
Lymphocytes Subset Phenotypes in Patients with Visceral Leishmaniasis

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Abstract:

Background: Visceral leishmaniasis is an endemic protozoan disease in Iraq Recovery from this disease confers a solid and permanent immunity.

Objective: Determining the type of lymphocyte phenotype by using; monoclonal antibodies against (CD 4, CD8).

Patients and methods: Indirect immunofluorescence technique analysis was performed to detect the percentage of CD4, CD8 and CD4/CD8 ratio positive lymphocytes.

Results: Our results showed that the patients groups have decrease in both the percentage of CD4 cells & CD4/CD8 ratio and increase in the percentage of CD8. Follow up of patients after treatment showed a significant increase in the percentage of CD4, CD4/CD8 ratio but they were still lower than their normal range.

Conclusion: CD4/CD8 ratio is reversed in VL infection then increase after treatment but it was less than its normal range.

Key words: CD-Markers profile, leishmania donovani, amastigote form.

Introduction:

Leishmaniasis is a spectrum of diseases caused by *Leishmania* species, protozoan of the order kinetoplastidia. They present a wide clinical spectrum ranging from cutaneous lesions to fatal visceral disease; they are distributed through 88 countries^[1].

There are about 30 species of sand flies, which can transmit different species of *Leishmania*^[2].

Leishmania is a genus of the family Trypanosomatidae, with two forms, an amastigote which occurs in the mammalian host and a promastigote which occurs in the insect host. Infection of man by this protozoan results in a disease called leishmaniasis^[3].

In Iraq, according to the reports of the Communicable Disease Control Center in Ministry of Health (MOH), infectious diseases including leishmaniasis remain on the list of major causes of morbidity and mortality^[4].

CD-markers are glycoproteins characterized in two populations of lymphocytes (T and B-lymphocyte) and natural killer cells. Most T-helper cells express CD4, whereas most T-cytotoxic cells express CD8, NK cells express CD16 and CD56, and B cells express CD19, CD21, CD32 and CD35. The CD4 cells are involved in the regulation function (helper/inducer) of the immune response and the T-CD8 cells have suppressive and cytotoxic activity. T-cell function releasing involves the respective recognition of CD4 and CD8 by class II and class I MHC molecules which represent their natural ligands. The count of CD4, CD8 lymphocyte in the peripheral blood is a major test in the hematological follow-up of the disease with immune dysfunction and patient after organ transplant or marrow graft. In subject with immune deficiency (virus), the count of CD4 lymphocytes is recommended for the clinical follow up patients and it intervenes in the definition of the disease evolution^[5].

In one study, CD4+ and CD8+ cells were within normal range in patient with active visceral

leishmaniasis but in acute visceral leishmaniasis patients had markedly reduced levels of memory T cells (CD3+/CD45ro+) and return to normal level following successful chemotherapy^[6]. Another study showed that pretreatment serum level of soluble CD4 and soluble CD8 were significantly higher in visceral leishmaniasis patients than in the healthy subjects^[7]. Croft^[8] reported that, the CD4 cell count was depressed in peripheral blood of acute and chronic visceral leishmaniasis cases as compared to the normal control, while the peripheral blood CD8 cell was normal in acute cases, was uniformly low in chronic cases. After treatment, the CD4 cell count in peripheral blood increased in contrast CD8 cell count remained unaltered in peripheral blood.

The aim is to determine the type of lymphocyte phenotype by using monoclonal antibodies against (CD4, CD8) in patient with VL.

Materials and Methods:

Patient's groups included in this study were divided as follows:

A-Confirmed VL group: Blood samples were collected from 50 children less than six years of age who had positive bone marrow smears for VL; those were diagnosed in Al-Mansoor Children Hospital, Al-Kadhimiya Children Hospital, Central Children Hospital and Al-Ilwia Children Hospital in Baghdad, Iraq.

B-Control groups:

B1-Endemicity control

Fifty blood samples were collected from children less than six years of age living in an endemic area (Al-Suwaira district) and who were apparently healthy.

B2-Healthy control: Forty blood samples were collected from children less than six years of age from different primary health centers in Baghdad with no history of living in endemic areas with VL and who were apparently healthy by physical examination.

