REPUBLIQUE DU CAMEROUN
Paix – Travail – Patrie
****

UNIVERSITE DE YAOUNDE I
FACULTE DES SCIENCES
DEPARTEMENT DE BIOLOGIE ET PHYSIOLOGIE ANIMALES
****

REPUBLIC OF CAMEROUN
Peace – Work – Fatherland
****

UNIVERSITY OF YAOUNDE I
FACULTY OF SCIENCES
DEPARTMENT OF
****

IMMUNOLOGICAL AND HISTOPATHOLOGICAL INVESTIGATIONS ON HUMAN CUTANEOUS LEISHMANIASIS ASSOCIATED WITH THE ACQUIRED IMMUNODEFICIENCY IN NORTHERN CAMEROON

Doctorate/PhD in Animal Biology

Par :

NGOUATEU KENFACK Omer Bébé
DEA/M Sc in Animal Biology

Sous la direction
KAMTCHOUING Pierre
Professor

Année Académique
2014-2015

DEDICATION

This thesis is dedicated to:

- my dear parents

Ngouateu Fogap Bernard (*RIP*) and **Letchige Christine;**

- my beloved kids

Ngouateu Ngouateu L.A., Ngouateu Letchige D.F. and **Ngouateu Tchinda M.M.**

ACKNOWLEDGEMENTS

For the fulfillment of this thesis, many individuals contributed in different ways. It is now the opportunity for me to present to all of them my deep gratitude.

I thank Pr **Kamtchouing P.,** who agreed to guide me since my Master's Degree. He always advises and encourages me. He agreed to supervise the present work despite his busy program. Pr **Dondji B.**, Central Washington University, USA, who is the coordinator of the Leishmaniasis Research Project in Northern Cameroon, offered to me all his know how. His permanent advice and confidence boosted me to realize this project. Pr **von Stebut E.**, Universitätsmedizin Mainz, Germany, integrated me into her research team for immunology, immunohistochemestry, histology part of this study. During my stay in her laboratory, she tirelessly provided me a very rich expertise. I extend my deep gratitude to Pr **Same-Ekobo A.**, Faculty of Medicine and Biomedical Science (UYI), who kindly accepted to guide me. The remarkable contribution of Pr **Maurer M.**, Charité-Universitätsmedizin Berlin, Germany, was invaluable. He deeply discussed with me to improve the quality of this work. Mast cells analysis was performed in his laboratory. Special thank to Pr **Dereure J.** and Dr **Ravel C.**, ''Centre Universitaire Hospitalier de Montpellier, France'' who characterized the *Leishmania* species.

I thank all the members of jury of my thesis defense for their contribution.

I am grateful to Drs **Kollo P.**, **Bissemou J.J.** and **Wawoua M** (MDs) as well as Messrs **Ndawakaï G.A.** and **Melkaï P.**, all of Mokolo District Hospital, for their tireless contribution to the realization of the first part of this project. I extend this gratitude to nurses of the same hospital and especially to misses **Kembou S.C.**, **Keunmegne K.D.D.**, Messrs **Kouaya T.H.R.**, **Tchapda R.**, **Golda** and **Valkada**. This part of the work was evenly possible by the help of administrative and traditional authorities of the Mayo-Tsanaga Division as well as the population of this Division, and many health centers. They are therefore highly acknowledged.

The first mentor to initiate me into the research world, Pr **Kamanyi A.**, University of Dschang, has never been discouraged to endlessly support me intellectually and morally. This encouragement was further intensified by Drs **Nguelefack T.B.** and **Watcho P.** and I shall never forget. Advice from Pr **Tchoumboué J.**, University of Dschang, and Dr **Sokeng S.**, University of Ngaoundéré were also of great help for me.

In Mainz, Mrs **Susanna L.K.** and Mrs **Claudia B.** initiated me respectively in immunology and, histology and immunohistochemestry techniques of analyses. I shall never forget their hospitality. Along the same lines, I add Mrs **Fleck S.** Some others persons in this the same research team were also for great help and we shared together research experience; they are: Mr **Dominik J.A.**, Dr **Kordula K-N.**, Miss **Diana V.**, Drs **Micheal F., Sven B.**, **Mohamed Z.**, Misses **Yashmin D.**, **Kirsten**, **Annemarie**, **Anja S.**, **Beate L.** and Mrs **Stefanie D.** I sincerely thank all of them and wish them to continue in the same spirit forever.

I thank the entire teaching staff and students of the Department of Animal Biology and Physiology (UYI) for their input during the seminar.

I am at a loss for words to express my gratitude to Drs **Frank S.** and **Metz M.,** as well as Miss **Sina H.** and Mrs **Johanna** who helped me in mast cells analysis in Berlin.

A special appreciation to my beloved **Lane M.** who supported my absence and took care of ours progeny. I cannot neglect the multiform contribution of **Djoudji** family, Mrs **Tsekeng K.R.**, as well as those of Messrs **Lekeufack J.C.**, **Awountsac T.**, **Konla S.C.**, **Nguezong W.M.R.**, **Teufack Y.**, **Lekeulem A.**, **Tepakbong L.**

My school-mates Drs **Ngoula F.**, **Kenfack A.**, **Dongock N.D.**, **Ateufack G.**, **Dzeufiet D.**, **Nguelefack M. P. E.,** and **Atsamo A.D.** always discussed with me and criticized my work to improve its quality. They are here deeply thanked.

My brothers and sisters all called **Ngouateu**, my uncles **Mbii Tanefouet** and **Konnang M.** as well as Messrs **Nguekeng B.**, **Mbouna C.M., Bidja G., Nguefack C.R.**, **Sontsa L.M.,** and Mrs **Momo M.**, are people who always encourage me. They are here thanked for their heartening advice and moral support.

Those who contributed anyway for the achievement of this work and whose names are not mentioned on this document have their names engraved rather in my mind, with the same consideration than the others.

This work received the financial support of the **Volkswagen Foundation** Hannover, Germany allocated to the Leishmaniasis Research Project in Northern Cameroon.

The **Cameroon National AIDS Control Committee** helped in the follow up of HIV positive patients.

The **Meteorology Station of Mokolo** provided data on the rainfall.

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LIST OF ABBREVIATIONS

Ab: Antibody

AB+M: Amphotericin B associated with Metronidazol

Ag: Antigen

AIDS: Acquired immune deficiency syndrome

APC: Antigen presenting cell

ARV: Antiretroviral (therapy)

BALT: Bronchus-associated lymphoid tissue

BLK: Blank

CD: Cluster of differenciation

CL: Cutaneous leishmaniasis

CR: Complement receptor

CRF: Circulating recombinant form (HIV)

CRP: C-Reactive protein

CRP-R: C-Reactive protein receptor

DAG: Diacylglycerol

DALT: Ducts-associated lymphoid tissue

DARC: Duffy antigen receptor for chemokine

DC: Dendritic cells

DCL: Diffuse cutaneous leishmaniasis

dDC: Dermal dendritic cell

ELISA: Enzyme linked immunosorbent assay

ERK: Extracellular signal related kinase

FACS: Fluorescent-activated cell sorting

FcR: Crystalisable fragment (of Ig) receptor

FFPE: Formalin-fixed paraffin-embedded (tissue)

GALT: Gastro-intestinal-associated lymphoid tissue

G-CSF: Granulocyte-colony stimulating factor

GIPL: Glycosylinositol phospholipid

GM-CSF: Granulocyte-Macrophage colony stimulating factor

GRO: Growth related oncogen

HAART: Highly active antiretroviral therapy

HIV: Human immunodeficiency virus

HIV-: Subject without HIV

HIV+: Subject with a positive HIV serology

IκB: Nuclear-factor-kappa B inhibitory protein

ICAM-1: Intercellular adhesion molecule-1

IHC: Immuno-histochemestry

IP3: Phosphatidyl inositol triphosphate

IP-10: Gamma interferon inducible protein

JAK: Janus kinase

LB: Bursodependent (B) Lymphocyte

LC: Langerhans cell

LCL: Localized cutaneous leishmaniasis

Leish-: Subject without leishmaniasis

Leish+: *Leishmania* positive subject

LFA: Lymphocyte function-associated antigen

LPG: Lipophosphoglycan

LSAB: Labeled Streptavidin-Biotin

LT: Thymodependent (T) Lymphocyte

LTR: Long terminal repeat

MAPK: Mitogen activated protein kinase

MBP: Mannose binding protein

MC: Mast cell

MCP: Monocyte chemoattractant protein

MCL: Mucocutaneous leishmaniasis

MFR: Mannose fucose receptor

MHC: Major histocompatibility complex

MIG: Monokine induced by IFN-γ

MIP: Macrophage inflammatory protein

MR: Mannose receptor

MΦ: Macrophage

NF-κB: Nuclear factor kappa B

NK: Natural killer

NTD: Neglected tropical disease

PAMPs: Pathogen-associated molecular patterns

PBS: Phosphate buffered solution

PCR: Polymerase chain reaction

PDGF: Platelet-derived growth factor

PGE2: Prostaglandine E 2

PIP2: Phosphatidyl inositol biphosphate

PKA: Protein kinase A

PKC: Protein kinase C

PLC: Phospholipase C

PMN: Polymorphonuclear

PRR: Pattern recognition receptor

PS: Phosphatidylserine

PTK: Proteine tyrosine kinase

PTP: Phosphotyrosine phosphatase

RT: Room temperature

STAT: Signal transducer and activator for transcription

STD: Standard

Tc or **T_{CTL}**: T cytolytic cell

T_{DTH}: T delayed type hypersensitivity

Th: T helper

TLR: Toll like receptor

Treg: T regulatory cell

VL: Visceral leishmaniasis

WBC: White blood cell

WHO: World health organization

ABSTRACT

It is estimated that 350 million people are at risk of acquiring leishmaniasis in 88 countries worldwide. The spread of HIV coupled with human population migrations due to war and natural disasters have expanded the endemicity of leishmaniasis significantly. Leishmaniasis is widely reported as an opportunistic infection in HIV-infected individuals. In Africa, few studies have focused on *Leishmania*/HIV co-infection. To evaluate the occurrence of such co-infection, we have conducted epidemiological, clinical, immunological and histopathological investigations on cutaneous leishmaniasis (CL) and *Leishmania*/HIV co-infection in the Mokolo focus of Northern Cameroon. A total of 32 466 persons were investigated. Amongst them 146 presented active CL lesions (0.5%) and an additional 261 (0.8%) had scars indicative of previous CL infection. Immunochromatographic test for HIV was carried out on serum samples of all CL active individuals and seven of them (4.8%) were HIV positive. All these seven subjects showed antibodies to HIV-1 while only two of them were positive for HIV-2. PCR identification of parasite strains successfully isolated from three individuals (2 Leish+HIV- and 1 Leish+HIV+) revealed *Leishmania major* to be transmitted in northern Cameroon. CL lesion numbers, surface area, and healing duration were significantly increased in HIV+ as compared to HIV^- CL patients. Next, in the German laboratories, we attempted to characterize the underlying humoral and cellular immune mechanisms for susceptibility to *Leishmania* and HIV co-infection in this endemic focus. The ELISA revealed in serum, an elevated level of *Leishmania*-specific IgG in all samples. HIV+ patients exhibited lower serum levels of *Leishmania*-specific IgG and decreased serum levels of IL-1b, IL-2, IL-6 and IL-8 but high level of IL-4. Immunohistochemecal analyses of skin biopsies obtained at different time points showed fewer epidermal Langerhans cells, CD1a+ dermal dendritic cells, CD68+ macrophages, CD 20+ B cells, as well as a markedly decreased numbers of $CD4^+$ T cells (~8-fold), but not CD8+ cells. Alongside fewer CXCR3+ Th1 cells, fewer Foxp3+ effector/regulatory T cells and reduced levels of IFN-γ expression were found *in situ* in CL patients co-infected with HIV. If the systemic response Th1/Th2 profile looked ambigous, in the the local response Th2 clearly predominated in immunodepressed patients. Co-infected patients also presented a very low mast cell degranulation. Our results confirm prior studies demonstrating worsened disease outcome in *Leishmania*/HIV

co-infected as compared to HIV negative patients indicating that an increased susceptibility to progressive disease after infection with this otherwise dermatotropic strain (*L. major*) is observed in the HIV+ patients. Finally, our immunological studies suggest severe alterations in the protective immune response initiated by antigen presenting cells and mediated by IFN-γ producing T cells in immunodeficient patients. Immune cells recruitment at the lesion site was also very affected. A detailed understanding of the immunological responses in *Leishmania*/HIV co-infected individuals may aid the development of optimized therapeutic regimens for this severely affected group. Our findings provide important data for the development and implementation of successful control programs against CL and HIV co-infection in this geographical area. The study is of great public health importance as both CL and HIV infection co-occur in the region and successful control programs against HIV in Cameroon should integrate infections such as leishmaniasis.

Key Words: *Cutaneous leishmaniasis, HIV, Immune reaction, Histopathology, Cameroon*

RESUME

La leishmaniose est une maladie parasitaire qui sévit dans 88 pays des zones tropicales et subtropicales. On estime à 350 millions le nombre de personnes à risque dans le monde. Elle est reportée comme une infection opportuniste chez les personnes infectées par le VIH. A cause des migrations des peuples liées aux guerres et aux catastrophes naturelles, les zones géographiques de l'infection au VIH et de la leishmaniose se sont chevauchées accentuant ainsi l'endémicité de la leishmaniose. En Afrique, peu de chercheurs s'intéressent à la leishmaniose et à la co-infection *Leishmania*/HIV ; c'est pour cela que nous avons focalisé notre attention sur l'étude épidémiologique, clinique, immunologique et histopathologique de la leishmaniose cutanée (LC) et de la co-infection *Leishmania*/HIV dans le foyer endémique de Mokolo au Nord Cameroun. Sur un total de 32 466 personnes recencées, 146 (0.5%) ont présenté des lésions actives de LC alors que 261 (0.8%) individus avaient des cicatrices typiques traduisant ainsi une infection antérieure. Cliniquement, les lésions étaient tantôt localisées tantôt disséminées sur le corps, avec un nombre variant de 1 à 20 lésions par sujet. Les tests immunochromatographiques de VIH effectués avec le sérum des 146 sujets enregistrés étaient positifs chez 7 (4.8%) personnes ; tous ces 7 sujets avaient dans leurs sérums des anticorps anti-HIV1 alors que deux parmi eux ont montré en plus des anticorps anti-HIV2. Les signes cliniques notamment le nombre, la surface totale des lésions par individu ainsi que la durée de guérison étaient plus marqués chez les sujets Leish+HIV+ comparés à ceux à sérologie HIV négative. L'identification du parasite par la PCR a montré qu'au foyer de Mokolo *L. major* peut être responsable de la LC aussi bien chez les sujets Leish+HIV- que chez ceux co-infectés. Plus tard, dans des laboratoires de recherche allemands, nous avons essayé de caractériser les mécanismes immunitaires cellulaires et humoraux qui soutendent la plus grande susceptibilité de certains sujets leishmaniens par rapport à d'autres dans un même foyer endémique. Dans le sérum de tous les sujets souffrant de la CL, le taux d'IgG anti-leishmaniennes ainsi que le titre de ses sous-types IgG1 et IgG2, déterminés par la méthode ELISA, étaient élevés par rapport aux sujets non leishmaniens, mais ce taux était plus faible chez les sujets Leish+HIV+. L'analyse multiplex du profil de cytokines sériques a révélé un faible taux d'IL-1b, IL-2, IL-6 et d'IL-8 dans les échantillons des co-infectés, mais un taux élevé d'IL-4 chez ces mêmes individus. L'examen immuno-

histochimique des biopsies cutanées prélevées à différentes périodes a montré une faible présence des cellules de Langerhans épidermiques, des cellules dendritiques dermiques-CD1a+, des macrophages-CD68+ aussi bien qu'une faible infiltration des lymphocytes T-CD4+ et B-CD20+ chez ces sujets co-infectés. Parallèlement une faible expression des cellules Th1 CXCR3, T effecteur/régulateur Foxp3 et d'IFN-γ a été notée *in situ* ; de même ces sujets immunodéprimés ont présenté une faible dégranulation des mastocytes. Même si au niveau systémique le profil Th1/Th2 semble mixte chez toutes les personnes souffrant de la leishmaniose cutanée, les sujets immunodéprimés ont montré localement une tendance à un profil Th2. Notre étude a montré que l'infection au VIH est un facteur d'aggravation de la LC et qu'une espèce dermatrope comme *L. major* peut, chez des personnes co-infectées, provoquer des lésions multiples, ectopiques larges et persistantes. Enfin nos études immunologiques ont révélé une altération sévère du rôle protecteur du système immunitaire, d'abord dans le recrutement des cellules immunitaires ensuite dans l'induction de la réponse immunitaire par les cellules présentatrices d'antigènes et dans la médiation par les lymphocytes T produisant l'IFN-γ. Les connaissances physiologiques apportées par notre étude permettent non seulement de bien comprendre le rôle de certaines cellules immunitaires dans la co-infection à *Leishmania*/HIV, mais aussi de développer un régime thérapeutique adéquat. L'impact de cette étude en santé publique n'est pas négligeable ; en effet, pour une bonne surveillance de l'infection au VIH dans le pays, il faut intégrer entre autre la leishmaniose car les leishmanies et le VIH coexistent au Cameroun.

Mots clés : *Leishmaniose cutanée, HIV, Réactions immunitaires, Histopathologie, Cameroun*

INTRODUCTION

Leishmaniasis is a range of diseases caused by protozoan parasites of the genus *Leishmania* (Hassam *et al*., 1991). It is transmitted to the vertebrate host by the female phlebotomine sand fly (Croft & Coombs, 2003). The disease is endemic in 88 countries throughout Africa, Europe, Asia, North and South America (Markle & Makhoul, 2004; Maurer *et al.*, 2009). The disease is known in three main forms namely cutaneous (CL), mucocutaneous (MCL) and visceral (VL) leishmaniases (Dedet, 1999a). More than 350 million men, women and children are at risk (Gumurdulu *et al*., 2004; Maurer *et al*., 2009). The number of individuals suffering from the pathology is 12 million worldwide (Desjeux, 1999; Croft & Coombs, 2003; Okwor *et al.*, 2009; Liese *et al*., 2010). It is reported 0.5 million new cases of visceral form and 1.5 million of cutaneous form annually (Croft & Coombs, 2003; Desjeux & Alvar, 2003; Gomes *et al.*, 2008). Sixty thousand to 70 000 annual deaths are attributable to leishmaniasis (von Stebut, 2007a; Torrico *et al.*, 2009). According to the Center for Food Security and Public Health (2009), leishmaniasis is one of the most important vector-borne diseases of humans. Today, it has gained a relevant position worldwide among the causes of death by infectious diseases (Patel & Shah, 2008). In the African continent, the prevalence and the clinical pictures of the disease vary from one country to another and even from one focus to another within the same country (Dondji, 2001). In some countries like Algeria, leishmaniases occupy the first place among parasite diseases and represent 35% of diseases with compulsory notification (Achour & Madiou, 2008).

Despite the fact that its epidemiology and its aetiopathology are well known, the disease remains generally poorly treated, untreated or neglected by peoples concerned (Dedet, 1999a). Like tuberculosis, Chagas disease, African trypanosomiasis, Buruli ulcer, leishmaniasis belongs to the group of condition known as the neglected tropical diseases or NTDs (Wacker & Lachat, 2006; Hotez *et al.*, 2009), and in this group it is one with a severe damage (Wacker & Lachat, 2006). As an orphan disease, leishmaniasis is scarcely studied for its physiopathology and for new therapeutic options (Randhawa, 2006) and the disease burden persists due to technical, managerial, financial and political constraints (WHO, 2002). It has become therefore one of the top five diseases targeted by the WHO Special Program for Research and Training in Tropical Diseases (Chaudhary *et al.*, 2008; Mehta *et al.*, 2009).

Nowadays, cutaneous and visceral leishmaniases cases reported in Cameroon are particularly severe in the northern part of the country where Mokolo has been revealed a focus of cutaneous form, while Kousseri is known to be a focus of visceral leishmaniasis (Dondji *et al.*, 2001).

Increasing risk factors are making leishmaniasis a growing public health concern for many countries around the world (Diza *et al.*, 2008). According to the WHO (2002) visceraal leishmaniasis is usually fatal when untreated, mucocutaneous leishmaniasis is a mutilating disease, while CL is disabling mainly in case of multiple lesions or diffusion. Approximately 90% of *Leishmania* infections are localised cutaneous form (von Stebut, 2007b). Cutaneous leishmaniasis affects the skin, an organ which is reported by Saladin (1998) as an important organ for the individual recognition, social acceptance, and self-image. In some circumstances the disfiguring outcome can cause social prejudice and harm the prospect of marriage and survival especially in women (Vlassoff, 1993; WHO, 1993; Pilcher, 2004). CL is therefore a stigmatizing disease (Alvar *et al*., 2008). On the other hand, there is currently a superposition of normally rural diseases, leishmaniasis, and a normally urban one, AIDS (Dedet, 1999a). The overlapping of leishmaniasis and AIDS has led to an emerging new entity *Leishmania*/HIV co-infection (WHO, 2007). Today, leishmaniasis has gained a relevant position worldwide among the causes of death by infectious diseases. This can be attributed to its risk of co-infection with HIV and rapid emergence of drug resistance (Patel & Shah, 2008). In Europe, intravenous drug users have been identified as the main population at risk of co-infection. In east Africa and India, the problem is frequently related with migrants, seasonal workers, refugees, sex workers and truck drivers. Individual risk factors such as malnutrition and immuno-suppression play an important role (WHO, 2002).

The dual infection of *Leishmania* and human immunodeficiency virus (HIV) is therefore obviously known, while AIDS by itself is a public health problem with a fatal issue, since an efficient drug for its cure is not yet available. In fact, Desjeux and Alvar (2003) reported that the total number of HIV-attributable deaths since the beginning of the epidemic is estimated to be more than 25 million, with most HIV-positive (>95%) cases found in the developing world; these authors added that HIV infection remains a challenge in industrialized countries. In the same report, the sub-Saharan Africa in 2002 registered 3.5 million individuals (adults and children) newly infected with HIV and 29.4

million living with HIV/AIDS. According to the report of the Cameroon National AIDS Control Committee (2008), the national prevalence of HIV infection in the year 2007 was >5% with a prevalence of 4.0% registered in the Far North Region, the site of the present study.

The relationship between CL and AIDS is poorly known (Guigemdé *et al.*, 2003). Few HIV-infected CL or MCL patients have been reported and observations on the treatment of CL and MCL in HIV-positive patients are largely anecdotal (Laguna, 2003); these need to be investigated. Nevertheless, despite the evidence of the presence of the disease in Cameroon as well as a case of visceral leishmaniasis associated with HIV reported by Kaptué and collaborators (1992), no current data are available. Couple of years ago, Mbuagbaw and co-workers (2006) presented a report on skin diseases affecting HIV/AIDS patients from different geographical locations in Cameroon without mentioning CL. On the other hand the last field survey on leishmaniasis was realized by Dondji (1997) more than ten years ago. This last investigator confirmed the presence of the disease in the area and elucidated some factors which lead to. Nevertheless, he performed his study using the mass sampling rather than the door-to-door sampling. Consequently, he could not provide the accurate prevalence of the disease.

To our knowledge, there are no cases of CL associated with HIV infection reported in Cameroon and in the Central Africa Sub-region as a whole. Moreover, data are scanty on the physiopathology, namely the characterization of cellular and humoral immune responses of *Leishmania/*HIV co-infection and on the identification of the different virus subtype involved in such a co-infection in human. In an attempt to fill this gap, the present investigation was undertaken on the immunological and histopathological aspects of the CL and the *Leishmania*-HIV co-infection, starting by epidemiological and clinical studies in northern Cameroon. Thus, we have planned to:

- update the epidemiological data and present clinical features of CL as well as the *Leishmania*-HIV co-infection in our study area;

- investigate the role of inflammatory cells in human CL lesion sites;

- characterize cytokines/chemokines that serve as potential predictors of clinical course of CL in humans and their correlation to the clinical outcome of the infection;

- evaluate the difference in the immune and therapy responses determined above in immunocompetent and immunodeppressed (e.g. HIV infected patients) CL patients.

CHAPTER I

REVIEW OF RELATED LITERATURE

I.1 THE IMMUNE SYSTEM: GENERAL ORGANIZATION AND PHYSIOLOGY

I.1.1 General organization of the immune system

I.1.1.1 Organs and tissues of the immune system

The body's immune response is its third line of defence against microorganisms (the first line includes the mechanical barriers of skin, mucous membranes, body hair, and body secretion; the second includes the inflammatory process) (Eisenhauer *et al*., 1998). Immune system also refers to a system of surveillance and maintenance of local or systemic homeostasis of the organism (Bosque *et al*., 1999). It is a collection of organs and vessels involved in producing and delivering the cells and molecules that protect the body from foreign invaders (Sell, 2001; Kelly, 2007).

The immune system is made of several special tissues, collectively designated as lymphoid or immune tissues (Virella & Goust, 2001). Organs of the immune system are divided into primary and secondary organs. Primary or central organs, such as bone marrow and thymus, are those within which immune cells mature. Secondary or peripheral organs are those at the level of which there are identification of antigens, and multiplication of immune cells. These organs include lymph nodes, tonsils, the spleen, amygdales (Schäffler and Menche, 2004; Kelly, 2007; Wikibooks Contributors, 2007). Lymphoid tissues include Peyer's patches of the intestine, the appendix and many mucosa-associated tissues such as the gastro-intestinal tract-associated lymphoid tissue (GALT), the bronchus-associated lymphoid tissue (BALT), the skin-associated-lymphoid tissue (SALT) and the ducts-associated lymphoid tissue (DALT). GALT and BALT have both central and peripheral functions (Sell, 2001).

I.1.1.2 Cells of the immune system

The major cells of the immune system are lymphocytes and macrophages (Saladin, 1998).

Lymphocytes are divided, on the basis of their function and phenotypic markers, into T lymphocytes (LT), B lymphocytes (LB) and Natural Killer T (NKT) cells (Sell, 2001; Schäffler & Menche, 2004).

Different T cells subpopulations are:

- T-helper (Th) cells which function is to help antibodies formation (Sell, 2001), activate plasmatic cells and Natural Killer (NK), and recognise the antigen presented by the antigen presenting cell (APC) (Schäffler &Menche, 2004).

- T-cytotoxic or cytolytic (Tc) cells kill target cells;

- T-delayed-type hypersensitivity (T_{DTH}) cells induce inflammation (Sell, 2001);

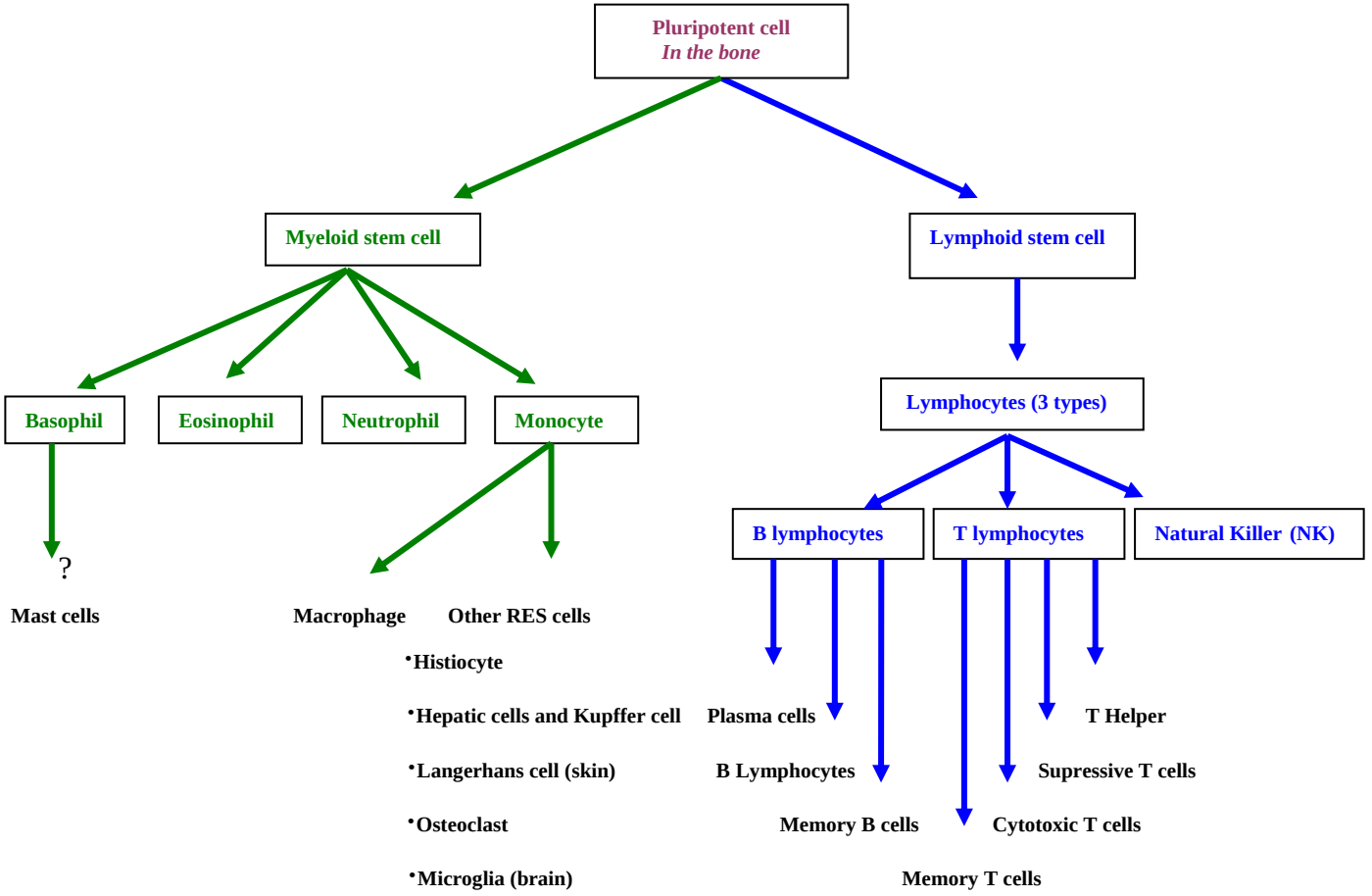
- T-regulatory (Treg) normal physiologic role is to mediate peripheral tolerance, thereby preventing autoimmunity, and to limit the damage caused by inflammatory responses to infectious agents (Macatangay *et al.*, 2010).

Subclasses of T-helper lymphocytes can be identified on the basis of their repertoire of cytokines (Borish & Steinke, 2003). From the naive lymphocytes (Th0) can derive Th1, Th2, Th17, Tfh, Treg dependently on the cytokine types present in the milieu (King *et al*., 2008).

Lymphocyte B cells are programmed to produce specific antibodies (immunoglobulines) and when a B cell encounters the kind of antigen that triggers it to become active, it gives rise to many large cells known as plasma cells, which produce antibodies (Kelly, 2007).

Natural Killer (NK) cells represent 10–15% of circulating lymphocytes and, by their cytotoxic potential and secretion of cytokines/chemokines, they are important mediators of both natural and adaptive immunity. Functionally, NK cells are defined by their ability to lyse the K562 erythromyelocytic leukemia cell line. NK cells play an important role in the innate defense against viral, intracellular bacteria and parasitic infections. They participate in immune surveillance against malignancies, in transplantation, in autoimmune diseases and immunosuppression, and also mediate physiological regulation of hematopoiesis, homeostasis of reproduction and the control of placentation (Dines *et al.*, 2002).

All the immune cells above derive from the pluripotent stem cells located in the red bone marrow. During their differentiation, stem cells have two main pathways namely the myeloid and the lymphoid pathways (Schäffler & Menche, 2004) (Figure 1).



(Souce: Schäffler & Menche, 2004 simplified)

Figure 1: Genealogy of the immune cells

RES: Reticulo-endothelial system; → Myeloid pathway; → Lymphoid pathway

Macrophages (MΦ) subpopulations include:

- histiocytes, wandering MΦ of the loose connective tissues;
- reticular cells in the stroma of lymphatic organs;
- microglia in the central nervous system;
- alveolar MΦ in the pulmonary alveoli;
- Kupffer cells lining the liver sinusoids (Saladin, 1998; Forget, 2004);
- osteoclast and chondroclast in bones and other connective tissue;
- dendritic cells (Sell, 2001; Forget, 2004).

Human dendritic cells (DC) represent a rare and heterogeneous population of cells that originate from CD34+ hematopoietic stem cells (Gogolak *et al*., 2007). DC are divided into two major groups: the conventional DC, these are non lymphoid tissue migratory and lymphoid tissue-resident DC; and plasmacytoid DC (pDC) also called natural interferon-producing cells. In the skin, there are epidermal DC also called Langerhans cells (LC) and dermal (interstitial) DC (dDC) (Palucka & Banchereau, 2006; Brosh *et al.,* 2008; Merad & Manz, 2009).

Others cells, namely myeloid stem cells, are also involved in the immune response. These cells include granulocytes or polymorphonuclear (PMN) which are neutrophils, eosinophils and basophils. Neutrophils, also known as microphages (Sell, 2001), are the most abundant leukocytes in the blood and are the first cells recruited to inflamed tissues. They have a pivotal role in immunity to infection not only by ingesting and destroying microbes, but also by secreting various cytokines. Neutrophiles also secrete Neutrophils extracellular traps (NETs), which are webs composed of chromatin and granular proteins that have been shown not only to ensnare bacteria and fungi, but also to provide a high local concentration of antimicrobial molecules (Guimarães-Costa *et al.*, 2009). Neutrophils are key players of the innate immune system providing a first line of defense against invading pathogens (de Souza Carmo *et al*., 2010). They contain granules with microbicidal molecules namely oxygen metabolites such as superoxide anion, hydrogen peroxide, and hydroxyl radicals. Eosinophils contain in their granules basic protein and aryksulfatase, which are toxic to parasitic worms. These cells also contain histaminase and may limit or modulate mast cell-mediated inflammation. Basophils granules contain heparin, histamine, and 5-hydroxytryptamine (serotonin). Mast cells, related to basophils, are located in loose connective tissue. Mast cells are

divided into two main subpopulations: those containing tryptase (MC^T) and those containing both tryptase and chymase (MC^{TC}). MC^T predominate in lung and mucosa while MC^{TC} predominate the connective tissue of bowel submucosa and skin (Sell, 2001).

Immune cells harbour on their surface or in their cytoplasm specific markers known as cluster of differentiation or CD. For example, CD1a is DC marker, while CD4 and CD8 are for T cell, CD56 for NK, CD20 for B cells, CD68 for MΦ (Geiger *et al*., 2009), CD62 (Selectin) for leucocytes, platelets, endothelial cells (Walzog & Gaehtgens, 2000), CD54 (ICAM-1) for leukocytes, DC and activated endothelial cells (Virella & Goust, 2001). Extravasated positive T memory cells present CD11a, CD18 and CD54 markers (Meymandi *et al.*, 2009).

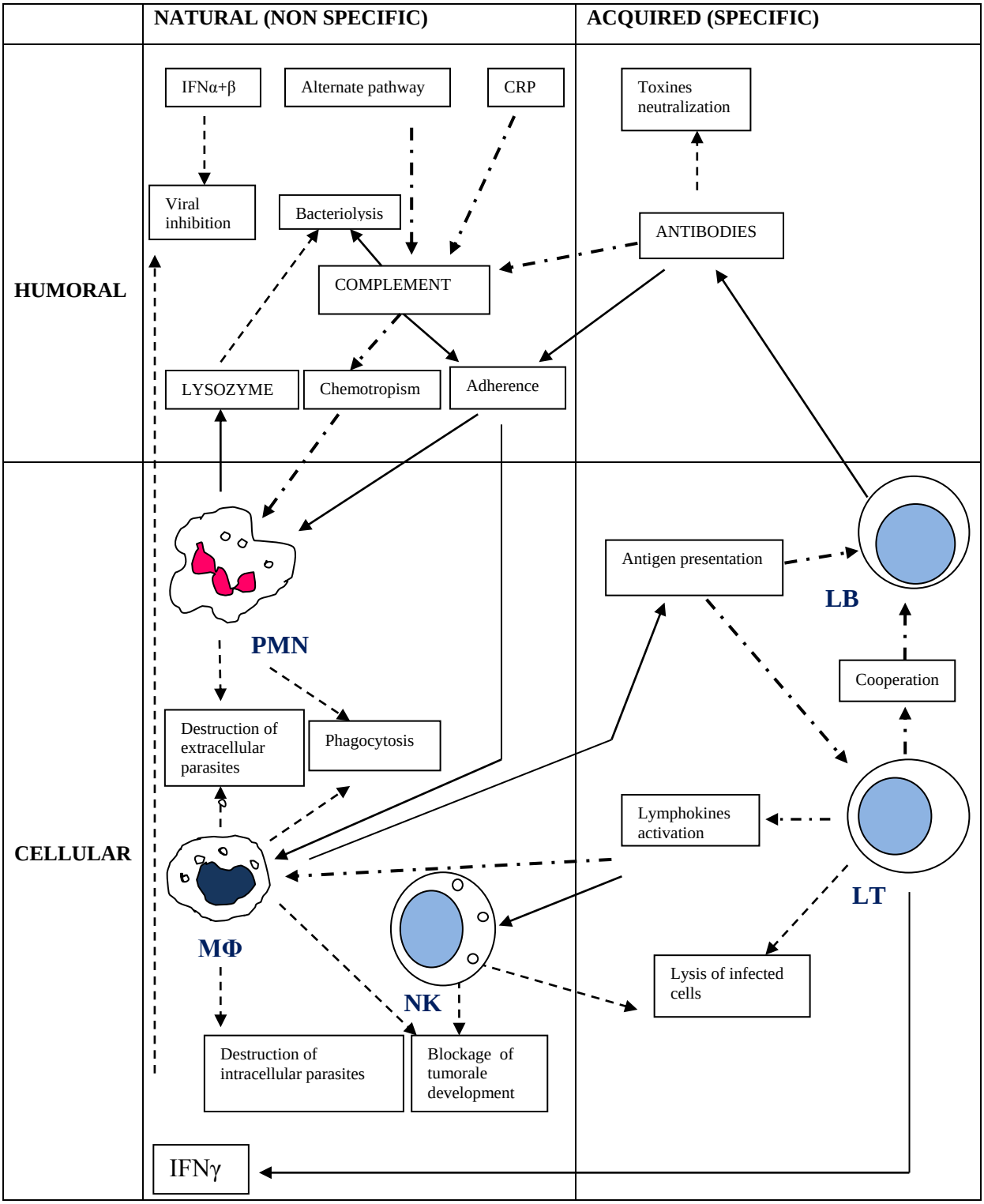
I.1.2 Physiology of the immune system

The immune reaction can be natural/innate or acquired/adaptive. Immune response is either cell mediated or humoral mediated (Figure 2) (Hole, 1993; Sell, 2001).

The natural immunity, also known as non specific is composed by species resistance (Hole, 1993), external barrier, WBC like phagocytes, NK, and many factors namely complements, cytokines and lysozyme (Hole, 1993; Schaffler &Menche, 2004).
The simplest mean to avoid an infection is to prevent the microorganisms from gaining access to the body. This is first done by the skin but the mucous also protects the epithelial glands (Roitt, 1990; Ferrera & Carro, 2001). Mechanical means used to trap and eliminate microorganisms and other foreign particles include ciliary movement, coughing and sneezing. Among other mechanical factors, which help protect the epithelial surfaces, one should also include the washing action of tears, saliva and urine. Many of the secreted body fluids contain bactericidal components, such as acid in gastric juice, spermine and zinc in semen, lactoperoxidase in milk and lysozyme in tears, nasal secretions and saliva. Another way to control invasion of the body by microorganisms is the antagonism with the natural flora (Roitt & Delves, 2001).

Phagocytosis is mainly performed by neutrophils and MΦ, but also by eosinophils and DC. These last cells have as primary role, the stimulation of the adaptative immunity (Campbell & Reece, 2005). In the phagocytes, the killing processes of pathogens can be oxygen-dependent (nitric oxide or NO and oxygen intermediates) or not (Roitt, 1990;

Sell, 2001). Also, cytotoxicity can be achieved by CD8 T, NK, CD4 T, and NKT cells, mainly through mechanisms involving antigen-dependent or –independent apoptosis of target cells (Ruiz and Becker, 2007).



(Adapted from Roitt, 1990)

Figure 2: Overlapping mechanisms of natural and acquired immune reactions

CRP: C Reactive protein, PMN: Polymorphonuclear, NK: natural killer, LT: T-lymphocyte, LB: B-Lymphocyte, MΦ: Macrophage, Stimulation: ⟶ Inhibition: ⟶ Induction: ⟶

The early concept of innate immunity was that it nonspecifically recognized microbes; however, the discovery of toll-like receptors (TLRs) in the mid-1990s showed that pathogen recognition by the innate immune system is instead actually specific, relying on germline-encoded pattern-recognition receptors (PRRs) that have evolved to detect components of foreign pathogens referred to as pathogen-associated molecular patterns (PAMPs). PAMPs recognized by TLRs include lipids, lipoproteins, proteins and nucleic acids derived from a wide range of microbes such as bacteria, viruses, parasites and fungi (Kawai & Akira, 2010). In humans, ten TLRs have been identified; more are still to be described (Maurer *et al.*, 2009). The recognition of PAMPs by TLRs occurs in various cellular compartments, including the plasma membrane, endosomes, lysosomes and endolysosomes (Kawai & Akira, 2010).

The acquired immunity also known as specific defence (Campbell & Reece, 2005) works by clonal selection. Independently on exposure to an antigen or pathogen, the immune system generates a repertoire of immune cell lineages or clones, each encoding a receptor with a predetermined shape and specificity. The human immune system creates in excess of 10^6 different clones. As a first approximation, those that react with self-antigens are deleted shortly after they mature. When the individual is infected with a pathogen, those clones that are specific for the pathogen will proliferate, producing a pathogen-specific immune cell population that is large enough to control that pathogen. This process is known as clonal expansion. After the pathogen is cleared, some of the pathogen-specific immune cells survive and confer immune memory (Bergstrom & Antia, 2006).

It is well known that DC are pacemakers of the immunity (Satthaporn & Emerin, 2001) they belong to the antigen-presenting cell (APC) family, which also includes B cells and macrophages (Merad & Manz, 2009). B and T lymphocytes are the mediators of immunity, but their function is under the control of DC which not only activate lymphocytes but also tolerize T cells to self-antigens thereby minimizing autoimmunity (Banchereau & Steinman, 1998).

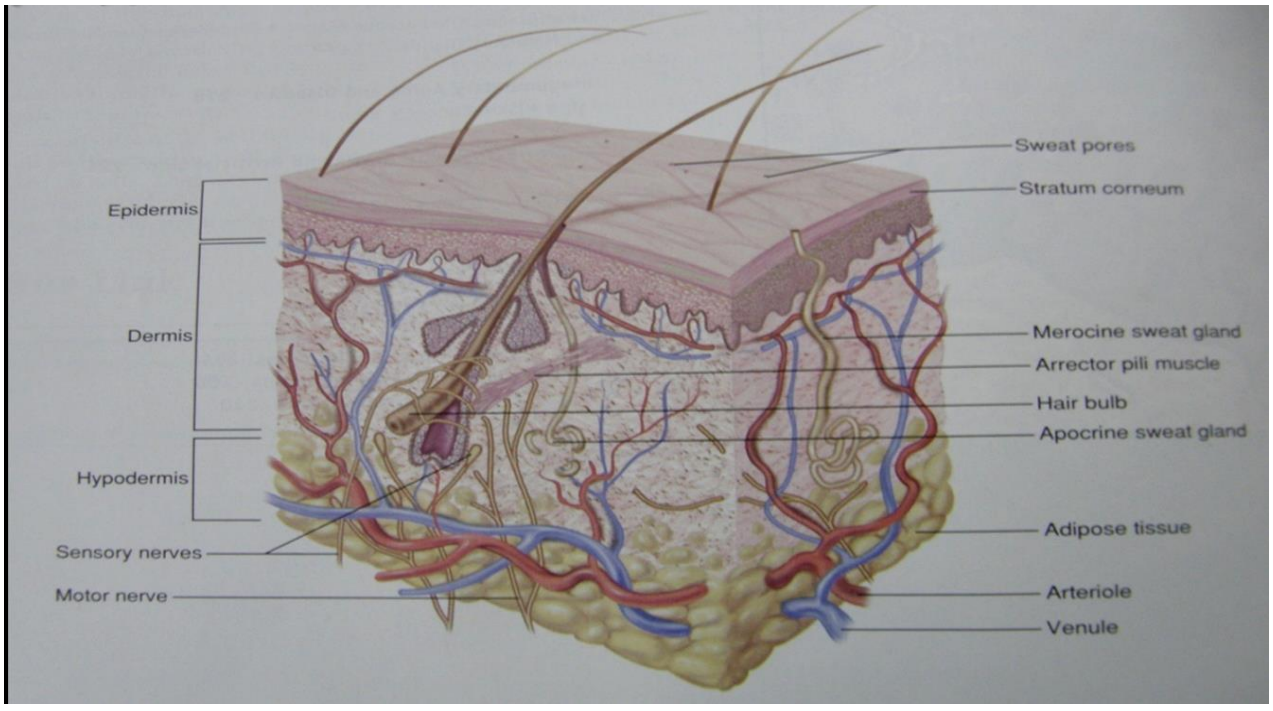
Immunity can be strong or weak, short-lived or long-lasting depending on the type of antigen, the amount of antigen, and the route by which the antigen enters the body. Immunity can also be influenced by inherited genes. When faced with the same antigen, some individuals will respond forcefully, others feebly, and some not at all (Kelly, 2007).

I.1 .3 Skin: Structure and role in the defence of the organism

I.1.3.1 Structure of the skin

The skin (Figure 3) or integument is the body's largest organ (Saladin, 1998; Eisenhauer *et al.*, 1998; Sell, 2001; Schäffler and Menche, 2004).With its appendages such as hair, nails, and glands, the skin forms the integumentary system (Saladin, 1998). It is the interface between the body's internal and external environments (Eisenhauer *et al.*, 1998), and the most common site for manifestations of immune reactions (Sell, 2001). In adults, the total area of the skin is 1.70 m^2 (Lacombe, 1988); it covers an area of about 15% of the body weight (Saladin, 1998).

Consisted of two main layers namely the epidermis and the dermis (Saladin, 1998; Angel *et al.*, 2001) below which the hypodermis, the skin is mostly thick from 1 to 2 mm. Its thickness ranges, however, from about 0.5 mm on the eyelids to 6 mm between shoulders. The difference in total thickness is due mainly to the variation in the thickness of the dermis. The epidermis accounts for only 0.007 to 0.12 mm of the total (Saladin, 1998).

