylen S, Singh OP, Sundar S, Sacks D. IL-27andIL-21 are associated with Tcell IL-10 responses in human visceral leishmaniasis. J Immunol. 2011;186: 3977-85.

48 Ganguly S, Das NK, Panja M, Pal S, Modak D, Rahaman M, et al, Increased Levels of Interleukin-10 and IgG3 Are Hallmarks of Indian Post-Kala-Azar Dermal Leishmaniasis. JID. 2008:197.