A sufficient amount (5ml) of blood was collected in an anticoagulant container and plain tube for cell mediated immunity from fifty children with disease (before administrating of sodium stibogluconate injection) and twenty five children who were followed-up until the end of therapy (28 days of sodium stibogluconate therapy). Each blood sample obtained in a plain tube centrifuged (for 30 minutes at 2500rpm at 20°C) as soon as possible to lymphocyte separation. The Isopaque- Ficol technique originally described by Boyum^[9] was used for isolation of mononuclear cells.

2-Methods Indirect Fluorescent Antibody Test (IFAT) For Counting Lymphocyte Subsets

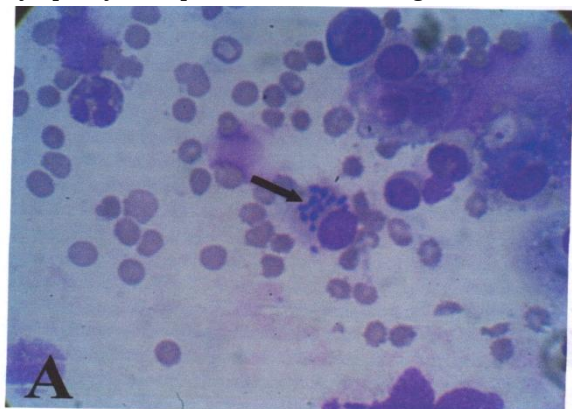
A-Principle:- Indirect immunofluorescence for the detection of CD-antigen depends on two steps; the first step leads to the binding of primary antibody to specific cell antigen. The second step allows the detection of specific CD-antigen when anti-mouse immunoglobulin IgG fluoresceinated conjugate is added to be examined by immunofluorescent microscope. The positive samples show an apple green fluorescence corresponding to areas of cell surface where primarily antibody bound.

Procedure: According to the method of ^[10].

B- Assay procedure:

1-Lymphocyte suspension was adjusted to contain 2×10^6 cell/ml, 45 μ l of lymphocyte suspension was transferred in tube and 5 μ l of monoclonal antibody (CD4, CD8) was added, mixed well and incubated at (2-8°C) for 30 minutes.

2-Lymphocyte suspension was centrifuged two times



at 1500g for 5 minutes and the supernatant was aspirated and discarded and cell pellet was resuspended in phosphate buffer saline (PBS) and bovine serum albumin (BSA).
Fig. (1): Bone marrow smears showing intracellular and extracellular *Leishmania donovani* bodies in patients with VL. A-viability of *Leishmania* amastigote in infected macrophage ($\times 100X$. B-amastigotes release from died macrophage to infect other macrophages (magnification power of a and b $\times 100X$.)

resuspended in phosphate buffer saline (PBS) and bovine serum albumin (BSA).

3-Fifty μ l of fluorescent conjugate (diluted 1:80 in PBS/BSA) was added and incubated for 30 minutes at (2-8°C) in the dark.

4-Washing was repeated as in the step 2.

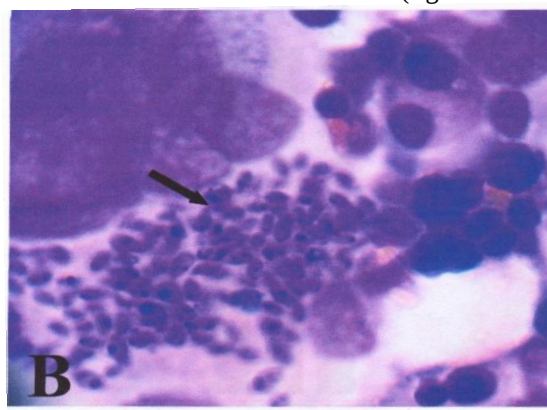
5-The cell pellets were resuspended in 200 μ l of PBS/BSA, a drop was delivered by Pasteur pipette and placed in the center of clean slide with coverslip.

C-Calculation of the results: Slides were examined under 40X-magnification of a fluorescent microscope. Their dark green staining identified positively labeled cells. Two hundred cells were counted to determine percentage of reactivity of the tested monoclonal antibodies.