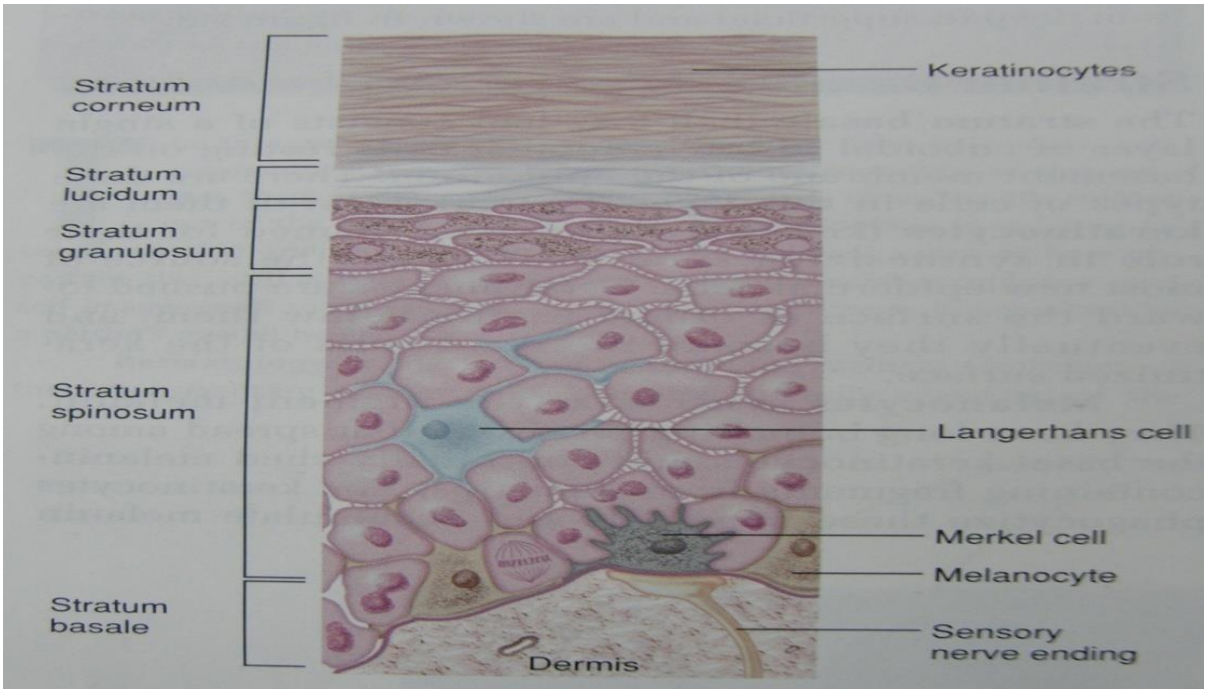


(Source: Saladin, 1998)

Figure 3: Skin structure, hypodermis and accessory organs

The epidermis (Figure 4) is a stratified squamous epithelium, and it usually consists of five layers which are, from the outside to the inside, the *stratum corneum*, the

stratum lucidum, the *stratum granulosum*, the *stratum spinosum* and the *stratus basale.* Like other epithelia, the epidermis lacks blood vessels and depends on diffusion of nutrients from the underlying connective tissue (Saladin, 1998).



(Source: Saladin, 1998)

Figure 4: Structure of the epidermis

The dermis is composed mainly of collagen but also contains elastic and reticular fibers, the usual cells of fibroconnective tissue, and an abundance of blood vessels, sweat glands, hair follicles, several kinds of sensory nerve endings, and nail roots in fingers and toes (Saladin, 1998).

I.1.3.2 Role of the skin in the defence of the organism

As the body's largest and most exposed interface with the environment, the skin has a central role in host defence (Kupper & Fuhlbrigge, 2004). One of its most important functions is to provide protection from infectious pathogens including intracellular parasites (Maurer *et al.*, 2009). Skin and epithelial surface of airways and the gastrointestinal tract represent the first line of defence; they constitute together the largest immune organ of the body (Palucka & Banchereau, 2006). The simple means to avoid infection is to avert the penetration of microorganisms in the body. This is first done by the skin, because this organ not only is impermeable to microorganisms but also it produces sweat containing lactic acid and sebum rich in fatty acids. Both lactic and fatty

acids are microorganism destroyers (Roitt, 1990; Ferrera & Caro, 2001). In addition, there are antimicrobial peptides (AMPs) which have broad antibacterial activity against gram-positive and negative bacteria and also show antifungal and antiviral activity. Human skin is a major source of antimicrobial peptides. The antimicrobial peptides produced in human skin include defensins, cathelicidins, dermicidin, and other short proteins first discovered for other biological activities such as neuropeptides and chemokines. Many other larger proteins with direct antimicrobial action also can be found in the skin such as lysozyme, elastase, complement, S100 proteins, and others (Kenshi & Richard, 2008).

The skin is evenly the most common site for manifestations of immune reactions. It is a lymphoepithelial organ that takes an active part in both non-specific and specific reactivity. Its immune system components include antigen processing Langerhans cells class II Major histocompatibility complexe (MHC), subcutaneous dendritic cells (class II MHC), and keratinocytes (class I and class II MHC); antigen reactive T and B cells, and granulocytes, mast cells, endothelial cells, and fibroblasts (Sell, 2001). Langerhans cells are unique APC present in the epidermis (Mizumoto & Takashima, 2004). They are precursors of dendritic cells in the lymph nodes. Langerhans cells process antigen and play a key role in induction and maintenance of immunity through activation of T and B cells. Keratinocytes have emerged as major producers of cytokines. They may provide continuing physiological signals for response to external stimuli with the production of inflammatory cytokines, adhesion molecules, chemotactic factors for inflammatory cells.

Located in the skin close to the endothelial cells are mast cells, MΦ, T cells, and dermal dendritic cells (dDC), which form the perivascular unit. This unit provides chemoattractive and adhesion molecules that signal inflammatory cells to enter the skin. Dermal T cells provide a rapid response to recall antigens and initiate cell-mediate immunity, dermal MΦ are activated to phagocytise and clear away products of inflammation as well as provide proliferation signals to dermal fibroblast and endothelial cells for repair (Sell, 2001).

In order to detect invading pathogens including parasites, the skin innate system has developed a large number and variety of “sensor systems” such as toll like receptors (TLR), complement receptors (CR) and others. In the skin, a few studies have shown that keratinocytes express TLR1 and TLR4, whereas DC pathways of TLR lead to the

induction of various genes involved in host defense, including inflammatory cytokines, chemokines, MHC and co-stimulatory molecules. In mammals, TLR activation induces multiple effector Figure 5*:* invading parasites, to recruit inflammatory cells to sites of invasion, and to facilitate and promote the induction of adaptative immunity (Maurer *et al.*, 2009).

I.1.4 Cytokines/chemokines and immunity

I.1.4.1 Definition and general classification of cytokines

Cytokines are polypeptides, secreted by many different kinds of cells but most prominently by activated white blood cells, that may act on the cell that secreted it or on other cells nearby or may be released systematically and act on distant cells in other tissue sites to regulate the function of the target cell. A chalone is classically defined as a cytokine, such as the transforming growth factor β1, that specifically decreases activity or proliferation of a cell type (Sell, 2001). Cytokines represent a universal language in the communication between different cells of the organism; and each cell which activity is controlled by a given cytokine has, on its surface, a specific receptor. Cytokines can be soluble constituents as well as membrane constituents. One of their common properties is that their production is not spontaneous, since they are essentially released when a cell is activated (Cavaillon, 1993).

There are several groups of cytokines. These include interleukins, cytokines produced by WBC to act on other WBC; growth factors, which act in haematopoiesis; interferons, which inhibit viral infections; ligands, which act on T and B cells, and factors which promote or inhibit inflammation (Sell, 2001). Lymphokines are a subgroup of cytokines produced by lymphocytes while monokines are a subgroup produced by monocytes/MΦ (Sell, 2001; Kelly, 2007).

I.1.4.2 Chemokines: definition and general classification

Chemokines are a subset of cytokines that cause the directed migration of leukocytes along a chemical gradient of ligand, known as the chemokine gradient (Luster, 1998; Balkwill, 2004; Burteau *et al*., 2007). They have low molecular weight (8–11kDa) (Matte and Olivier, 2002) and they are induced by inflammatory cytokines, growth factors and pathogenic stimuli (Balkwill, 2004). Chemokine receptors comprise a large family of

seven transmembrane domain G protein-coupled receptors differentially expressed in diverse cell types (Murphy *et al*., 2000; Bacon *et al*., 2001). To date, more than 44 chemokines are known (Teixeira *et al*., 2006).

The two major structural subfamilies are distinguished by the arrangement of the two amino terminal cysteine residues, which are either separated by a single amino acid (CXC) or are in adjacent (CC) positions. C chemokines (which lack two out or four canonical, cysteines) and CX3C chemokines (with three intervening aminonacids between the first two cysteines) are minor structural subfamilies (Teixeira *et al*., 2006; Wood, 2006).

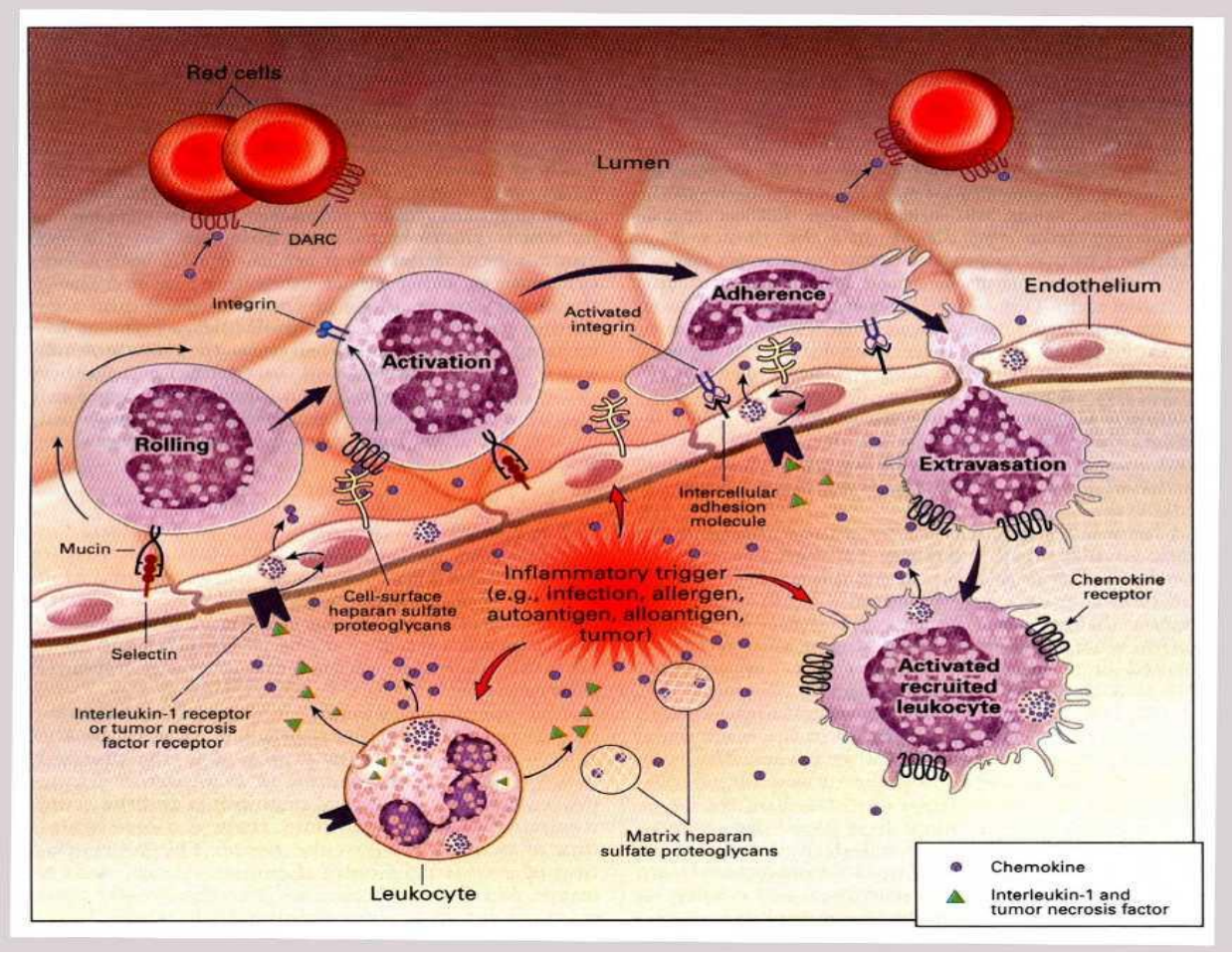
Chemokines have their receptors on the target cells. On resting T cells there is CXCR4 while on activated T cells there are CCR1, CCR2, CXCR1. Dendritic cells have CCR1, CCR2, CCR4, etc. (Luster, 1998).

I.1.4.3 Role of cytokines/chemokines in immunity

Cytokines play an important role in the communication between cells of multicellular organisms. Cells movement and extravasation are under the control cytokines/chemokines and cell adhesion molecules (Figure 5). There are several stages in extravasation: rolling, firm adhesion, and diapedesis. Cytokines such as tumor necrosing factor-α (TNF-α) are released during inflammation and stimulate the endothelium of veins to express the surface adhesion molecule P-selectin (also E-selectin). E and P-selectins bind reversibly to glycoproteins on leukocytes causing them to roll along the endothelial surface. Intercellular adhesion molecules (e.g. ICAM-1) are up-regulated and bind to the leukocyte integrins lymphocyte function-associated antigen 1 (LFA-1) and complement receptor 3 (CR3). Adhesion of leukocytes results in arrest of leukocyte motion, allowing secreted proteases to disrupt endothelial tight junctions and the basement membrane, subsequently resulting in diapedesis. L-selectins expressed by leukocytes are also involved in the process (Klein & Enders, 2007).

As intercellular mediators acting in nanomolar to picomolar concentrations, cytokines regulate survival, growth, differenciation and effector functions of cells. They are key players in the regulation of the immune response, particularly during infections, inflammatory joint, kidney, vessel and bowel diseases, or neurological and endocrinological autoimmune diseases. Unlike hormones, cytokines are not stored in

glands as preformed molecules, but are rapidly synthesized and secreted by different cells mostly after stimulation. Most cytokines are difficult to detect in serum because their producer cells are often adjacent to the target cells and usually only small amounts of cytokines are released. Cytokines act on many different target cells (pleiotropism) and frequently affect the action of other cytokines in an additive, synergistic or antagonistic manner. Besides their pleiotropic effects, cytokine actions are often redundant, i.e. similar biological responses can be achieved by several different cytokines. They exert their actions, which can be auto-, para- or endo-crine, *via* specific cell surface receptors on their target cells (Heinrich *et al.*, 1998).



(Source: Luster, 1998)

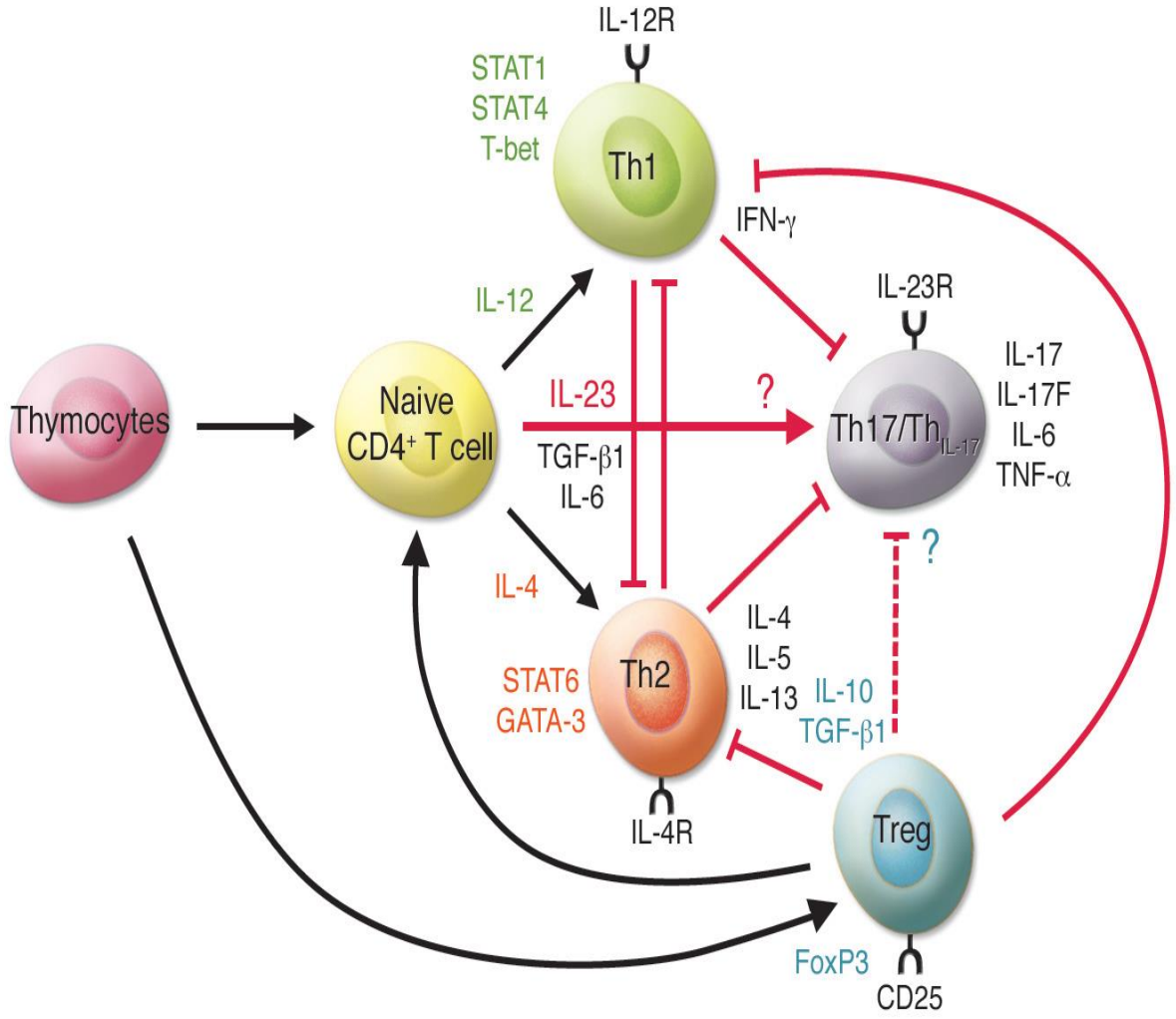
Figure 5: Chemokine regulation of leukocytes movement
DARC: Duffy antigen receptor for chemokine

Chemokines are secreted at sites of inflammation and infection by resident tissue cells, resident and recruited leukocytes, and cytokine-activated endothelial cells. Chemokines are locally retained on matrix and cell-surface heparan sulfate

proteoglycans, establishing a chemokine concentration gradient surrounding the inflammatory stimulus, as well as on the surface of the overlying endothelium. Leukocytes rolling on the endothelium in a selectin-mediated process are brought into contact with chemokines retained on cell-surface heparan sulfate proteoglycans. Chemokine signaling activates leukocyte integrins, leading to firm adherence and extravasation. The recruited leukocytes are activated by local proinflammatory cytokines (such as IL-8) and may become desensitized to further chemokine signaling because of high local concentrations of chemokines. The Duffy antigen receptor for chemokines (DARC), a nonsignaling erythrocyte chemokine receptor, functions as a sink, removing chemokines from the circulation and thus helping to maintain a tissue-bloodstream chemokine gradient (Luster, 1998).

Although the effects of cytokines are in general exerted locally at the site of their production, TNF-α and TNF-β, as well as interleukin-1 (IL-1) and IL-6, have major systemic (endocrine) effects when either produced acutely in large amounts, as in the case of bacterial sepsis, or chronically in lesser amounts, as in the case of chronic infections (Feghali & Wright, 1997). Cytokines have a central role in influencing the type of immune response needed for optimal protection against particular types of infectious agents, and they may also normally reduce allergic and autoimmune responses (Delves & Roitt, 2000). It is reported that CD4+ T cell subsets designated as Th1 produce IFN-γ, IL-2, IL-12, IL-15, IL-18 which are responsible for protective effects against pathogens mediated primarily through cellular immunity, whereas Th2 subsets produce IL-4, IL-5, and IL-10 involved in the protective effects against pathogens mediated primarily through humoral immunity (Ezra *et al*., 2010). Th0 produce primarily IL-2 but may also synthesize cytokines characteristic of both Th1 and Th2 lymphocytes. In human beings, Th1 lymphocytes produce IFN-γ and TNF-β but not IL-4 and IL-5. Th2 lymphocytes produce IL-4, IL-5, IL-9, and IL-25 but not IFN-γ or TNF-β. Both classes produce GM-CSF, TNF-α, IL-2, IL-3, IL-10, and IL-13. Th3 (Tr1) lymphocytes produce the immunosuppressive cytokines TGF-β and IL-10 and may be important in actively suppressing or terminating immune responses (after pathogens clearance) (Borish & Steinke, 2003). IL-23 induces the differentiation of naive CD4+ T cells into IL-17–producing helper T cells (Th17/ThIL-17) via mechanisms that are distinct from the Th1

and Th2 differentiation pathways. The transcriptional factors critical for the development of Th1 (STAT1, STAT4, and T-bet) and Th2 (STAT6) cells may not be required for the induction of Th17/ThIL-17 cells. The transcriptional factor(s) essential for the development of Th17/ThIL-17 cells remain unknown (Iwakura & Ishigame, 2006) (Figure 6).



(Source: Iwakura and Ishigame, 2006)

Figure 6: T helper (Th) and T regulatory (Treg) differenciation from naive CD4+ T cell
CD: Cluster of differenciation, GATA: Transcription factors which bind to the DNA sequence ''GATA'', IFN: Interferon, IL: Interleukine, TGF: Transforming growth factor, STAT: Signal transducer and activator for transcription.

Both Tc and Th1 lymphocytes can mediate killing of the infected cells against which they became sensitized. Cytotoxic T cells kill the infected cells directly, stopping the replication of the intracellular organism, while activated Th1 cells release cytokines,

such as IFN-γ, which activate macrophages and increase their ability to destroy the intracellular infectious agents (Virella, 2001). Fully committed Th1 cells express chemokine receptors, CXCR6, CXCR3, and CCR5. They are involved in cell-mediated immunity against intracellular bacteria and viruses. Th2 cells express chemokine receptors, CCR3, CCR4, and CCR8, and are important in humoral immunity against parasites and helminthes. Th17 cells require a combination of TGF-β1 and proinflammatory cytokines (IL-1b, IL-6, and/or IL-21) to differentiate from naive CD4+. Human Th17 cells produce, for example, IL-17A, IL-17F, IL-22, and IL-26, and are important in host protection against extracellular pathogens and in autoimmunity. Their surface markers include chemokine receptors, CCR4, CCR6, and CD16. In addition to effector T cells, naive CD4+ T cells can also differentiate into induced Treg (iTreg) in the presence of IL-2 and TGF-β1 or IL-10. iTreg produces immunosuppressive cytokines, transforming growth factor -β1-(TGF-β1), IL-10, and IL-35, and express surface marker CD25. Similar to thymus-derived naturally occurring Treg (nTreg, not shown), iTreg also expresses the master regulator transcription factor, Foxp3 (Di Cesar *et al*., 2009).

The main functions of chemokines are cell activation and stimulation of leukocyte migration (Burteau *et al*., 2007). Chemokines are secreted in a stimulus-specific manner from a variety of cell types, including leukocytes, fibroblasts, epithelial cells, and endothelial cells. Although each type attracts competent cells to the inflammatory site, their actions are specific to particular cellular groups. For example, members of the CXC class, such as IL-8, growth-related oncogene (GRO), and platelet factor-4, act mainly on neutrophils, whereas members of the CC class, such as monocyte chemotactic protein (MCP)-1, monocyte inflammatory protein (MIP)-1α, MIP-1β, and regulated on activation normal T cell-expressed and -secreted protein (RANTES), act on a larger group of cells, including monocytes, basophils, eosinophils, and lymphocytes, but not neutrophils. Lymphotactin, the only C chemokine, acts solely on specific subgroups of B and T lymphocytes. Recently, a CX3C branch member termed fractalkine was discovered and was reported to attract monocytes, neutrophils, and T lymphocytes, although this remains controversial (Matte & Olivier, 2002). Some pathogens use chemokines receptors to inter their host cells. The human immunodeficiency virus for example inters the T cell only when there is the simultaneous presence of the CD4 marker and CXCR4, and to enter the MΦ this virus uses both CD4 marker and CCR5 (Luster, 1998).

I.1.5 Inflammation and wound healing

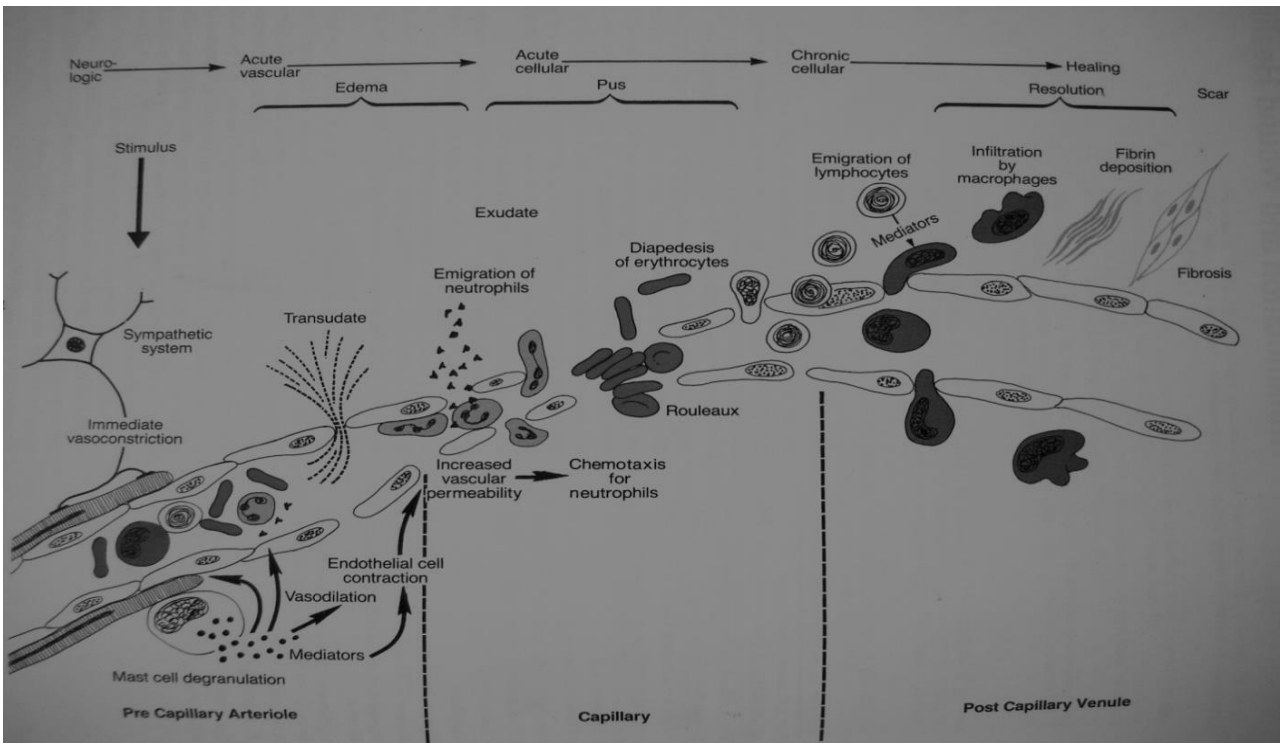
I.1.5.1 Inflammatory process

Inflammation is a response to tissue injury of any kind including trauma and infection (Saladin, 1998). It is the primary process through which the body repairs tissue damage and defends itself against infection (Sell, 2001). The inflammatory reaction is characterized by the localized redness, swelling, heat, pain (Lacombe, 1988; Saladin, 1998; Ferrera & Caro, 2001), and even the loss of function (Saladin, 1998; Sell, 2001; Wikibooks Contributors, 2007). When an injury occurs, a capillary and several tissue cells are apt to rupture, releasing histamine and kinins. These cause the capillaries to dilate, become more permeable, and leak fluid into these tissues. Dilation and fluid leaking into the tissues causes swelling, redness, and heat. The swelling and kinins stimulate nerve endings, causing pain. If there has been a break in the skin due to the injury, invading microbes may enter (Wikibooks Contributors, 2007).

Inflammation may be immune when initiated by a specific reaction of immunoglobulin or sensitized T lymphocytes with antigen; or non-immune when it is initiated by release of bacterial products, foreign bodies, or components of dying tissue. Inflammation can also be acute or chronic. Chronic inflammation follows the acute inflammation if the acute response is not adequate to clear the tissue.

The manifestations of inflammation depend upon the severity and on the location of the reaction as well as the nature of stimulus. Systemic effects of inflammation include fever and leucocytosis.

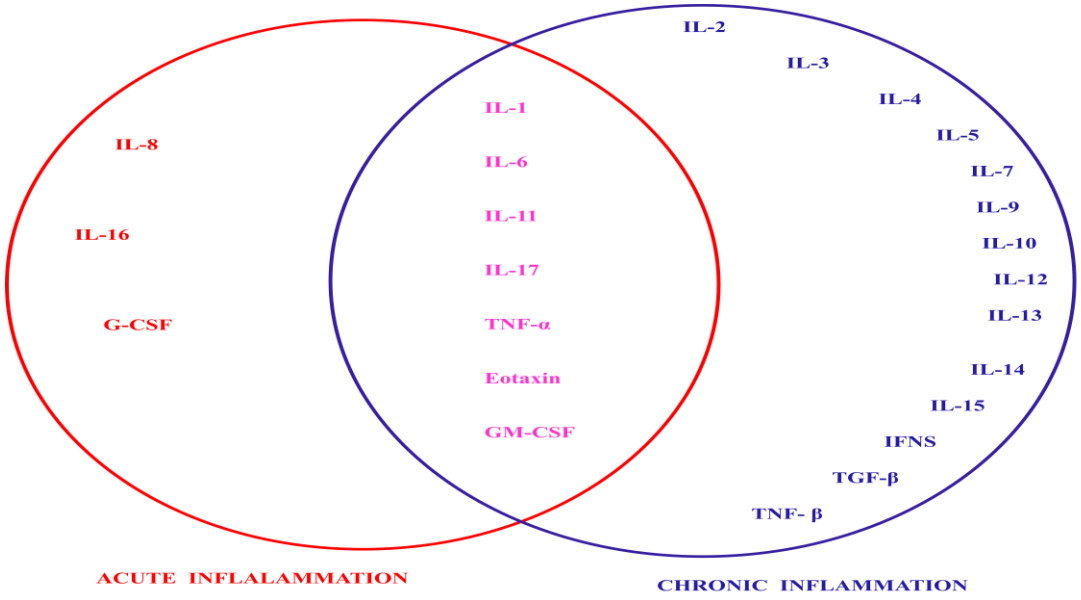
As it is illustrated in the Figure 7, the cellular players in the process of acute inflammation include mast cells/basophiles, platelets, neutrophils, and eosinophils. Granulocytic cells here present are activated by a variety of chemical processes and in turn produce and release a number of chemical mediators. The cells of chronic inflammation are lymphocytes, macrophages, and plasma cells. These are mononuclear cells in contrast to PMN which are the hallmark of the acute inflammation. Plasma cells are present in many forms of chronic inflammation and represent antibody-producing cells that migrate from lymph nodes to inflammatory sites (Sell, 2001).



(Source: Sell, 2001)

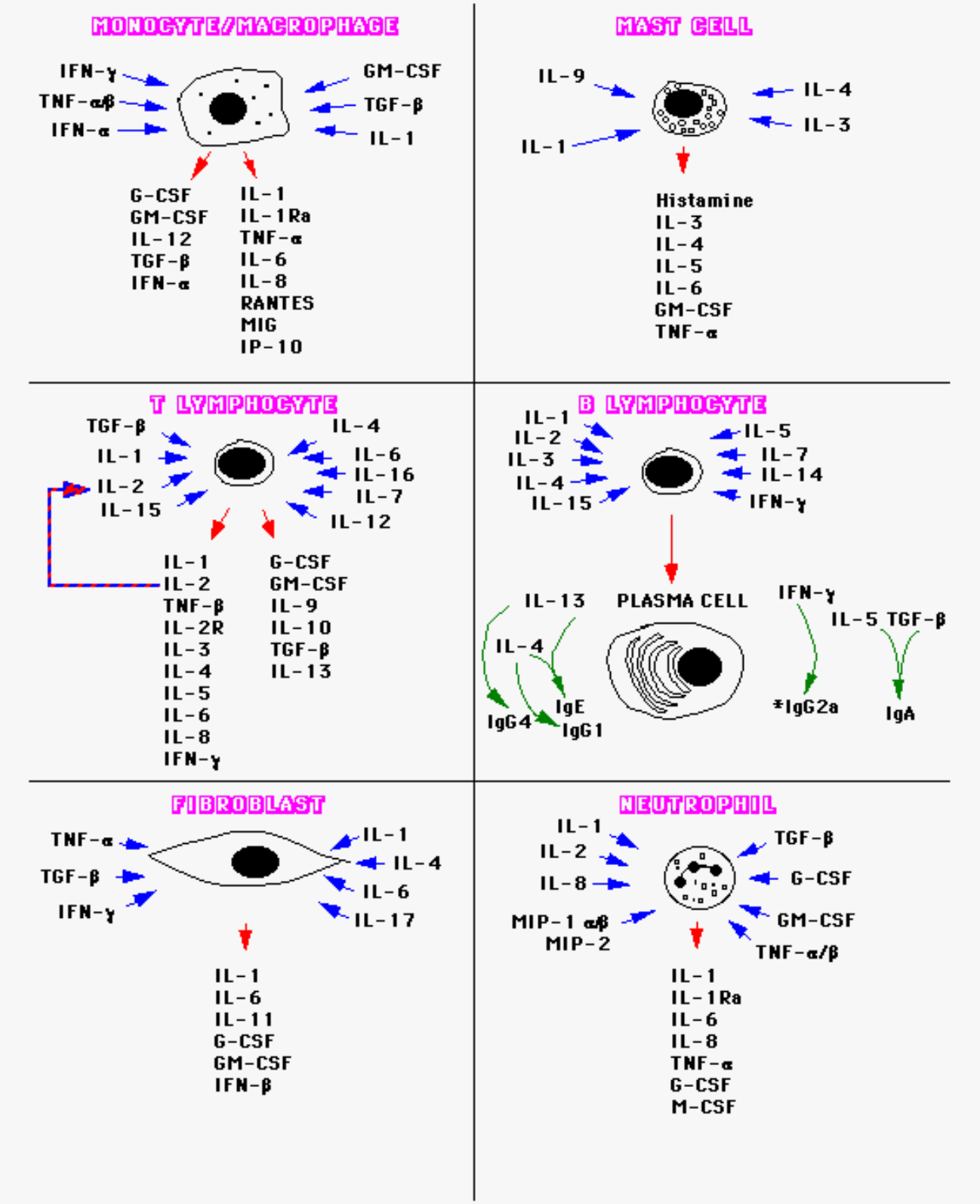
Figure 7: The sequence of events in the process of inflammation and wound healing

Amongst cytokines produced during the inflammatory process, there are those produced early but others are up-regulated later (Figure 8). Their main sources and their targets are depicted on the Figure 9.



(Source: Feghali & Wright, 1997)

Figure 8: Cytokines involved in acute and chronic inflammatory responses
G-CSF: Granulocyte-colony stimulating factor, GM-CSF: Granulocyte macrophage-colony stimulating factor, IFN: Interferon, IL: Interleukine, TGF: Transforming growth factor, TNF: Tumor necrosis factor



(Source: Feghali & Wright, 1997)

Figure 9: Inflammatory cytokines, their primary sources and target cells

G-CSF: Granulocyte- colony stimulating factor, GM-CSF: Granulocyte-macrophage colony stimulating factor IFN: Interferon, Ig: Immunoglobuline, IL: Interleukine, M-CSF: Macrophage colony stimulating factor, MIG: Monokine induced by interferon gamma, MIP: Macrophage inflammatory peptide, TGF: Transforming growth factor, TNF: Tumor necrosing factor

I.1.5.2 Mechanism of wound healing

The wound healing process is a normal physiological response to injury and generally leads to restoration of normal structure and function in damaged tissues (Bayat *et al*., 2008). The process of wound healing can be considered to begin during the earliest phase of injury or inflammation (Sell, 2001). Normal human wound healing is usually described as occurring in 3 successive and interrelated phases: inflammation, migration, and remodelling (Jones *et al*., 2004). The time of onset of healing depends on the extent of damage and whether or not inflammatory has been enhanced by infection, immune mechanisms, or chronic injury (Sell, 2001). The cell types which interact in the healing include platelets, lymphocytes, endothelial cells, macrophages, fibroblasts (Figure 7) (Sell, 2001) as well as mast cells (Bayat *et al*., 2008). In uncomplicated sterile wounds, platelets limit hemorrhage and are source of platelet-derived growth factor (PDGF) which is most likely the first growth factor to take place in the healing process. PMN and MΦ clear necrotic tissue by phagocytosis. Once lymphocytes and MΦ enter the wound, the healing process is controlled not only by cytokines but also by various growth factors as well as by cell matrix interactions mediated by integrins and cell adhesion molecules. The sequence of release of various factors and activation at the site of injury controls cell proliferation, chemotaxis, cell adhesion molecules, and interaction as well as differenciation and extracellular matrix formation (Sell, 2001).

I.2 BACKGROUND ON LEISHMANIASIS

I.2.1 Definition and classification of leishmaniasis

I.2.1.1 Definition

Amongst parasitic diseases, leishmaniases belong to those known since the Antiquity and they are rich by their events (Jarry, 1999). According to the Dictionnaire de Médecine (2001), leishmaniasis is a reticulo-endotheliasis caused by a zooflagellated of the genus *Leishmania* which is transmitted by an insect of the genus *Phlebotomus*. It is a complex of diseases caused by at least 20 species of protozoan parasite of the genus *Leishmania* (Salman *et al*., 1999).

I.2.1.2 Classification

Leishmaniases have the same mode of transmission but they differ from their clinical forms which depend upon the parasite species concerned (Dondji, 2001). Globally the disease encompasses a broad range of manifestations which can be divided into cutaneous leishmaniasis and visceral leishmaniasis (Santos *et al*., 2009). The cutaneous (or tegumentary) leishmaniasis (CL) can be divided into localized cutaneous leishmaniasis (LCL), diffuse cutaneous leishmaniasis (DCL), or muco-cutaneous leishmaniasis (MCL) (Dedet, 1999c; Alvar *et al*., 2008). The MCL is an uncommon manifestation of cutaneous leishmaniasis that may present years after the initial skin ulcer has healed. This metastatic complication of the primary lesions result in disfiguring and ulceration of mucous membranes of the nose, mouth and throat cavities (Patel & Shah, 2008). According to the healing evolution, CL can be classified as acute, chronic, recidivans, disseminated and post-kala-azar forms (Puig & Pradinaud, 2003).

The visceral leishmaniasis, which is generally more severe than CL, has two epidemiological forms. It is characterized by fever, substantial weight loss, hepatomegaly, splenomegaly, and anaemia. It is fatal without treatment and may be fatal despite of treatment (Patel & Shah, 2008). In the zoonotic form of VL, found mainly in the Mediterranean basin, the parasites infecting humans have generally come, via a sand fly, from dogs. In the anthroponotic form, which occurs, sometimes in severe and deadly epidemics, in East Africa, Bangladesh, India and Nepal, there is human-human transmission via the sand fly vector (Desjeux &Alvar, 2003).

Other names of leishmaniasis are:

CL is known as *Alep's sore, Bagdad sore, Orient sore, Jericho's sore, Bouchir's sore, Gafsa sore, Bouma sore, Dehli sore, Kantara sore, one year sore, Zibans sore, Sahara chancre, date sore.*

MCL is also called the South American forest leishmaniasis, *pian-bois, Bahia's sore, or gum trees ulcer;*

VL is also known as *kala-azar, black disease, doum-doum fever, death fever, tropical splenomegalia, ponos, Assam's disease, Sahib disease* (Dictionnaire de Médecine, 2001).

In Mokolo, Cameroon, CL is known as *Mblegoyave* or *Mwele* (Dondji, 1997).

I.2.2 Geographical distribution and prevalence

Human leishmaniasis is found in five continents and is endemic in 88 countries mainly in tropical and subtropical regions. Amongst these countries concerned, 16 are developed and 72 are developing countries. It is thought an approximate prevalence of 12 million, with 1.5 million to 2 million new cases of CL and 500 000 new cases of VL each year. Sixty-two countries have endemic VL, and the geographical distribution of this disease is expanding in several areas of the world urbanization (e.g. in north-eastern Brazil) or massive international migrations (e.g. on the Indian sub-continent) (Desjeux & Alvar, 2003).

Leishmaniasis is endemic from northern Argentina to southern Texas (not in Uruguay, Chile, or Canada), in southern Europe, Asia (not southeast Asia), the middle east, and Africa (particularly east and North Africa, with sporadic cases elsewhere), but not in Australia or Oceania. Over 90% of worldwide cases of leishmaniases are reported in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, and Syria (Old World), and in Brazil and Peru (New World). The geographical distribution of cases evaluated in developed world reflects travel and immigration patter (Herwaldt, 1999). In northern Africa, the disease is prevalent in Morocco, Algeria, Tunisia, Egypt and Libya. Comparatively, there are low prevalence rates of the disease in West African countries including Cameroon, Ghana, Guinea, Burkina Faso, Niger, Mali, Nigeria and Senegal. Cutaneous leishmaniasis is proposed to be endemic in a belt running from Mauritania, Gambia and Senegal in the west to Nigeria and Cameroon in the east (Kimutai *et al*., 2009). VL is reported in Asia (India, Bangaladesh, Nepal, China), in southern Europe, America (Médecins sans frontières, 2003) and even in Africa (Cameroon) (Kaptué *et al.*, 1992; Dondji *et al*., 2001). Fifty to 100 cases of New World CL are diagnosed each year in the United States. They are contracted in any country from Mexico to Argentina, excepted Uruguay and Chile. There is also an endemic focus in Texas (Markle & Makhoul, 2004).

I.2.3 Germ, Vectors, Reservoir hosts

I.2.3.1 Germ

Leishman and Donovan first described the *Leishmania* protozoan in 1903. Since then, this organism has been found to be a complex grouping of species, at least 20 of

which cause infections in humans. Some species cause VL, some cause CL and some cause the both (Herwaldt, 1999; Markle & Makhoul, 2004).

The place of the genus *Leishmania* in classification is:

Kingdom: Protista Haeckel, 1866
Sub-kingdom: Protozoa Goldfuss, 1817 emend Vickerman, 1848
Phylum: Sarcomastigophora Honigberg & Balamuth, 1963
Sub-phylum: Mastigophora Diesing, 1866
Class: Zoomastigophora Calkins, 1909
Order: Kinetoplastida Honigberg, 1963 emend Vickerman, 1976
Sub-order: Trypanosomatina Kent, 1880
Family: Trypanosomatidae Döflein, 1901 emend Grobben, 1905
Genus: *Leishmania* Ross, 1903

(Pratlong and Lanotte, 1999).

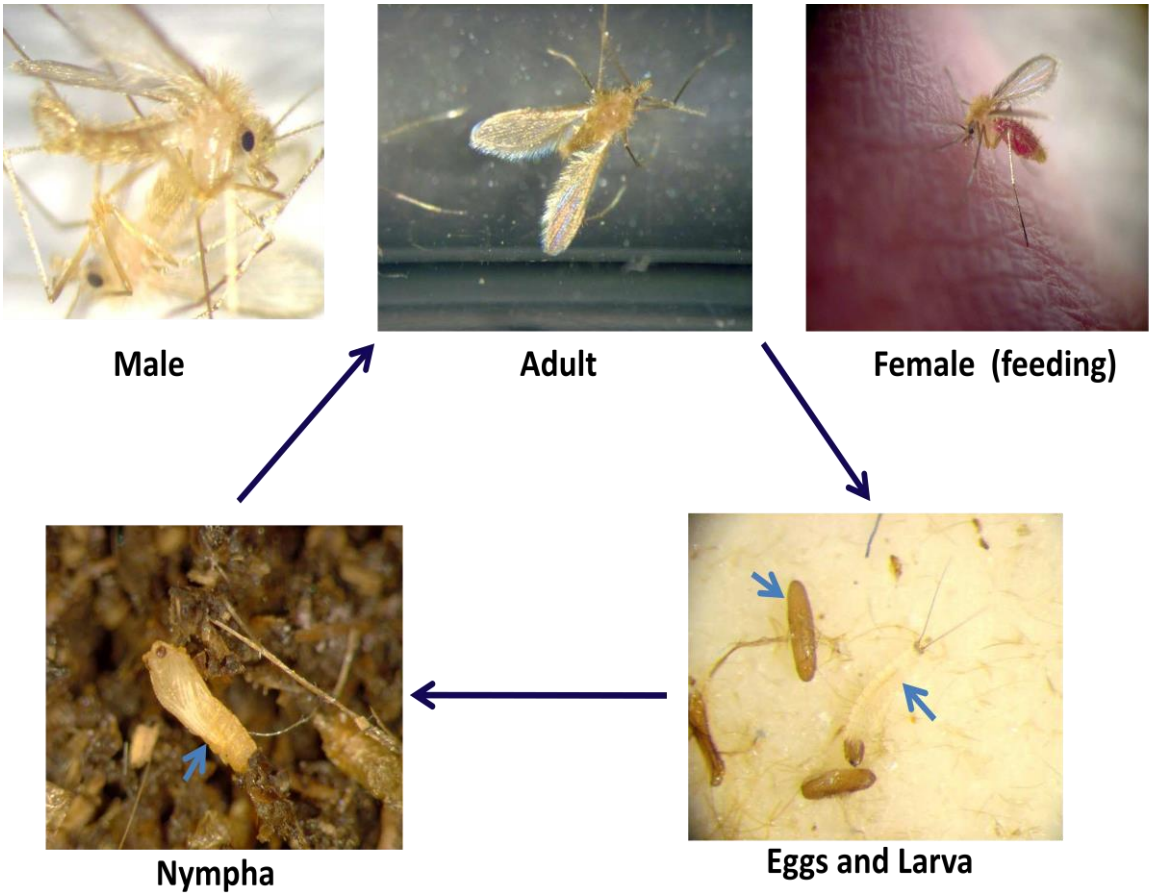
Based on its development pattern in the sand fly vectors, the genus *Leishmania* has two sub-genera: the sub-genus *Leishmania* Ross, 1903 in which the parasite growth is restricted to the fore and mid guts of the sandfly; and the sub-genus *Viannia*, Lainson and Shaw, 1987 in which *Leishmania* development is peripylorian. Each sub-genus is divided into complexes of species. The first sub-genus is distributed in the Old and the New worlds while the second is restricted in tropical and subtropical zone of the New world (Pratlong and Lanotte, 1999).

I.2.3.2 Vector

Leishmaniases are transmitted by the phlebotomine sand fly (Figure 10) which is a small, hairy and soundlessly flying insect (Sharma & Singh, 2008). Around 30 species are incriminated in this transmission (Herwaldt, 1999).

Six genuses of sand fly are known. They are: *Brumptomyia*, *Lutzomyia*, *Warileya*, *Phlebotomus, Sergentomyia* and *Chinius* but only two genuses are involved in the transmission of leishmaniases (Léger & Depaquit, 199) namely the genus *Phlebotomus* in the Old World and the genus *Lutzomyia* in the New World. The sand fly becomes infected when feeding on the blood of an infected individual or an animal reservoir (Awasthi *et al.*, 2004; Sharma & Singh, 2008). The protein from the blood is necessary for the female to develop their eggs. If *Lutzomyia wellcomei* usually bites during day

time, most female sand flies are known to bite at night and dusk. In their search for blood they cover a radius of a few to several hundred meters around their habitat. Some of the Old World species breed in peridomestic situations and enter human habitations, whereas disease transmission in the New World is associated with humans living or working near the forest (Sharma & Singh, 2008).



(Source: Boussaa, 2008)

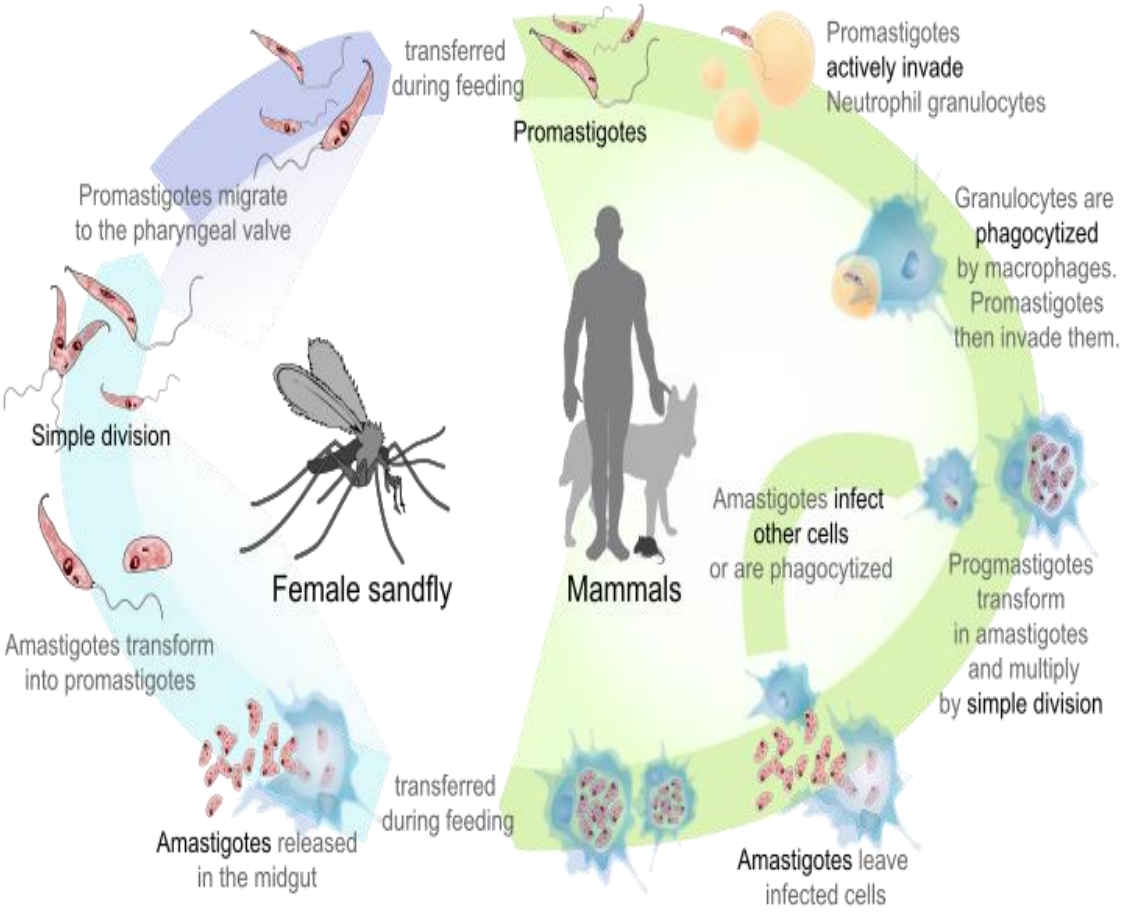
Figure 10: **Phlebotomus duboscqi** ***life cycle***

I.2.3.3 Reservoir hosts

Depending on the type of animal reservoir host, leishmaniases are classified into two groups: zoonotic and anthroponotic leishmaniases. In zoonotic type, wild, peridomestic and domestic animals (dog) are reservoirs. Some animals incriminated are foxes, jackals, wolves, rats, sloths, marsupials, dogs. In anthroponotic type of leishmaniases, man is the only reservoir (Dondji, 1999).

I.2.3.4 Leishmania life cycle

The life cycle of *Leishmania* (Figure 11) is simple and it involves two stages without sexual stage. In insect vector, the parasite is a promastigote form (elongated, motile and extracellular stage) while in vertebrates the parasite is found in amastigote form (ovoid, nonmotile and intracellular stage). The insect vector injects promastigotes into the host's skin and soon after the parasite is taken-up by skin macrophages where the promastigotes transform into amastigotes within 12–24 h of inoculation. After transformation, the amastigotes multiply within the macrophage and ultimately the macrophage bursts releasing the amastigotes to infect other macrophages. The opportunity to transmit the amastigotes from infected host to uninfected host of the same species or other species is provided by sand fly insect vector. It is postulated that in cases of CL, the infected macrophages ooze out with the pool of blood and are taken up by the sand fly. However, it is debatable in cases of VL, where the parasite is concentrated in the spleen, liver and bone marrow, but how it is made available to the sand fly which can penetrate only skin deep. It is believed that some infected macrophages are released in the blood circulation and it is a chance that the same macrophage is taken up by the sand fly. This chance factor holds further strong as only a few sand flies will be found infected even in a kala-azar household. Vice-versa is also true. In spite of, the fact that the sand fly will remain infected for whole life (few weeks), it can successfully transmit the infection only to a few patients. As the amastigotes are taken up by the sand fly, the transformation of amastigotes to promastigotes starts within hours of ingestion and completely transformed into motile promastigotes within 24–48 h and keep on dividing by binary division. The mature metacyclic promastigotes are accumulated in the midgut and foregut. The sand fly transmits the infection during another blood meal on the same or another host species (Sharma & Singh, 2008).



(Source: http://en.wikipedia.org/wiki/File:Leishmania_life_cycle_diagram_en.svg)

Figure 11: **Leishmania *life cycle***

I.2.4 *Leishmania* parasite and the immune response

I.2.4.1 Inoculation of Leishmania by sand fly and subsequent uptake by immune host cells

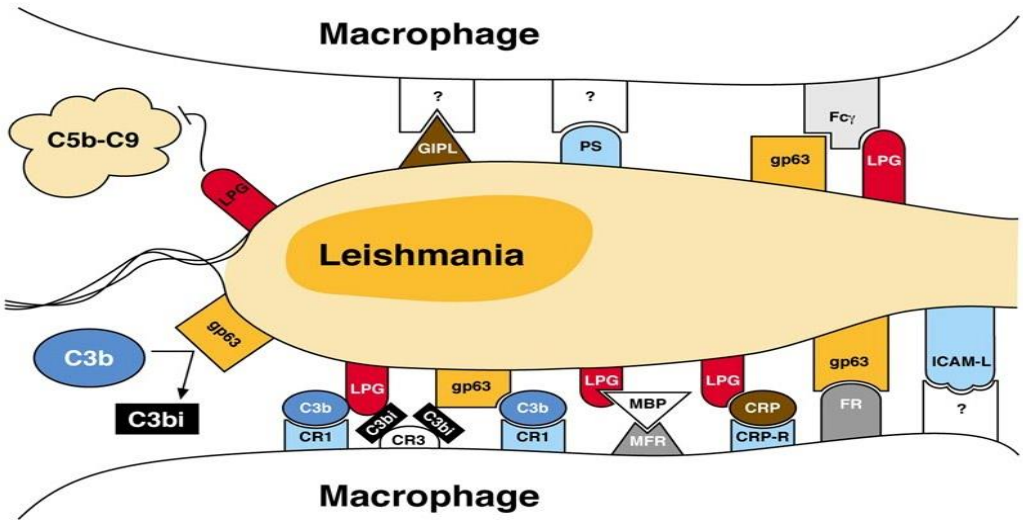
When the infected haematophagous sand fly bites a vertebrate host, it introduces its mouthparts into the skin, lacerates blood vessels causing haemorrhages and then feeds in the haemorrhagic pool formed. *Leishmania* promastigotes are regurgitated at this point in the vector saliva. Without the vasodilator activity of its saliva, the sand fly would hardly achieve the dermal capillaries. At this point, a battle takes place between the host resistance and the insect's strategies to obtain blood. At the host's side, the natural defense mechanisms will be activated but the sand fly saliva has potent vasodilatatory agents (de Almeida *et al*., 2003).

Even if after inoculation most *Leishmania* promastigotes are rapidly killed in the extracellular tissue environment, some can escape the toxic extracellular milieu and

survive if they gain access to phagocytic cells (van Zandbergen *et al.*, 2004). Promastigotes release a chemotactic factor for neutrophils (van Zandbergen *et al.*, 2002) and these cells are rapidly and massively recruited to the site of *Leishmania* inoculation, where they phagocytose the parasites, some of which are able to survive within these first host cells (Aga *et al.*, 2002; Awasthi *et al.*, 2004; John & Hunter, 2008; Peters *et al.*, 2008; Ritter *et al.*, 2009; Charmoy *et al.*, 2010). Importantly, *Leishmania*, by their lipophosphoglycan (LPG) induce NET (neutrophils extracellular trap) production by neutrophils. NETs are known to destroy *Leishmania* parasites, but also neutrophils by NETosis (Guimarães-Costa *et al.*, 2009). Inside neutrophils, the parasites which survive do not undergo multiplication. The life span of neutrophils is within 6–12 h. But *Leishmania* can delay the apoptotic death program of neutrophils up to 42 h and, therefore, promotes longevity. However, after 42 h even the infected neutrophils die (by apoptosis, necrosis, and NETosis). The time point at which infected neutrophils become apoptotic coincides with the peak migration of MΦ into the infected tissue. Infected human neutrophils secrete high levels of MΦ inflammatory peptide-1β (MIP-1β), thus attracting MΦs (van Zandbergen *et al.*, 2004). In *L. major* infection, neutrophils apoptosis is induced by MΦ through membrane TNF-α (von Stebut, 2007a). Early after sand fly bite, MΦ and neutrophils are present in similar numbers at the site of infection, but macrophages are not the predominant cell type involved in phagocytosis of these pathogens. This could be due to compromised activity of the macrophages that are involved in clearing apoptotic neutrophils. Interestingly, in the absence of these PMN, MΦ can be recruited in the wound site, phagocytise parasites but the *L. Major* ability to establish infection is compromised. Even if the process is not clearly understood, it is then suggested that the parasites released from apoptotic neutrophils are better adapted to survive in MΦ (John & Hunter, 2008).

In physiological conditions, *Leishmania* promastigotes bind C3b and C3bi (Figure 12). This allows the parasite to bind to complement receptor 1 and 3 (CR1&3) of macrophage before it is internalized (Antoine *et al.*, 1999; Forget, 2004). Systems like toll-like receptors (TLRs) and complements receptors help to detect the presence of infection and induce activation of inflammatory and innate response. Innate immunity against *Leishmania* involves recognition receptors (TLR), cell types (myeloid dendritic cells, plasmatoid DC) cytokines (IL-12, IFN-α/β) and signaling pathways (Tyk2 kinase)

that are necessary for the initial sensing of the parasites and the subsequent development of an efficient NK cell response (Ezra *et al.*, 2010). CR1 constitutes the major macrophage receptor for mature promastigote, though additional parasite surface glycoprotein (*e.g.*, gp63 membrane protease) and other macrophage receptors (*e.g.*, CR3, mannose fucose receptor) have been mentioned (Awasthi *et al.*, 2004). DC take up *L. major* via a different receptor than the MΦ. They acquire the parasite mainly through Fcγ receptor (FcγR)I and FcγRIII-mediated uptake of amastigotes (Woelbing *et al.*, 2006; von Stebut, 2007b).



(Source: Forget, 2004)

Figure 12: Using of surface molecules and soluble proteins in* Leishmania-*Macrophage interaction
CR: complement receptors, CRP: C-reactive protein, CRP-R: C-reactive protein receptor, FR: fibronectine receptor, FCγ: Crystalisable fragment of gamma immunoglobuline, GIPL: Glycosylinositol phospholipid, gp: glycoprotein, ICAM: intercellular adhesion molecule, LPG: lipophosphoglycan, MBP: mannose binding protein, MFR: mannose fucose receptor, PS: phosphatidylserine.

I.2.4.2 Immune reactions towards Leishmania

Leishmania infection develops in a wide spectrum of clinical findings, ranging from asymptomatic, subclinical and self-resolving infection to progressive VL characterized by fever, splenomegaly and pancytopenia (Casio *et al.*, 2004). This depends not only on the parasite species but also on the type of the host's immune response (Moll & Berberich, 2001). The *Leishmania* infection is accompanied by a complex immune response that begins with the innate response, where innate receptors such as TLR2

present on macrophages, DC and NK cells recognize molecules present on the parasite surface, such as LPG, and induce the production of proinflammatory cytokines such as TNF-α, IFN-γ and IL-12, as well as costimulatory molecules. In leishmaniasis, macrophages play a triple role since they are host cells, antigen-presenting cells (APC) that activate specific T cells and effector cells whose leishmanicidal efficacy depends on the presence of activating cytokines such as IFN-γ and TNF-α (Ruiz & Becker, 2007). Neutrophils appear to have an important role in *Leishmania* elimination through phagocytosis of amastigotes in the later stages of the disease process (Daboul, 2010). *Leishmania* and LPG stimulate NET release from naïve neutrophils. These webs possess leishmanicidal activity (Guimarães-Costa *et al*., 2009).

A Th1/Th2 paradigm in leishmaniasis has been largely established (de Souza et al., 2000). It is known that the establishment of a Th1 response protects against intracellular pathogens, while a Th2 host response is linked to allergy or protection against extracellular pathogens (de Almeida *et al*., 2003). In human and experimental cutaneous leishmaniasis, development of protective immunity is dependent on the generation of IFN-producing T cell. The host response to infection appears to be regulated by specific patterns of local cytokine production. Resistance to *Leishmania* is associated with a Thl cytokine profile, IL-2 and IFN-γ; whereas susceptibility to infection is associated with production of Th2 cytokines, IL4, IL-5, and IL-10 (Pirmez *et al*., 1993, Nateghi *et al*., 2010; Ezra *et al*., 2010). CD8+T (Tc) cells and NK also contribute to destroy *Leishmania* by producing IFN-γ (Ezra *et al*., 2010). CD8 T cells can produce IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-10 and TGF-β (Ruiz & Becker, 2007). The goal of the infected host's immune system is therefore to generate antigen-specific T-dependent immunity (Th1/Tc1) to fight against *Leishmania* (von Stebut, 2007a; Geiger *et al*., 2009).

Regarding the humoral reaction, generally, elevated levels of IgG, IgM, IgE and IgG subclasses are found during leishmaniasis. In CL, usually they are present at low levels during the active phase of the disease. Contrastingly, strong antileishmanial antibody titers are well documented in VL (Patel & Shah, 2008).

I.2.4.3 Mechanisms of evasion used by **Leishmania**

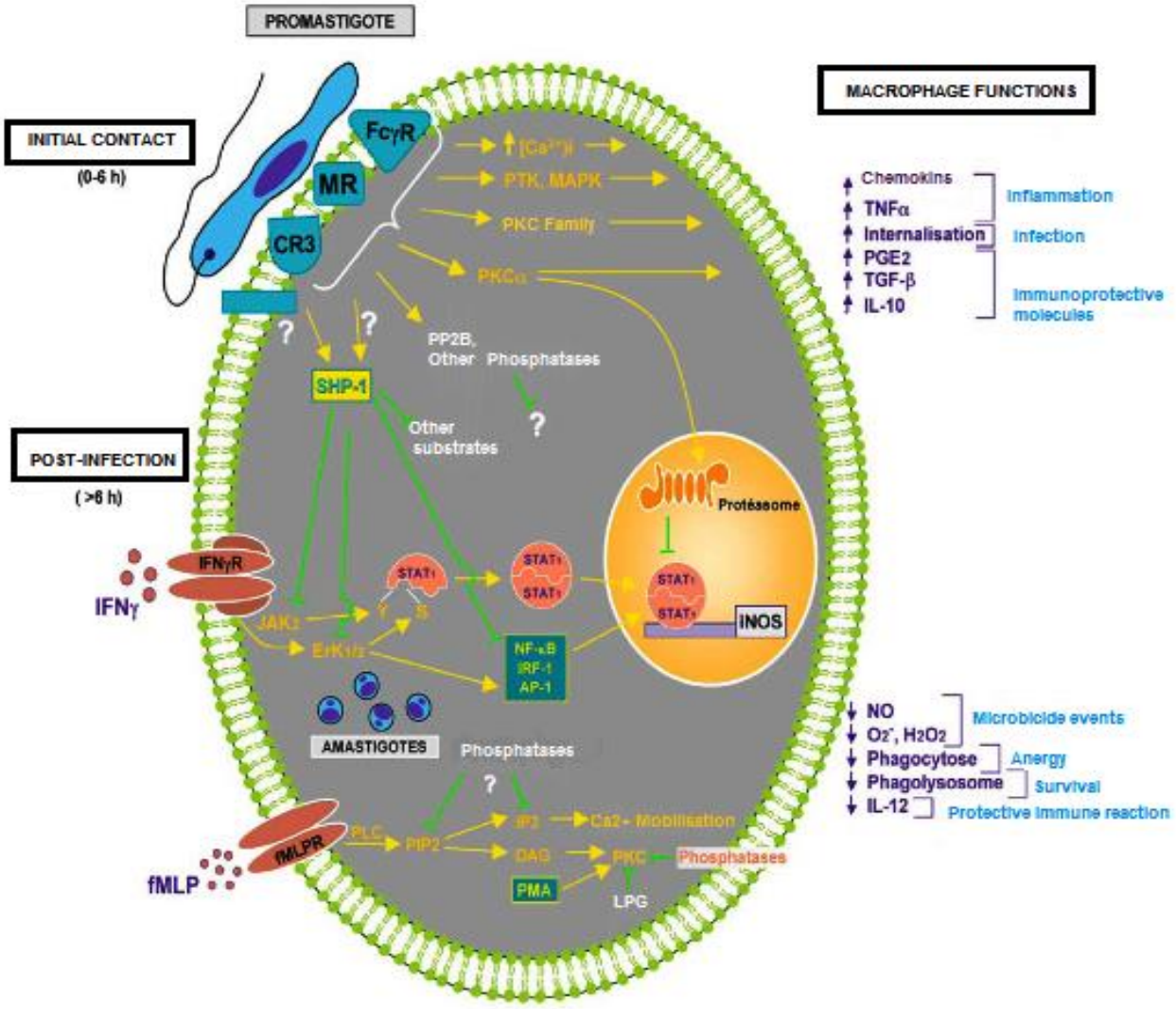
Leishmaniasis presents their host with varied immunological problems and the net outcome. The infection tends to be long and chronic with host mounting varied and

sometimes unsuccessful immunological responses. Similar to other chronic infections, it is the cell mediated immune response that has critical importance in leishmaniasis. It is responsible for the elimination or protection of the parasite and perhaps modulation of the clinical features (Simeen & Arfan, 2006). The presence, at the site of inoculation, of agents like platelets, thrombin (coagulation), kinins (vasoconstriction), complement system, natural antibodies, and phagocytes could decrease the vectorial competence, hampering or even preventing the insect ability to blood-feed. However, by the vector's side, the pharmacological agents of the saliva especially maxadilan (*Lutzomyia)* and adenosine (*Phlebotomus*), antiplatetelet aggregation, apyrase (*Lutzomyia* and *Phlebotomus*), and prostaglandin E2 (vasodilatatory and immunossupressive actions), IL-2 binding factor, can counterbalance the host response (de Almeida *et al.*, 2003). It is reported that dendritic cells uptake amastigotes rather than *L. major* promastigotes (Brosh *et al.*, 2008). Promastigotes are known to inhibit DC motility (Jebbari *et al.*, 2002). The parasite may therefore subvert DC from their potentially protective role during leishmaniasis. When the parasite is in the macrophages, the amastigotes multiply ultimately and the macrophage bursts releasing these amastigotes to infect other macrophages. This stage is chronic in nature and may continue for months to years and even for the life time without noticeable signs and symptoms (Sharma & Singh, 2008).This depending upon a number of factors , including the specie and strain of the parasite, as well as the host's genetically determined immune responses (Okwor *et al.*, 2009).

Leishmanial contact induces the release of IL-8 and inhibits the production of gamma interferon-inducible protein 10 (IP-10) by PMN. This production of IP-10 can lead to the prevention of NK activation (van Zandbergen *et al*., 2002).

The intracellular protozoan parasite *Leishmania* has been known for its ability to evade its host immune response principally by inhibiting phagocyte functions. Indeed, infected macrophages show a loss of microbicidal (NO, oxygen intermediates) and immunological activities (IL-1, IL-12, MHC). This allows for its replication and invasion of the host. These dysfunctions are correlated by alterations in signalling cascades depending on Ca^{2+}, protein kinase C (PKC), janus kinase2/signal transducer and activator for transcription1α (JAK2/STAT1α) and mitogen activated protein kinase extracellular signal related kinase (MAPK ERK1/2) (Figure 13). It has also been reported that

Leishmania infection could induce the macrophage phosphotyrosine phosphatase (PTP) activity, a negative regulator of tyrosine kinase-dependent pathways. Moreover, the use of PTP inhibitors showed their essential role in parasite (Forget, 2004).



(Source: Forget, 2004)

Figure 13: Signalisation pathways and macrophage functions modulated during the infection by* Leishmania *parasite

AP-1: Activator protein-1, CR3: Complement receptor-3, DAG: Diacylglycerol, ErK1/2: Extracellular signal related kinase1/2, FcγR: Fc receptor for gamma immunoglobuline:, fMLP: N-formyl –methionyl-leucyl-phenylalanine, IRF-1: Interferon regulated factor-1, IFNγR: Interferon gamma receptor, IL: Interleukine, IP3: Inositol triphosphate, iNOS: inducible NO synthethase, JAK: Janus kinase, LPG: Lipophosphoglycan, MAPK: Mitogen activated protein kinase, MR: Manose receptor, NF-κB: Nuclear factor kappa B, NO: Nitric oxide, PGE2: Prostaglandine E2, PIP2: Phosphatidyl inositol biphosphate, PKC: Protein kinase C, PKT: Protein tyrosine kinase, PLC: Phospholipase C, PMA: Phorbol myristate acetate, PP2B: Protein phosphatise 2B, SHP-1:Src homology 2 domain phosphatise, STAT: Signal transducer and activator of transcription, TGF: Transforming growth factor, TNF: Tumor necrosis factor.

The three major evasion mechanisms used *by L. donovani* are: (i) use of complement receptors on MΦ, (ii) inhibition of intracellular killing by MΦ, (iii) modulation of T-cell immunity or immune deviation (Sell, 2001).

In the draining lymph nodes of mice that had healed a cutaneous infection with *Leishmania major,* 40% of the persisting parasites are associated with fibroblasts forming the reticular meshwork of the lymph nodes. *In vitro,* both promastigotes and amastigotes of *L. major* infected primary skin or lymph node fibroblasts. Compared with macrophages, cytokine-activated fibroblasts had a reduced ability to express type 2 nitric oxide synthase and to kill intracellular *L. major*. These data identify fibroblasts as an important host cell for *Leishmania* during the chronic phase of infection and suggest that they might serve as safe targets for the parasites in clinically latent disease (Bogdan *et al*., 2000).

I.2.5 Evolution of the pathology

Cutaneous leishmaniasis often involves only the skin, and may be characterized by one to dozens of lesions. Depending on the species of *Leishmania*, ulcers, smooth nodules, flat plaques or hyperkeratotic wart-like lesions may be seen. The initial lesions, which occur on skin that was exposed to sandflies, are usually papules. Many lesions remain localized, but in some cases, the parasites may spread via the lymphatics and produce secondary lesions on the skin, or occasionally the mucosa, of other parts of the body. Regional lymphadenopathy sometimes occurs. Cutaneous leishmaniasis is usually painless unless the lesions become secondarily infected, and except in the ear, the ulcers tend to remain confined to the skin and do not affect the subcutaneous tissues (CFSPH, 2009).

Visceral leishmaniasis is usually an insidious, chronic disease among the inhabitants of endemic areas; however, the onset may be acute in travelers from *Leishmania*-free areas. In some cases (especially in Africa), a primary granuloma appears on the skin before the systemic signs. The most common symptoms of visceral leishmaniasis are a prolonged undulant fever, weight loss, decreased appetite, signs of anemia, and abdominal distension with splenomegaly and hepatomegaly. Thrombocytopenia may cause bleeding tendencies, including petechiae or hemorrhages on the mucous membranes, and leukopenia can result in increased susceptibility to other

infections. Other symptoms may include coughing, chronic diarrhoea, darkening of the skin, lymphadenopathy, and in many cases, signs of chronic kidney disease (Deniau & Houin, 1999; CFSPH, 2009).

The histopathological presentation of CL shows a great variability, a predominant pattern characterized by the presence of unorganized granuloma without necrosis (Andrade-Narvaez *et al*., 2005). Generally, in the granuloma, the cellular profile is Th1 type for localized CL, Th2 for the diffused CL and mix Th1-Th2 for MCL (Dedet, 1999c; Huerre & Esterre, 1999). Th2 is also observed in the VL. In LCL and MCL there is more CD4+ cells infiltration while MΦ dominate in DCL and VL. Consequently, the CD4+/CD8+ ratio is high in LCL and MCL but low in DCL and VL (Huerre & Esterre, 1999) (Table I).

Tableau I: Histopathology and physiopathology of leishmaniases

	LCL	**MCL**	**DCL**	**VL**
Site	Skin	Oro-pharyngeal cavities, Nose	Skin	Bone marrow, Liver, Spleen, Digestive system
Main histological type	TCD4 dominant	TCD4 dominant	Macrophages dominant	Macrophages dominant
Immunological status	Hypersensitivity	Hypersensitivity	Anergy	Anergy
Ratio CD4+/CD8 in the infiltrat	↗	↗	↘	↘
HB15+ (dendritic cells)	↗	↘	↘	**?**
Th1/Th2 profile?	Th1	Th1 and Th2	Th2	Th2

Source: Huerre &Esterre, 1999, simplified)

DCL: Diffuse cutaneous leishmaniasis, LCL: Localised cutaneous leishmaniasis, MCL: Muco-cutaneous leishmaniasis, VL: Visceral leishmaniasis

I.2.6 Incubation period, diagnosis and therapy of leishmaniasis

I.2.6.1 Incubation period of leishmaniasis

People can carry some species of *Leishmania* asymptomatically for long periods, without becoming ill. In humans, the reported incubation period for CL can be as short as 1 to 2 weeks or as long as several months when it is caused by New World species, and up to 3 years when Old World species are involved. The incubation period for VL is 10 days to several years; most cases seem to become apparent in 2 to 6 months (CFSPH, 2009). Nevertheless, some extreme cases can occur in 40 hours (Deniau & Houin, 1999).

I.2.6.2 Diagnosis of leishmaniasis

In most countries in which CL is endemic, diagnosis is based on clinical and epidemiological criteria only (van der Meider *et al.*, 2007).The presence of single or multiple nodular/ulcerative skin lesion(s) can be considered indicative of CL (Molina *et al*., 2003). Examination of Giemsa stained slides of the relevant tissue is still the technique most commonly used to visualize the parasite. The sample should be examined by light microscopy under oil immersion for amastigotes. To ensure that the visualized structures are amastigotes, rather than other "dot"-like organisms, an experienced observer should look for the characteristic size (2–4 μm in diameter), shape (round to oval), and internal organelles (the nucleus and kinetoplast). With Giemsa staining, the cytoplasm typically is pale blue and the nucleus and kinetoplast pinkish red or violet blue. Other conventional methods for parasitological diagnosis include *in vitro* culture of infected tissue or inoculation into animals (e.g. golden hamsters). Species identification can be accomplished by isoenzyme analysis of cultured promastigotes or with various molecular methods or monoclonal antibodies, which also can be used for *in situ* diagnosis. Polymerase chain reaction (PCR), which is currently a research tool, has the potential to increase sensitivity. Immunodiagnostic methods include serological tests to detect antibody or antigen, and assays to detect *Leishmania*-specific cell-mediated immunity, such as intradermal skin testing and detection of proliferative responses of circulating lymphocytes to leishmanial antigens. The usefulness of such methods depends on the clinical syndrome and the assay. Another issue is that the methods may not reliably differentiate remote from recent or current infection. Advances in molecular methods (e.g. production of recombinant and synthetic antigens) have the potential to lead to the development of improved and field-applicable diagnostic techniques (Herwaldt, 1999). The rK39 rapid diagnostic test is used to detect in the sample tested antibodies against recombinant K39 antigen of *Leishmania* (WHO, 2010).

I.2.6.3 Therapy of leishmaniasis

Leishmaniasis is fortunately treatable. However, antileishmanial therapy is a bewildering subject, largely because of the complexities of the disease and the inadequacies of published information (Herwaldt, 1999). Early diagnosis and effective treatment would aid in the clinical management and eventual outcome of the disease (van

der Meider *et al.*, 2007). Although CL is usually a self healing skin disease (Nateghi *et al.*, 2010), there are some reasons to treat it. Treatment can be recommended in cases of:

- cosmetically unacceptable lesions;
- chronic lesions;
- large lesions;
- lesions in immunosuppressed patients;
- lesions over joints;
- mucosal disease;
- multiple lesions;
- nodular lymphangitis;
- worsening lesions (Markle & Makhoul, 2004).

For a given antileishmanial drug to target and kill the parasite, it has to cross many membrane barriers, namely the MΦ membrane; the low pH parasitophorous vacuole probably by passive diffusion, and the membrane of parasite itself via membrane transporters, endocytosis, or diffusion (Croft & Coombs, 2003). In the case of topical administration, the drug must obviously first cross the skin barrier.

Some important medicines used in this leishmaniasis therapy are recapitulated in Table II.

Apart from medicines mentioned in this table, cotrimoxazole is also used to treat leishmaniasis (Niamba *et al.*, 2006). Metronidazole and sterol biosynthesis inhibitors (ketoconazole, fluconazole, itraconazole and terbinafine) are well-tolerated drugs that are potentially active against *Leishmania* when given by mouth (Bahashwan, 2011). Local treatment modalities including topical paromomycin, cryotherapy, localized controlled heat (thermotherapy), carbon dioxide laser therapy, or intralesional meglumine antimoniate can be effective against *Leishmania major* or *Leishmania tropica* (Minodier *et al.*, 2005). Radiotherapy and exeresis are used by some people (Dedet, 1999b). Many plant extracts has also shown antileishmanian activities (Luize *et al.*, 2005).

Avoidance of sand flies is important but difficult. The use of insecticides in endemic areas is important. House and space spraying reduces sand fly populations. The use of fine-weave pyrethroid-impregnated bed nets and curtains is also important. Destruction of rodent reservoirs by pumping insecticides into rodent burrows has a limited success (Markle & Makhoul, 2004).

Tableau II*: Usual drugs in leishmaniasis therapy*

Name of drug (chemical type)	Voice of Administration	Syndrom	Mechanism of action
Pentavalent antimony Meglumine antimonial or N-méthyl glucamine(Glucantime); Sodium stibocluconate(Pentostan)	IM, IV	VL CL	Activated within the amastigote, but not in promastigote, by conversion in a lethal trivalent form. The mechanisms of action of these products are still unknown .Antimony are reputed to inhibit ATP synthesis, glycolytic and fatty acids oxidations. Antimony might be concentrated into the MØ or might be transformed into their metabolites to be efficient.
Amphotericin B (Fungizone, Ambisome)	Topical (ointment), IV (suspension)	CL VL MCL	AB complexes with 24-sbstituted sterols, such as ergosterol in cell membrane, causing pores which alter ion balance. It inhibits lanosterol demethylation. Lanosterol then accumulates abnormally and provokes modifications of parasite membrane and then lose of vital substances. Amphotericine B might also stimulate the production of MΦ and increase their phagocytosis capacity
Pentamidine isethionate (Pentam 300, Pentamidine, Pentacarinat, Aventis)	IM, IV	VL	It inhibits DNA synthesis by blocking thymidine synthetase and by binding to transfer RNA
Paromomycin (Aminoglycoside antibiotic) also known as **Aminosidine or Monomycine (Gabbromicina)**	Topical (ointment), IM, IV	CL	Effective with *L. major* and *L. Mexicana* ; it inhibits parasite protein synthesis by linking to ribosome
Allopurinol (Zyloprim, Zyloric)	*Per os*	CL	Incorporates into parasite RNA with lethal effects. It has a synergetic activity with antimonial
Miltefosine	*Per os*	CL VL	Miltefosine has been shown to block the proliferation of *Leishmania* and to alter phospholipid and sterol composition
Sitamaquine (8-Aminoquinoline)			Might affect mitochondrial electron transport chain
Imiquimod (Imidazoquinoline)	Topical	CL	Stimulates Th1 response, thus increasing the production of NO from MØ, as well as the production of TNF-α, IFN-γ and IL-12
Recombinant IFNγ (Imukin)	SC, IM	CL VL	Activation of MØ enhances their O_2 radicals and NO derivates production; Increasing of MHC II molecules at the surface of MØ; induction of differentiation of Th0 to Th1 and the proliferation of Th1 as well as the stimulation of cytolytic cells NK and CD8
Imidazol (Ketoconazol or Nizoral , Itraconazol or Sproranox)	*Per os*	CL	They inhibit the cytochrome P450 and then jam the synthesis of the parasite cell membrane sterols ,the disorganization of parasite organelles

(Source: Croft & Coombs, 2003 altered refering to Dondji, 1998; Dedet 1999b; Herwaldt, 1999; Markle et Makoul, 2004; Patel & Shah, 2008, Almeida & Santos, 2011)

AB: Amphothericin B, CL: Cutaneous leishmaniasis, IM: Intramuscular (injection), IV: Intravenous (injection); MCL: Mucocutaneous leishmaniasis; MHC: Major histocompatibility complex; MØ: Macrophage, NK: Natural killer, SC: Subcutaneous (injection); VL: Visceral leishmaniasis

As far as the vaccine is concerned, Markle & Makhoul (2004) added that an ideal vaccine against leishmaniasis should obey to the following criteria:

- inhibition of the penetration of the promastigotes in histio-monocytes;
- prevention of the survival of the infectious promastigotes within phagolysosome;
- inhibition of the multiplication of amastigotes in lysosomal compartment;
- neutralization of the infectivity of amastigotes released after the host cell burst

Despite this rich background on leishmaniasis, to date no vaccine is available against any form (Markle & Makhoul, 2004; von Stebut, 2007b) and historically, the most effective tool to protect against CL is leishmanization (Nateghi *et al*., 2010). Protective immunity against this obligate intracellular protozoan parasite is dependent on the development of antigen-specific CD4+ and CD8+ T cells capable of releasing IFN. This cytokine in turn, activates phagocytic host cells to generate oxidative radicals and to eliminate the parasite (Kronenberg *et al.*, 2008). Vaccination with killed parasites or defined leishmanial antigens generally induces only short-term protection. The reasons for this difference are currently not known but may be related to differences in the quality of the early immune responses to live and killed parasites, since the killed and the live parasites (*L. major*) do not induce the same early cytokines response even if the early inflammatory response induced by both forms is comparable (Okwor *et al*., 2009). Strong correlation has been found between delayed type hypersensivity response to *Leishmania* antigens and anti-salivary gland IgG1 and IgE levels from children living in endemic areas of visceral leishmaniasis. Thus, previous non-infected phlebotomine bites may confer a degree of protection against infection. Effective vaccination approaches able to protecting the host against *Leishmania* infection, using maxadilan or other protective proteins in the insect saliva have been reported in mice (de Almeida *et al*., 2003). Recently, Yamamoto and collaborators (2010) studied a transgenic anopheline mosquito, known as flying vaccinator, susceptible to deliver *Leishmania* vaccine via blood feeding. These authors reported that the transgenic mosquito expressed the *Leishmania* vaccine candidate, SP15, and mice bitten repeatedly by this mosquito raised anti-SP15 antibodies.

I.2.7 Leishmaniasis in Cameroon

Despite the fact that CL cases were suspected since the Antiquity (Jarry, 1999), the first cases (overall 326) were recorded in Cameroon, mainly in Garoua and its

environments since 1930s by Hervé. In 1951, Rageau mentioned that the vector was likely *Phlebotomus roubaudi*. Nevertheless, in Mokolo, an important focus of CL, the first cases were reported in 1972 by Buerles and Starrach (Djibrilla *et al.*, 1979). Djibrilla and collaborators (1979) undertook a prospective study between December 1975 and January 1976 and reported 58 cases of CL in Mokolo. They therefore concluded that this focus was always active.

In 1983, Deniau and co-workers realized the first parasitological confirmation of VL (kala azar) in a young girl from the south part of the country but who previously lived in Kousseri (in the Far North Region) (Deniau *et al.*, 1986).

In 1992, Kaptué and collaborators confirmed 13 cases of kala azar amongst which 11 were from Kousseri, 2 from Yaoundé (Center Region), and one from Fontem (South-West Region). One of the patients was declared HIV positive. The conclusion was that, apart from the Far north, others leishmaniasis foci exist in the country. It is reported that in Cameroon, Mokolo is likely amongst others, a focus of CL while Kousseri is a VL focus (Dondji *et al*., 2001).

The first identification of the causative agent of CL in Mokolo is the issue of Dondji and co-workers researches (1998). The authors reported that the causative agent (or one of the agents) is *Leishmania major*, zymodeme MON-26.

As far as concern the vector, Dondji and collaborators (2000) reported in Mokolo *Sergentomya schwetzi, S. africana, S. bedfordi, S. squamipleuris, S. clydei, Phlebotomus duboscqi* (with an important epidemiological role in leishmaniasis), and *P. rodhaini.* But phlebotomine sand flies species were previously collected in other parts of Cameroon namely in Yaoundé (*S. africana*); Bafia (*S. africana*, *S. schetzi*, *S. grenieri*); Evodoula (*P. duboscqi*, *S. similima*, *S. schoutedeni*); Kousseri (*S. africana, P. duboscqui, S. logonensis*); Logone Birni (*S. africana, S. antennata, P. duboscqi*); Garoua (*S. africana, S. clydei*); Douala (*S. africana*) (Dondji, 2001).

In 2009, Ngouateu and collaborators reported the first cases of CL associated with HIV infection in the country precisely in Mokolo endemic focus.

I.3 HIV INFECTION AND AIDS

I.3.1 Germ

AIDS is the acquired immuno-deficiency syndrome. Its germ (Figure 14) is the acquired immuno-deficiency virus (HIV) described for the first time in 1981. It belongs to the Family Retroviridae; this is the family of RNA viruses which need the host DNA for their multiplication (Medecins sans Frontières, 2003).

Two serotypes of HIV exist namely HIV-1 and HIV-2. Both types are transmitted by sexual contact, through blood, and from mother to child and the AIDS produces by them is clinically indistinguishable. However, it seems that HIV-2 is less easily transmitted, and the period between initial infection and illness is longer than in the case of HIV-1 (*http://www.avert.org/hiv-types.htm*).

Worldwide, the predominant virus is HIV-1, and generally when people refer to HIV without specifying the type of virus they will be referring to HIV-1. The relatively uncommon HIV-2 type is concentrated in West Africa and is rarely found elsewhere.

There are four groups of HIV based on their genetic materials. These are:

- Group O appears to be restricted to West-Central Africa;
- Group N discovered in 1998 in Cameroon is extremely rare;
- Group M the most common and represent more than 90% of HIV-1 on infected patients;
- Group P discovered in Cameroon recently in 2009.

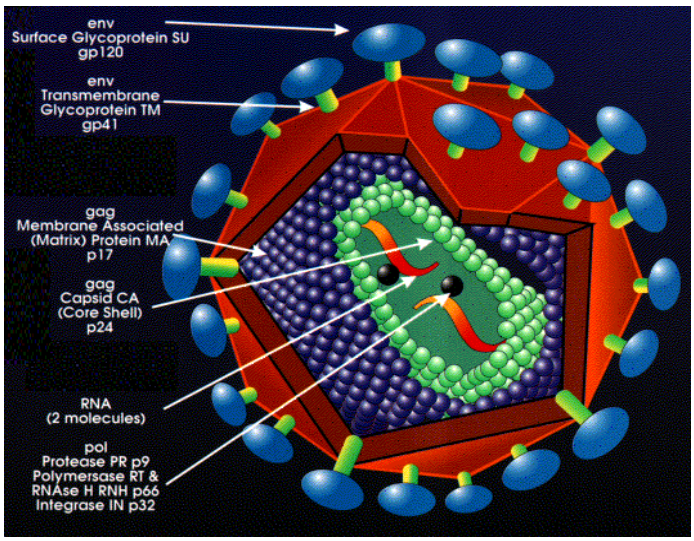
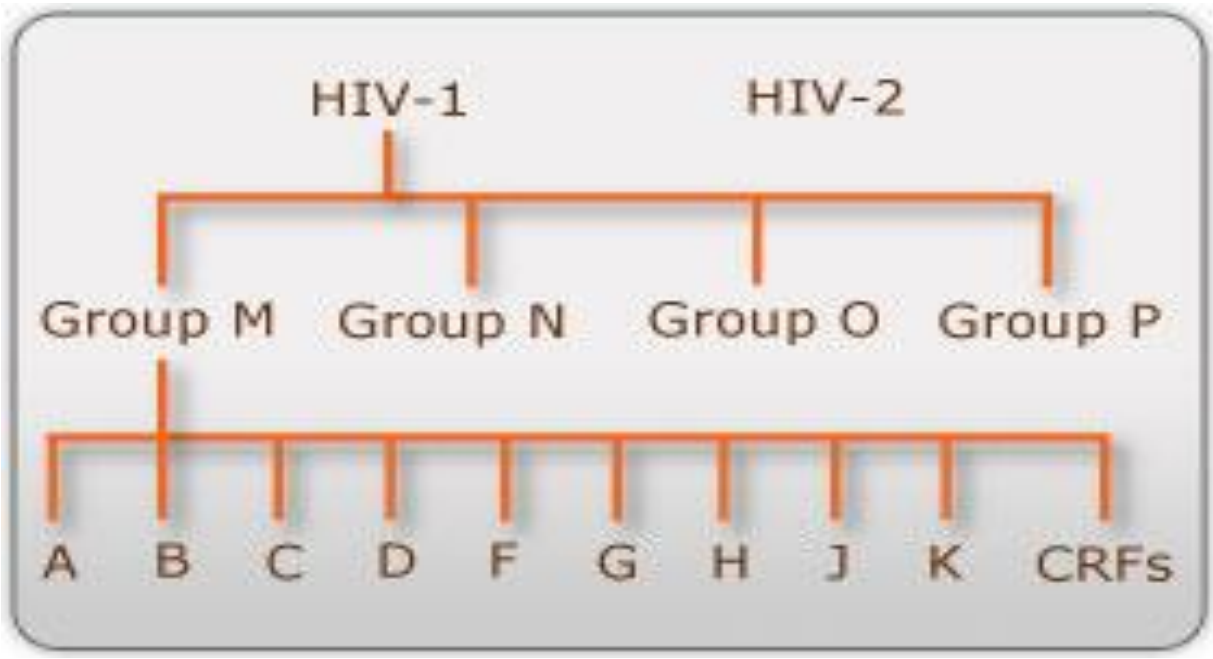


Figure 14: Illustrative structure of HIV

Within group M there are at least nine genetically distinct subtypes (or clades). These are subtypes A, B, C, D, F, G, H, J and K. Occasionally, two viruses of different subtypes can meet in the cell of an infected person and mix together their genetic material to create a new hybrid virus. Many of these new strains do not survive for long, but those that infect more than one person are known as "circulating recombinant forms" or CRFs (Figure 15) (*http://www.avert.org/hiv-types.htm*).