Statistical analysis: Data were analyzed statistically using SPSS program version 10. Results were expressed using simple statistical parameters. Analysis of quantization data was done using t-test and ANOVA (analysis of variance). Acceptable level of significance was considered to be less than 0.05

Results:

Results of bone marrow smears Fifty bone marrow smears showed moderate to severe megaloblastosis, an increased number of plasma cells and megakaryocytic hyperplasia with abnormal morphology. Amastigotes appeared as round or oval bodies found intracellular in monocytes and macrophages, extracellular leishman bodies are also seen in the stained smears (figure 1)



Lymphocyte subsets enumeration by IFAT: One method for enumeration of lymphocyte subsets by immunofluorescence was compared in healthy children using CD4, CD8, of monoclonal antibody against antibody of VL.

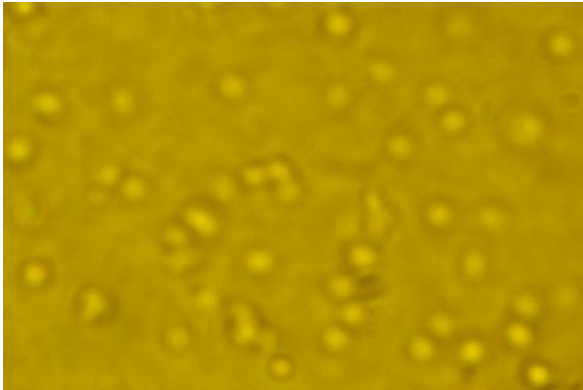


Fig.(2): Slides of indirect immunofluorescence microscope at 490 nm without exposure to UV-light to see lymphocyte subsets (400 x).

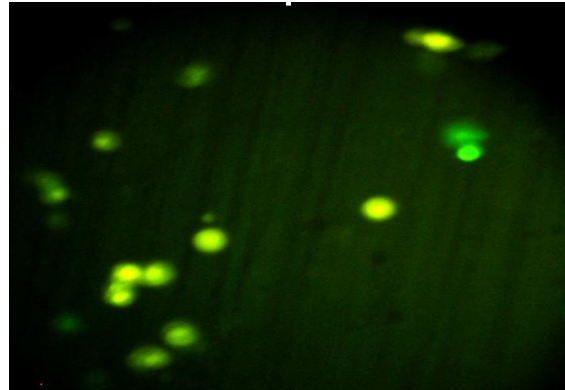


Fig.(3): Slides of indirect immunofluorescence microscope at 490 nm. Positive cells give green-apple when stained with FITC-labeled antibodies after exposure to UV-light to see lymphocytes. (400 x) Lymphocytes subsets counting by Indirect Fluorescent Antibody Test (IFAT) .

T-helper cells (CD4+)

CD4 was lower in pretreated VL group ($25 \pm 0.49\%$) than in control group ($33 \pm 0.39\%$) with significantly difference ($p < 0.01$). CD4 in the post treated VL group was ($31.1 \pm 0.42\%$) significantly

higher in comparison with Pretreated group ($p < 0.01$). There was a significant difference between post treated and control group ($p < 0.01$) (Table1) (fig.4).