(Source: http://www.avert.org/hiv-types.htm)

Figure 15: Different levels of HIV classification
CRFs: Circulating recombinant forms

I.3.2 Epidemiology of HIV infection

AIDS is a pandemic disease. According to the last estimates from the Joint United Nations Programme on HIV/AIDS and the World Health Organization (WHO), 42 million people were living with HIV/AIDS at the end of 2002. During 2002, some 5 million people became infected with HIV and 3.1 million died as the result of HIV infection, despite the availability, in the richer countries, of highly active, antiretroviral therapy (HAART). The total number of HIV-attributable deaths since the beginning of the epidemic is estimated to be more than 25 million. Although most HIV-positives

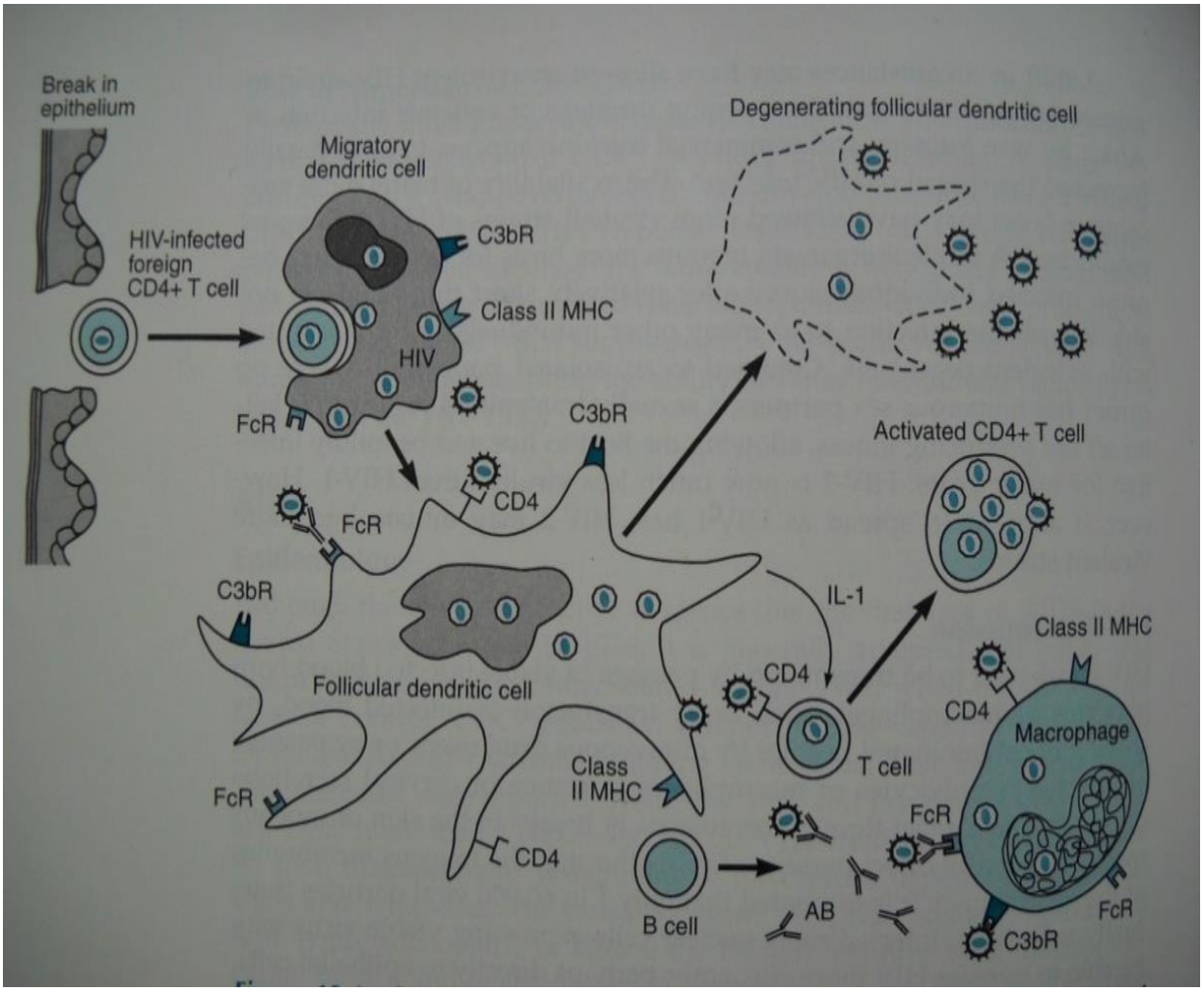
(>95%) are to be found in the developing world, HIV infection remains a challenge in industrialized countries. In sub-Saharan Africa, in 2002, 3.5 million individuals (adults and children) were newly infected with HIV and 29.4million were living with HIV/AIDS (Deniau *et al.*, 2003). Of 4.3 million new HIV infections in 2006, 2.8 million (65%) occurred in sub-Saharan Africa, which continues to be the most severely affected part of the world. In this region, HIV has had a marked negative impact, reducing adult life expectancy by 50% in several countries (Alvar *et al*., 2008).

According to the report of the Cameroon National AIDS Control Committee (2008), the prevalence of HIV infection in the country in the year 2007 was more than 5% (3.7% for male and 7.3% for female). The Far North Region, one of the most populated of the country (*http://www.citypopulation.de/Cameroon*), registered a lower prevalence of 4.0% (3.0% for male vs 8.2% for female).

I.3.3 Transmission and immunopathology of HIV infection

HIV is believed to be transmitted by passage of viable infected blood lymphocytes or macrophages, such as by transfusion of infected blood, by sharing of contaminated needles by drug users, or by passage of infected lymphocytes or macrophages in semen or cervical secretions during sexual contact through contusions of breaks in the skin or mucous membranes or by direct passage of virus through the mucous membranes of the genital tract (Sell, 2001).

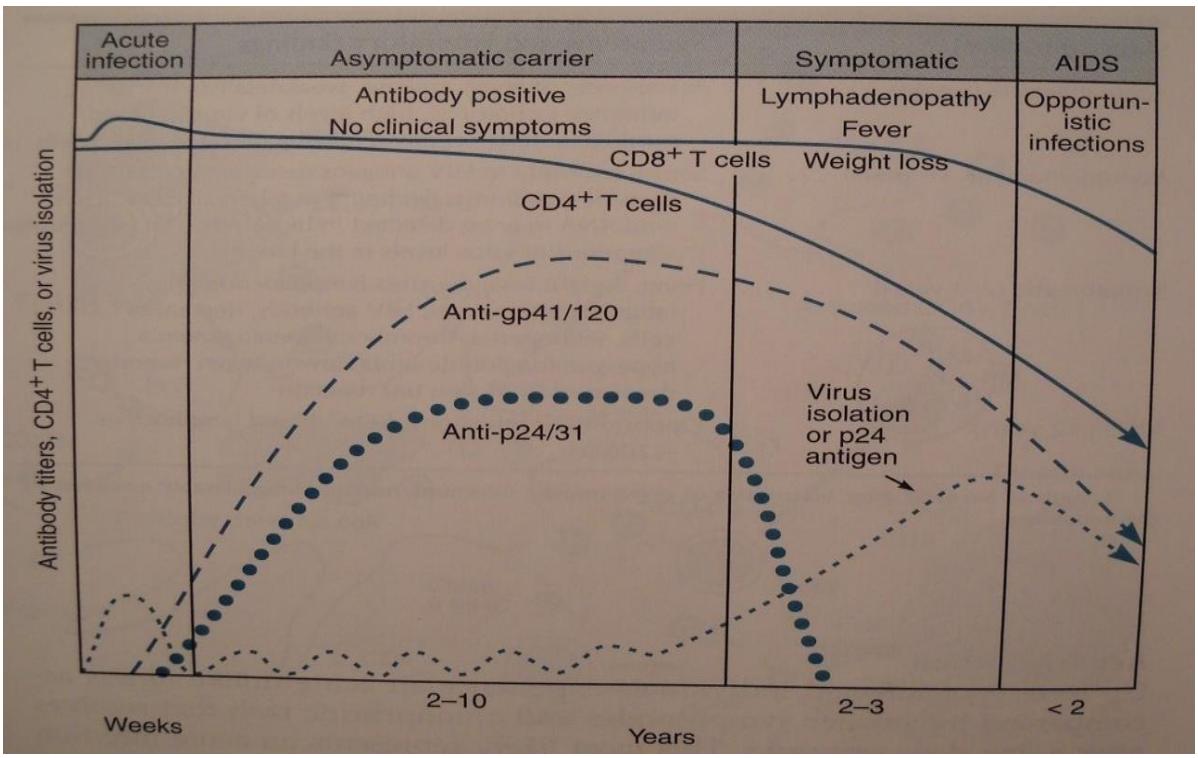
HIV infects CD4+ T helper, macrophages and dendritic cells (Ezra *et al*., 2010) but a wide range of cells; follicular DC are particularly affected (Sell, 2001) (Figure 16). To enter the host cell, a region of viral envelope gp120 first binds to a domain on CD4 on the cell. This interaction causes a conformational change in gp120, resulting in binding of another domain of the virus to co-receptors CCR5 or CXCR4 (Sell, 2001) namely on monocytes and macrophages (Okwor and Uzonna, 2013). The infected host immune cell is eventually destroyed leading to higher susceptibility of the organism to diseases (Ezra *et al*., 2010).



(Source: Sell, 2001)

Figure 16: HIV invasion in the organism and infection of immune cells
AB: Antibody, CD: Cluster of differenciation, C3bR: Complement receptor, FcR: Crystalisable fragment receptor, MHC: Major histocompatibility complexe

One of the most striking clinical features of the HIV infection remains the long latency period between the initial viral invasion and the onset of AIDS, and, eventually the establishment of immunodeficiency state (Figure 17). Progression to AIDS among HIV infected individuals has been shown to be associated with the active replication of HIV. Critical events that occur during the virus life cycle, such as reverse transcription, integration of the viral genome into host DNA, and subsequent viral replication, are all tightly associated with the host machinery. Virus gene expression relies heavily on a number of host cell transcription factors such as NF-κB etc. (Zhao *et al.*, 2004).



(Source: Sell, 2001)

Figure 17: Changes occurred in the organism from the virus acquisition to AIDS

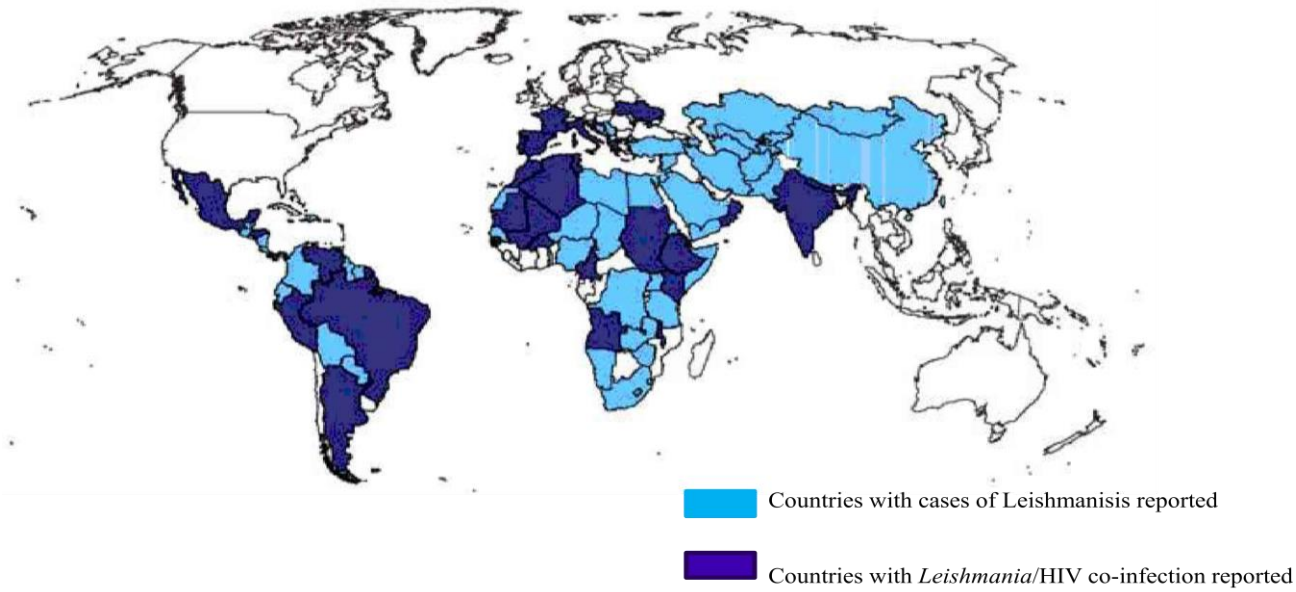
I.4 *LEISHMANIA* AND HIV CO-INFECTION

I.4.1 Epidemiology and geographical distribution

Leishmaniases are diseases associated with rural areas and poverty, but it has adapted to urban environment as well (Markle & Makhoul, 2004). Economic development, behavioural and environmental changes such as new settlements, intrusion into primary forest, deforestation, massive migration from rural to urban areas, fast and unplanned urbanization, building of dams, new irrigation schemes are conditions which increase exposure to sand fly so that in several areas of the world there is a clear and disturbing increase in number of cases of leishmaniasis (WHO, 2002).

The HIV pandemic in the tropics is expanding into rural and remote areas endemic for which human leishmaniasis (WHO, 2010); consequently, the *Leishmania*-HIV co-infection becomes evident, especially among adults (Puig & Pradinaud, 2003). CL, MCL and VL associated with HIV/AIDS cases have been reported since 1987 (Rosatelli *et al.*, 1998). Today, leishmaniasis is emerging as the third most frequent opportunistic infection in AIDS patients in various parts of the world, particularly in *Leishmania*-endemic countries where, in 25–70% of adult patients with VL, there is HIV coinfection (Pourahmad *et al.*, 2009). Epidemiological data reveal that around 50% of all adult cases

of VL are HIV+ and 1.5-9 % of patients with AIDS in south-western Europe suffer from newly acquired or re-activated VL (Puig & Pradinaud, 2003; Paredes *et al.*, 2003).To date, co-infection with *Leishmania* and HIV has been reported in 35 countries in Africa, Asia, Europe, and South America (Figure 18) but most of the cases notified by the WHO are from four countries in south-western Europe namely France, Spain, Italy and Portugal (Desjeux & Alvar, 2003; WHO, 2010). Over 700 cases with concurrent infections of HIV and leishmaniasis (predominantly VL) have been described with more than 90% cases registered in South-Western Europe (Salman *et al.*, 1999). Injectable drugs users are at the highest risk by inoculating themselves intravenously with the used syringes (Salman *et al.*, 1999; Ezra *et al.*, 2010). In Africa, the number of cases is expected to rise and is further impaired by social adversities like mass migration, displacement, civil unrest, and war (Parades *et al.*, 2003). Countries in which cases are reported in the continent are Algeria, Angola, Burkina Faso, Cameroon, Guinea Bissau, Kenya, Malawi, Mauritania, Morocco, Sudan, Tunisia, Mali (Desjeux et Alvar, 2003) as well as Senegal and Ghana (Lartey *et al.*, 2006). The real impact of HIV/*Leishmania* co-infection is probably being underestimated owing to constraints in surveillance and reporting of cases (Paredes *et al.*, 2003).



(Source: Desjeux & Alvar, 2003)

Figure 18: World-wide distribution of leishmaniasis and countries reporting* Leishmania*/HIV co-infection cases in 2001

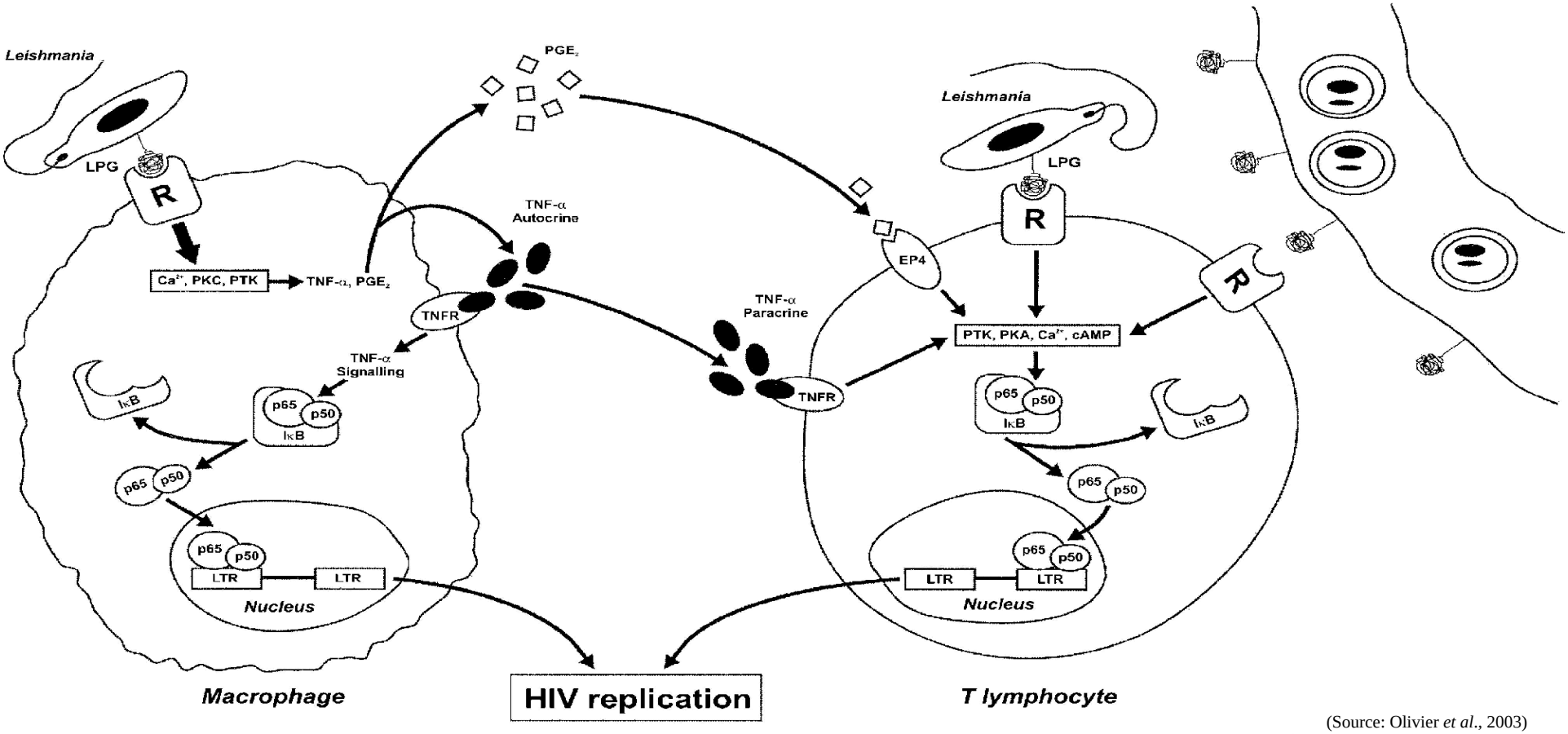
I.4.2 Immune response of *Leishmania* /HIV co-infected patients

Diffused cutaneous leishmaniasis (DCL) has been observed in patients infected by *Leishmania* species such as *L. infantum*, *L. braziliensis* and *L. infantum*, which normally do not induce DCL in immunocompetent persons (Dedet *et al*., 1999). In co-infected patients then, dermatropic species of *Leishmania* have reported to often induced VL and *vice versa* (Torrico *et al.*, 2009).

Co-infection may amplify the immune defect against both leishmaniasis and HIV and increase disease severity and morbidity (Chaudhary *et al*., 2008). HIV infection can increase the risk of developing leishmaniasis by 100 to around 2000 times (Santos *et al.*, 2009; Ezra *et al.*, 2010). It is reported that both leishmaniasis and HIV infection exert a synergistic detrimental effect on the cellular immune response because they target similar immune cells, the monocyte/MΦ (Bossolasco *et al.*, 2007; Alvar *et al*., 2008; Parades *et al.* 2003; Ezra *et al.*, 2010). In humans, both HIV and *Leishmania* interact with, invade, and multiply within cells of myeloid or lymphoid origin (Figure 19) (Olivier *et al.*, 2003).

Thus, infection of the same cell type by two different pathogens may have important effects on the immune response and influence the biology of both microorganisms (Zhao *et al*., 2004). Co-infection of HIV and *Leishmania* produces cumulative deficiency of the cell mediated immunity, a key factor for primary protection against infection, recurrences, or metastasis of parasites (Chaudhary *et al*., 2008). HIV promotes *Leishmania* multiplication *in vitro* and impairs the ability of MΦ to control the growth of this parasite (Bossolasco *et al.*, 2007). On the other hand, *Leishmania* parasite is reported to increase the HIV replication (Preiser *et al*., 1996). In addition, both HIV and *Leishmania* induce a shift from Th1 to a Th2 type cytokine profile, with reduced IL2 and IFNγ production (Bossolasco *et al.*, 2007).

Compared to HIV-infected patients without leishmaniasis, co-infected patients show a cytokine profile with significant elevations in IL-4, IL-10 and IL-2-receptors, and decreased post-stimulation production of IFN. It has been shown that *Leishmania infantum*-derived lypophosphoglycan (LPG) can induce HIV-1 expression in latently infected peripheral blood mononuclear cells, probably mediated by the secretion of TNF (Paredes *et al.*, 2003). LPG induces the secretion of TNFα and IL1α, proinflammatory cytokines, which have been revealed to play a major role in the up-regulation of HIV1 gene expression via a positive action on NF-κB signalling pathway (Zhao *et al*., 2004).



(Source: Olivier *et al*., 2003)

Figure 19: Schematic representation of the signaling mechanisms involved in* Leishmania*-lipophosphoglycan-mediated HIV-1expression by monocytes/macrophages and T-lymphocytes

EP4: Prostaglandine E2 receptor type 4; IκB: Nuclear-factor-kappa B inhibitory protein; LPG: Liphosphoglycan; LTR: Long terminal repeat; PGE2: Prostaglandine E2; PKA: Proteine kinase A; PKC: protein kinase C; p65/p50: Active elements of Nuclear-factor-κB; PTK Proteine tyrosine kinase; R: receptor.

In addition, IL-6 has been reported by Garg *et al*. (2009) to stimulate HIV-1 replication in macrophages again via a NF-kB mediated signal transduction pathway. An increased production of IL-6 and TNF-α has been detected in *Leishmania* infected macrophages. Thus, in the case of HIV-1/*Leishmania* co-infected patients, it is likely that HIV-1 uses a part of the *Leishmania*-mediated cytokine network to its own advantage (Garg *et al*., 2009). The role of the transcriptional transactivator (Tat) protein, an essential viral gene product for HIV-1 replication, in the exacerbation of *Leishmania* proliferation in human macrophages has been mentioned. Tat is secreted in large amounts by cells infected by HIV-1 and is able to stimulate secretion of IL-1β, IL-4, IL-6, IL-8, TGF-β, TNF-α and TNF-β. The results showed that Tat protein was able to override the leishmanicidal effect of IFN-γ and to enhance *Leishmania* replication in human macrophages through the production of prostaglandin E2 in the macrophage, which in turn increases the synthesis of TGF-β1 and the augmentation of *Leishmania* growth (Alvar *et al*., 2008).

I.4.3 Diagnosis of leishmaniasis in HIV positive individuals

In HIV-infected patients, diagnosis of leishmaniasis may be delayed because clinical manifestations typical of the disease in the immunocompetent do not develop (Deniau *et al.*, 2003).

Although the majority of *Leishmania* infections in HIV-positive individuals display clinical features of classic kala-azar, cutaneous and mucocutaneous leishmaniasis, as well as VL in many atypical locations have been increasingly reported. HIV-associated leishmaniasis has five major clinical characteristics:

- parasitic dissemination, to the skin in diffuse cutaneous leishmaniasis, or throughout the reticulo-endothelial system in visceral and visceralizing syndromes;
- atypical locations, as a consequence of this parasitic dissemination and a defect in cell-mediated immunity;
- a chronic and relapsing course, with each patient typically experiencing two or three relapses despite proper treatment;
- poor response to standard therapy;

- lack of anti-*Leishmania* antibodies, which is seen in many endemic areas (Paredes *et al.*, 2003).

I.4.4 Therapy of leishmaniasis on HIV positive patients

The best way to treat leishmaniasis in HIV+ patients is still the subject of controversy. Experience in the therapy of these patients has been more-or-less limited to countries in Mediterranean basin.

The co-infected cases are also far more likely to respond poorly to antileishmanial treatment (Deniau *et al.*, 2003). The absence of immune response has an impact in the treatment of DCL cases, and HIV and VL co-infection cases (Croft & Coombs, 2003).

Cutaneous leishmaniasis has been treated successfully on HIV+ patients using only cotrimoxazole without ARV (Niamba *et al*., 2006). Previously, Amato and collaborators (2000) after failure of antimonial therapy, they treated successfully with liposomal amphotericin B (Ambisome) a HIV positive patient suffering from MCL and who developed diabetes mellitus. This medicine is efficient in both immunodepressed and immunocompetent patients (Minodier *et al*., 2005) even if Schraner and collaborators (2005) found a case of resistance in a co-infected patient treated rather with miltefosine. Bossolasco and collaborators (2007) reported that the administration of human recombinant IL2 concurrently with highly active antiretroviral therapy (HAART) increased the CD4 cell counts and function in HIV positive patients, including those with advanced disease. This recombinant IL2 also reduced the local parasite load in human CL on these patients. But recently, the National Institute of Allergy and Infectious Diseases (2009) reported that the increase of CD4 cells in this case does not translate into reduced risks of HIV-associated opportunistic diseases or death when compared with the risks in patients under only antiretroviral therapy.

CHAPTER II

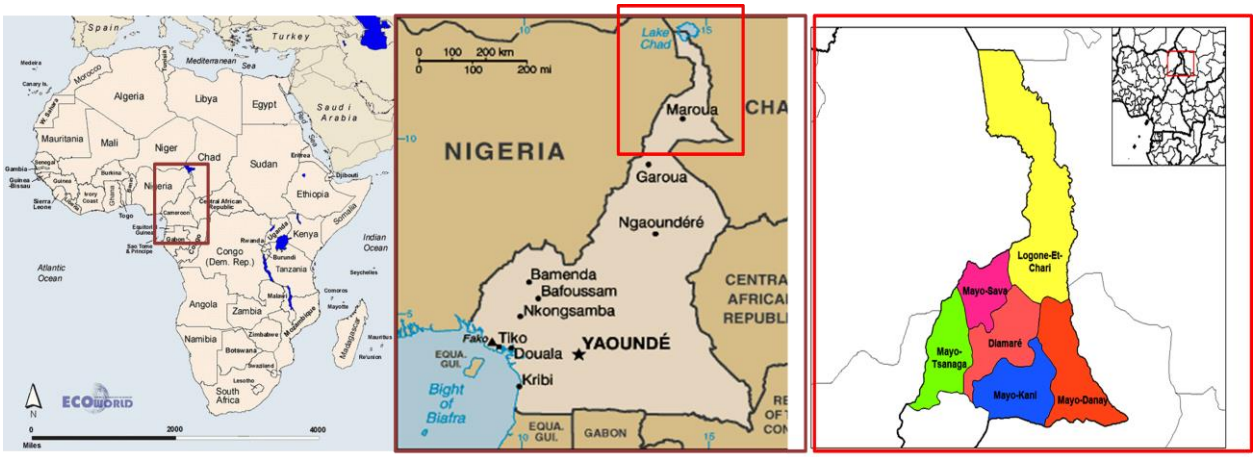
MATERIALS AND METHODS

II.1 STUDY SITE

II.1.1 Overview of the Far North Region

The field study was carried out in the Far North Region of Cameroon (Figure 20) which head quarter is Maroua. It is one of the most populated regions of the ten that constitute the whole country.

The region is divided administratively into six divisions. They are: Diamaré, Logone et Chari, Mayo Danaï, Mayo Kani, Mayo Sava, and Mayo Tsanaga. This last division was the main field of our study.



(Source: Wikipedia)

Figure 20: Localization of the study site
The arrow shows the Mayo Tsanaga Division which headquarter is Mokolo

II.1.2 The Mayo Tsanaga Division

The Mayo Tsanaga Division is divided into seven sub-divisions namely: Mokolo (Headquarter), Koza, Soulédé-Roua, Bourha, Mogodé, Hina, and Mayo-Moskota. The Division covers a total area of 4.393 km^2 with a total population of 574.864 inhabitants estimated in 2001 (*http://fr.wikipedia.org/wiki/Mayo-Tsanaga*). Mokolo is situated in 10°44'25''North latitude and 13°48'10'' East longitude (*http://fr.wikipedia.org/wiki/Mokolo*) and is divided into cantons constituted by the villages and villages by the quarters. At the head of each canton there is a Lamido. Villages have at their respective heads Lawanes while quarters are headed by Djaoros.

Mokolo landscape is rocky, hilly (Figure 21a) and dominated by Mandara Mounts (1200-1500 m). The climate is soudano-sahelian type with characteristic vegetation, rainfall and temperature. There are two main seasons: the rainy season (June to October) and the dry season (November to May). The daily temperature generally fluctuates between 24°C and 35°C. However, the temperature could go lower than 24°C during the harmattan which begins in December and terminates in January. This period is characterized by dusty and violent winds which are caused by the significant temperature difference between the cold air masses that occur at night and the heat stored by rocks during the day (Dondji *et al.*, 2000).

Ethnic groups found in Mayo Tsanaga are Mafa, Mufu, Kapsiki, Fulbé, Gawar, Turu.

Languages spoken in Mokolo are the two national languages (French and English), the regional language, Fulfulde, spoken by people of the three regions of the northern part of the country; local dialects like Mafa, Kapsiki, Mufu are also spoken.

The habitat is called “saré”, and is more often made up of compounds of small houses (generally round shape) built by mud with thatched roof; the stems of maize and sorghum are used for this purpose (Figure 21b).

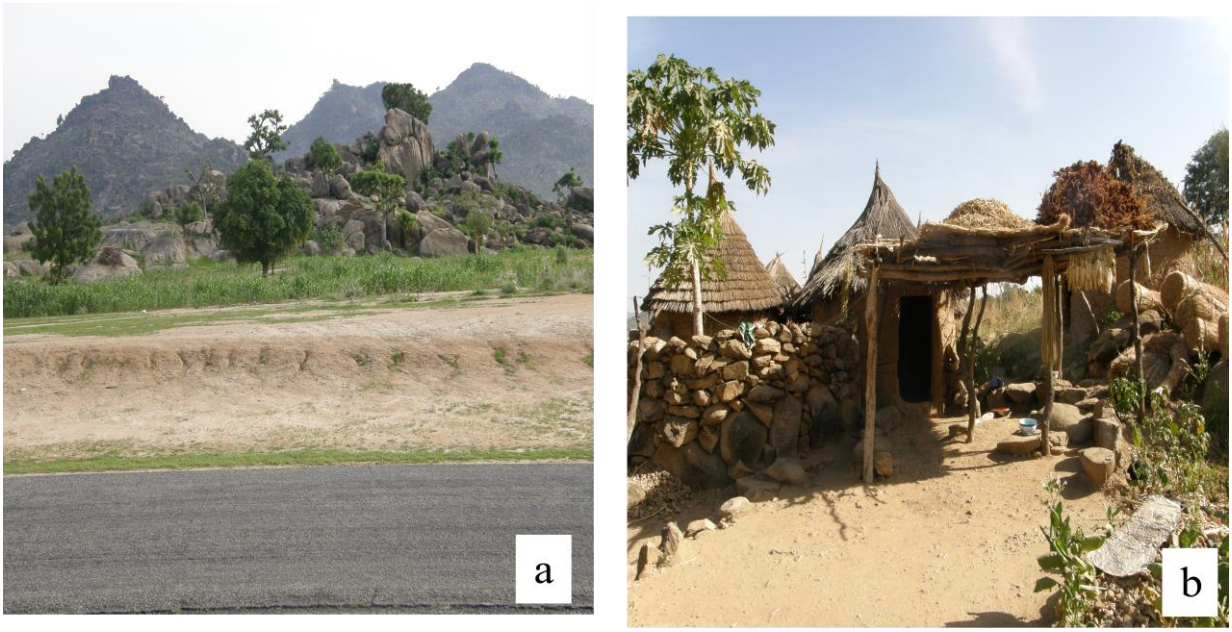


Figure 21: Mokolo landscape (a) and Habitat (b)

II.2 METHODS

II.2.1 Data collection during the field survey

Data were collected from December 2007 to March 2009. During the first weeks of the field investigation we were concerned to sensitization of peoples, who were informed on the aims of the project. This was performed through administrative authorities, traditional heads, health personnel, churches and mosques, schools, radio etc. An announcement was prepared for this purpose.

Before starting the door-to-door survey, a questionnaire was prepared. This document allowed to collect socio-demographic and health information as well as some important information. The research team made three field visits per week. Once on the field, the team first met Lawanes who were supposed to be previously informed by their Lamido or through an announcement. The Lawanes authorised the meeting with their Djaoros. A trained nurse from the Far North Region was involved in this door-to-door survey. However, to get access to each house, Djaoros of each locality provided guides.

Information on the etiology of the lesions was collected to discard lesions due to other causes. Pictures of CL lesions and local names of the disease were also used to facilitate its recognition by local people. In fact, in the Mayo-Tsanaga Division cutaneous leishmaniasis is well known as "*Mblegoyave*", "*Mbleplesh*" or "*Mbleteplesh*" by Mafa peoples, "*Gourleng*" by Mufu, "*Gourlenle*" or "*Houdoure goyave*" by Fulbe, "*Jéova*" or "*Djambrem*" by Kapsiki. Suspected scars were also investigated. Nevertheless, for a given scar to be typical and to be taken into consideration, it should obey to some criteria such as: the person bearing it must have got the active lesion in his current residence, it aetiology may not be from an injury or a burn, the scars might be round or oval shape with pronounced line of demarcation.

During the investigation we interviewed people to know if they were aware of the origin of the disease. We also questioned them about the use or not of the mosquito net, insecticide/insect repellent, the number of persons using a mosquito net.

II.2.2 Patients and control subjects recruitment

Each leishmaniasis suspected subject was invited in the Laboratory of Leishmaniasis Research Project located in Mokolo District Hospital. Patients or their

parents/guardians (for children) who reached the laboratory were deeply informed about the objectives of the study, the different tests to be performed as well as the confidentiality of their results, and their free of charge follow up. They were free to participate and only those who consented and who signed the consent form were involved.

Another questionnaire was used in the laboratory to collect socio-demographic information as well as the former therapy used, the number, size, site and the age of the lesions. The previous HIV status of the patients was also requested by asking them if they have already been tested for HIV or not.

HIV+ controls were volunteers recruited from the HIV Treatment Unit of the Mokolo District Hospital, a division of the National Committee of the Fight against HIV/AIDS.

Double negative controls (neither *Leishmania* nor HIV infection) were participants who have never stayed in a leishmaniasis endemic area.

All non-volunteers were not not included in the study.

II.2.3 Collection and preservation of sera and skin biopsies samples

In each visit, venous blood (5 ml per patient) was collected under strict aseptic conditions (alcohol 70%). After one hour at room temperature (RT), the blood was centrifuged and the supernatant (serum) was collected and preserved at -80°C in ependorf tubes. For HIV positive patients, another sample of blood was collected in tubes contening ethylene diamine tetraacetic acid (EDTA) for the CD4, CD3, and CD8 count.

After a local anesthesia (Lidocaine injection) and cleansing (Povidone iodine 10%) of a lesion, an approximately 5 mm biopsy was taken from the reddened and swollen edge by excising with a surgical blade. The control biopsy was collected on the opposite part of the body under the same conditions of anesthesia. Biopsies were preserved in 10% formaline.

II.2.4 Diagnosis of cutaneous leishmaniasis and evaluation of lesions parameters

The diagnosis of cutaneous leishmaniasis was based on clinical and parasitological criteria, namely the macroscopic observation of lesions and the confirmation through the microscopical identification of *Leishmania* amastigotes on Giemsa-stained smears.

The number of lesions was recorded. For each lesion, the location on the body and the diameter were noted. The surface of a given lesion was estimated as follow:

- for round shape lesions, $\mathbf{S = \pi.R^2}$, where **S** is a surface in mm^2 and **R** a radius in mm;

- for ovoid shape lesions, $\mathbf{S = \pi.R.r}$, where **S** is a surface in mm^2, **R** is a big radius in mm, and **r** a small radius in mm.

The total surface of lesions per a given patient was the sum of all the individual lesions surfaces.

Lesions age was estimated according to the patient declaration. Nevertheless, some periodical events or festivities such as Christmas, the first of January (New Year festival), the National Youth day (11^{th} February), the International Woman day (8^{th} March), the National day (20^{th} May), the beginning of the rainy season etc. were used as indicative to conjecture the period of the lesion onset.

II.2.5 Diagnosis of HIV infection

The HIV status of each patient suffering from CL was determined on sera, using commercially available kits. These tests belong to rapid assays recommended by the Cameroon government. Abbott Determine®-HIV1/2 Illinois, USA or Retrocheck HIV® (1+2), Qualpro Diagnostics, Goa, India were used as the first rapid immunochromatographic tests, and if positive, Bioline SD HIV1/2 3.0, Standard Diagnostics Inc., Kyonggi-do, South Korea, was used as confirmative test (HIV_1 and/or HIV_2). Determine® has a high sensitivity but low specificity while Retrochek® and Bioline® are less sensitive but present high specificity (Aghokeng *et al.*, 2009).

Patients were considered HIV positive when both immunochromatographic tests were positive.

For double *Leishmania* and HIV positive (Leish+/HIV+) patients, in order to evaluate the level of severity of the pathology, the CD4, CD3, CD8 count was performed in the whole blood using a BD FACS Count ™ kit. The whole blood was introduced in tubes containing CD4/CD3 and CD8/CD3 reagents respectively. After 1h incubation, the fixative solution was introduced in each tube for 30 min incubation period. The result was read using the BECTON DICKINSON FACS-Count.

II.2.6 Therapy regiment

Patients whose lesions persisted more than two months (or those with more severe symptoms) from the first visit received antileishmanian drugs. The main therapy regimen was Amphotericine B (Fongizone) local application associated with Metronidazol 1.5 mg/kg body weight/day. Due to the supply shortage of Amphotericin B in the country, this regimen was replaced by Allopurinol (Zyloric 300) 20 mg/kg body weight daily.

Cotrimoxazol was used in two cases, while antiretroviral (ARV) (Triomune) therapy was used in one case of HIV infection (patient with blood CD4 less than 200 cells/µl).

Some patients received, as a placebo, only multivitamin (A, B1, B2, B3, C, and D3) while others, depending on their lesions evolution, did not receive any drug.

II.2.7 *Leishmania* species characterization

For *Leishmania* species identification, samples of biopsies were collected on the lesions and preserved in the RPMI (Roswell Park Memorial Institute) medium. Molecular identification was based on the PCR amplification and sequencing of a 486 bp polymorphic region of the RNA polymerase II largest sub-unit gene. Sequences comparison against an internal database showed a 100% similarity with the LEM62 *L. major* reference strain (MHOM/YE/76/LEM62).

II.2.8 Data on the rainfall

The meteorology station of Mokolo provided data on the rainfall, but due to the damage of some of its material, it lacked other information such as the annual temperature variation, the wind rate, the hygrometry etc.

II.2.9 Histology and histology

II.2.9.1 Slides preparation

Biopsies samples were put in cassettes and introduced in Tissue-Tek VIP 5Jr for automation during which, they were incubated 1h in Formalin 4%, 1 h in EtOH 70%, 1 h in EtOH 80%, 1 h in EtOH 96%, 1 h in EtOH 96%, 1 h in EtOH 100% (x 2), 1 h in Xylol (x 2), 1 h in Paraffin (x 2), and 1.5 h in paraffin.

After this, materials in the cassettes were embedded into paraffin using the blocker Thermo Shandon Histocentre2. Care was taken to lay the biopsy according to the desired direction of the cut.

After cooling down the embedded samples, a microtome (LEICA RM 2245) was used to cut 0.4 µm thick tissues. Sections floating on water were picked up on slides. Care was taken to flatten (without folds) the sample to the slide which was laid on a hot plate (40 °C) for 10 min and then in the incubator at 70°C for 1 h. Slides containing formalin-fixed paraffin-embedded (FFPE) tissues were then ready for usage.

II.2.9.2 Routine histology staining

The Hematoxylin-Eosin (H&E) and the Giemsa staining were realised using the protocol illustrated in Figure 22. In general, deparaffinization (in xylol and alcohol solutions, respectively) and hydration were followed by staining (H&E or Giemsa), washing and dehydration (alcohol solutions and then xylol). After the staining process, slides were covered by cover slips followed by the microscopical examination (Magnification 400X).

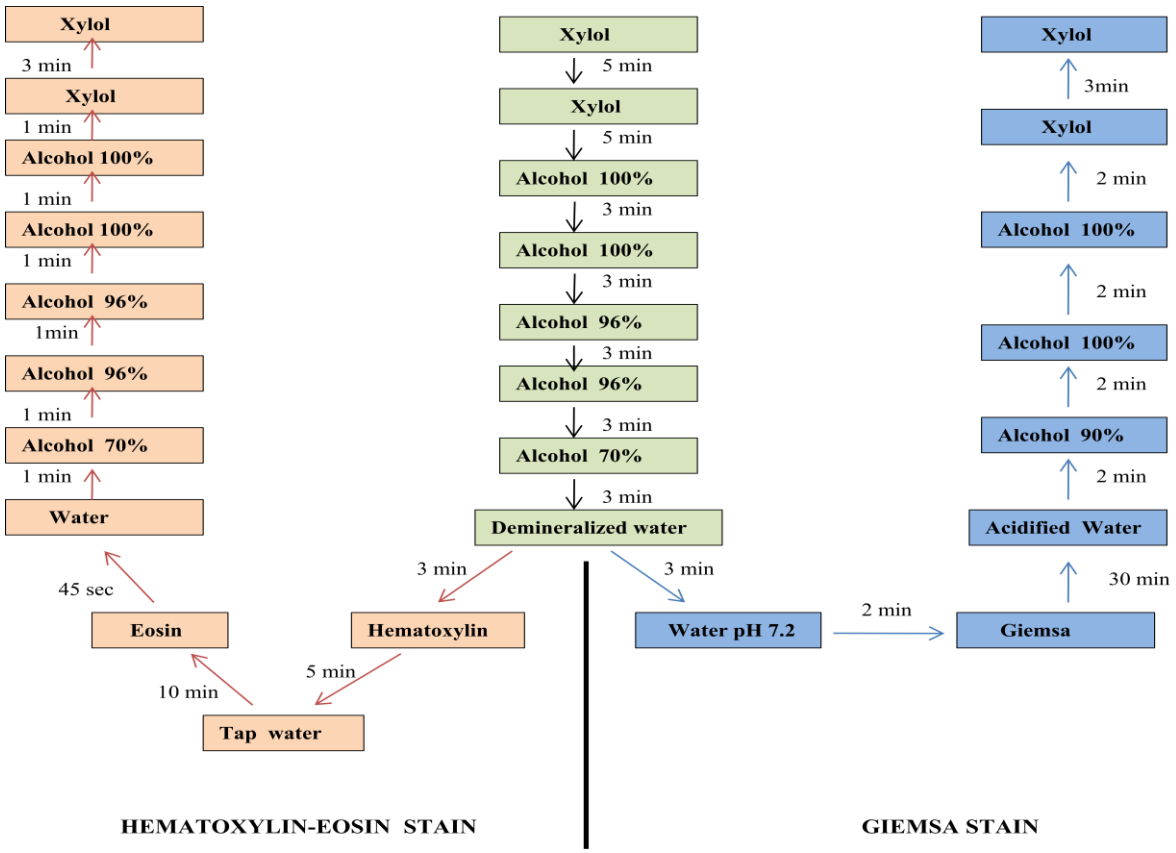


Figure 22: Protocol of Hematoxylin-Eosin and Giemsa staining

II.2.9.3 Immunophenotypic characterization of inflammatory cells at the lesion sites

Immunohistochemestry (IHC) technique was performed to characterize inflammatory cells infiltration at the CL lesions sites. IHC is a wide-used biological technique that combines anatomy, physiology, immunology and biochemistry. Developed from the antigen-antibody binding reaction, immunohistochemistry can be considered as a method that visualizes distribution and localization of specific antigen or cellular components in separated tissues, or tissue sections. Immunohistochemistry provides *in situ* information which promises a more convincing experimental result. Major components in a complete immunohistochemistry experiment are:

- Primary antibody binds to specific antigen;
- The antibody-antigen complex is formed by incubation with a secondary, enzyme-conjugated, antibody;
- With presence of substrate and chromogen, the enzyme catalyzes to generate colored deposits at the sites of antibody-antigen binding.

In the present study, the Labeled Streptavidin-Biotin (LSAB) method was performed.

i) Characterization of inflammatory cells infiltration

In preheat (15 min) retrieval solution, containers holding the slides with FFPE tissue, including the positive control slide, were exposed to heat (20 min) in the water bath. After this, cooling was performed during 20 min at room temperature.

Slides were then washed in the tap water and preserved in 10% Tris-HCl buffer (pH 7.60).

After this stage, each slide was laid on a cover plate and the whole was introduced in the cover plate rack.

CD antibodies were then diluted with the antibody diluents as follow: anti-CD4 (clone 1F6, Novocastra (UK), dilution 1:40), CD8 (clone 23G12, Novocastra (UK), dilution 1:100), CD20 (clone 7D1; Novocastra^{TM} , United Kingdom, dilution 1:200), and CD68 (clone PG-M1; Dako, Denmark, dilution 1:50), CXCR3 (clone 1C6; Pharmingen; dilution 1:50), Foxp3 (clone 236A/E7, Abcam, dilution 1:500), CD1a (clone 010; Novocastra^{TM} , United Kingdom, dilution 1:100), CD83 (clone 1H4B; Novocastra^{TM}, dilution 1:10), CD54 (clone 23G12; Novocastra^{TM}, dilution 1:25), IL-5 (clone 9906; R&D Systems, dilution 1:1000), IFN-γ (clone IFN-γ (H-145):sc-8308; Santa Cruz Biotechnology, dilution 1:100), TGF-β (clone TGF-β(V):sc-146; Santa Cruz Biotechnology; dilution 1:200), and IL-6 (clone 1936; R&D Systems, dilution 1:200). For each experimentation, after two times (5 min x2) washing by the Tris-HCl solution, 100 µl of each diluted antibody were added to the preparation for 1h (overnight for CD54) incubation at room temperature.

During the incubation period samples were always covered and protected from light. The first antibody was then washed (5 min x2) in the Tris-HCl solution. After this washing, one drop of the biotinylated secondary antibodies was added for 30 min incubation at RT. The second antibody was washed (5 min x2) in the Tris-HCl solution and one drop of Streptavidin Alkaline Phosphatase added for 30 min incubation. During this incubation time, the chromogen solution was prepared as followed: for an end volume of 2250 µl (prepared for 20 slides) 2000 µl of alkaline phosphatase substrate was introduced in a tube and completed respectively by 80 µl

Chromogen Red1 + 80 µl Chromogen Red2 + 80 µl Chromogen Red3 + 10 µl Levamisol.

The Alkaline Phosphatase was washed (5 min x2) in the Tris-HCl solution. Each Cover plate was then taken out from the rack and the slide was removed. After this, 100 µl of chromogen solution was spread over the tissue on the slide for 6 min. The microscope was used to verify any eventual color change starting by the positive control. This was followed by washing (4 x) in the distilled water and the staining in Hematoxylin (30 s). After this staining, the slide was washed in the tap water (5 min) and once more in the distilled water (1 min). The preparation was then air dried (≈15 min) and introduced in xylol (4 x 3 min). The slide was finally covered using a cover slip.

This was followed by microscopic observation. Cells count was performed in each skin layer (epidermis and dermis) by a blinded investigator at a magnification of 400X. For each sample, antigen-expressing cells were counted in five randomly-selected high power fields (HPF; X400) and one value was the mean number of all the 5 fields.

ii) Mast cells characterization

Immunophenotyping was realised using the Labeled Streptavidin-Biotin (LSAB) method. The procedure was as in *II.8.3.1* with a slight alteration. The different steps were as follow:

All the slides containing samples were immersed respectively into the xylol (10 min x2), absolute alcohol (EtOH) 100% (10 min), alcohol (EtOH) 30% (10 min). After this step, they were first washed with the tap water (2 min) and then with the tris-HCl 1X (5min). After preheating the retrieval solution, the slides were introduced in this solution and heat up during 20 minutes and then cooled down during 20 others minutes at RT. Distilled water and Tris-HCl solution 1X (5min) were respectively used to wash the slides. After this, the slides were incubated with the first antibody, antitryptase (1:2000), during 30 min at RT. This was followed by another washing with the Tris-HCl solution 1X (5min) before the introduction of LSAB for 20 min incubation. After another washing with the buffer, the second antibody (A) was introduced for 20 min incubation and the slides were then washed again with the same

buffer. One drop of the solution B was put on each slide for 15 min incubation and the preparation was then stained with hematoxylin (2 min). After washing with tap water, all the slides were covered using the cover slips. This was followed by a microscopic observation and cells count. Marked cells were counted per five high power field (HPF; X400) and the mean number was calculated for each sample.

The MC degranulation was estimated as follow:

- no degranulation (ND): granules loss < 10%;
- moderate degranulation (MD): granules loss [10-50%];
- extensive degranulation (ED): granules loss > 50% (Maurer *et al.*, 2006).

The percentage of degranulation was calculated as follow:

$$\textbf{Percentage of Degranulated cells (\%)} = \frac{\textbf{Number of Degranulated Cells}}{\textbf{Total Number of Cells}} \textbf{ X 100}$$

II.2.10 Evaluation of antibodies and cytokines/chemokines levels in the serum

During this step of the study, in addition to double positive subjects for leishmaniasis and HIV infection (Leish+HIV+) and individuals presenting only leishmaniasis (Leish+HIV-), we included two control groups: the first group was constituted of 16 individuals without cutaneous leishmaniasis but with a positive HIV serology (Leish-HIV+), and the second group was made up of 8 individuals without CL and with a negative HIV serology living in non endemic zone (Leish-HIV-).

II.2.10.1 Determination of Leishmania-specific antibody levels

Indirect Enzyme Linked Immunosorbent Assay (ELISA) was performed to determine anti *L. major* human IgG and IgG subtypes (IgG1a/b, IgG2). In ELISA, an unknown amount of antigen is affixed to a surface, and then a specific antibody is washed over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substrate is added that the enzyme can convert to some detectable signal. Thus in the case of fluorescence ELISA, when light of the appropriate wavelength is shone upon the sample, any antigen/antibody complexes will fluoresce so that the amount of antigen in the sample can be inferred through the magnitude of the fluorescence.

Sera were pre-diluted with the PBS 1X (1:1000 for the whole IgG, and 1:10, 1:100, 1:1000 for IgG subtypes).

A 96 well Immune-Maxisorp plate was coated with 100 µl/well of *L. major* lysate (0.05 mg/ml in phosphate-buffered saline-PBS) and incubated overnight. After three successive washes, with washing buffer (PBS 1X + 0.05% Tween 20), 180 µl/well of block buffer (PBS 1X +2% Bovine Serum Albumin- BSA + 0.05% Tween 20) was introduced for one hour incubation at room temperature (RT).

The standard (STD), Aliquot Standard "Dillenkofer" (5.5 ng/ml), was first diluted serially in the assay diluent to obtain respectively 1000, 500, 250, 125, 62.5, 31.25, and 15.63 ng/ml (for IgG subtypes, no STD was available).

After washing out the block buffer, the STD (100µl/well) was introduced in the two first rows in the descending with the blank (BLK) (assay diluents) in the last well of each row. In the remaining others wells were diluted 10 µl/well of different serum samples (pure or pre-diluted) in 90 µl/well of essay diluents.

This was followed by 2 h incubation at room temperature.

During the incubation period, detection antibodies were diluted (Goat anti-human IgG, γ-chain spez for total IgG 125 ng/ml, dilution 1:10 000; anti-human IgG1 Fc Biotin and anti-human IgG2 Fc Biotin for IgG subtypes 1 and 2 respectively, dilution 1:250) was introduced in each well (100 µl/well) for 1h incubation.

Not bound standard was washed out and the detection antibody (100 µl/well) was then added and incubated for 20 min.

This was followed by washing and subsequent introduction of prediluted (1:200) Streptavidin-HRP (100 µl/well) for 20 min incubation at RT.

After this, Streptavidin was washed and 100 µl/well of mixed substrate A+B (1/1) was added. The eventual development of a colour (blue) in each well was followed by the introduction of 50 µl/well of H_3PO_4 1M to stop the reaction.

Results were read using a Microplate reader Elx808™ Fa BioTek, λ=520 nm.

II.2.10.2 Serum cytokines/Chemokines profile

The Fluorescent -Activated Cell Sorting (FACS) method was used to determine the level of some pro-inflammatory and inflammatory cytokines up-regulated in sera

of all CL patients. The kit used was FlowCytomix Human Th1/Th2 11 plex Kit Bender MedSystem BMS810FF.

Bender MedSystems bead-based assays follow the same principle as a sandwich immunoassay:

- fluorescent polystyrol beads are coupled with antibodies specific to the analytes to be detected;
- a mix of coupled beads is incubated with the samples or standard mixture to be tested;
- analytes in the sample bind to the antibodies coupled to the beads;
- a biotin-conjugated second antibody mixture is added, which binds to the analytes bound to the capture antibodies;
- streptavidin-phycoerythrin (PE) is added which binds to the biotin conjugates;
- beads are differentiated by their sizes and distinct spectral signature by flow cytometry;
- FlowCytomix Pro 2.3 Software enables calculation of analyte concentration in the tested samples.

During the present study, we proceeded as follow:

In order to avoid beads agglutination by fats, each serum was first centrifuged (2000 rpm, 5min).

Seven STD tubes (the first STD two times) were prepared. The first tube was 1:20 dilution of reconstituted standards (STD of each analyte). The next six tubes were obtained by a serial dilution of the STD. Thus, 100 µl of assay buffer was previously introduced in each tube and 50 µl of the solution of one tube was transferred to the next starting with the first tube. The blank was the assay buffer.

In sample tubes, was introduced a volume of 25 µl of each serum sample.

Beads mixture was previously prepared (for 50 tests, 50 µl of each bead + 1350 µl of Reagent Diluent). In each tube, 25 µl of beads mixture was introduced and the biotine-conjugate mixture (50 µl) was added in this previous volume. The whole was mixed and incubated during 2 h (protected from light).

During the incubation time, the Streptavidine-Phycoerythrin (Streptavidine-PE) solution was prepared (for 50 tests, 58 µl of Streptavidine-PE solution + 2800 µl of Assay Buffer).

After the incubation, 500 µl of assay buffer was added in each tube and all the tubes were spun down (200 g, 5 min); this step was repeated twice.

The Streptavidine-PE (50 µl) was added in each tube and incubated at room temperature during 1 h (protected from light).

After the incubation, 500 µl of assay buffer was added in each tube and all the tubes were spun down (200 g, 5 min); this step was repeated twice.

At the end, 50 µl of Assay Buffer was added in each tube.

Results were read using the flow cytometer BD LSRII. The cytometer setup was performed using provided setup beads (SB). Before reading, the content of each tube was well mixed using the vortex.

Analysis was started by STD curve (STD1 to STD7 and BLK).

II.2.11 Data analysis

Analysis was performed using StatView© and unpaired Students t-Test. Data are presented as mean ± SEM.

Z-test correlation was used in bivariate analysis of Th1:Th2 cytokines, IgG:IgG1, IgG:IgG2, IgG1:IgG2.

Regression equations was performed between IFN-γ and some others cytokines.

II.2.12 Ethical consideration

The study was approved by the National Ethics Committee, Cameroon (Yaoundé, 19th April 2006), and administratively authorized by the Division of Health Operations Research, Ministry of Public Health, Yaoundé, Cameroon (N° D30-44/AAR/MSP/DROS/CRC, 2nd February 2007). It was conducted in accordance with the Declaration of Helsinki, guidelines from the International Conference on Harmonization (ICH) and Good Clinical Practice (GCP). All patients (or their guardians for minors) provided written informed consent to participate to the study. The participation of patients was free and their follow-up was free of charge.

CHAPTER III

RESULTS AND DISCUSSION

III.1 RESULTS

III.1.1 Issue of the epidemiological investigation

III.1.1.1 Outcome of the field survey

In 4 872 houses of 19 villages, a total number of 32 466 persons with 47.6% males versus 52.4% females were surveyed. In this population, 146 subjects (0.5%) presented active cutaneous leishmaniasis (CL) lesions and an additional 261 subjects (0.8 %) showed scars, indicative of previous CL infections. This is a total 407 (1.3 %) for old and new cases.

On the field, the distribution of CL cases was not homogenous in Mokolo focus. The number of individuals affected varied from one locality to another. In fact, during the survey, Mandaka village registered the highest number (71) of old cases as indicated by the presence of scars, while Mboua and Mofolé registered the highest number of individuals with active lesions (28 and 27 respectively). In Gouzda village no leishmaniasis case was recorded (Figure 23).

However, apart from Mokolo subdivision, four cases came from other subdivisions: Hina (1), Soulédé-Roua (2), Gazawa (Diamaré Division) (1).

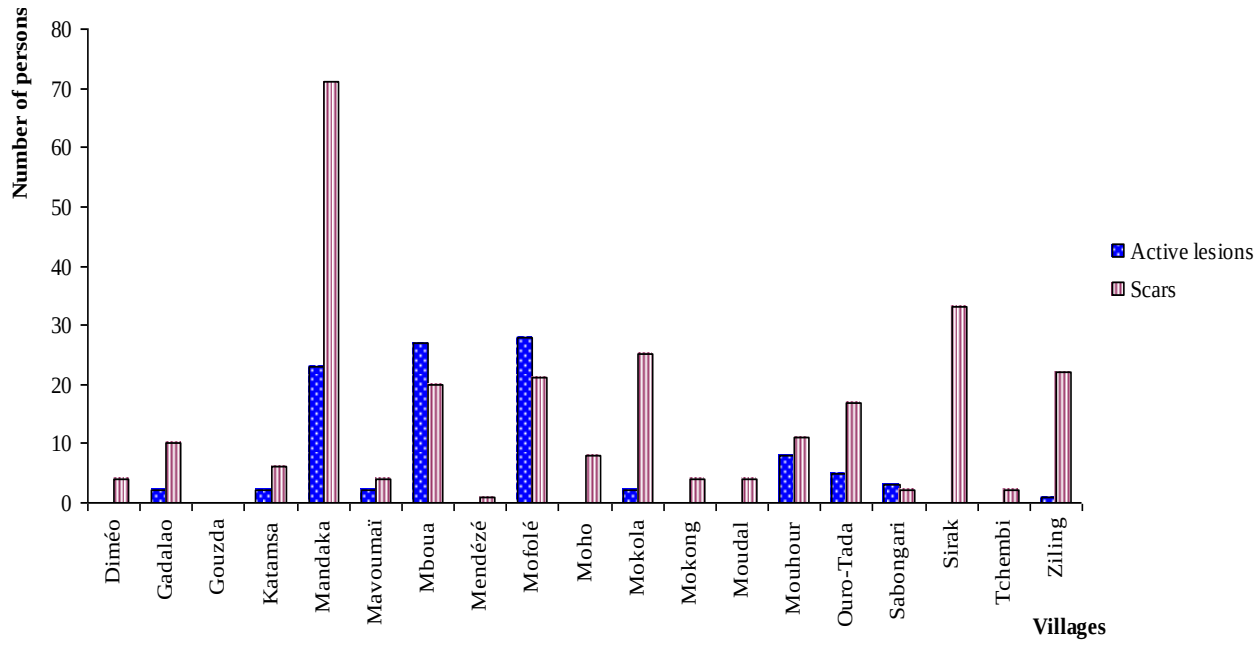


Figure 23: Distribution of cutaneous leishmaniasis victims in villages of Mokolo focus

In general, the number of CL patients varied from 0 to 5 per house. Seven individuals who during the field survey were suffering from CL were further diagnosed as HIV positive; this represented 4.8% of the subject with active CL infection and 0.02% as the prevalence of *Leishmania*/HIV co-infection in the whole population surveyed. In this co-infected group, six patients were females while one was a male.

The age of subjects presenting cutaneous leishmaniasis ranged from 4 months to 72 years (mean value 16.59 ± 14.16 years). Individuals less than 20 years were the most affected and represented 80% (Figure 24). Amongst them, 44.5% were recorded in the age range of teenagers. The CL prevalence in adults is lower than that of teenagers. In people from 11 to 40 years, the prevalence of the disease is higher in males than in females. Overall, we registered 71 (48.4%) males vs 75 (51.6%) females but the prevalence of the diseases according to the patient gender was higher in male than in female (0.48 and 0.43% respectivelly).

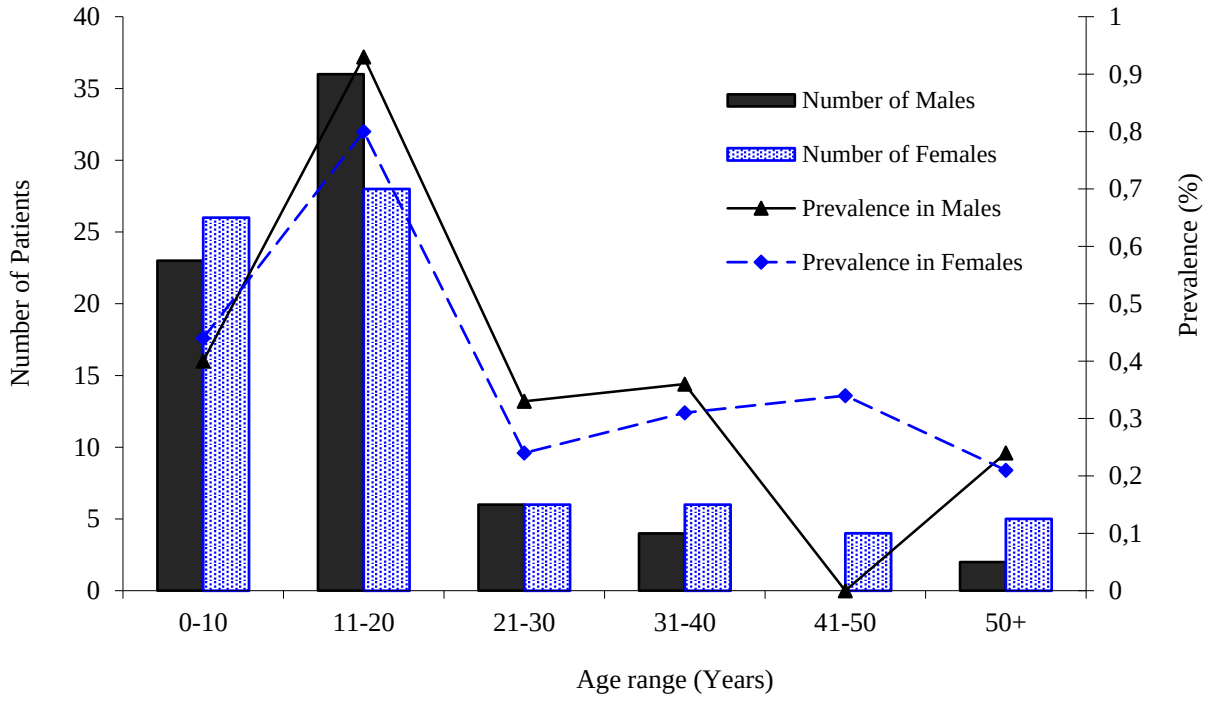


Figure 24: Age range and sex distributions of CL patients in Mokolo
n=146

The youngest co-infected patient was a woman of 17 years while the eldest was a man of 50 years (mean value = 30.60±13.07). They were farmers and housewives.

Regarding their marital status, the only man was polygamist. Of the six females, two were widows, two were abandoned by their respective husbands because of leishmaniasis, and two were married. Thus, four co-infected women were husbandless.

About the occupation of the patients, students (49.7%) were the most concerned followed by farmers (23.5%). Unemployed people, these are those without a specified occupation and children, represented 15.9 %. Civil servants, mason etc. were rare in this study (Figure 25).

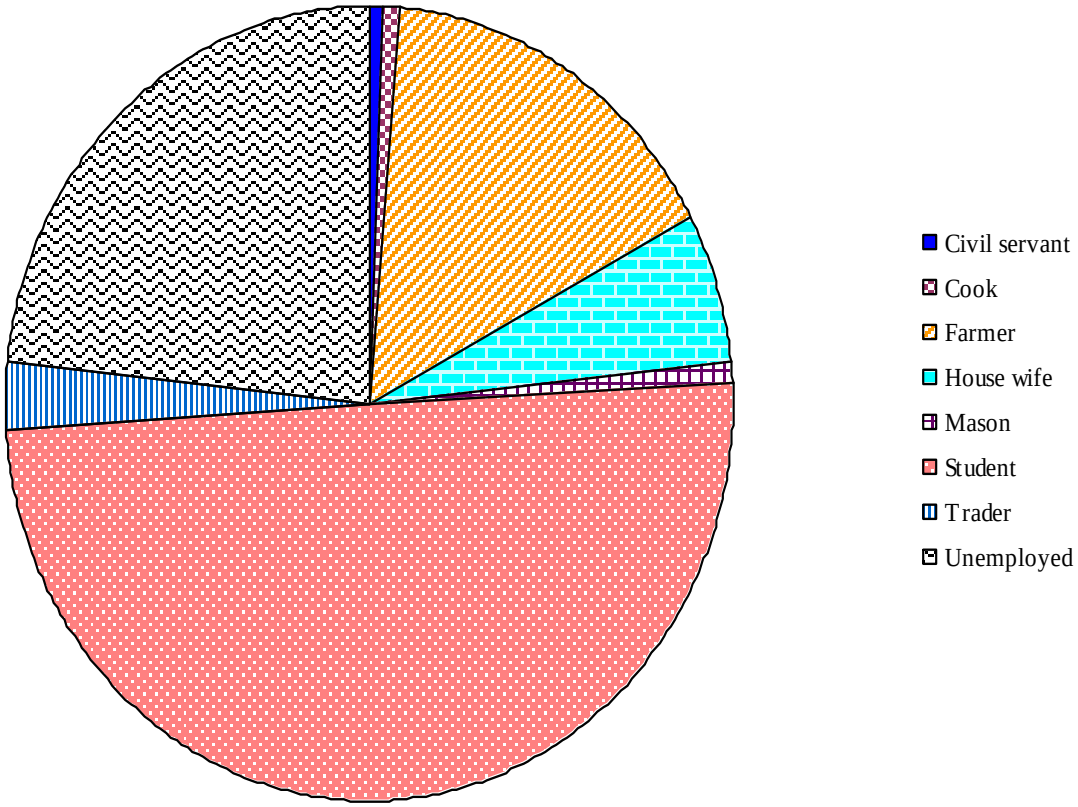


Figure 25: Distribution (percentage) of cutaneous leishmaniasis patients according to their occupation
n=146

We wanted to know if in the population surveyed some individuals were aware of the origine of the disease. It came out that no one knew that leishmaniasis is an insect borne disease, but they speculated on other causes like eating of guava, witchcraft etc.

Mosquito net users represented 31.4% of the whole population counted. In 621 (12.7%) houses of our study site, people declared to use sporadically insecticides or insect repellents.

III.1.1.2 Rainfall and annual distribution of cutaneous leishmaniasis

As it is depicted in Figure 26, in year 2008, the rainy season started in May and ended in October. August was the rainiest and 407.5 mm of rainfall were registered in the region. Remarkably, we recorded the highest monthly prevalence in October (1.5%), followed by September (1.1%), this is months after the beginning of the rainy season. Around May CL cases were rare and May was the month of the lowest prevalence.

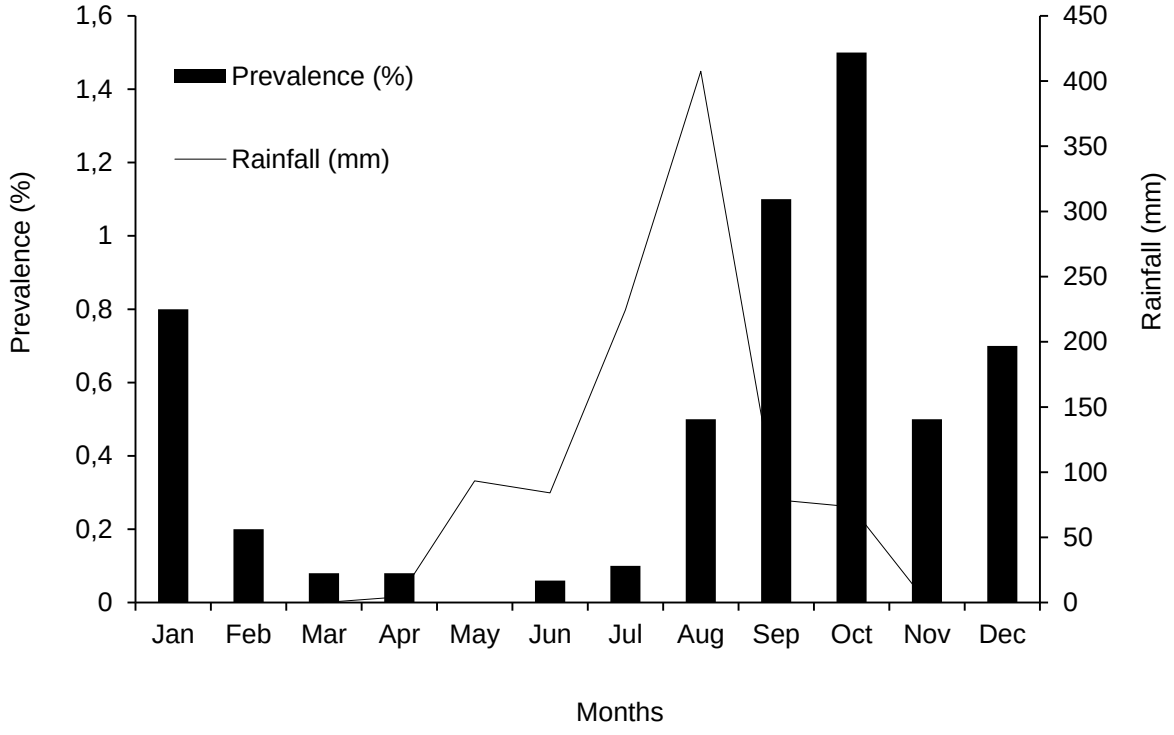


Figure 26: Relationship between occurrence of annual cutaneous leishmaniasis cases and rainfall in Mokolo endemic focus
From November to April it is the dried season, from May to October it is a rainy season
n=146

III.1.2 Clinical features of cutaneous leishmaniasis lesions

III.1.2.1 Characteristics of common lesion types

A lesion usually began by a nodule (Figure 27a) which could ulcerate or not. A ‘’volcano” sign was the characteristic of many lesions; nevertheless some other signs such as the presence of papules around the ulcer (Figure 27c), lymphadenitis characterised by the swelling of lymph nodes (Figure 27g) were often diagnosed. Others forms of lesions such as the verruca like were observed. Crusted plaques were also noticed. Clinical healing was characterised by a typical scar sometimes with a demarcation line.

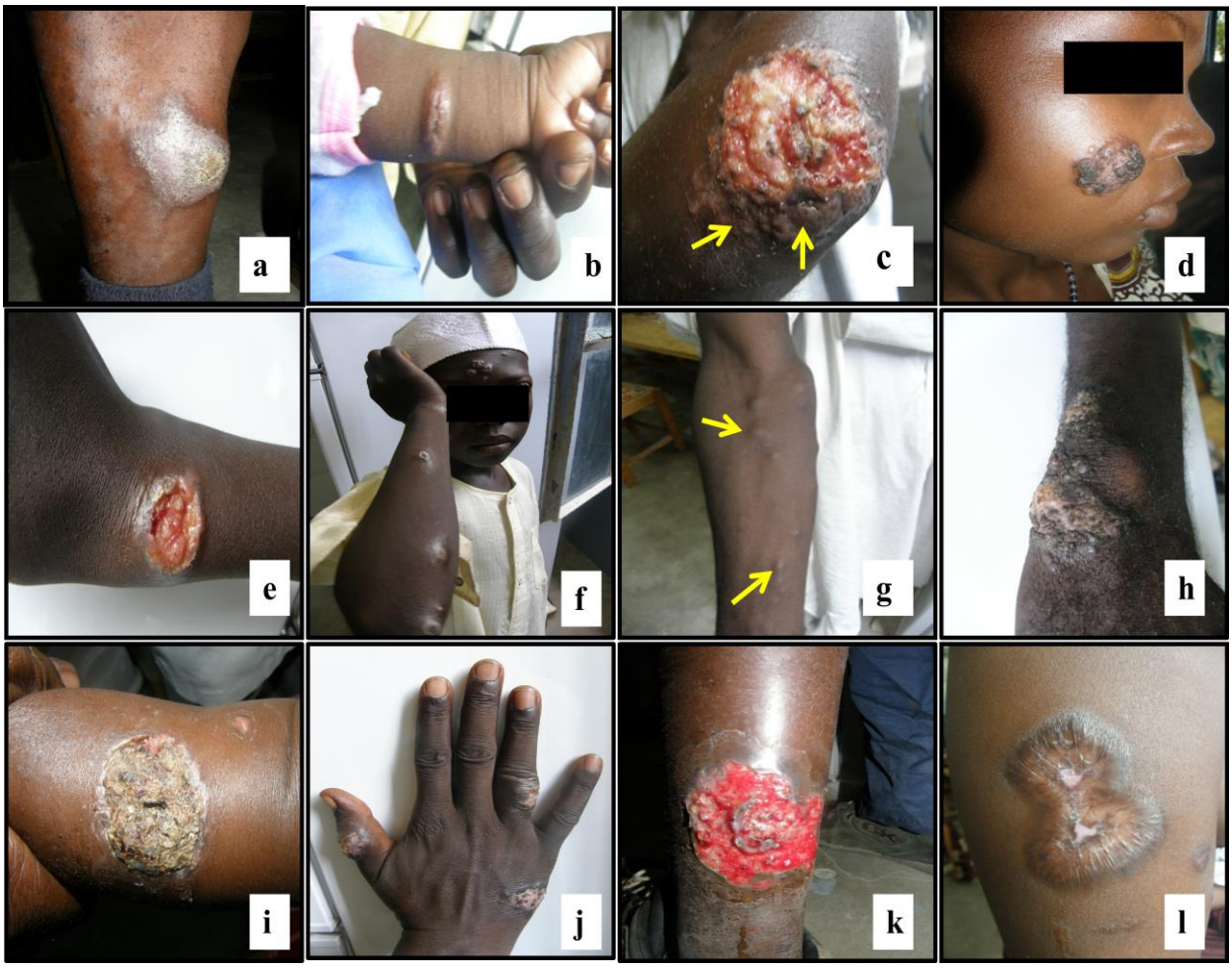


Figure 27: Illustration of common lesion types in Mokolo
a: Nodule, **b**: Nodule at the beginning of ulceration, **c**: Lesion on a elbow with satellite papules (arrows), **d**: Verruca-like lesion on cheek, **e**: Typical volcano sign with a central depression and raised border, **f**: Multiple lesions disseminated on limb and face with the affection of the eyelid, **g**: Lymphadenitis characterised by lymph nodes swelling (arrows), **h**:Lesion that induced lymphangitis in **g**, **i**: Crusted lesion, **j**: Lesions on the hand with the affection of fingers, **k**:Wet lesion on a leg, **l**: Typical scars with demarcation line.

III.1.2.2 Lesions number and distribution on the body

The number of lesions per CL subject varied from 1 to 20 (mean value 3.5 ± 0.3). Patients presenting 1 to 3 lesions were the most frequent (modal class) and represented more than 45% of the whole. Subjects with more than 12 lesions were rare in the study even if two patients presented 19 and 20 lesions respectively (Figure 28).

A positive and significant correlation (*r = +0.36, p = 0.0009*) was observed between the number of active lesions and the healing duration.

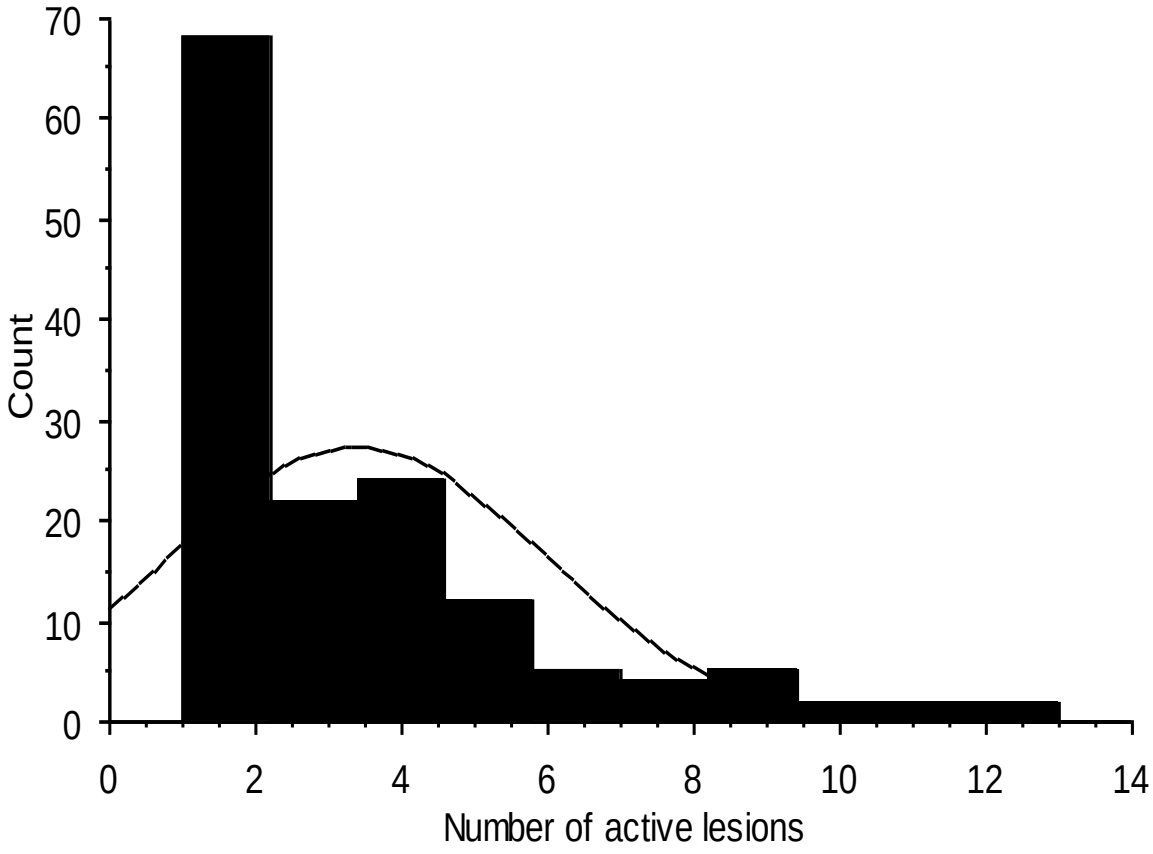


Figure 28: Frequency distribution of cutaneous leishmaniasis patients according to the number of active lesions
Total number of patients n =146

Table III recapitulates the distribution of cutaneous leishmaniasis lesions over the body for each anatomical site. It emerges from this table that, parts of the body more exposed to sand fly bite are more vulnerable. In fact, the majority of lesions occurred on the fore arms (26.3%) and the legs (24.4%). On the forehead and arms we registered

8.2% and 7.5% respectively. The buttock which is scarcely bitten by the sand fly showed consequently few lesions. In general, the limbs (with 79.2% of lesions) were the most affected parts of the body followed by the head (14.3%). It is remarkable that ears, eyelids, leaps, chin and fingers were also affected.

Tableau III: Lesions distribution on the body

Part of the body		**Number of lesions**	**Percentage (%)**
Head	Fore head	40	8.15
	Eye lids	4	0.81
	Nose	5	1.02
	Cheek	16	3.26
	Ear	1	0.20
	Chin /lips	4	0.81
		70	**14.25**
Trunk /Neck	Neck	1	0.20
	Thoracic region	18	3.67
	Abdominal region	13	2.65
		32	**6.52**
Upper limb	Shoulder	7	1.43
	Arm	37	7.54
	Elbow	34	6.92
	Fore arm	131	26.68
	Hand/Wrist	12	2.43
		221	**45.01**
Lower limb	Hip/Buttock	1	0.20
	Thigh	22	4.48
	Knee	11	2.24
	Leg	119	24.24
	Ankle/Foot	15	3.05
		168	**34.22**
TOTAL		**491**	**100**

These data were collected from a number of patients n=146

In the present study, it was often possible to register more than one active lesion on the same subject. Evenly, multiple lesions could be found on the same body part.

As it is illustrated on Figure 29, the majority of CL patients (73, 3%) presented lesions localized on only one part of their body. While 8 (5.5%) registered lesions disseminated simultaneously on the head, trunk and limbs. On 21.1% of participants, lesions were observed on two different anatomical parts of the body.

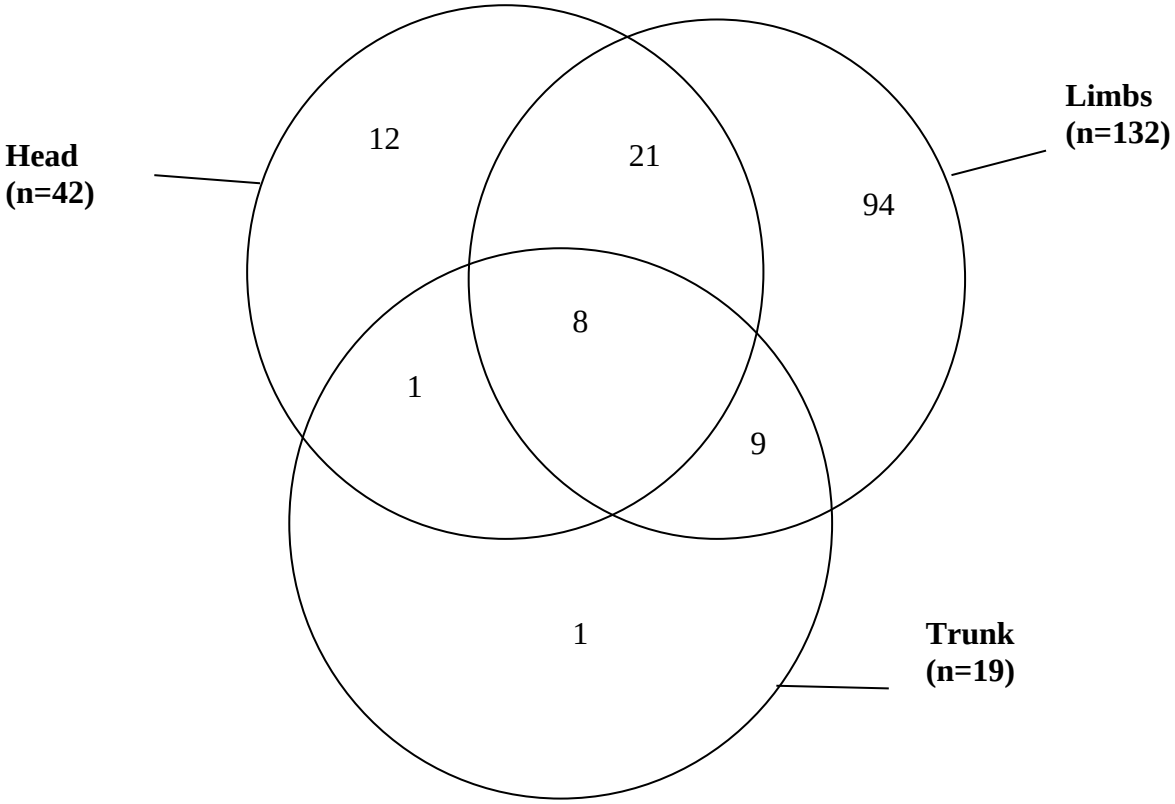


Figure 29: Distribution of patients according to active lesions dissemination on their body
Total number of patients n=146

III.1.2.3 Lesions surface

Regardless the health status, the gender and the age of CL subjects, the total surface of lesions per individual varied from 94.99 to 25 036.02 mm^2 (mean value = 1 367.15 ± 198.17 mm^2). The majority of patients showed a total surface of lesions lower than 3 000 mm^2 (Figure 30). There was a positive and significant correlation ($r = +0.79$, $p < 0.0001$) on one hand between the total surface of the lesion and the healing duration, on the other hand ($r = +0.4$, $p < 0.0001$), between the number of active lesions and their total surface per patient.

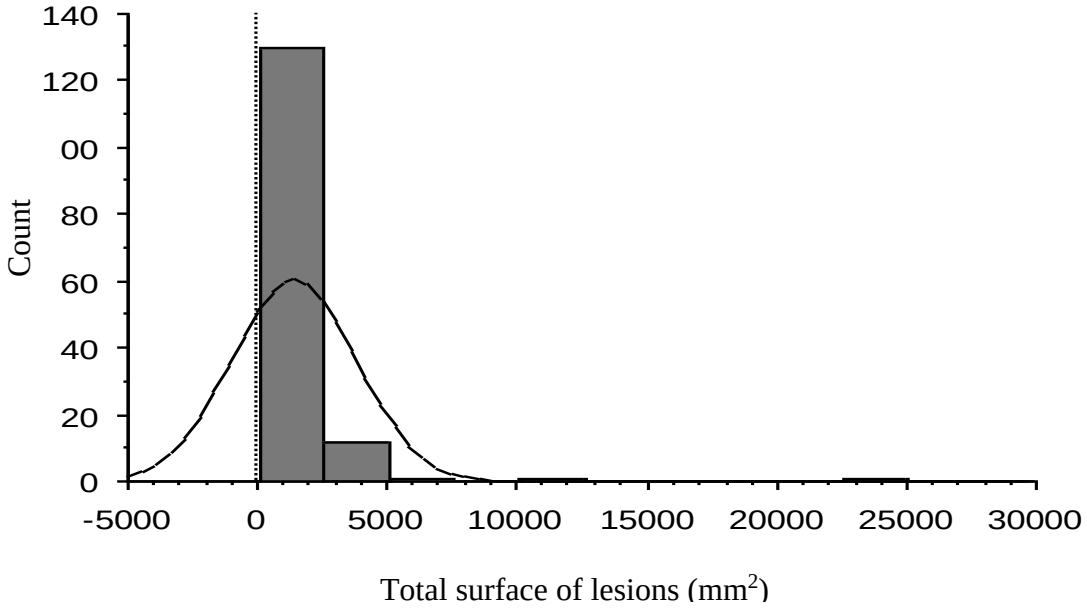


Figure 30: Distribution of patients in function of the total surface of active lesions
n=146 patients

The majority of leishmaniasis lesions registered in this study measured less than 500 mm^2. A given lesion scarcely measured more than 1 500 mm^2 even if some lesions measured around 2 000 mm^2 (Figure 31).

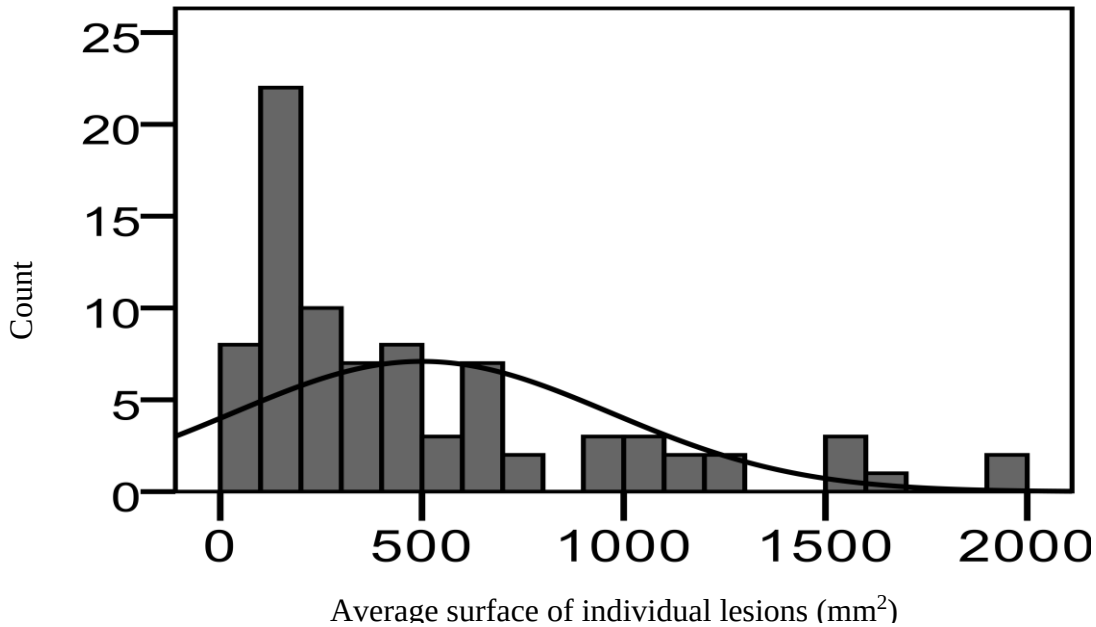


Figure 31: Average surface per cutaneous leishmaniasis lesion
n=146 patients

III.1.2.4 Gender and age of a patient and clinical features of cutaneous leishmaniasis

The sex of the patient didn't influence clinical manifestations of CL. Hence, no significant difference ($p > 0.05$) was observed between clinical signs of males and those of females. In fact, the number, the total surfaces of the lesions as well as the healing duration were not statistically different according to the patient gender (Figure 32).

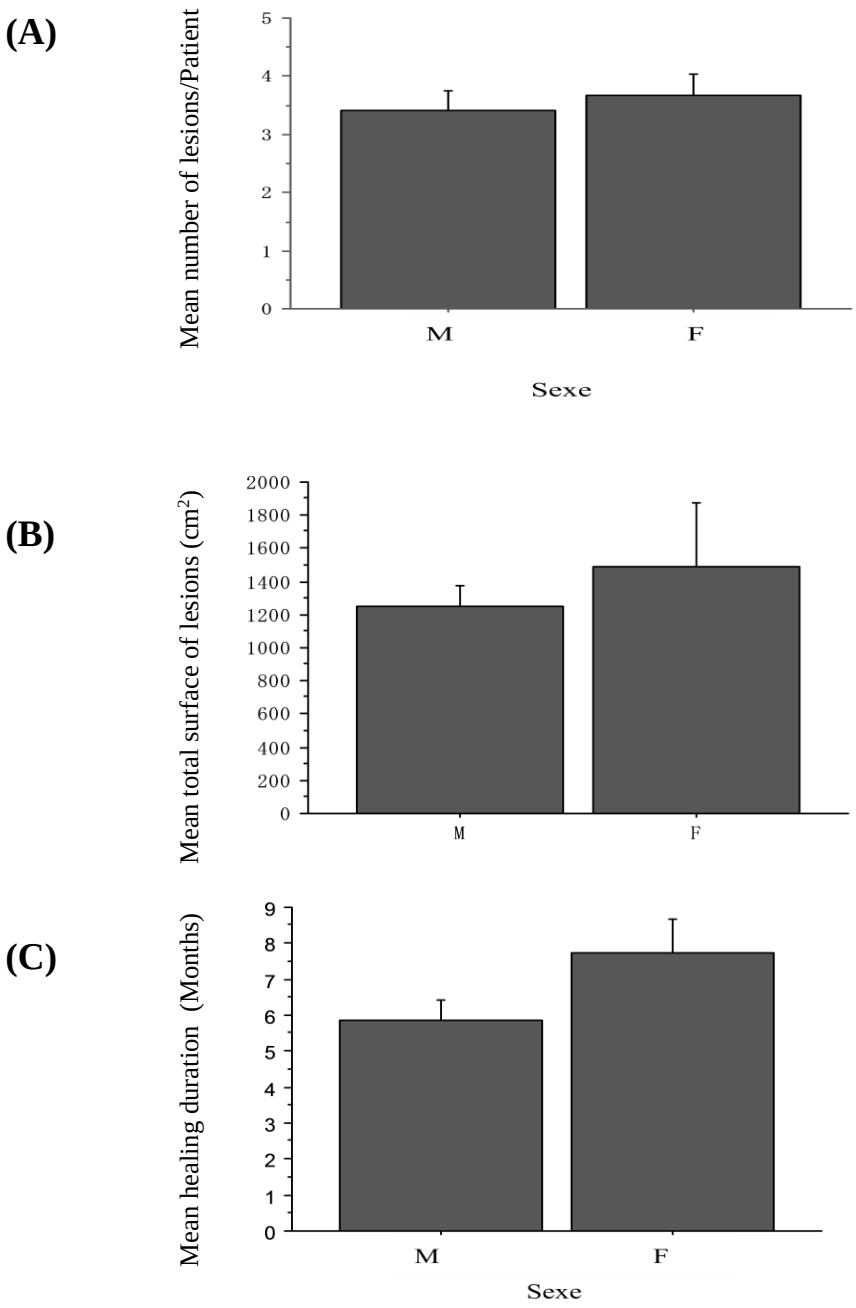


Figure 32: Clinical features of cutaneous leishmaniasis based on the patient gender
(A): Number of lesions, (B): Total surface of lesions, (C): Healing duration, M: Male (n=71), Female (n= 75), data are mean ± SEM.

In subjects with CL only, gender and patient age did not show a significant influence ($p>0.05$) on the healing duration (range 2–14 months, mean 5.6 ± 0.5 months) (Figure 33). Nevertheless, it was remarked that younger patients (less than 18 years) presented few ($p<0.006$) and significantly ($p<0.03$) smaller lesions as compared to elder individuals. A positive correlation was observed between the age of the patient and the number of active lesions ($r=+0.5$, $p=0.02$), the age and the total surface of the lesions ($r=+0.5$, $p=0.006$).