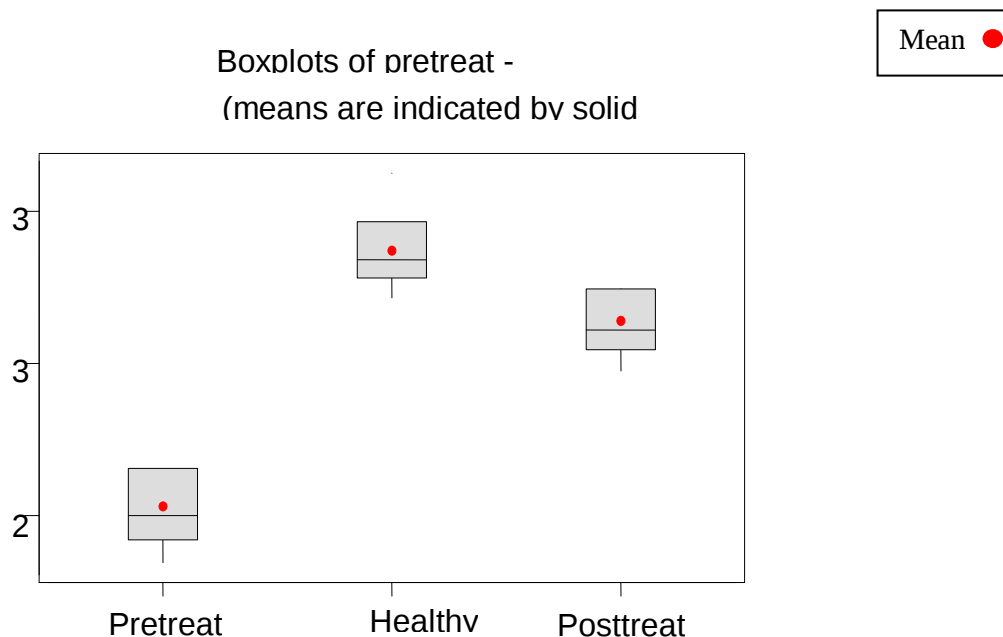
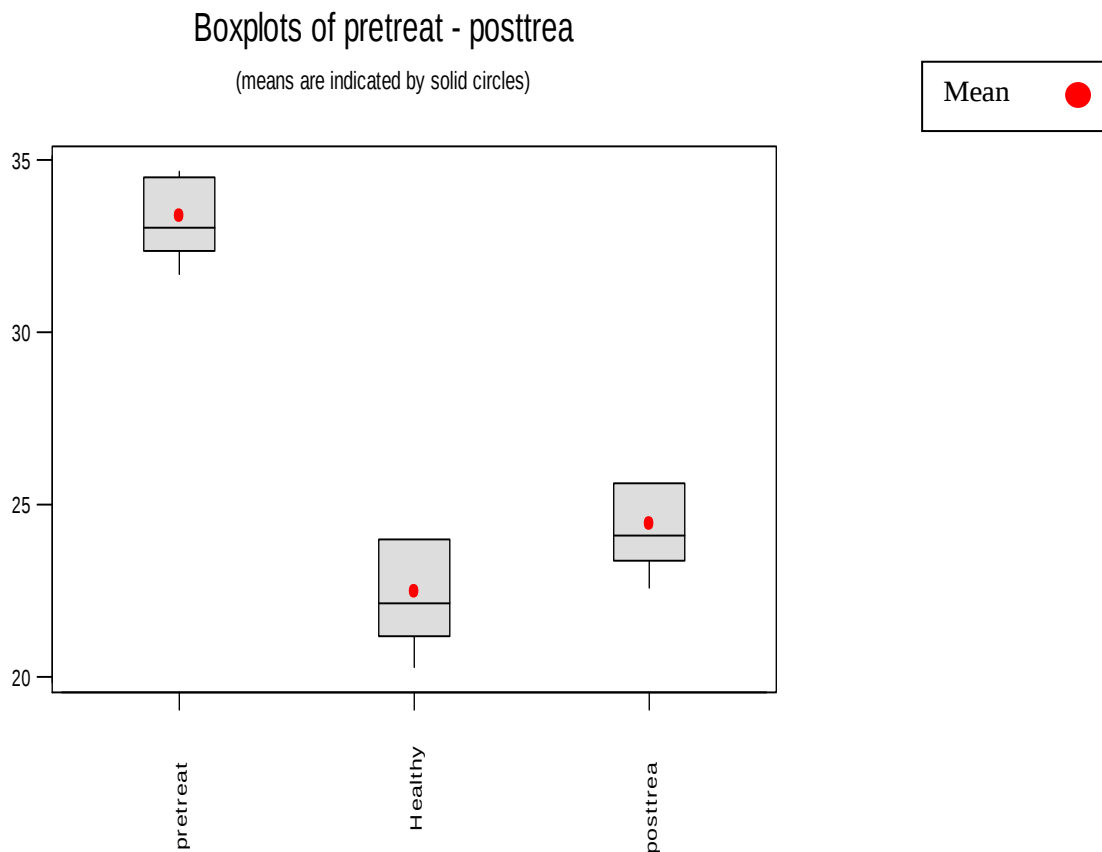


Fig.(4):Percentage of (CD4) among pretreated VL, healthy control and post treated VL by immunofluorescence test.