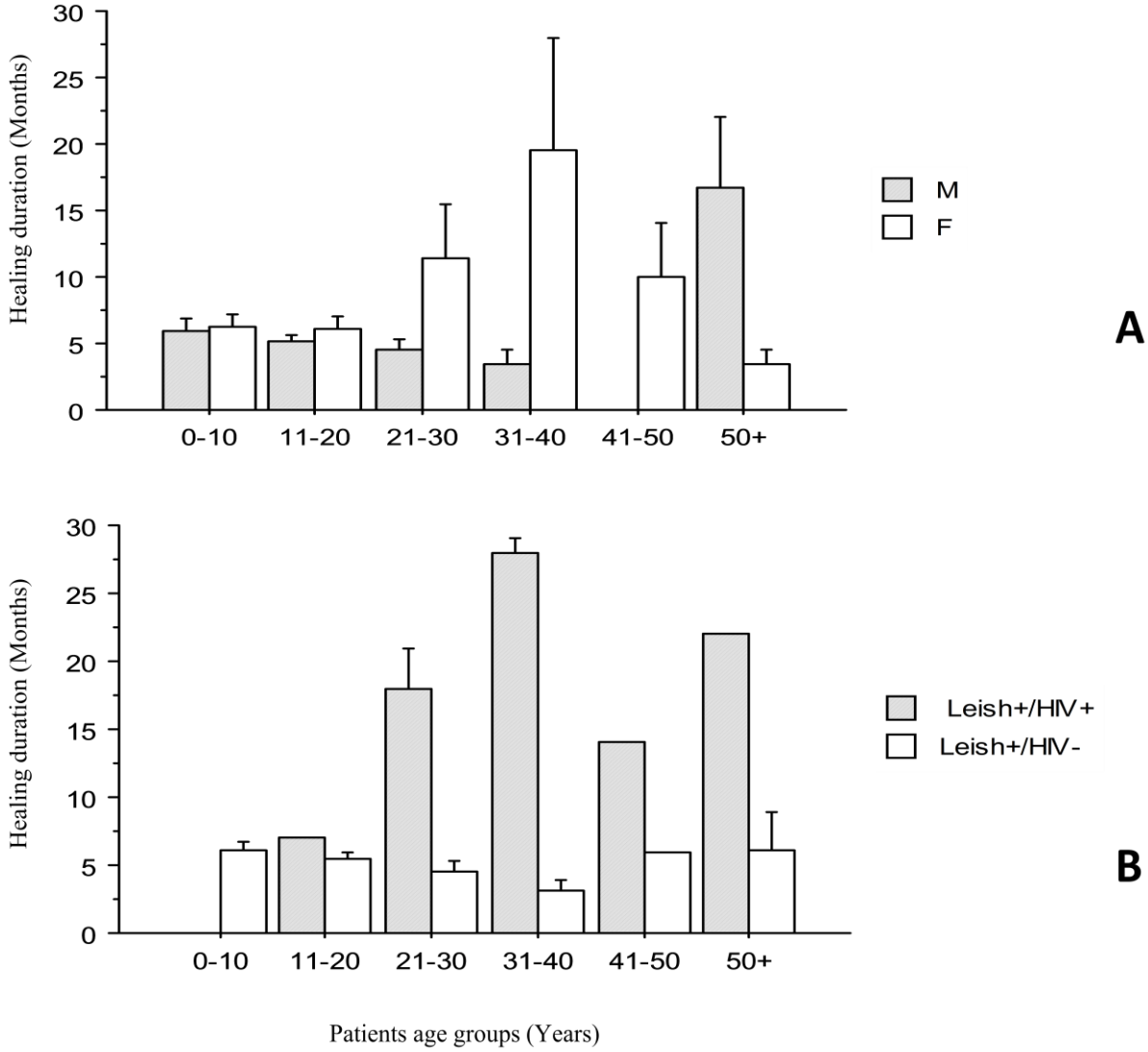


Figure 33: Healing duration of cutaneous leishmaniasis lesions in different age groups based on (A) gender and (B) HIV status.

F: Female, M: Male, total nuber of patients n=146, Leish+/HIV+: *Leishmania*-HIV co-infected patients (n=7), Leish+/HIV-: patients with CL only (n=139), data are mean ± SEM.

III.1.3 Therapeutic outcomes

The main therapy regimen used was Amphotericine B (Fongizone®), local application, associated with Metronidazole *per os* (1.5 g/kg body weight daily x 15 days). Due to the supply interruption of Amphotericine B in the country, we further replaced the regimen by Allopurinol (Zylloric®) *per os* (20 mg/kg body weight daily) which also provided good results. Metronidazol, Cotrimoxazol, Amphotericin B and Allopurinol were considered as "specific treatment" for leishmaniasis. All other therapies used by the participant were counted as "non specific treatment."

During our study, we observed that many individuals (27%), before their first visit, intended to treat CL using non-conventional drugs such as antibiotics and plant products. Sometimes (5% of cases) the drug used was unknown. On the other hand, the majority of subjects (73%) declared to not previously use any drug (Figure 34). Sixty percent of Leish+HIV+ patients declared to have used some drugs before their first visit.

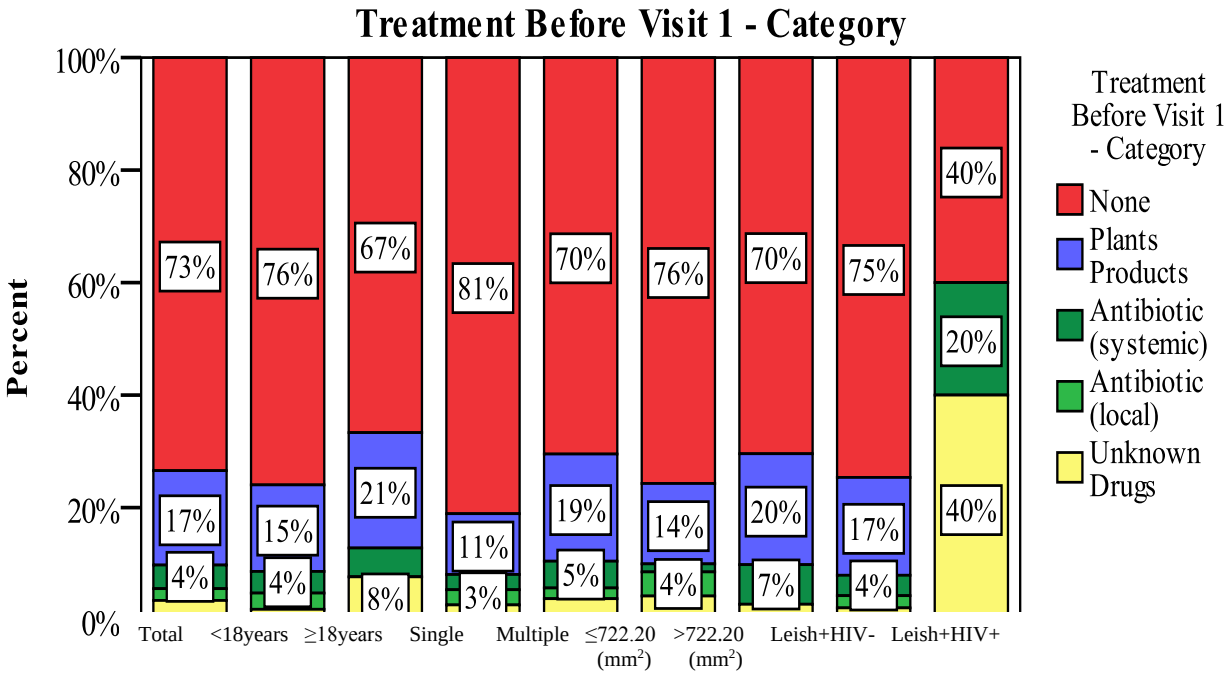


Figure 34: Patients distribution and some parameters recorded according to the therapy used before the first visit

Patients ages is splited into two groups (<18 years and ≥18 years); single and multiple refer to the number of lesions; ≤or >722.20, this refer to the total surface of lesions (mm^2); Leish+HIV+: *Leishmania*-HIV co-infected patients (n=7), Leish+HIV- : patients with CL only (n=139).

Remarkably, patients who previously tried to combat the disease by using the nonconventional therapy before being involved in this study were generally those with more severe clinical signs. Thus, their number and total surface of active lesions were significantly ($p = 0.04$) high. Their lesion resolution was also significantly ($p = 0.01$) slower (Figure 35). Along the same lines of nonconventional therapy, during our survey, some few people declared to resolve their CL lesions by a local application of boiled oil (a sort of thermotherapy) or by scraping very often their lesions (a sort of exaeresis or a curettage).

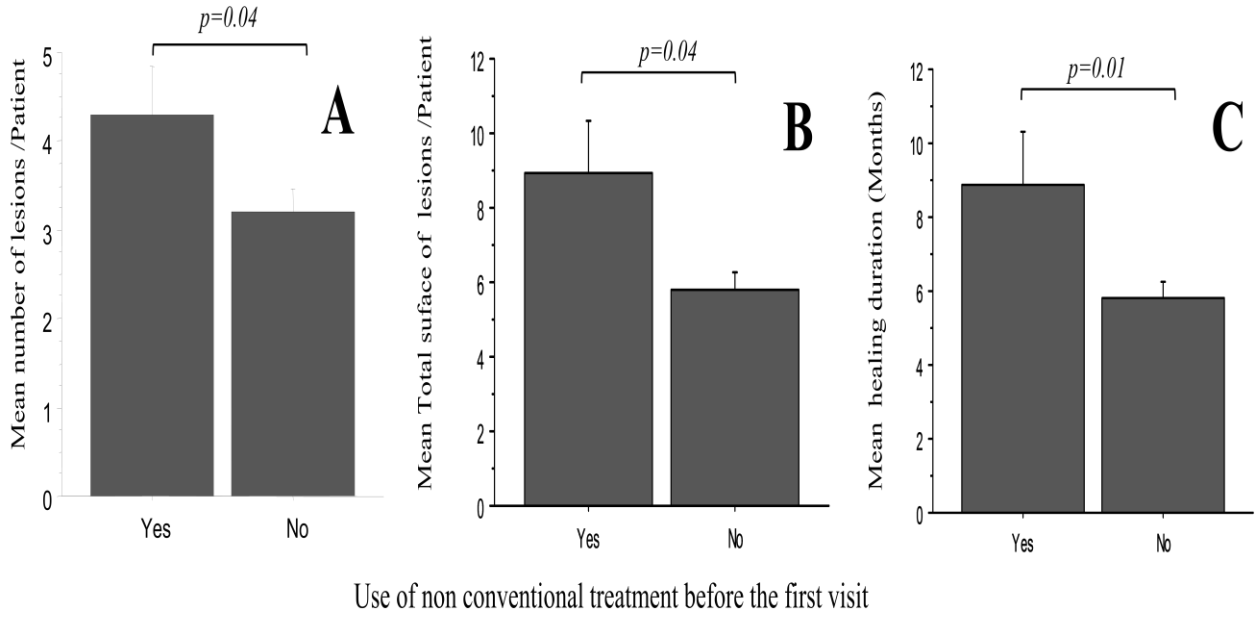


Figure 35: Effect of the use of non conventional treatment before the first visit on clinical features
A: Number of active lesions (Yes: n =44; No: n=102), B: total surface of active lesions (Yes: n =44; No: n=102), C: Healing duration from the disease onset (Yes: n=26, No: n=58), data are mean ± SEM.

From the first visit, self healing was observed on 74.3 % of patients 3 months later; consequently these participants didn't receive the antileishmanian drugs. Self healed patients were generally those with a significant low number ($p = 0.01$) and reduced total surface ($p = 0.03$) of active lesions. In general, the healing duration varied from 2–29 months (6.7 ± 0.6 months) from the onset of the disease.

Antileishmanian therapy was needed only in cases of large, persist, badly localised (e.g. face) lesions. The therapy boosted lesions resolution so that healing occurred in general three weeks from the beginning of the therapy.

III.1.4 Diagnosis measures and *Leishmania* species characterization

III1.4.1 Diagnosis measures

The observation of the Giemsa stained smear through the light microscope showed amastigotes (ovoid and purple bodies with a darker nucleus) on many slides. The Figure 36 illustrates one *Leishmania* positive slide. It is remarkable that after host cell disruption, amastigotes could be free or grouped into the rosaceous form.

The highest parasite load was generally observed on immunodeficient subjects.

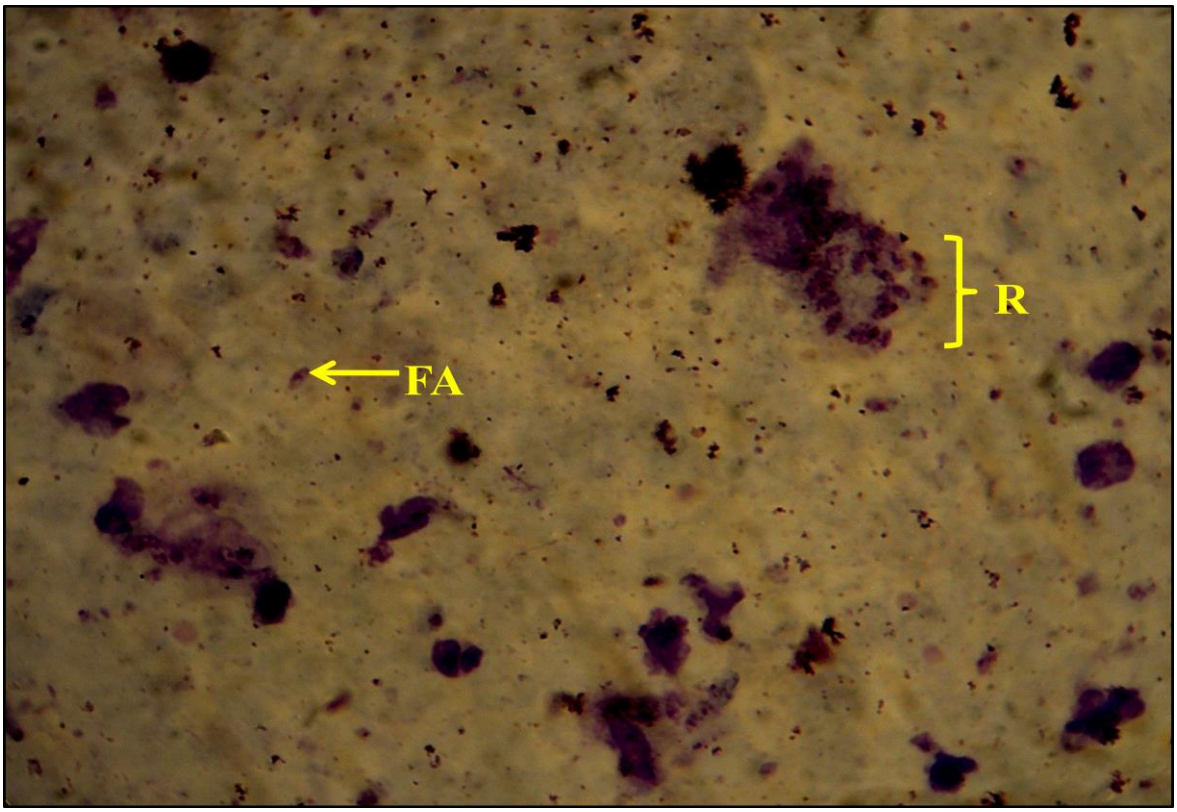


Figure 36: Giemsa stained smear showing amastigotes of* Leishmania major *(X1000)
FA: Free amastigote (oval shape, purple colour with a darker nucleus, no extracellular flagellum) which is susceptible to be phagocytosed, R: Rosaceous from a burst macrophage.

III.1.4.2 Leishmania species characterization

Parasites isolation was attempted in six subjects but due to bacterial contamination, isolates from only three subjects were identified by PCR. Sequences comparison against an internal database showed 100% similarity with the LEM62 *Leishmania major* reference strain (MHOM/YE/76/LEM62). So, our isolates identified by molecular techniques were those of *Leishmania major*. Interestingly, this species was responsible of leishmaniasis in both *Leishmania* mono-infected and *Leishmania*/ HIV co-infected subjects (Table.IV).

Tableau IV: Biological molecular identification of* Leishmania *isolates from cutaneous leishmaniasis and* Leishmania*/HIV co-infected individuals from the Mokolo endemic focus

Patient sex	**Age (Years)**	**Body site of infection**	**HIV Serology**	**PCR Identification results**
F	35	Thigh	HIV+	Contaminated
M	51	Thigh	HIV+	Positive (*L. major*)
F	11	Arm	HIV-	Positive (*L. major*)
M	12	Thigh	HIV-	Contaminated
F	09	Arm	HIV-	Positive (*L. major*)
M	10	Arm	HIV-	Contaminated

Biopsies collected in Mokolo was preserved in RPMI milieu and sent to Montpellier (France) for parasite identification using PCR

Our data showed that in Mokolo, there are individuals suffering from cutaneous leishmaniasis only and people showing *Leishmania*/HIV co-infection. Ever since, it became more interesting to investigate the influence of HIV infection on the clinical signs of leishmaniasis.

III.1.5 Influence of the HIV infection on cutaneous leishmaniasis clinical features

III.1.5.1 Confirmation of HIV serology testing

We analyzed the CD3, CD4, and CD8 blood levels of co-infected patients. It came out that all these patients showed a CD4 serum level less than the normal value expected (520–2000 cells/µl). One patient presented a critical CD4 count (112 cells/µl) and was submitted to the antiretroviral therapy (Triomine). Overall, the CD4/CD8 ratio was always lower than the normal value (1.5–2.5) (Figure 37).

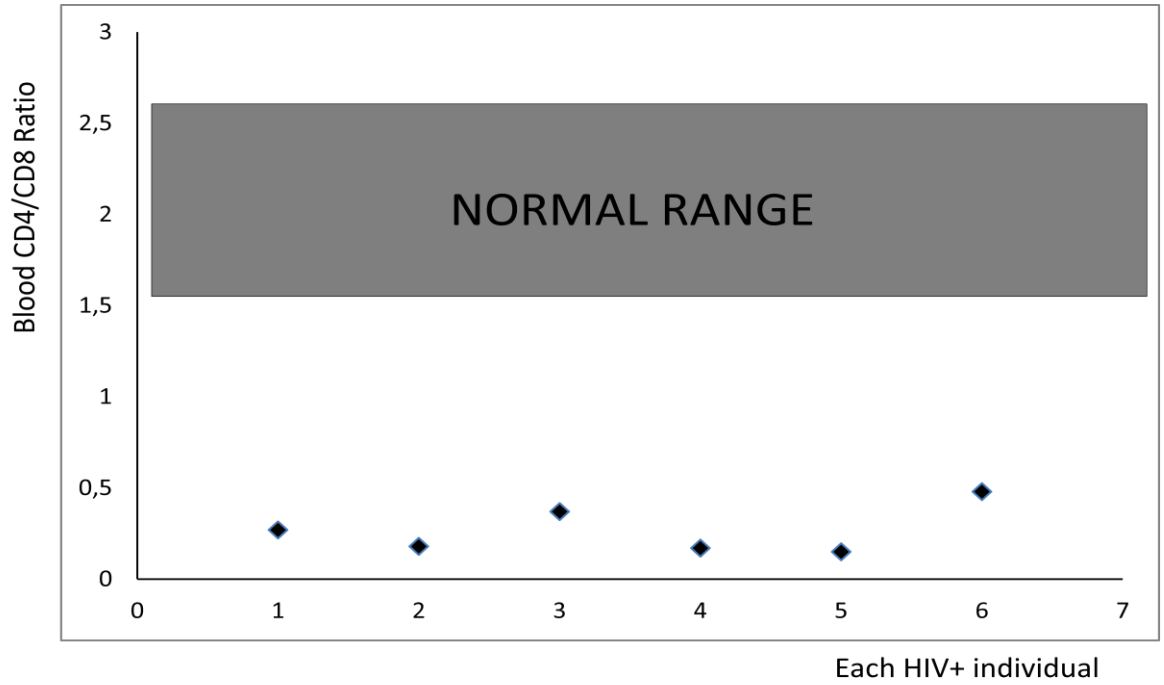


Figure 37: Blood CD4/CD8 ratio of* Leishmania *and HIV co-infected patients
n=6 (1 male, 5 females), Normal range of CD4/CD8 ratio:1.5–2.5

III.1.5.2 Influence of the HIV infection on the number, surface and localisation of CL lesions

As it is illustrated on the Figure 38, Leish+HIV+ patients' clinical features were more pronounced than those of *Leishmania* mono-infected subjects. Three patients presented disseminated lesions. In this group of patients, atypical forms (necrotizing,

cheloid) and an ectopic localization (e.g. armpit) of lesions were recorded. The necrotizing lesion showed a swollen border and a central hemorrhagic area.

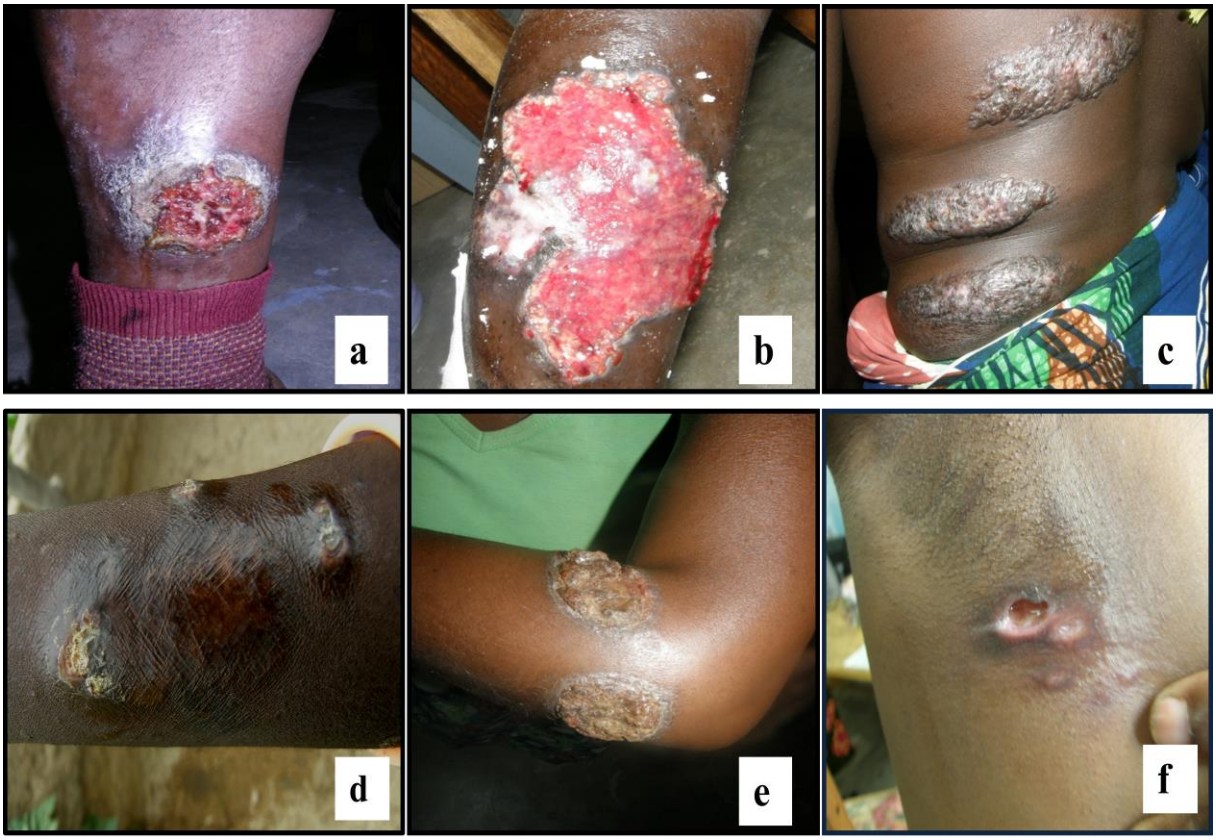


Figure 38: Representative lesion types in immune depressed patients
a: A lesion on the lone co-infected male, **b**:Necrotizing lesion on the leg, **c**: Multiple Cheloid-like lesions on the trunk, **d**: Reactivation on a scar, **e**: Two of twelve disseminated lesions on a patient and **f**: An atypical localization (armpit) of a lesion on the same patient in **e**.

Leishmania mono-infected patients presented fewer lesions as compared to co-infected subjects. During our study, individuals who presented more than 10 active lesions were rare. Amongst the five participants with more than this number of lesions, three showed a positive serology for HIV. In addition, no HIV patient showed less than three lesions. These co-infected individuals also showed a total surface of active lesions range of 343.830 –71 101.5 mm^2 (mean = 15 665.37 ± 9 783.73 mm^2). In general, the number and the total surface of lesions were significantly ($p \leq 0.0002$) high (2.5 and 7-fold respectively) in Leish+HIV+ subjects as compared with *Leismania* monoinfected individuals (Figure 39).

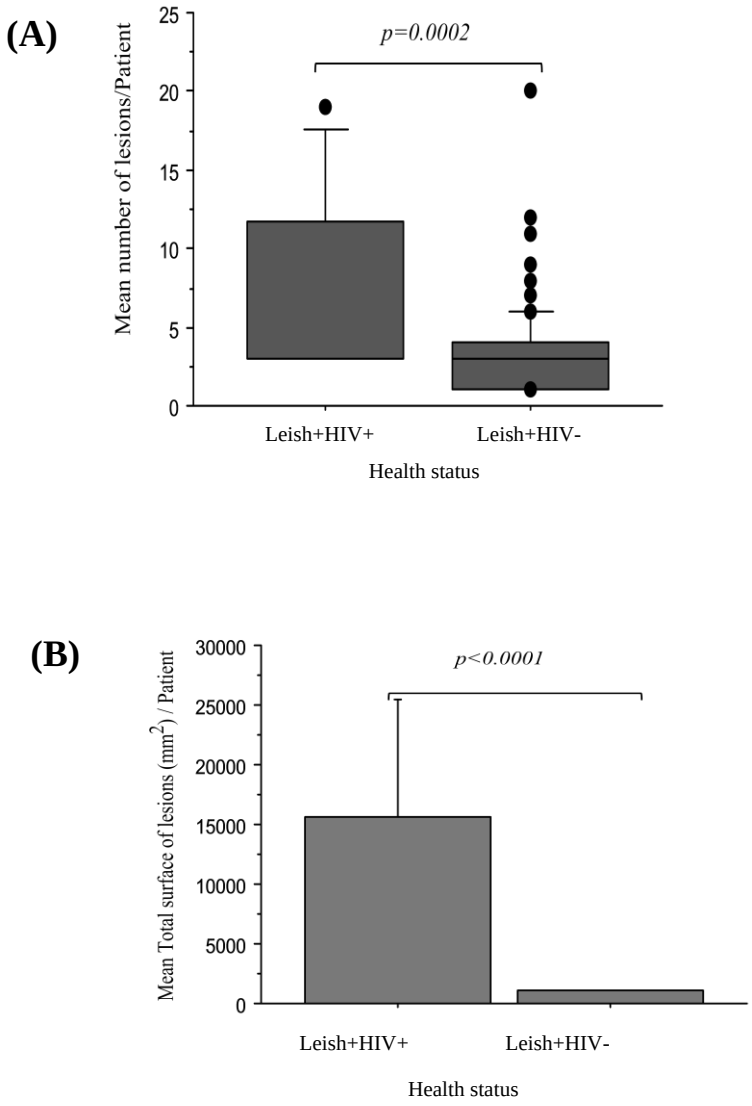


Figure 39: Comparative representation of clinical features of cutaneous leishmaniasis in Leish+HIV- and Leish+HIV+ patients

(**A**): Number of lesions, (**B**): Total surface of lesions, Leish+HIV+: *Leishmania*-HIV co-infected patients (n=7), Leish+HIV- : patients with CL only (n=139), data are mean ± SEM.

III.1.5.2 Influence of the HIV infection on the cutaneous leishmaniasis lesions resolution

As it is depicted on Figure 40 illustrating the comparative evolution of a chronic lesion in Leish+/HIV- and Leish+/HIV+ subjects, we can observe that even treated with antileishmanian drugs, lesion resolution was significantly ($p < 0.0001$) delayed (4-fold) on immunosuppressed patients as compared to Leish+/HIV- individuals. Two cases of recrudescence after healing were recorded in co-infected patients.

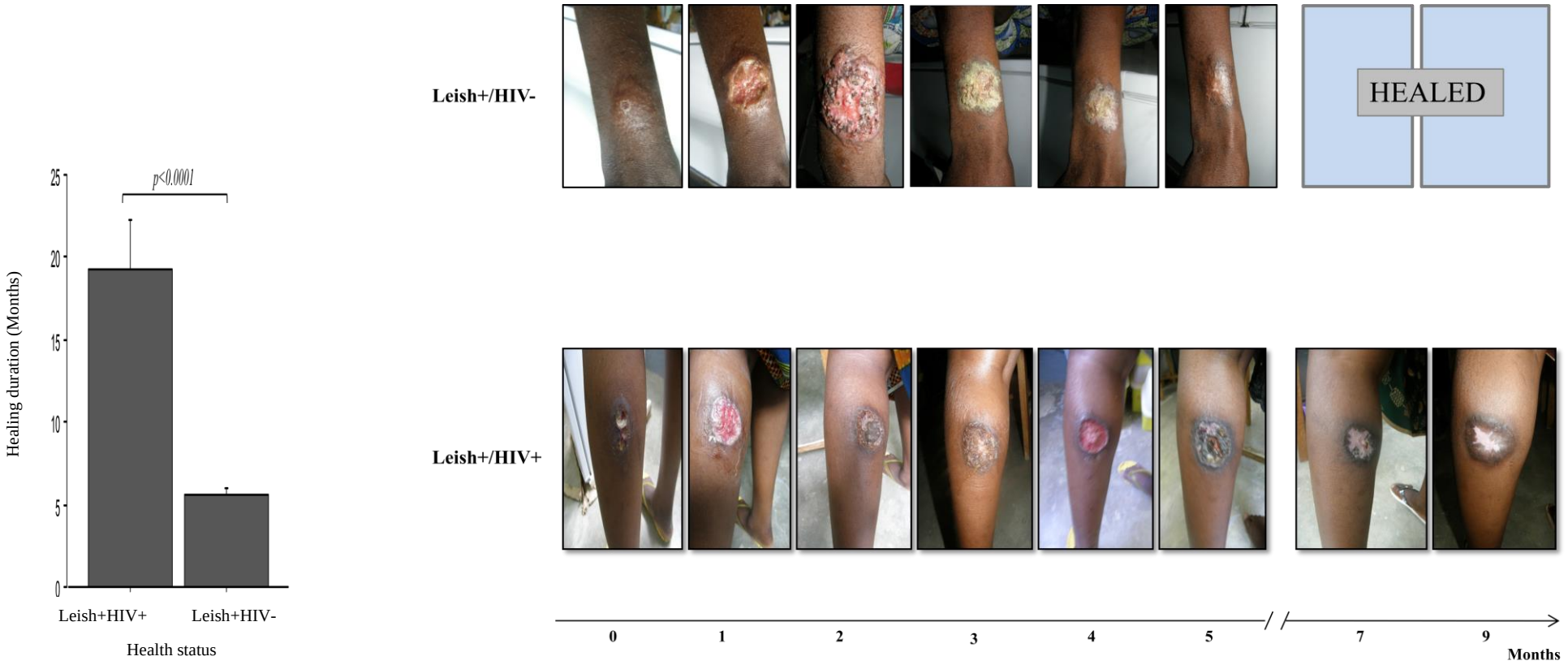


Figure 40: Comparative evolution of a chronic lesion in Leish+HIV- and Leish+HIV+ patients

Interestingly, both patients were registered at the same date (08th -08-08). Leish+/HIV- patient's lesion was at the beginning (three weeks old) while Leish+/HIV+ patient's lesion was one month old. They first received the same therapy (Amphoterecin B + Metronidazol) but the Leish+HIV+ patient was subsequently (from the 4th month) submitted to anti-retroviral therapy. Leish+HIV+: *Leishmania*-HIV co-infected patients (n=7), Leish+HIV- : patients with CL only (n=139), data are presented as mean ± SEM.

The Table V bellow recapitulates information recorded from every single *Leishmania*/HIV co-infected individual.

Tableau V*: Recapitulative information on* Leishmania*/HIV co-infected individuals*

	Leish+HIV+ patients						
	1	2	3	4	5	6	7
Sex	♀	♀	♀	♀	♀	♂	♀
Age (Years)	25	17	37	36	45	51	25
Number of lesions	3	19	12	11	3	3	3
Total surface of lesions (mm^2)	352,47	4001,18	25036,02	11101,50	343,83	3806,47	5016,15
Part(s) of the body concerned	Upper limb	Fore head Upper limb Lower limb	Upper limb Lower limb	Upper limb Lower limb Trunk	Head Lower limb	Upper limb	Trunk
Duration before the 1st visit (months)	12	3	13	1	2	14	10
Treatment before the first visit	Unknown antibiotic	No	M	Penicillin	C	No	Unknown
Treatment	AB+M	AB+M	AB+M+A	AB+M+C+ARV	AB+M+A	AB+M+A	A
Healing durarion (weeks)	28	16	60	28	12	31	18
CD3+ cells/µl	1084	1384	1108	834	2627	1414	nd
CD4+ cells/µl	211	200	278	112	292	434	nd
CD8+ cells/µl	782	1117	749	647	>2000	907	nd
CD4/CD8 Ratio	0.27	0.18	0.37	0.17	<0.15	0.48	nd
HIV serotypes	1 + 2	1 + 2	1 + 2	1	1	1 + 2	1 + 2

A: Allopurinol, AB: Amphotericine B, ARV: Antiretroviral therapy, C: Cotrimoxazole, M: Metronidazole, Leish+/HIV+: *Leishmania* and HIV co-infected patients, nd: non determined, ♀: Female (n=6); ♂: Male (n=1); Normal values: CD3 = 804–3600 cells/µl, CD4 = 520–2000 cells/µl, CD8 = 260–1200 cells/µl, CD4/CD8 = 1.5–2.5.

In general, antileishmanian therapy used (Amphotericin B + Metronidazol, or Allopurinol) provided good results mainly on Leish+/HIV- patients. Leish+/HIV+ patients needed a longer duration and sometimes antiretroviral therapy for their lesions to heal.

From the present study, it is clear-cut that, in Mokolo leishmaniasis endemic focus, clinical manifestations of the pathology varied from one patient to another. In fact, the age group, the HIV status etc. influenced clinical features of the disease as well as the therapeutic response. Since the immune system protects the organism against attacks, we therefore predicted that such a difference might be basically attributable to the immune response of each individual. Indeed, cells infiltration at lesions sites, antileishmanian antibodies and cytokines, in short, both cellular and humoral immune reactions can be at the base of such a difference. This difference need to be physiologically examined in order to understand immunological processes underlining it.

III.1.6 Histhopathology and immunohistopathology of cutaneous leishmaniasis and the influence of the HIV status

III.1.6.1 Routine histology assessment

During the present study, cutaneous leishmaniasis lesions were characterized by cells infiltration at the site of parasite inoculation. In fact, the number and the type of cells changed at this zone. The direct consequence was the increase of the skin thickness. Both epidermis and dermis were concerned by this modification. As compared to the healthy skin, the dermis showed a considerably change in cell types at the level of CL lesion. This change was mainly due to leukocytes infiltration (Figure 41).

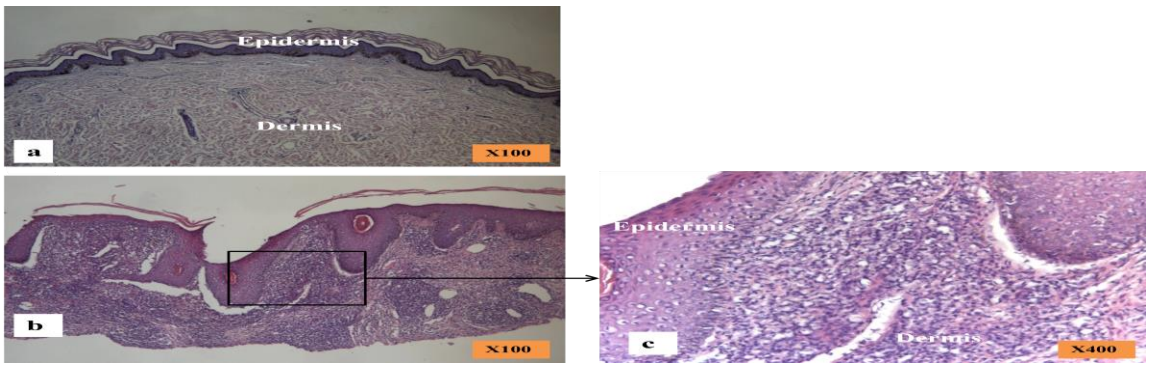


Figure 41: Epidermal hyperplasia and dermal infiltration at the level of the CL lesion (HE, x 100 and x400)
a: Control (healthy skin), b: Over view, b: Magnifie

On the other hand, the Giemsa stained biopsies also showed cells infiltration, Leishman-Donovan bodies were cleary observed in some samples as it is illustrated on Figure 42.

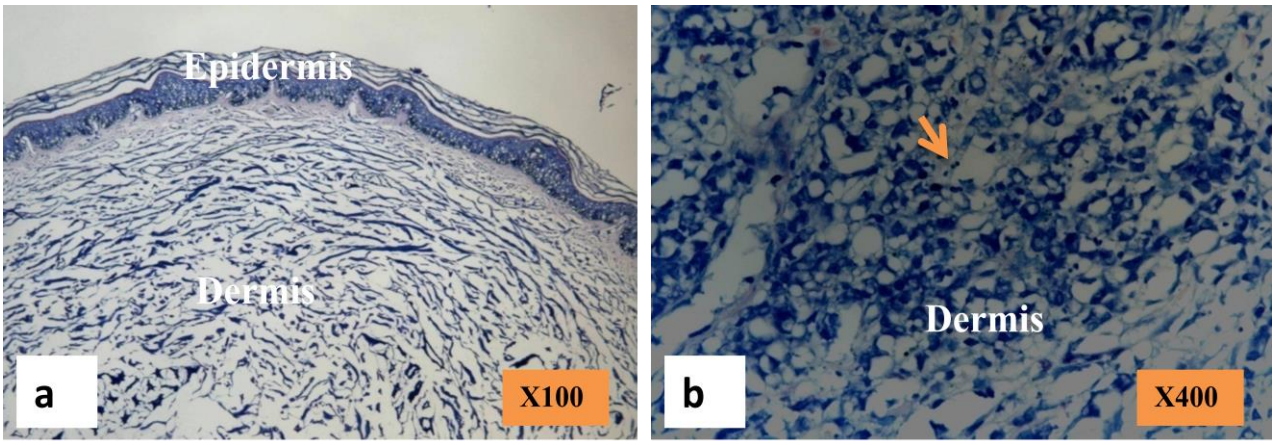


Figure 42: Giemsa stained skin showing cell infiltration and amastigotes (arrow)
a: Control (healthy skin), **b**: Lesion site

III.1.6.3 Characterization of immune cells at the cutaneous leishmaniasis lesion site

Representative stainings to illustrate cellular sublocalization of expression are depicted on the figure 43. The normal (healthy) skin is used as control. This figure shows the presence of some marked cells in both skin compartments (CD1a+, CD8+, etc.), but also the presence of some other cells only in the dermis (CD4+, CD20+, etc.). The very abundant and widely distribution of some cells types (CD1a+, CD8+, etc.) can be cleary observed. CD54 marker was not only expressed on infiltrated cells, but also on the endothelium of blood vessels.

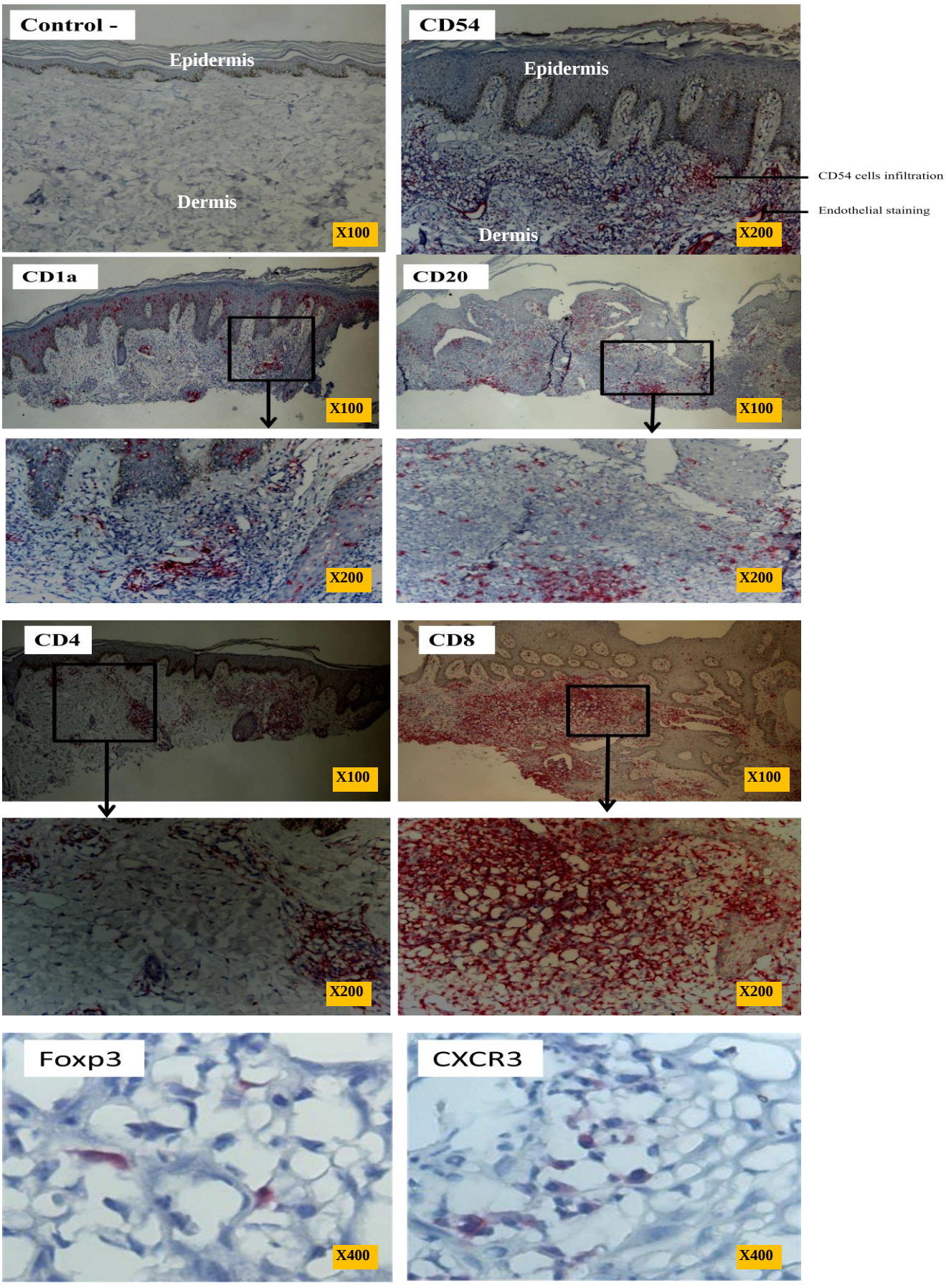


Figure 43: Illustrative representation of some immunohistological staining for epidermal and dermal markers in cutaneous leishmaniasis

The negative control is a healthy skin, positive cells for each marker are red

Be it in the healthy skin or at the lesion site, mast cells were absent in the epidermis but present in the dermis. Their granules were almost compact in the cells of normal skin (Figure 44a). However, at the level of CL lesion, MC looked more granulated but also they exocytosed many of their granules which were observable around the cell (Figure 44b). Degranulation could be moderate or extensive.

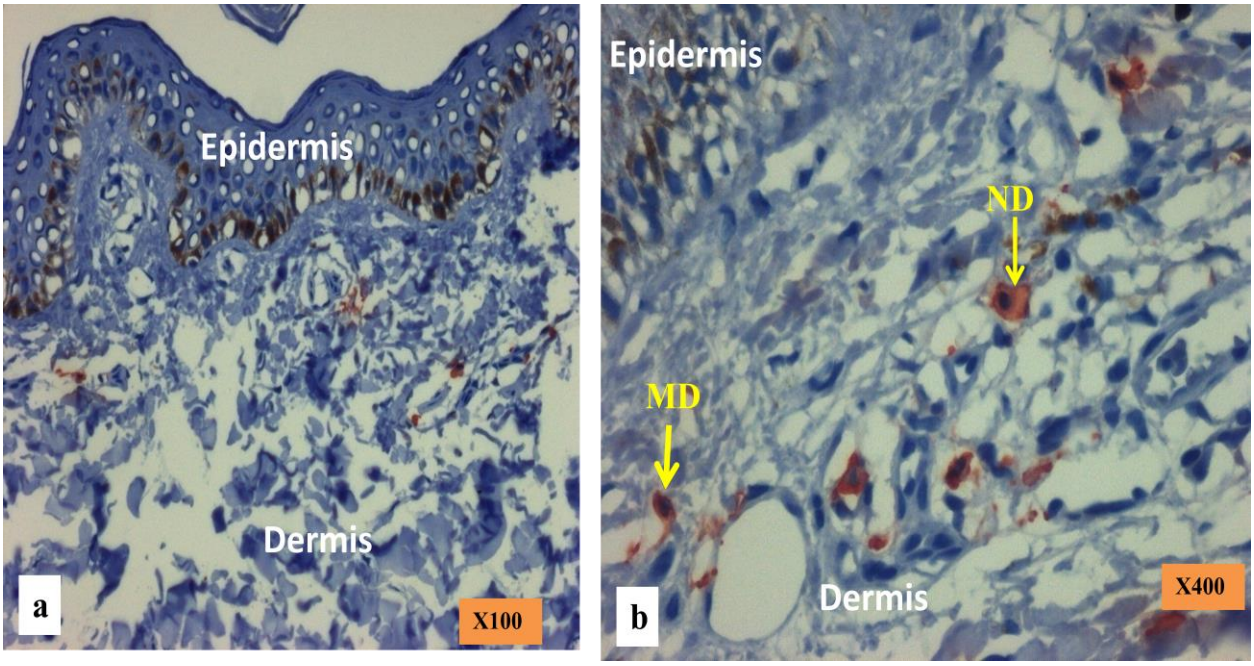


Figure 44: Illustration of the immunological staining of mast cells in the skin of cutaneous leismaniasis patients (x400)
a: Healthy skin or negative control, b: Lesion, MD: Moderate degranulation, ND: No Degranulation, Granules in this picture are brown.

III.1.7 Local cells alteration in cutaneous leishmaniasis and/or HIV infected patients

III.1.7.1 Antigen presenting cells

i. Dendritic cells

In the quiescent skin, few resident CD1a+ cells were observed in both epidermis (Langerhans cells) and dermis (dermal dendritic cells). Nevertheless, at the CL lesions site, there was a high CD1a+ cells hyperplasia and/or infiltration and consequently more

cells at this level. In general, immunodeficient individuals presented a significant ($p = 0.02$) lower number of CD1a+ cells in the epidermis (Figure 45). However, in the dermis no statistical difference was observed.

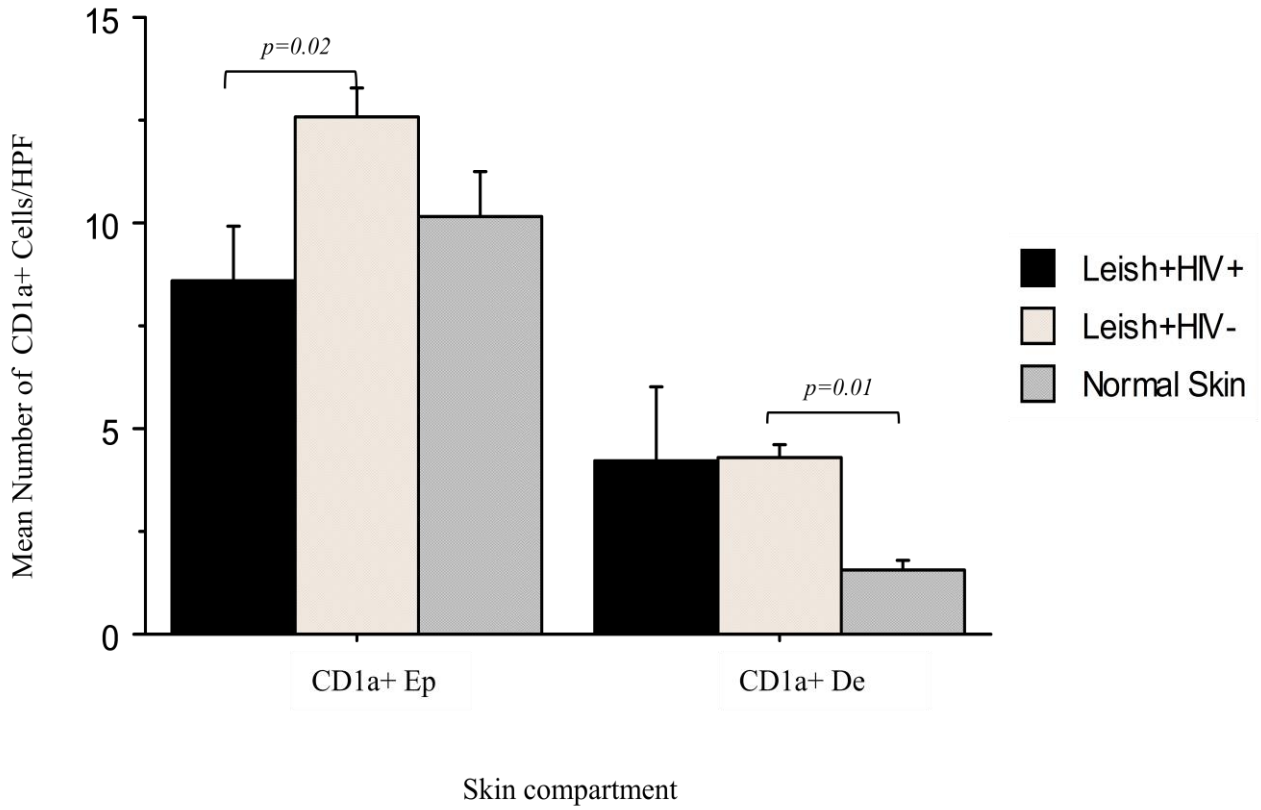


Figure 45: Representation of CD1a+ cells infiltration in the epidermis (Ep) and the dermis (De) of immunocompetent and immunodeficient patients
Normal skin (n=10), Leish+HIV+: *Leishmania*-HIV co-infected patients (n= 25), Leish+HIV-: patients with leishmaniasis only (n=119), n is the total number of samples analysed, data are mean ± SEM.

Be it in the dermis or in the epidermis and regardeless the health status as well as the antileishmanial therapy used, at the lesion site the number of CD1a+ cells fluctuated in general during the lesion resolution. In the course of leishmaniasis evolution, HIV+ patients showed a delay in the expression of this cell type (Figure 46).

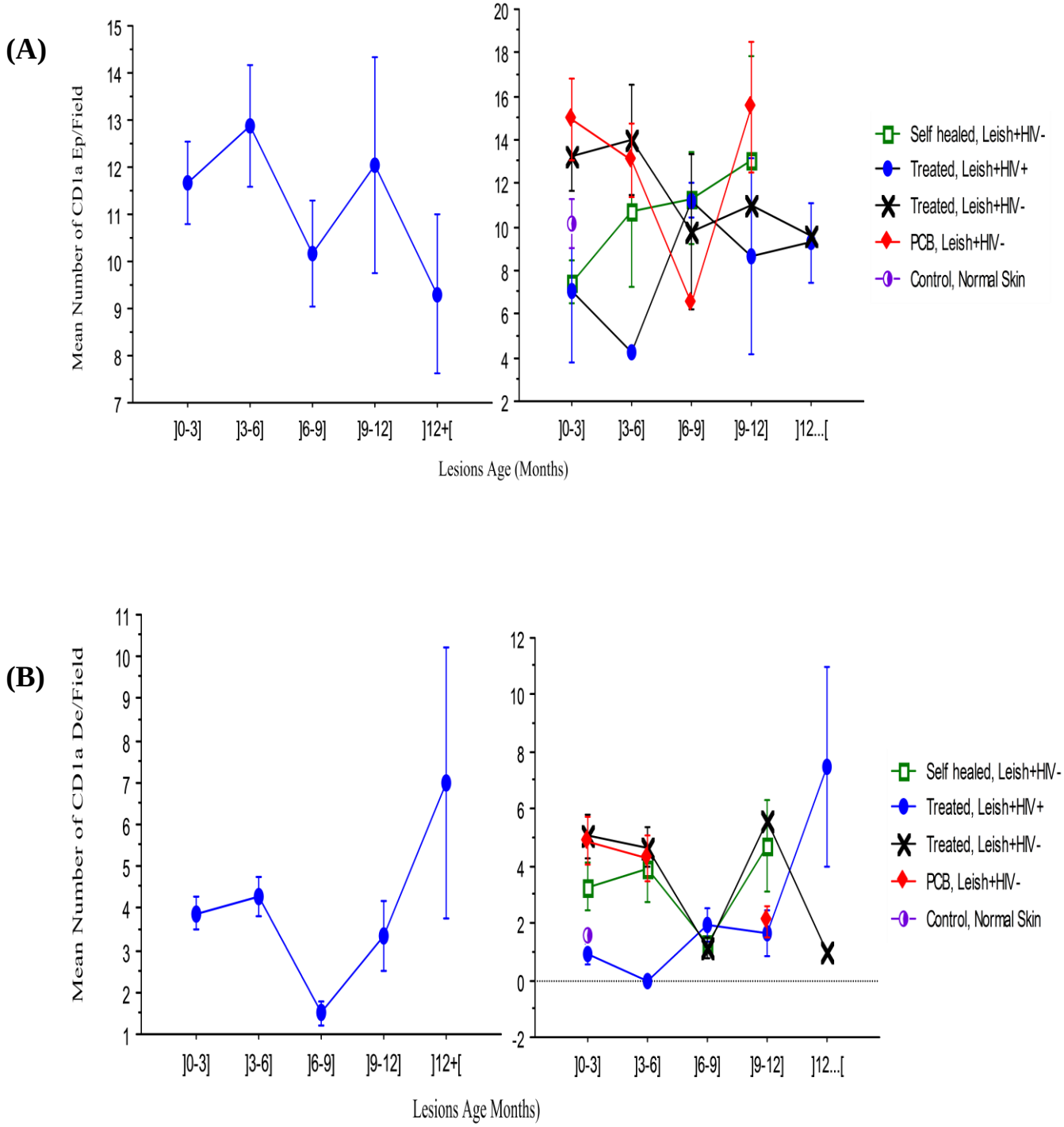


Figure 46: Changes occurred in the skin CD1a+ cells amount during leishmaniasis evolution and influence of the therapy and the HIV status

(A) Epidermis (Ep), (B) Dermis (De), Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, PCB: Placebo, data are mean ± SEM, for each age range $8 \leq n \leq 79$

ii. Activated dendritic cells

The number of activated dendritic cells (CD83+), more expressed in the dermis than the epidermis, was significantly ($p = +0.03$) lower in *Leishmania*/HIV co-infected patients as compared to subjects suffering from leishmaniasis only (Figure 47).

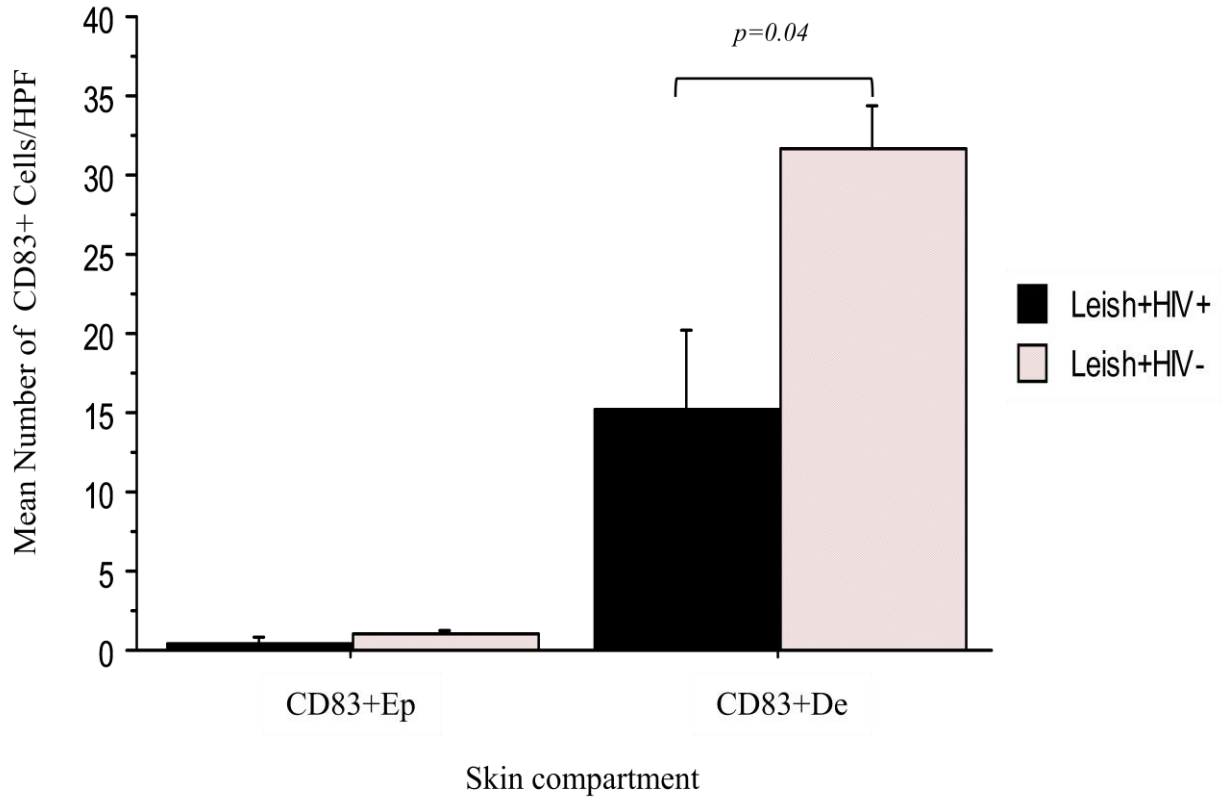


Figure 47: Representation of CD83+ cells infiltration in the epidermis (Ep) and the dermis (De) of immunocompetent and immunodeficient patients
Leish+HIV+: *Leishmania*-HIV co-infected patients (n= 20), Leish+HIV-: patients with leishmaniasis only (n=116), n is the total number of samples analysed, data are mean ± SEM.

In the course of the lesion resolution the number of activated dendritic cells droped in all the groups of CL subjects (Figure 48). Leish+HIV+ patients usually showed very fluctuated CD83+ cells number during the lesion progression with a very low number during the period of high severity (the second trimester).

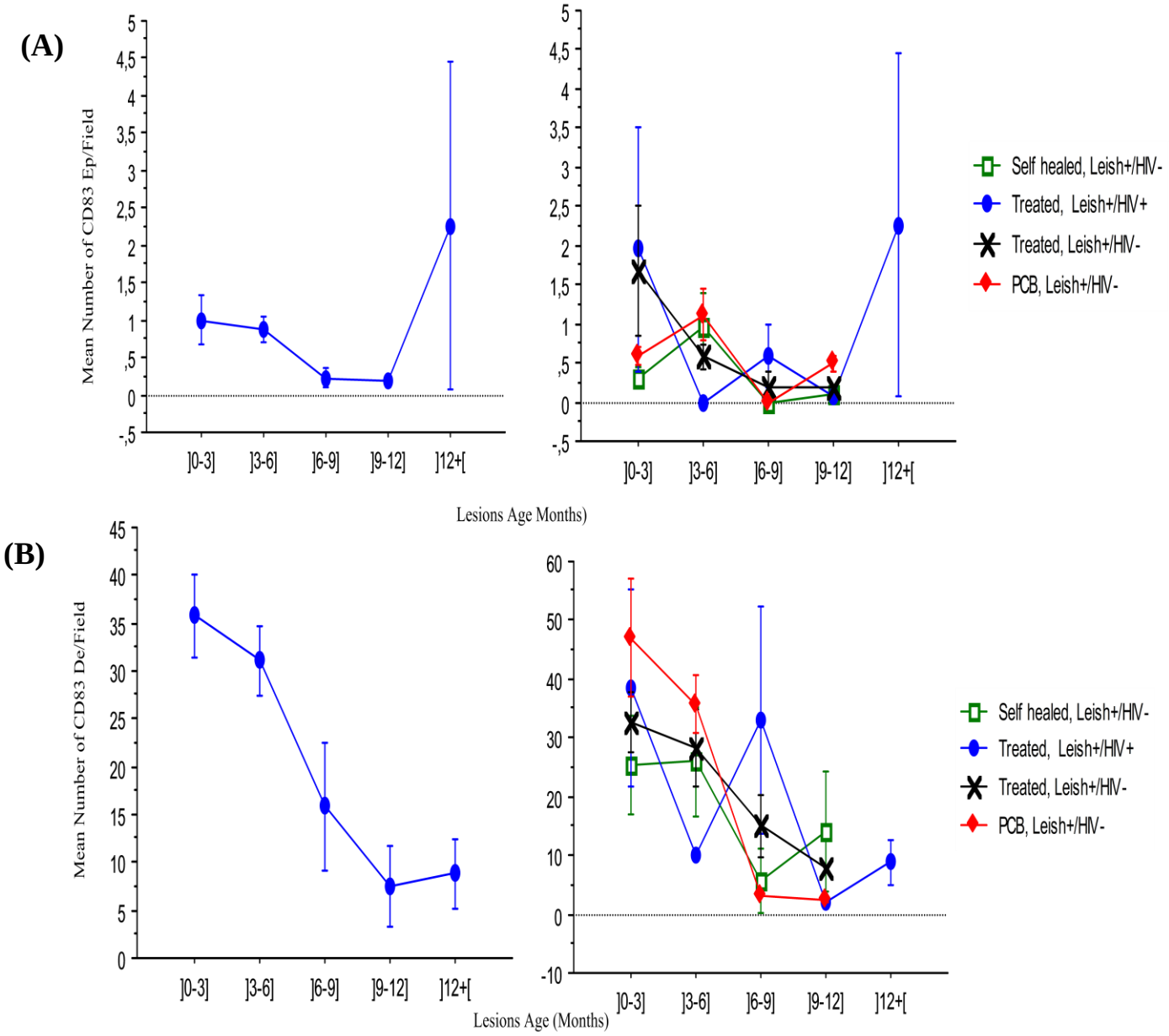


Figure 48: Changes occurred in the skin CD83+ cells amount during leishmaniasis evolution and influence of the therapy and the HIV status

(A) Epidermis (Ep), (B) Dermis (De), Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, PCB: Placebo, data are as mean ± SEM, for each age range 8≤ n ≤66.

The normal skin did not show any CD54+ (ICAM-1+) cells (CD54+ is marker of activated DC as well as extravasated T and B memory cells). At the CL lesion site, there were ICAM-1+ cells in the dermis (but not in the epidermis). Despite the huge number of dermal CD54+ cells, there was no significant ($p > 0.005$) difference observed between Leish+HIV- and Leish+HIV+ subjects (Figure 49).

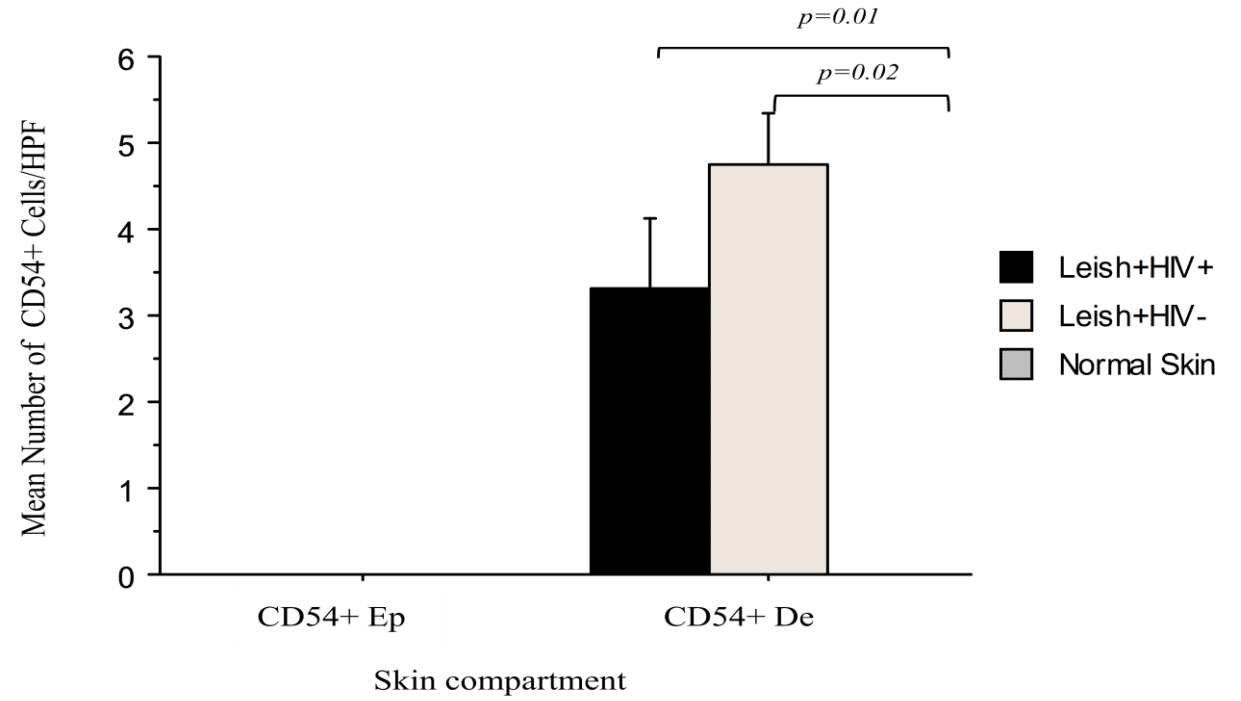


Figure 49: Representation of CD54+ cells extravasation in the dermis (De) of immunocompetent and immunodeficient patients
Normal skin (n=10), Leish+HIV+: *Leishmania*-HIV co-infected patients (n= 25), Leish+HIV-: patients with leishmaniasis only (n=119), n is the total number of samples analysed, data are mean ± SEM.

During lesion evolution, more vulnerable patients (those with persisted lesions and Leish+HIV+) tended to present more cells expressing CD54+ marker. In all the groups, healing was marked by a decrease in CD54+ cells amount at the lesion site (Figure 50).

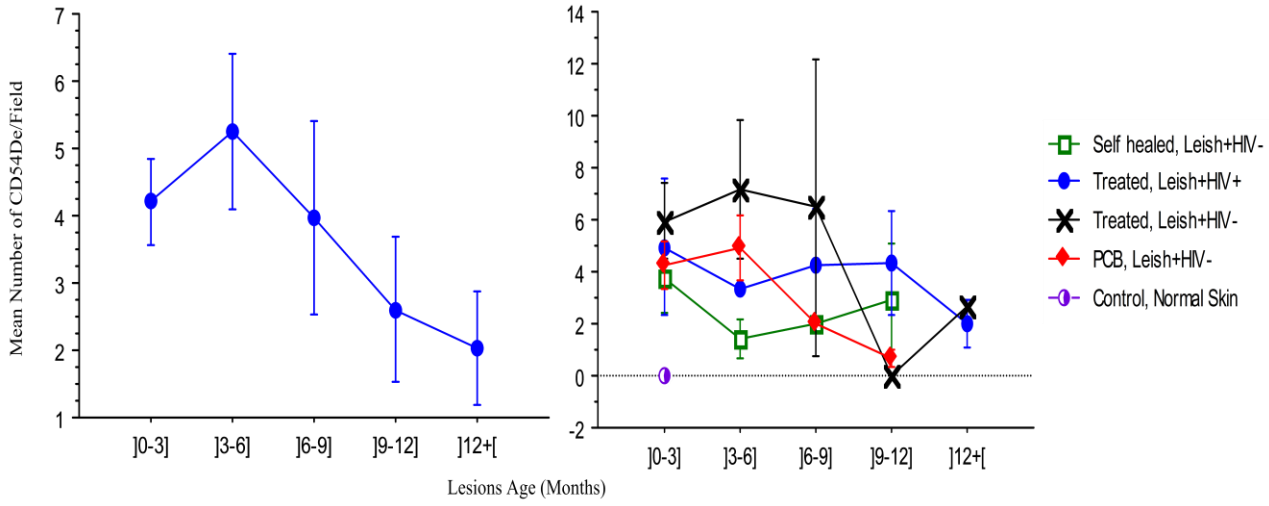


Figure 50: Changes occurred in the dermal (De) CD54+ cells amount during leishmaniasis evolution and influence of the therapy and the HIV status
Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, PCB: Placebo, data are mean ± SEM, for each age range $8 \leq n \leq 79$

iii. Macrophages

As it is discriminated on Figure 51, the number of CD68+ cells was significantly ($p = 0.003$) lower in the dermis of immunodeficient patients as compared to immunocompetent ones. In the epidermis, no significant difference was observed between these two groups.

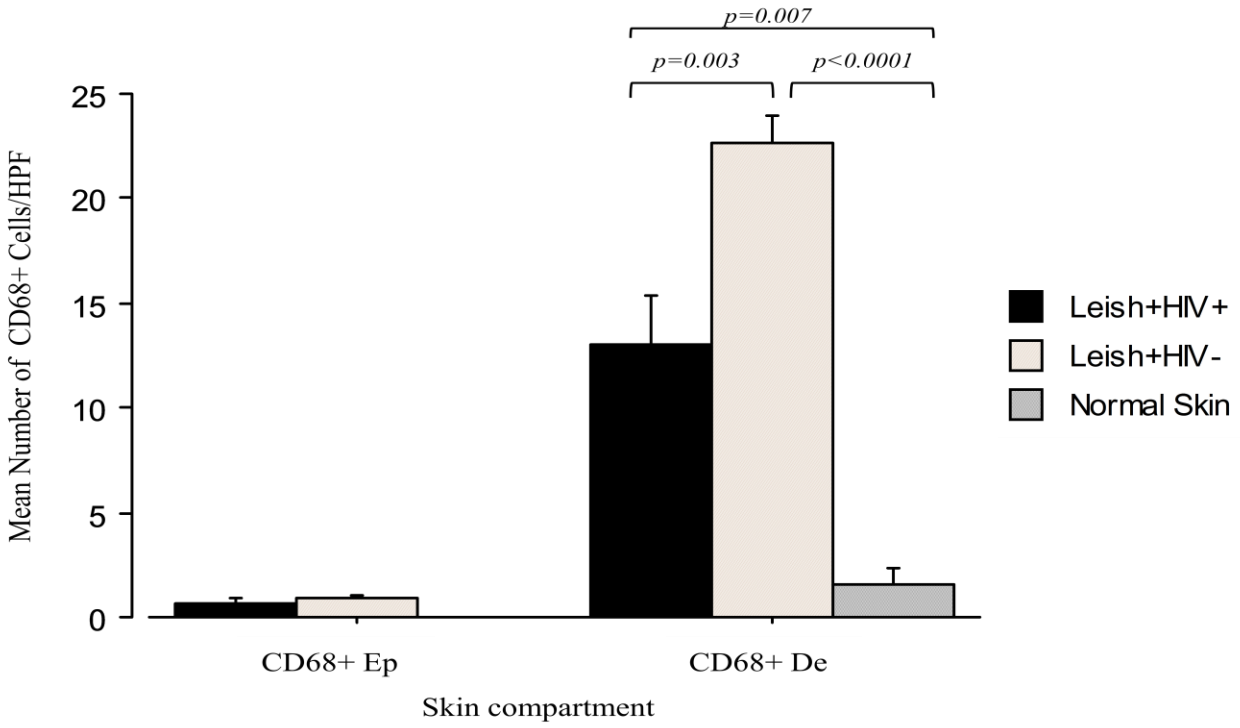
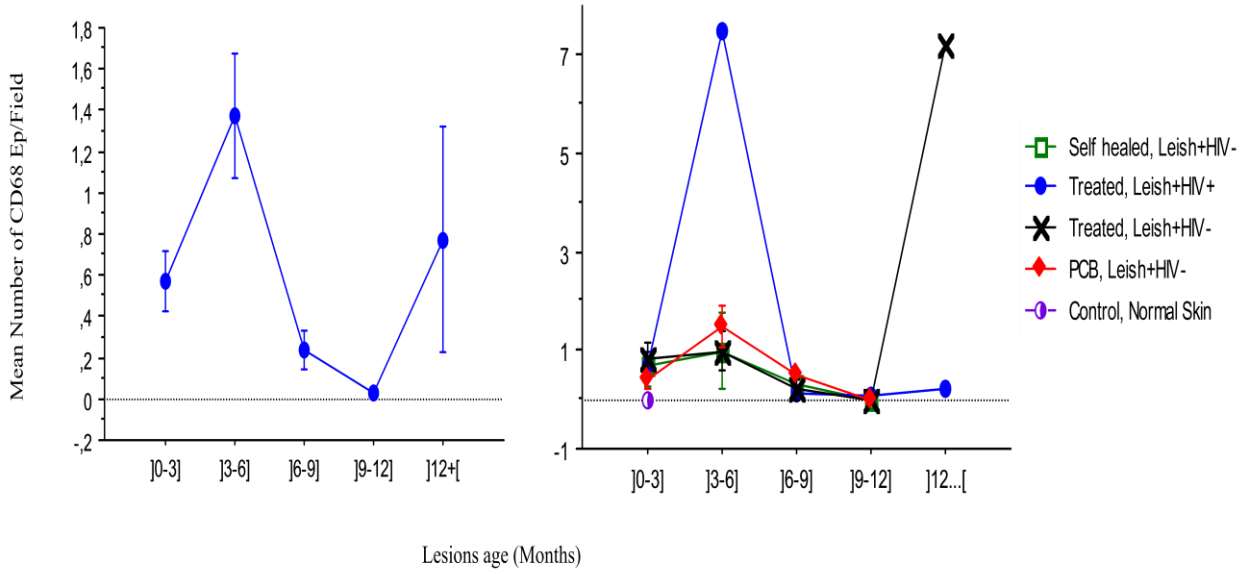


Figure 51: Representation of CD68+ cells infiltration in the epidermis (Ep) and the dermis (De) of immunocompetent and immunodeficient patients

Normal skin (n=10), Leish+HIV+: *Leishmania*-HIV co-infected patients (n= 25), Leish+HIV-: patients with leishmaniasis only (n=119), n is the total number of samples analysed, data are mean ± SEM.

During lesions progression, the number of dermal CD68+ cells reduced in both co-infected patients and treated CL mono-infected individuals. Self healing depended on the permanent presence of CD68+ in the dermis (Figure 52). Nevertheless, in patients who, because of the persistence of their lesions, needed antileishmanian therapy to cure and HIV+ patients, this number reduced remarkably between 6 and 9 months from the begginning of lesions. In all the groups, the lesion resolution was characterized by the decrease of CD68+ cells count to the normal skin value .

(A)



(B)

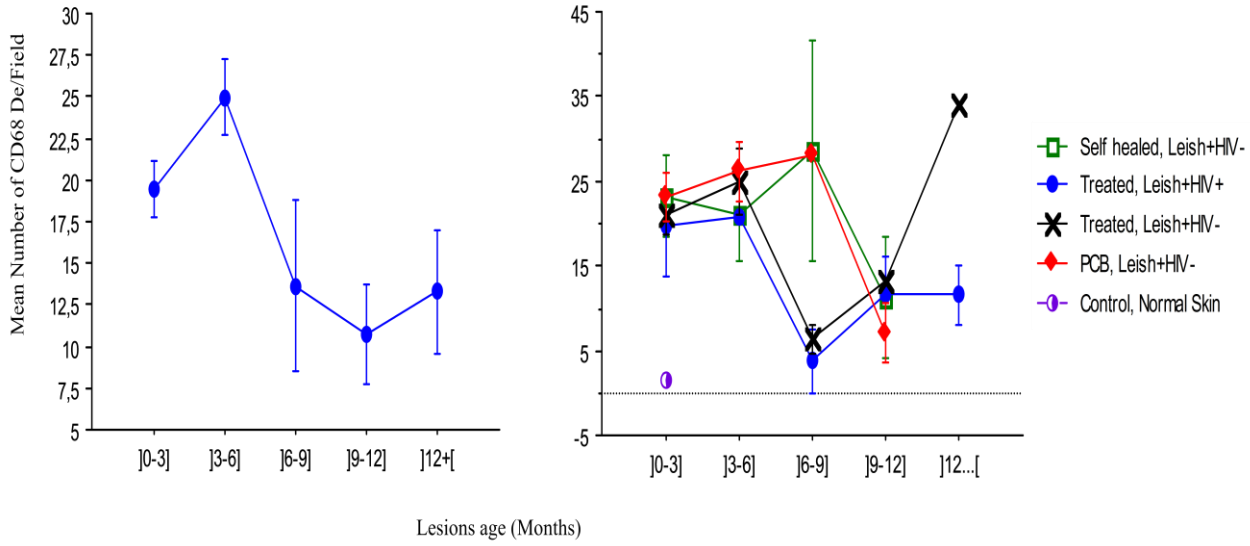


Figure 52: Changes occurred in CD68+ cells amount in the skin during leishmaniasis evolution and influence of the therapy and the HIV status

(A) Epidermis (Ep), (B) Dermis (De), Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, PCB: Placebo, data are mean ± SEM, for each age range 8≤ n ≤79.

III.1.7.2 T lymphocytes

i. T helper cells

CD4+ cells were absent in the normal skin. However, at the CL lesion site, the expression of these cells phenotype in the dermis (but scarcely in the epidermis) was evident (Figure 53).

In general, Leish+HIV+ patients presented less dermal CD4+ cells infiltration at their lesion sites as compared to Leish+HIV- subjects.

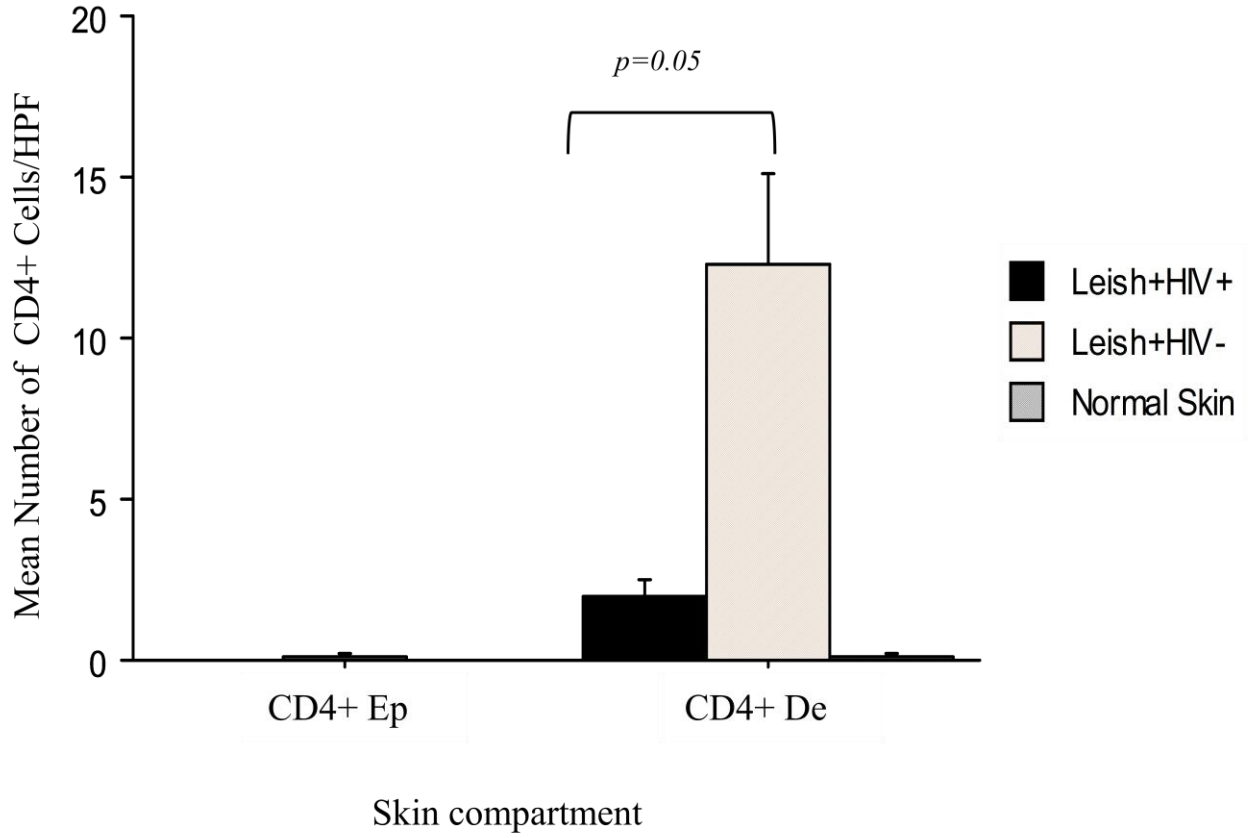


Figure 53: Representation of CD4+ cells infiltration in the epidermis (Ep) and the dermis (De) of immunocompetent and immunodeficient patients
Normal skin (n=10), Leish+HIV+: *Leishmania*-HIV co-infected patients (n= 25), Leish+HIV-: patients with leishmaniasis only (n=119), n is the total number of samples analysed, data are mean ± SEM.

As it is illustrated in Figure 54, during the lesion evolution, Leish+HIV+ subjects always showed few CD4+ cells while Leish+HIV- presented an abundant infiltration in the second trimester. Healing was characterized by the tendency of CD4 count to drop to the initial value. One year after the beginning of the disease, the amount of CD4+ cells was significantly low (*p=0.04*).

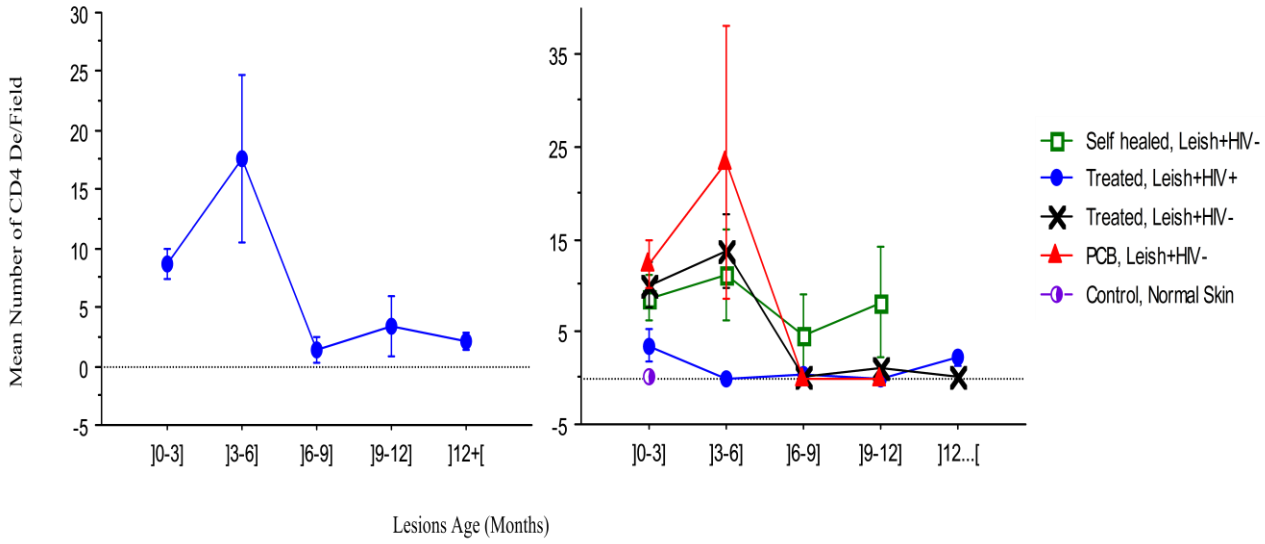


Figure 54: Changes occurred in CD4+ cells in the dermis (De) during leishmaniasis evolution and influence of the therapy and the HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, PCB: Placebo, data are mean ± SEM, for each age range 8≤ n ≤79.

ii. T helper 1 cells

The number of dermal Th1 (CXCR3+) cells was clearly lower in Leish+HIV+ as compared to HIV-negative CL patients(Figure 55).

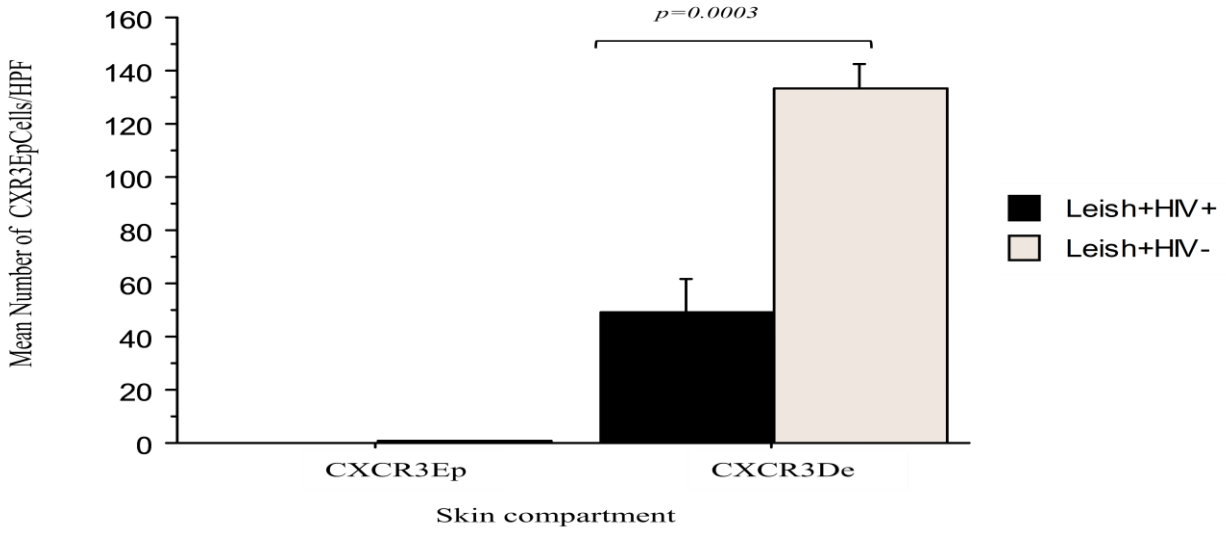


Figure 55: Representation of CXCR3+ cells infiltration in the epidermis (Ep) and the dermis (De) of immunocompetent and immunodeficient patients

Leish+HIV+: *Leishmania*-HIV co-infected patients (n= 20), Leish+HIV-: patients with leishmaniasis only (n=115), n is the total number of samples analysed, data are mean ± SEM.

More produced in the second trimester of the lesion evolution, CXCR3+ cells amount droped later in all the groups in both epidermis and dermis (Figure 56).

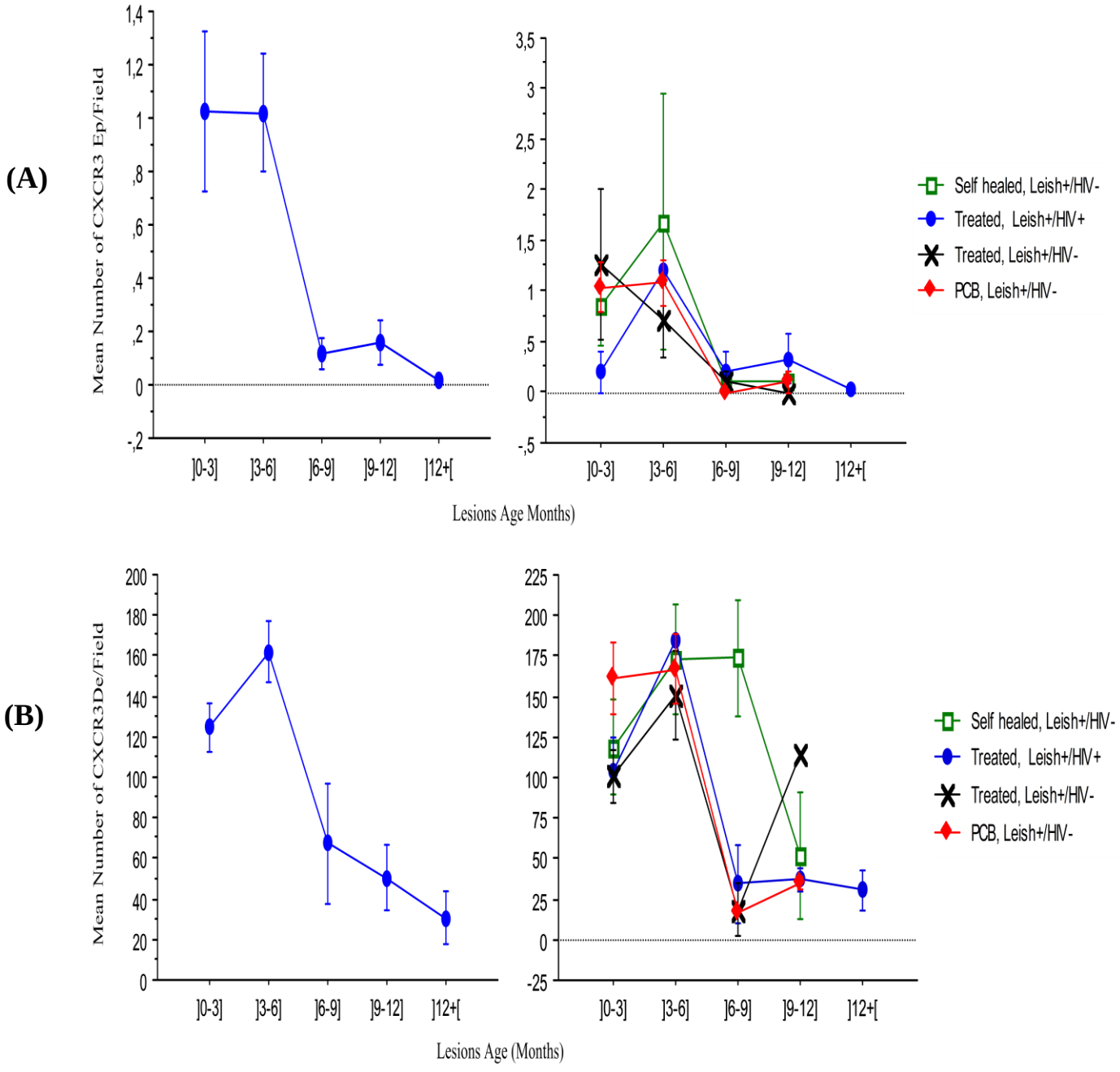


Figure 56: Changes occurred in CXCR3+ cells in the skin during leishmaniasis evolution and influence of the therapy and the HIV status

(A) Epidermis (Ep), (B) Dermis (De), Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, PCB: Placebo, data are mean ± SEM, for each age range $8 \leq n \leq 65$

iii. Cytolytic T cell

CD8+ cells were absent in the normal epidermis. Nevertheless, at the CL lesion site these cells were observed in both epidermis and dermis layers of the skin (more abundant in the dermis than in the epidermis).

Despite a huge CD8+ cells infiltration at lesion sites, in general, there was no significant ($p > 0.005$) difference observed in CD8+ cells count in Leish+HIV+ individuals as compared to Leish+HIV- subjects (Figure 57).