T-cytotoxic cells (CD8+):

CD8+ was significantly higher in the pretreated VL group (33 ± 0.42)% than in the control group (22.1 ± 0.59)% ($p < 0.01$). in the post treated VL pretreated group ($p < 0.01$). There was a significant difference between post treated and control group ($p < 0.01$) (Table 1). (Fig.5)

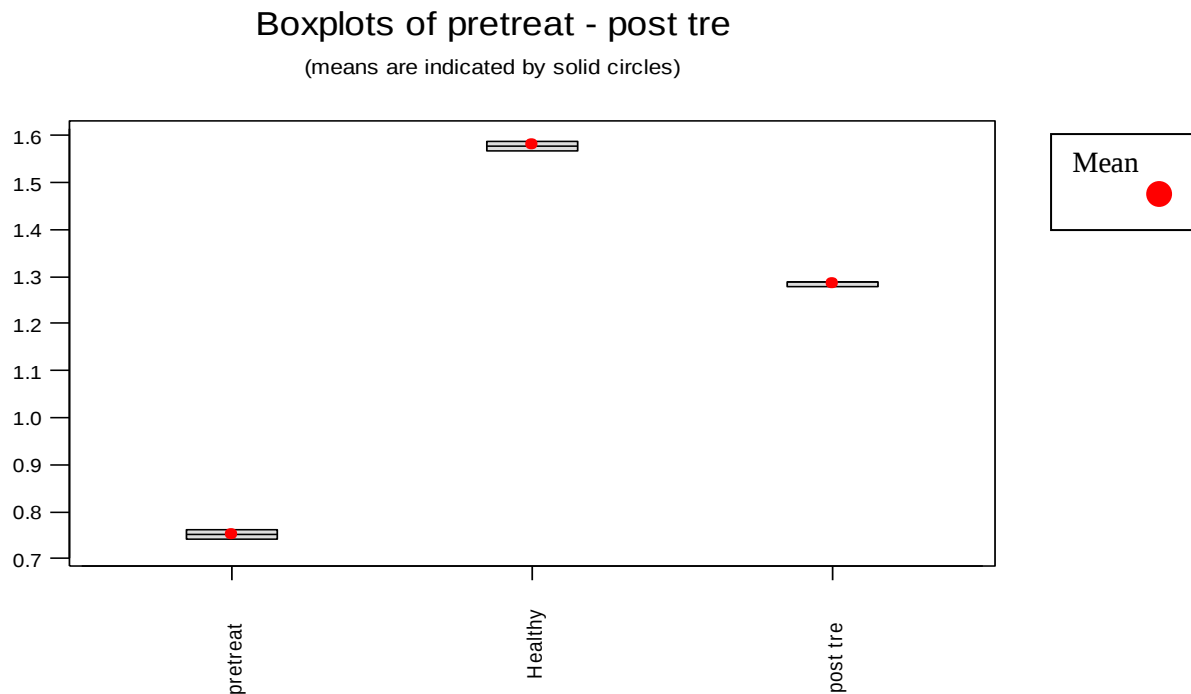
Fig.(5) :Percentage of (CD8) among pretreated VL, healthy control and post treated VL by immunofluorescence test.

**CD4+/CD8+ ratio:**

CD4+/CD8+ ratio was significantly lower in pretreated VL group (0.75) than in the control group (1.59). While in the post treated group the ratio was (1.29) higher than that in the pretreated group ($p < 0.01$) and lower than that in the control group. (Table1).(Fig.6).

Table(1)Percent of total T-lymphocyte subsets in patients with VL before and after sodium stibogluconate therapy.

CD-Marker	Pretreated VL Group (Mean $\pm$ SE)	Healthy control group (Mean $\pm$ SE)	Post treated VL group (Mean $\pm$ SE)
CD4	25 $\pm$ 1.56	35 $\pm$ 1.25	31.1 $\pm$ 1.35
CD8	33 $\pm$ 1.33	22.1 $\pm$ 1.87	24..1 $\pm$ 1.52
CD4/CD8ratio	0.75	1.59	1.29



(Fig.(6) :Percentage of (CD4/CD8) among pretreated VL, healthy control and post treated VL by immunofluorescence test.

Discussion:

Lymphocyte subset enumeration by IFAT

Suppression may be mediated by macrophage, either by defective antigen processing and presentation, or by elaboration of suppressive mediators like IL-10, TGF- β and prostaglandin E2^[11].

Other results further showed that both CD4⁺ and CD8⁺ T cells contribute in producing IL-10 during the disease and IFN- γ at a successful cure. IL-10 is produced by many cell types including B cells and macrophages. Subsets of T cells, including CD4⁺ and CD8⁺, have been reported to produce IL-10, and our studies herein confirm these observations in Kala-azar^[12, 13, 14]. It is known that NK cells, acting as innate immune effector cells and may play an important role in the early stages of *Leishmania* infection, they respond rapidly and serve as source of activating cytokine like IFN- γ and TNF- α , which not only inhibit the growth of parasite during initial stages of infection but also influence the differentiation of CD4⁺T-cells towards Th1phenotype^[15].

The results of this study have revealed that there was a significant increase of CD8⁺T-cells in patient with VL in comparison with control. These results in agreement with results of^[16] who confirmed the increase of CD8⁺ T-cells in VL cases, however^[17] stated that peripheral blood CD8⁺Tcell count was normal in acute and uniformly low in the chronic

cases. This may be explained by the presence of IL-2 from macrophage and Th1 which increase CD8⁺ division.

The role of CD8 T-cells against *Leishmania* infection was suggested by^[18] who reported that CD8⁺ T-cells were shown to be responsible for the conversion of susceptible BALB/C mice into resistant phenotype after depletion of CD4-T-cells against *L. major* infection. Despite the fact that CD8⁺T-cells also produce IFN- γ on activation and can directly destroy the infected macrophage^[19].

A different observation was in CD4⁺T-cells in patients with VL, it was significantly lower than control. These results are in agreement with Rohtagi et al.,^[17] who confirmed that acute and chronic VL cases are depressed in peripheral blood CD4 cell count. This lowering of CD4⁺ may result from apoptosis as reported by^[20] who suggested that CD4 T-cells derived from susceptible mice undergo rapid apoptosis, produce less IL-2 and IFN- γ and fail to mediate DTH.

Results showed that CD4⁺/CD8⁺ ratio was significantly lower than control group, this may be considered as an Index of immune suppression in VL patients. This result agrees with Ghosh et al.,^[21] who reported that at diagnosis CD4⁺ cells showed a significant decrease while CD8⁺ cells were significantly increased when compared with control and CD4⁺/CD8⁺ ratio was inverted.

Treatment resulted in a significant increase in the percentage of CD4, and CD4/CD8 ratio compared with pretreated group but still lower in comparison with control. In contrast the percentage of CD8 showed a significant decrease in comparison with pretreated group and still higher than control.

These results confirmed the results of previous studies (21) who showed that CD4+/CD8 +ratio returned to normal value three months after recovery

CD4/CD8 ratio is reversed in VL infection then increase after treatment but it was less than its normal range.

References:

- 1- WHO (World Health Organization) (2005): Leishmaniasis burden http://www.who.int/vaccine-research/documents/new_vaccine/ent/index5.html/.
- 2- WHO (World Health Organization) (2000): The Leishmaniasis and Leishmania/HIV co-infections. WHO fact sheet no.116. <http://www.who.int/inffs/fact116.htm/>.
- 3- Aleexander,J.,Satoskar A.R., Russel D.G.(1999): *Leishmania species*:models of intracellular parasitism.J.Cell Sci.;112 (18):2993-3002.
- 4- WHO (World Health Organization) (2003): Communicable diseases [who/cds/2003.17](http://www.who.int/infectious-disease-news/IDdocs/whocds2003.17/1).<http://www.who.int/infectious-disease-news/IDdocs/whocds2003.17/1>.
- 5- Levy.J.A.(1996): Infection by human immunodeficiency virus-CD4 is not enough .N.Engl.J.Med;335:1528-1530.
- 6- Cillari, D.(1995):In vivo and in vitro cytokine profiles and mononuclear cell subsets in Sicilian patients with active visceral leishmaniasis .Cytokine;774-775.
- 7- Schriefer,S.(1995):Serum soluble markers in the evaluation of treatment in human visceral leishmaniasis .Clin. Ex. Immunol;102:535-540.
- 8- Croft, S. L., S. Sundar, A. H. Fairlamb. 2006. Drug resistance in leishmaniasis. *Clin. Microbiol. Rev.* 19: 111-126.
- 9- Boyum,A.(1968):Separation of leukocytes from blood and bone marrow. *Scand.J. Clin.Lab. Invest.*; 21(suppl 97).
- 10- Slaper-Cortenbach,I.C.M.(1988):The flow cytometric detection of Terminal deoxynucleotidyl Transferase (TdT) and other intracellular antigens in combination with membrane antigens in acute lymphatics leukemia. *Blood*,72.
- 11- Saha, B., Das,G., Vohra, H., Ganguly, N. K. and Mishra,G. C.(1995):Macrophage T-cell interaction in experimental visceraalleishmaniasis: failure to express constimulation molecules on *Leishmania*-infected macrophages and its implication in the suppression of cell mediated immunity. *Eur. J. Immunol*; 25:2492-2498.
- 12- Holaday, B. J., M. M. Pompeu, S. and Jeronimo, M. J. (1993): Potential role for interleukin-10 in the immunosuppression associated with kala azar. *J. Clin. Invest.* 92: 2626-2632.
- 13- Mary, C., V. Auriault, B. and Faugere, A. J. (1999): Control of *Leishmania infantum* infection is associated with CD8⁺ and IFN- γ - and interleukin-5-producing CD4⁺ Ag-specific T cells. *Infect. Immun.* 67: 5559-5566.
- 14- Nylen, S., R. Maurya, L. and Eidsmo, K. D. (2007): Splenic accumulation of IL-10 mRNA in T cells distinct from CD4⁺CD25⁺ (Foxp3) regulatory T cells in human visceral leishmaniasis. *J. Exp. Med.* 204: 805-817.
- 15- Sharon,T.M. and Scott,P.(1993): Natural killer cells are a source of interferon -gamma that drive differentiation of CD4 T-cells subsets and induces early resistance to L. major in mice.J.Exp.Med.;178:577-587.
- 16- Colmenares,M., Kima, P.E., Samoff,E., and McPratt,D.(2003):Perforin and gamma interferon are critical CD8 T-cell-mediated responses in vaccine-induced immunity against *Leishmania amazonensis* infection;71:3172-3182.
- 17- Rohtagi,A., Agarwal, S.K., Bose, M., Chattopadhyay, D.and Sahak.(1996):Blood, bone marrow and splenic lymphocyte subset profiles in Indian VL.Trans.R. Soc. Trop.Med.Hyg.; 90(4):431-434.
- 18- Stenger, S. and Modlin, R.L.(1998):Cytotoxic T-cell response to intracellular pathogens. *Current Opinion in Immunology*;10:471-477.
- 19- Maash, K., Sanchz, F, Hailu, A. and Akuffo, H.(1998):Indication of the protective role of natural killer cells in human cutaneous leishmaniasis in an area of endemicity.Inf. Immun;66 (6):2698-2704.
- 20- Das, G., Vohrn, H., Saha, B. And Mishra, G.(1999):*Leishmania* infection of a susceptible hosts results in CD4 T-cells apoptosis and decreased Th1 cytokine production.Scan. J. Immunol.; 49:307-310.
- 21- Ghosh, M.K., Ghosh,A.K., ddy,M.,Nandy,A.and Ghosh,A.C.(1996): Subpopulation of T-lymphocytesin the peripheral blood and lymph nodes of Indian Kala-azarpatients. *Med. Microbiol. Immunol.*;185(3):183-187.

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