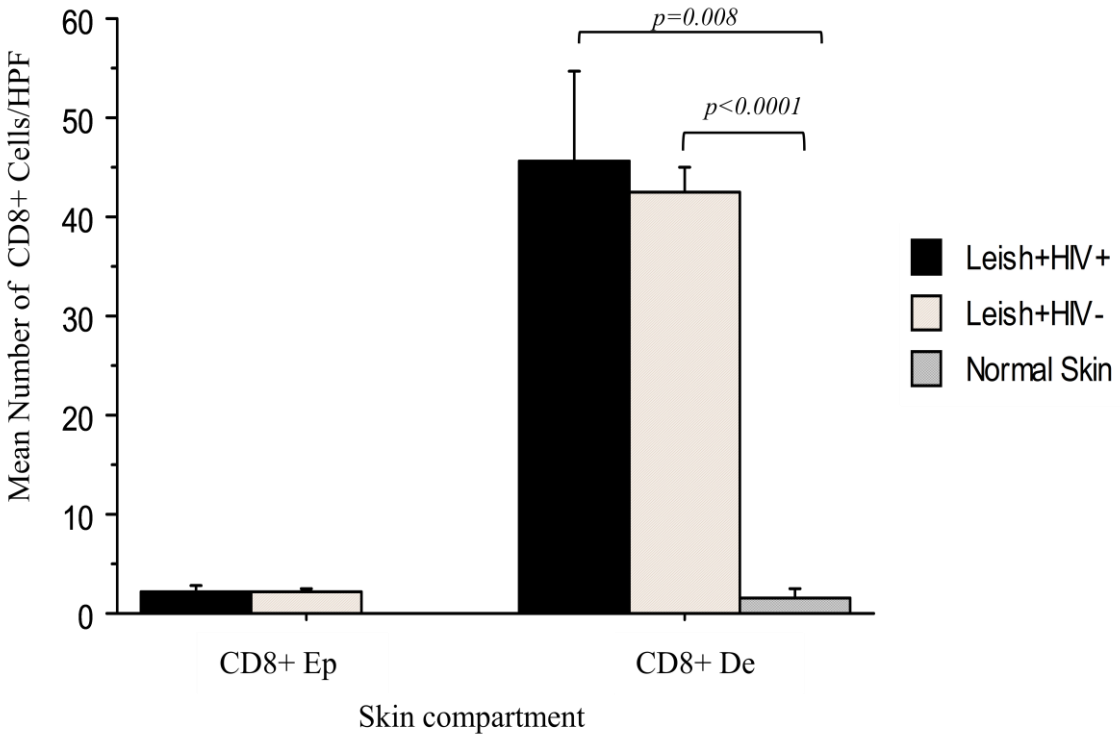


Figure 57: Representation of CD8+ cells infiltration in the epidermis (Ep) and the dermis (De) of immunocompetent and immunodeficient patients
Normal skin (n=10), Leish+HIV+: *Leishmania*-HIV co-infected patients (n= 25), Leish+HIV-: patients with leishmaniasis only (n=119), n is the total number of samples analysed, data are as mean ± SEM.

Interestingly during lesions evolution, it was observed that *Leishmania*-HIV co-infected patients generally tended to present more CD8+ cells in both epidermis and dermis. Independently on the skin compartment, patients with advanced healing process were characterized by the decrease in the number of CD8+ cells in all the groups (Figure 58).

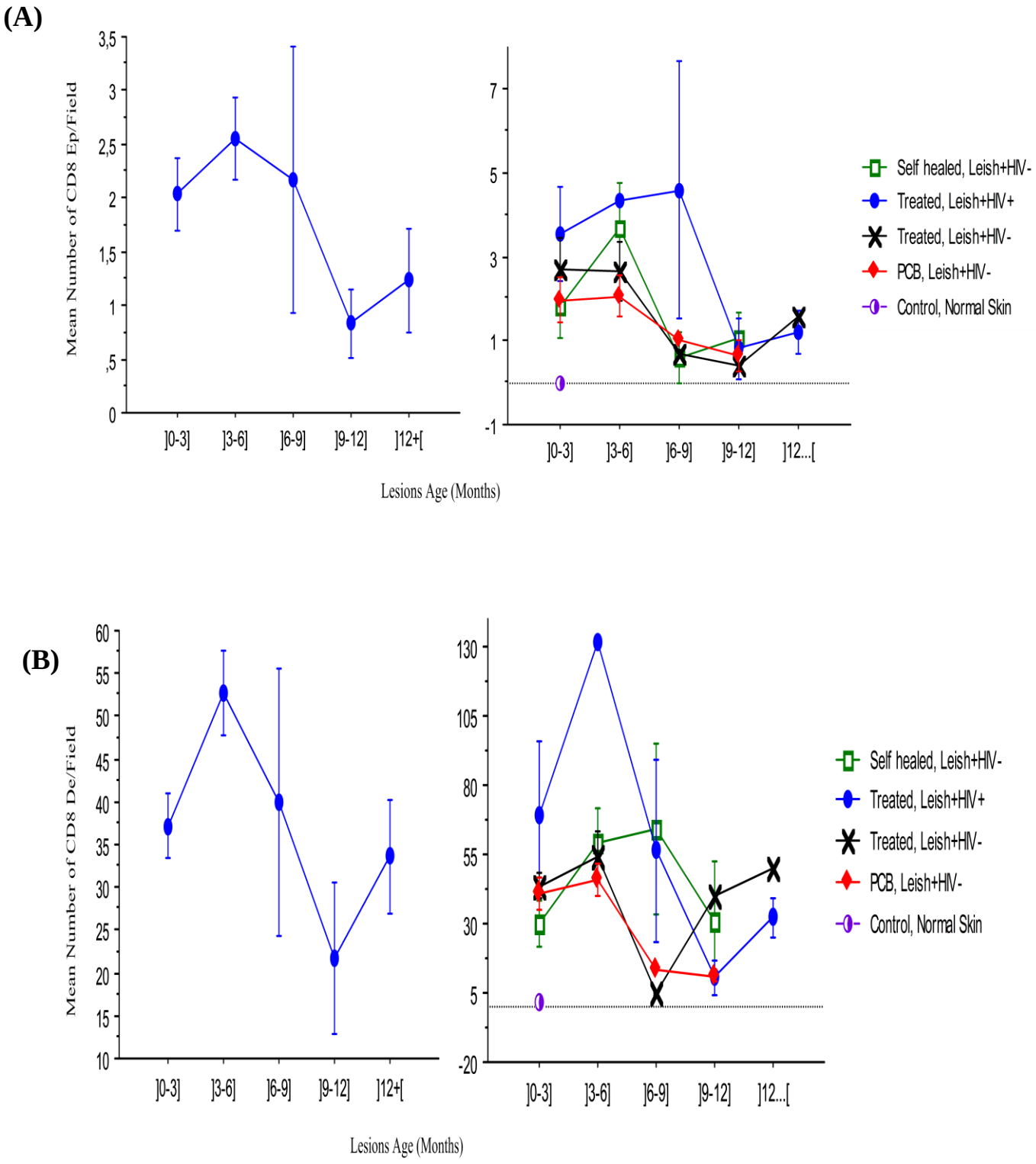


Figure 58: Changes occurred in CD8+ cells amount in the skin during leishmaniasis evolution and influence of the therapy and the HIV status

(A) Epidermis (Ep), (B) Dermis (De), Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, PCB: Placebo, data are mean ± SEM, for each age range $8 \leq n \leq 79$.

iv. Regulator and effector Tcells

The number of dermal Treg (Foxp3+) cells was lower in Leish+HIV+ as compared to HIV-negative CL patients (Figure 59).

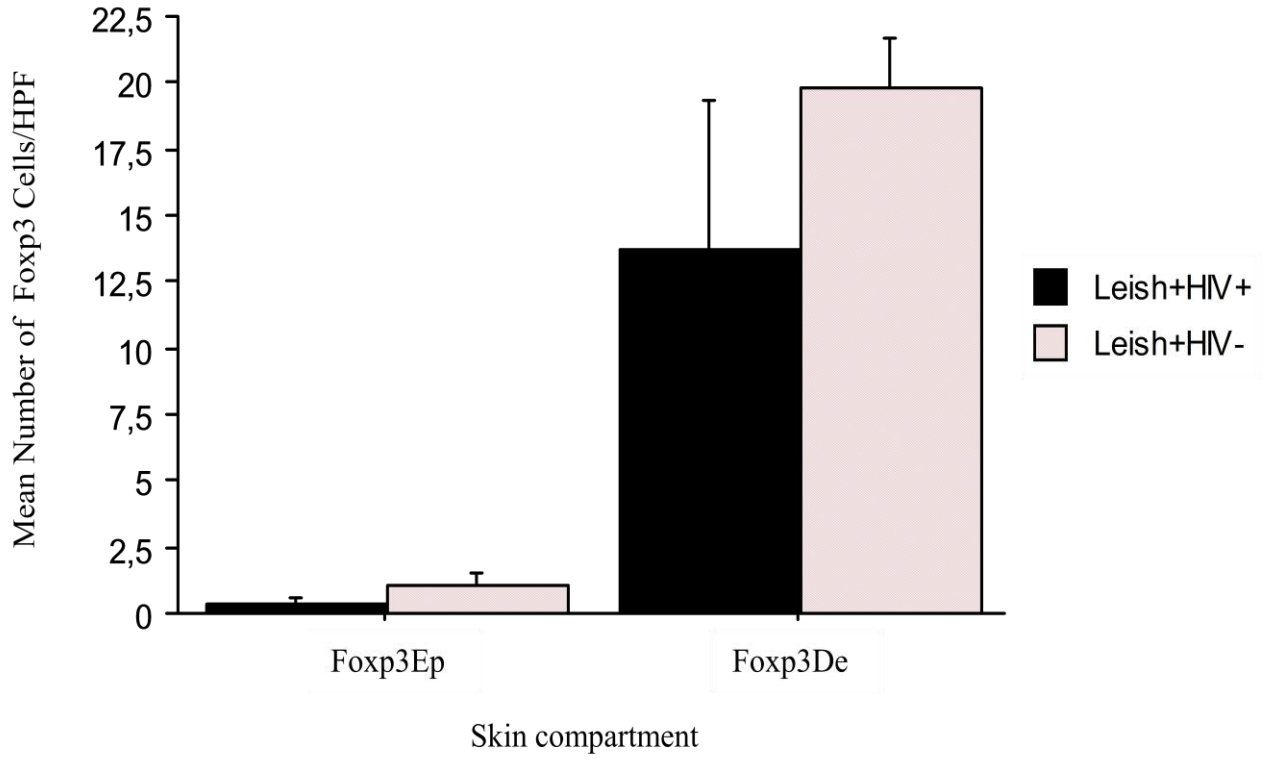


Figure 59: Representation of Foxp3+ cells infiltration in the epidermis (Ep) and the dermis (De) of immunocompetent and immunodeficient patients
Leish+HIV+: *Leishmania*-HIV co-infected patients (n=20), Leish+HIV-: patients with leishmaniasis only (n=116), n is the total number of samples analysed, data are mean ± SEM.

The number of Foxp3+ cells in both epidermis and dermis showed a tendancy to reduce during leishmaniasis healing process (Figure 60).

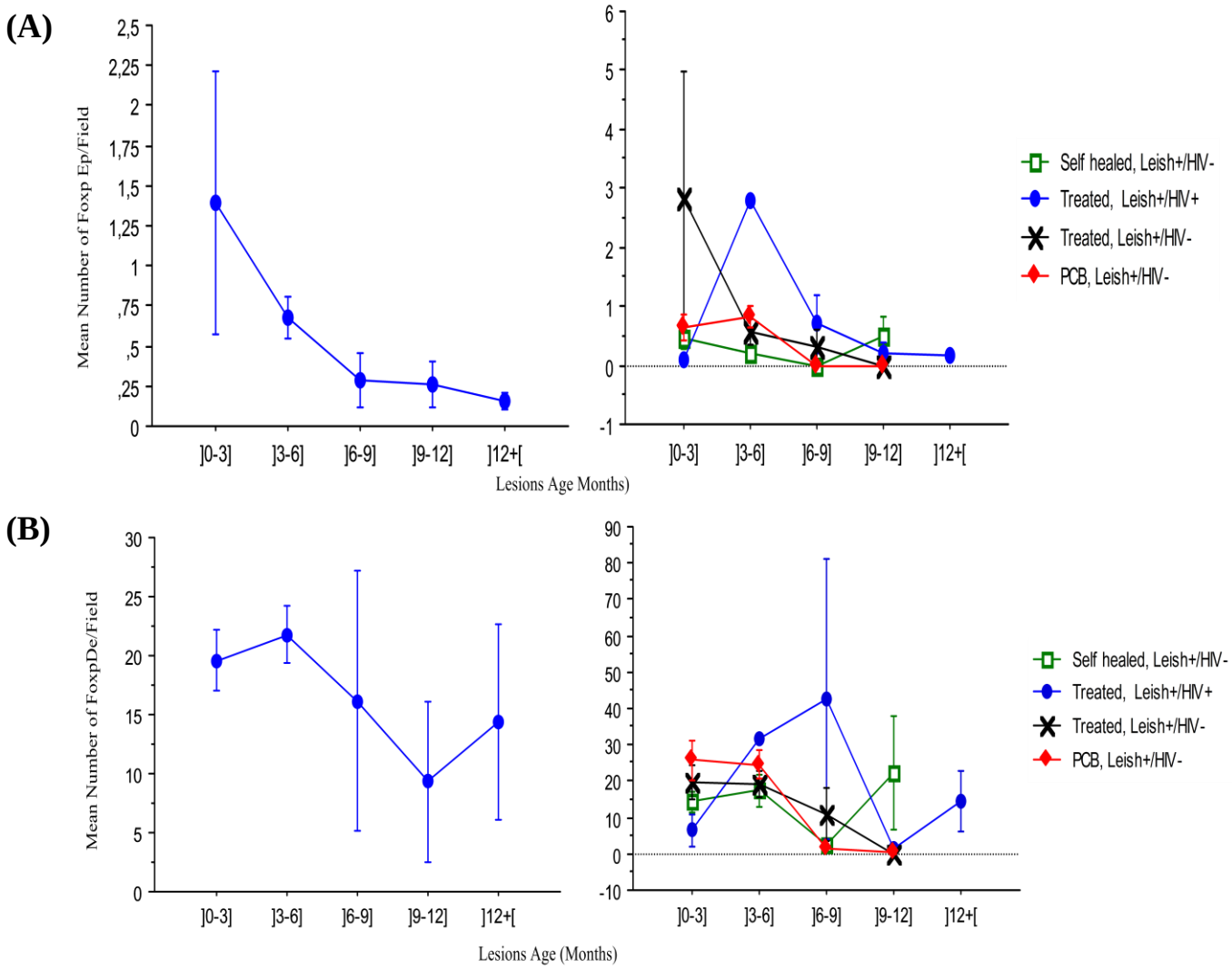


Figure 60: Changes occurred in Foxp3+ cells amount in the skin during leishmaniasis evolution and influence of the therapy and the HIV status

(A) Epidermis (Ep), (B) Dermis (De), Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, PCB: Placebo, data are mean ± SEM, for each age range $8 \leq n \leq 66$.

III.1.7.3 B Lymphocytes

CD20+ cells were normally absent in the healthy skin. Due to the CL lesion, these cells infiltrated the skin, but they were generally limited in the dermis compartment. Their distribution was not often homogenous. As it is shown on Figure 61, a significant ($p = 0.002$) low number was observed in dermal infiltration in Leish+HIV+ subjects as compared to CL mono-infected patients.

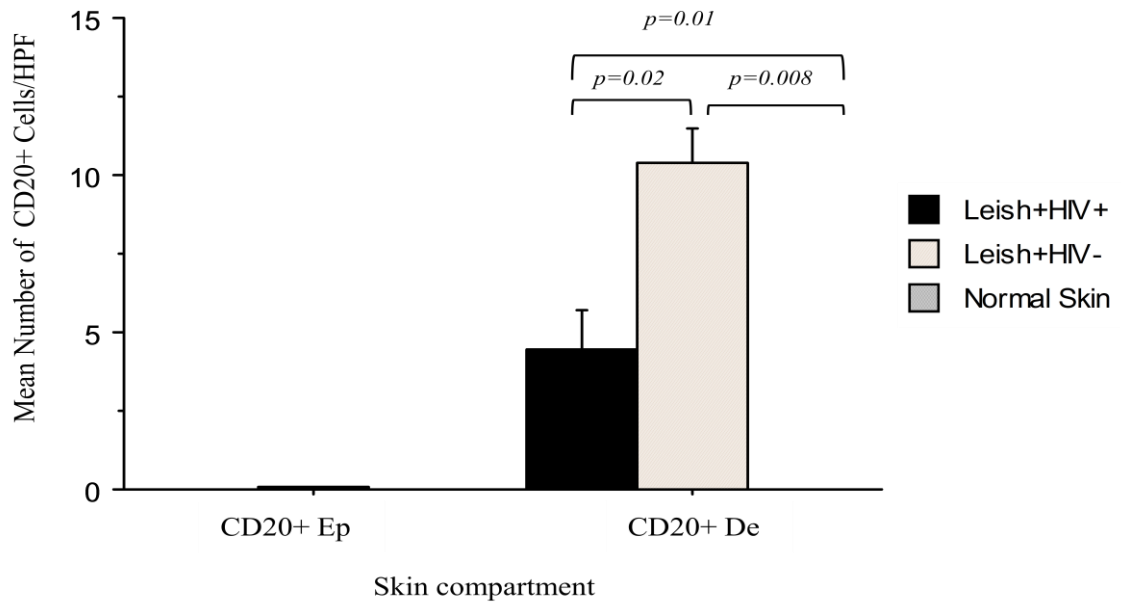


Figure 61: Representation of CD20+ cells infiltration in the epidermis (Ep) and the dermis (De) of immunocompetent and immunodeficient patients

Normal skin (n=10), Leish+HIV+: *Leishmania*-HIV co-infected patients (n= 25), Leish+HIV-: patients with leishmaniasis only (n=119), n is the total number of samples analysed, data are mean ± SEM.

In the second semester from the onset of CL, the CD20+ cells amount was the highest in all groups. The lesion resolution was characterized by the decrease in CD20+ cells number. As it is illustrated in Figure 62, during lesions evolution, the amount of these cells was almost low in Leish+HIV+ group but maintained even after one year.

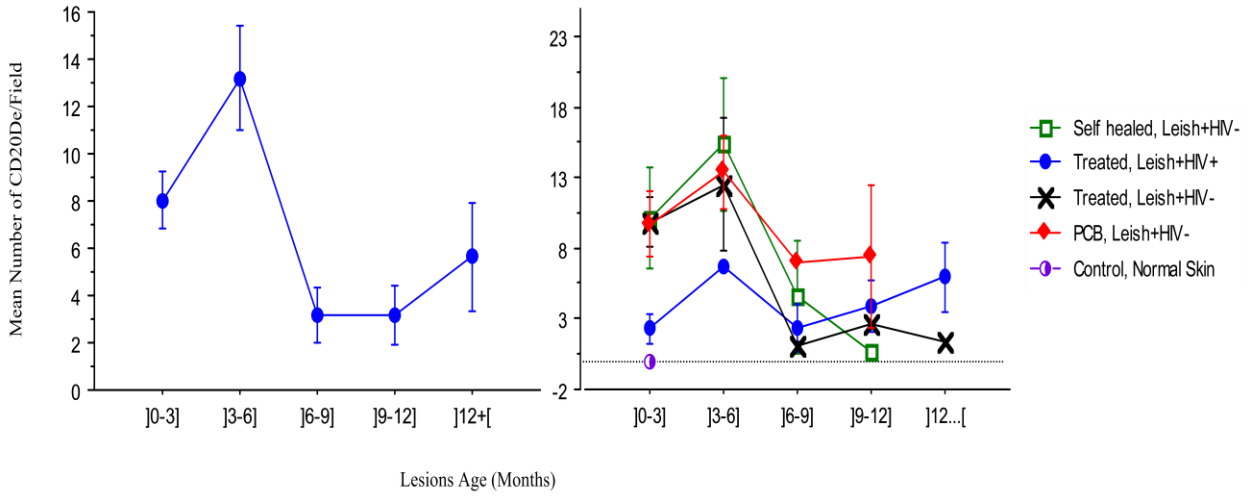


Figure 62: Modifications observed in dermal CD20+ cells amount during cutaneous leishmaniasis evolution and influence of the therapy and HIV status

(A) Epidermis (Ep), (B) Dermis (De), Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, PCB: Placebo, data are mean ± SEM, for each age range $8 \leq n \leq 79$.

III.1.7.4 Mast cells infiltration and degranulation

Leish+HIV+ patients presented a significant ($p = 0.02$) increase in MC number at the lesion sites compared to the normal skin. Despite this tendency, there was no statiscal difference observed between co-infected and patients with CL only (Figure 63).

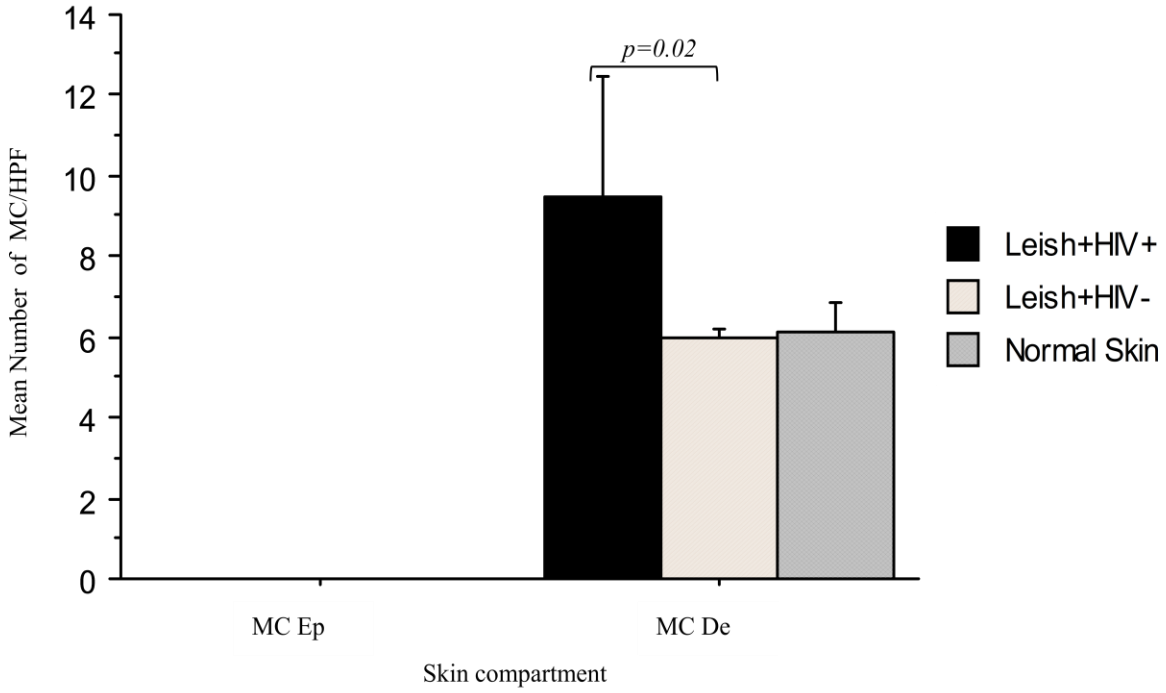


Figure 63: Representation of mast cells infiltration in the dermis (De) of immunocompetent and immunodeficient patients
Normal skin (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n= 23), Leish+HIV-: patients with leishmaniasis only (n=110), MC: Mast cell, n is the total number of samples analysed, data are presented as mean ± SEM.

Whether treated or not and independently on the health status, in more chronic lesions the number of MC was significantly ($p < 0.05$) high; and so there was a positive correlation ($r = 0.4$, $p = 0.0005$) between lesions age and MC infiltration. Patients with chronic lesions, whether HIV positive or not, presented high number of MC at the lesion site even one year after the beginning of the disease. MC infiltration was more marked in the third trimester of lesions evolution mainly in self-healed patients. In general, more vulnerable patients (i.e those who needed antileishmanian drugs to heal and Leish+HIV+

subjects) showed few MC at this period as compared to self healed individuals. When healing was complete, the number of MC tended to come to the initial value (Figure 64).

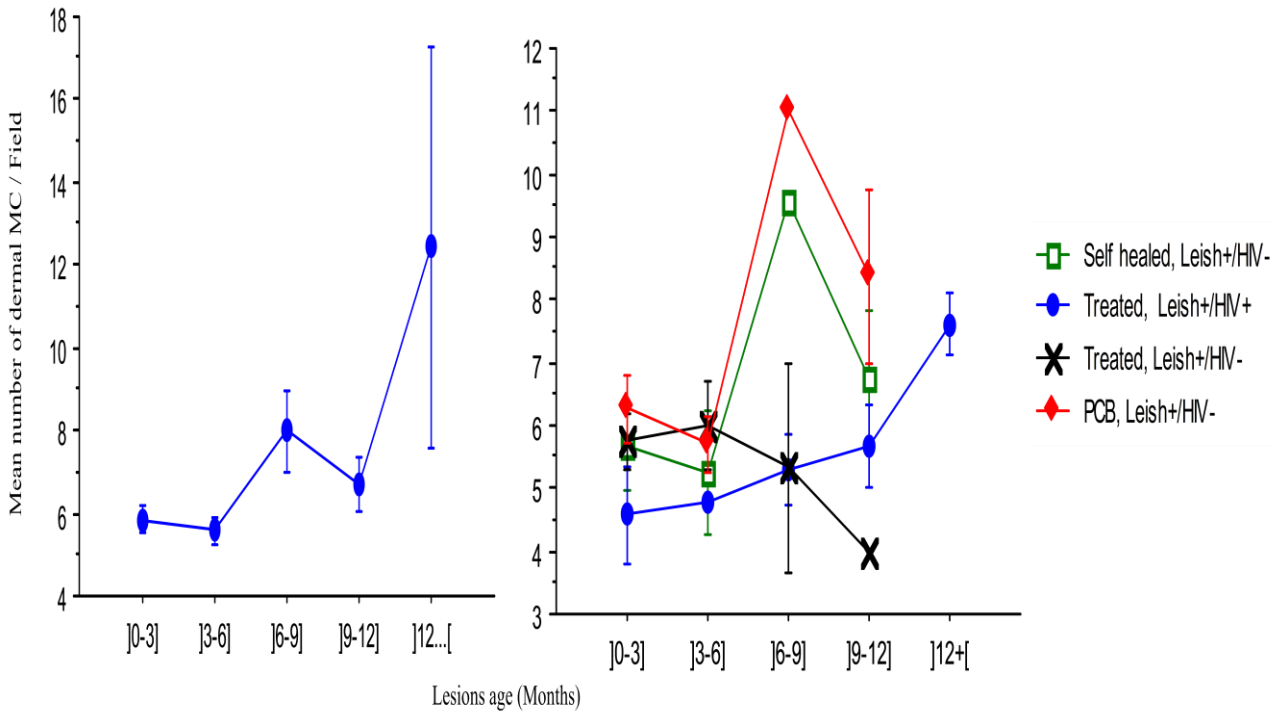


Figure 64: Changes occurred in mast cells infiltration during lesions progression and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, PCB: Placebo, data mean ± SEM, for each age range $8 \leq n \leq 80$.

We observed that MC infiltration was not generally influenced by the patient age. Thus, no significant difference was noticed between MC infiltration in the youth as compared to adults.

As far as the degranulation rate is concerned, MC degranulation was influenced by the health status as well as by the patient age and the lesion age. In Leish+HIV- patients, there was a significant extensive MC degranulation ($p = 0.0005$) compared to Leish+HIV+ subjects who rather presented more not degranulated mast cells ($p < 0.0001$). The degranulation was very low or almost absent in the normal skin (Figure 65).

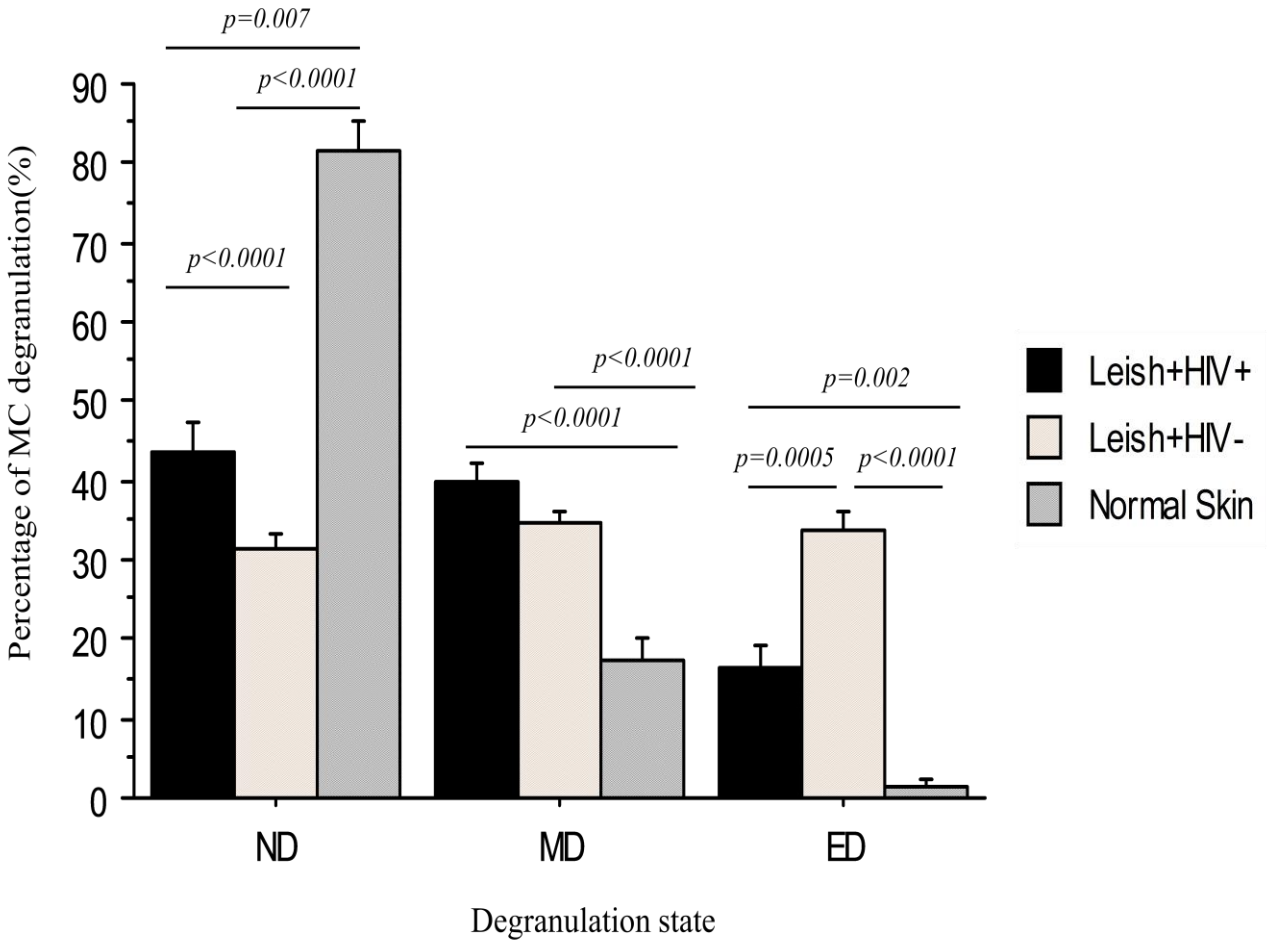


Figure 65: Representation of the intensity of mast cells degranulation on immunocompetent and immunosuppressed CL patients

ND=No degranulation, MD= Moderate degranulation, ED=Extensive degranulation, Normal skin (n=10), Leish+HIV+: *Leishmania*-HIV co-infected patients (n= 25), Leish+HIV-: patients with leishmaniasis only (n=119), n is the total number of samples analysed, data are presented as mean ± SEM.

As it is depicted in Figure 66, during CL resolution MC degranulation tended to increase in immunocompetent subjects as compared to immunodeficient patients who showed rather a fluctuation in MC degranulation. Extensive degranulation was observed in both groups, but during lesions evolution it was always higher in Leish+HIV-. Independently on the HIV serology, negative correlations were observed between MC degranulation and clinical features namely the number of lesions ($r = -0.25$, $p = 0.03$), the total surface of lesions ($r = -0.30$, $p = 0.01$), and the healing duration ($r = -0.32$, $p = 0.02$).

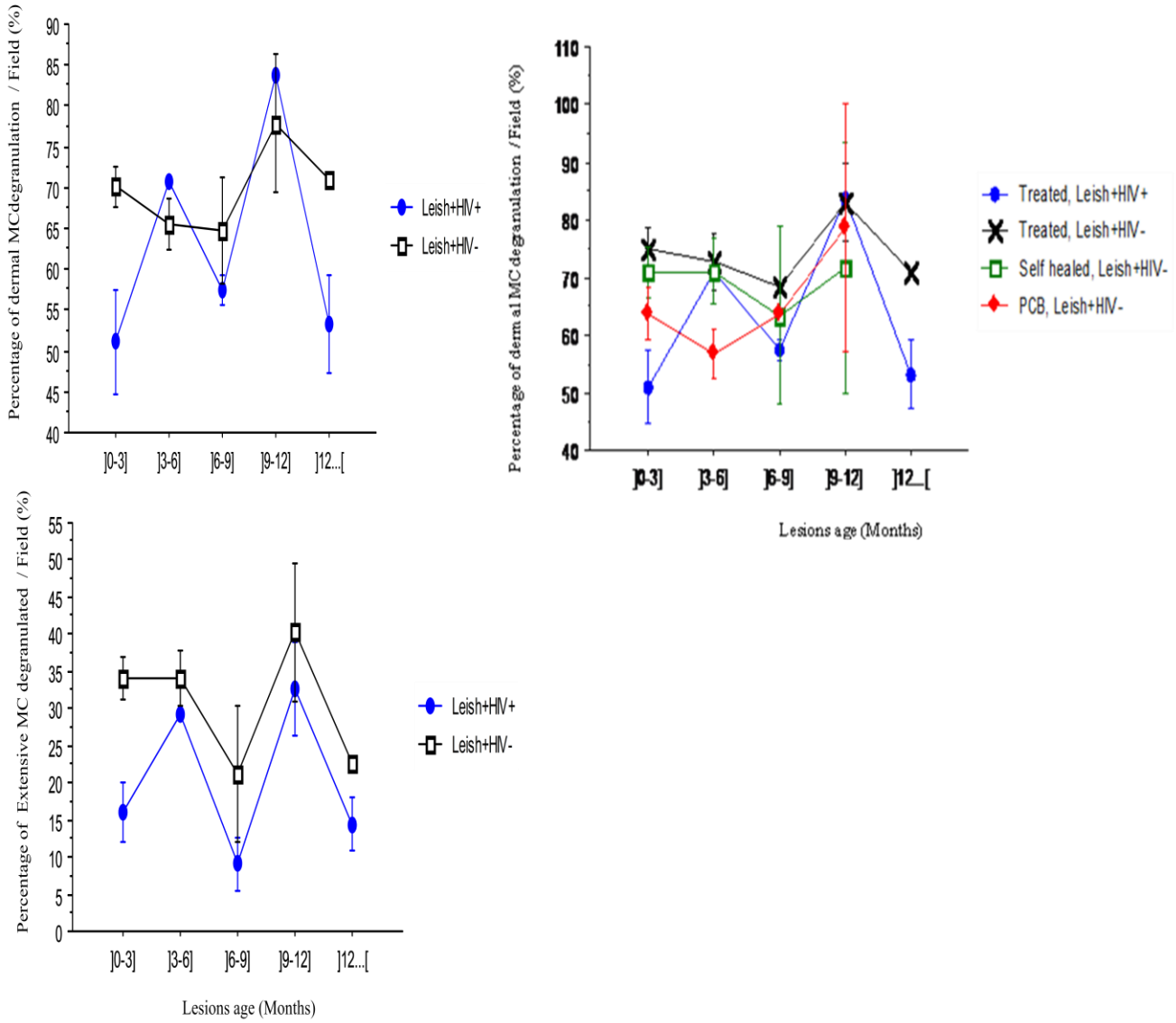


Figure 66: Mast cells degranulation changes during cutaneous leishmaniasis evolution in HIV positive or negative patients

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, PCB: Placebo, data are mean ± SEM, for each age range $8 \leq n \leq 80$.

It was noticed that independently on the HIV serology of the patient, MC degranulation was significantly ($p < 0.05$) more pronounced in the youth as compared to aged individuals (Figure 67).

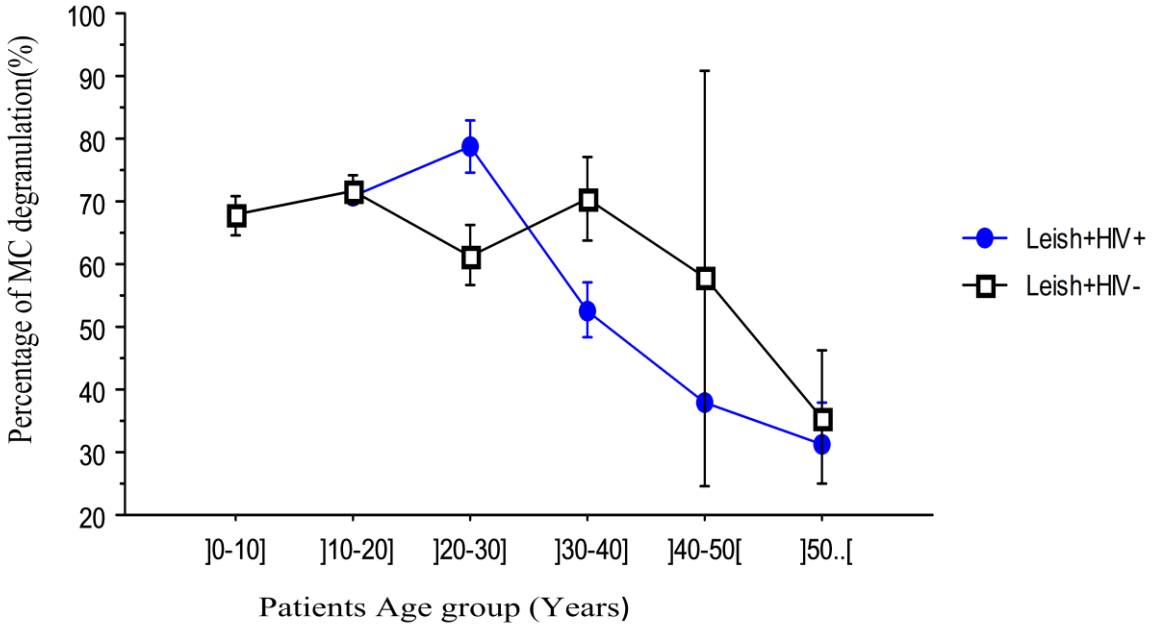


Figure 67: Influence of the patient age on Mast cells degranulation
Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, data are mean ± SEM, for each age range 20≤ n ≤100.

Some immune cells acts by engulfing foreing organisms which subsequently undergo a destruction, but all immune cells generally acts via substances they release in their environment or in the internal milieu. This is why during our study we also looked at the humoral reaction in human CL.

III.1.8 The systemic humoral response towards cutaneous leishmanisis

III.1.8.1 Serum antibodies expression

i. Serum level of total antileishmanial IgG

The whole anti *Leishmania* antibody was evaluated in the serum. As compared to control groups subjects (Leish-HIV- and Leish-HIV+), IgG level was significantly high in sera of patients with active CL lesions. On the other hand, Leish+HIV+ subjects presented a significant ($p = 0.02$) low amount of total serum IgG as compared to Leish+/HIV- individuals (Figure 68).

In general we found a weak but significant positive correlation ($r = 0.3$, $p = 0.02$) between total IgG and the number of lesions. When we took into consideration only Leish+HIV- patients, this correlation was more marked ($r = 0.37$, $p < 0.0001$) and even extended to total surface ($r = 0.48$, $p < 0.0001$). Surprisingly, when the group of co-

infected patients was considered alone, no real correlation was observed between IgG and clinical features.

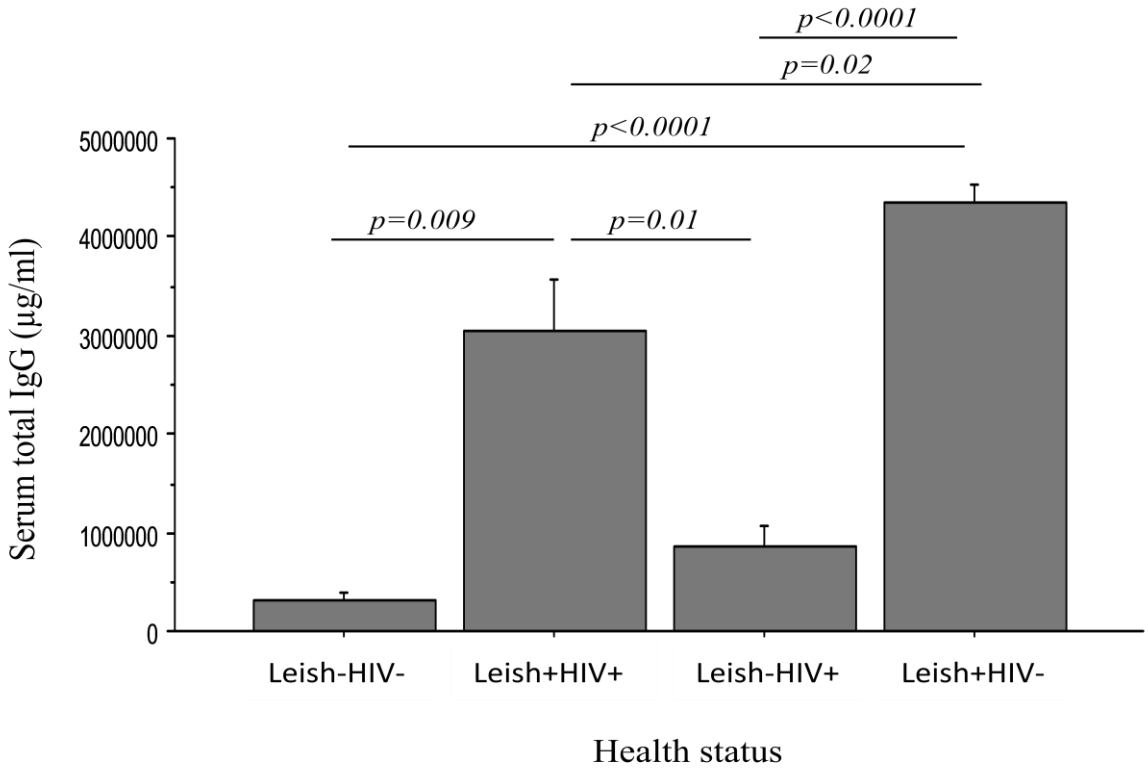


Figure 68: Serum level of total antileishmanian IgG in patients with cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=11), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=38), Leish-HIV+: patients with HIV only (n=16), Leish+HIV- : patients with leishmaniasis only (n=315), n is the number of samples analysed, data are mean ±SEM.

Independently on the therapy regimen and the health status, the total anti leishmanial IgG production depended on the lesion age. The earlier the lesion the lower the serum level of total IgG. The optimal prodution was registred when clinical signs were more marked, this is in the second trimester of lesions evolution. During the resolution period the antibody level reduced significantly ($p = 0.002$) but remained, even after one year, higher than in CL negative control groups (Figure 69). Interestingly, subjects with persistent lesions, who needed antileishmanial therapy to heal, produced more IgG than those who received only placebo or those who self healed. But during the lesion evolution, Leish+HIV+ patients always showed a deficient or delayed total IgG upregulation.

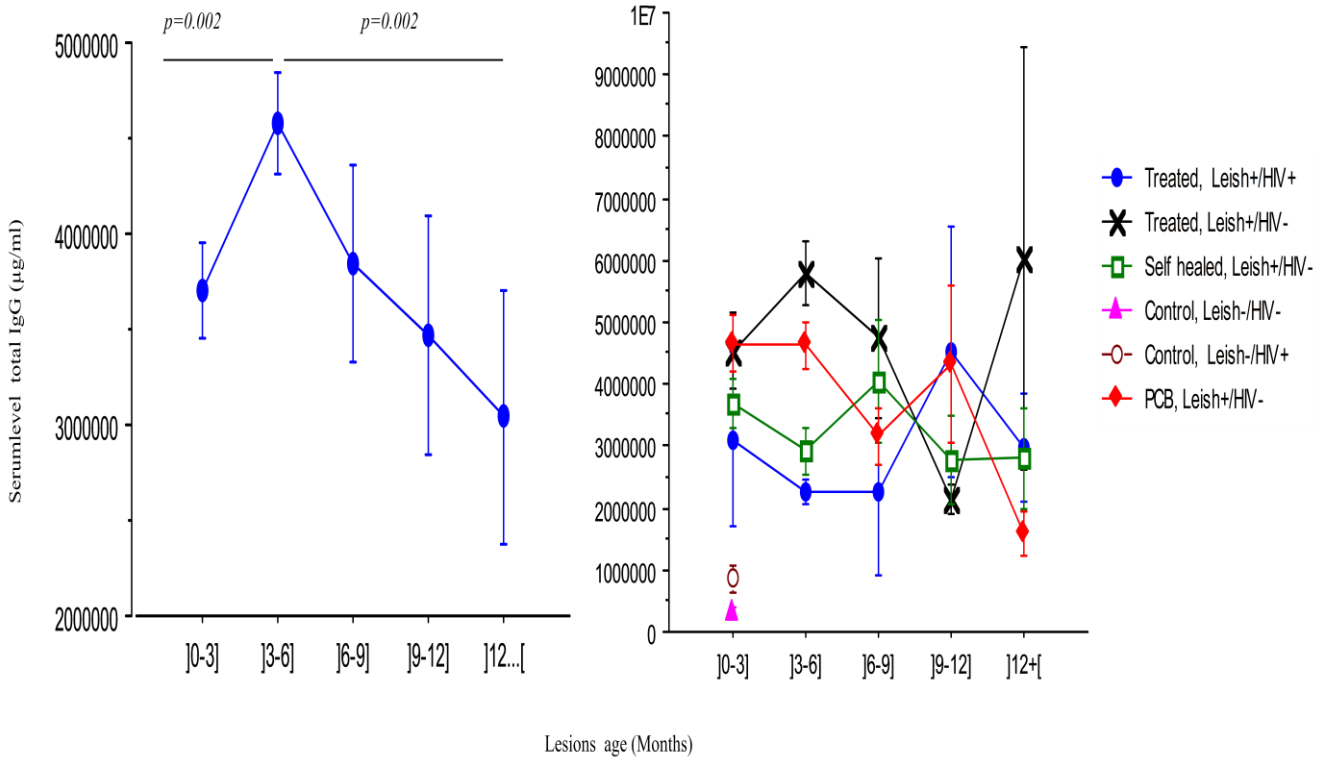


Figure 69: Influence of the lesion age , therapy and HIV status on the serum level of total antileishmanian IgG

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV- : patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM, for each age range 24≤ n ≤171.

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As far as the therapy is concerned, wether treated or not, independently on the HIV serology, in general, the patient's age did not show a real influence on the total antileishmanian antibody secretion.

ii. Serum level of antileishmanian IgG subtypes 1 and 2

IgG subtypes 1 and 2 titers were evaluated. It came out that both subtypes are upregulated in CL patients whether co-infected or not (Figure 70). *Leishmania*-HIV co-infected showed a tendancy to produce low IgG2 as compared to Leish+HIV- patients; precisely and significantly ($p \leq 0.01$) when more concentrated sera (dilution 1:10, 1:100) were used. In general, the IgG1 titer was higher than that of IgG2.

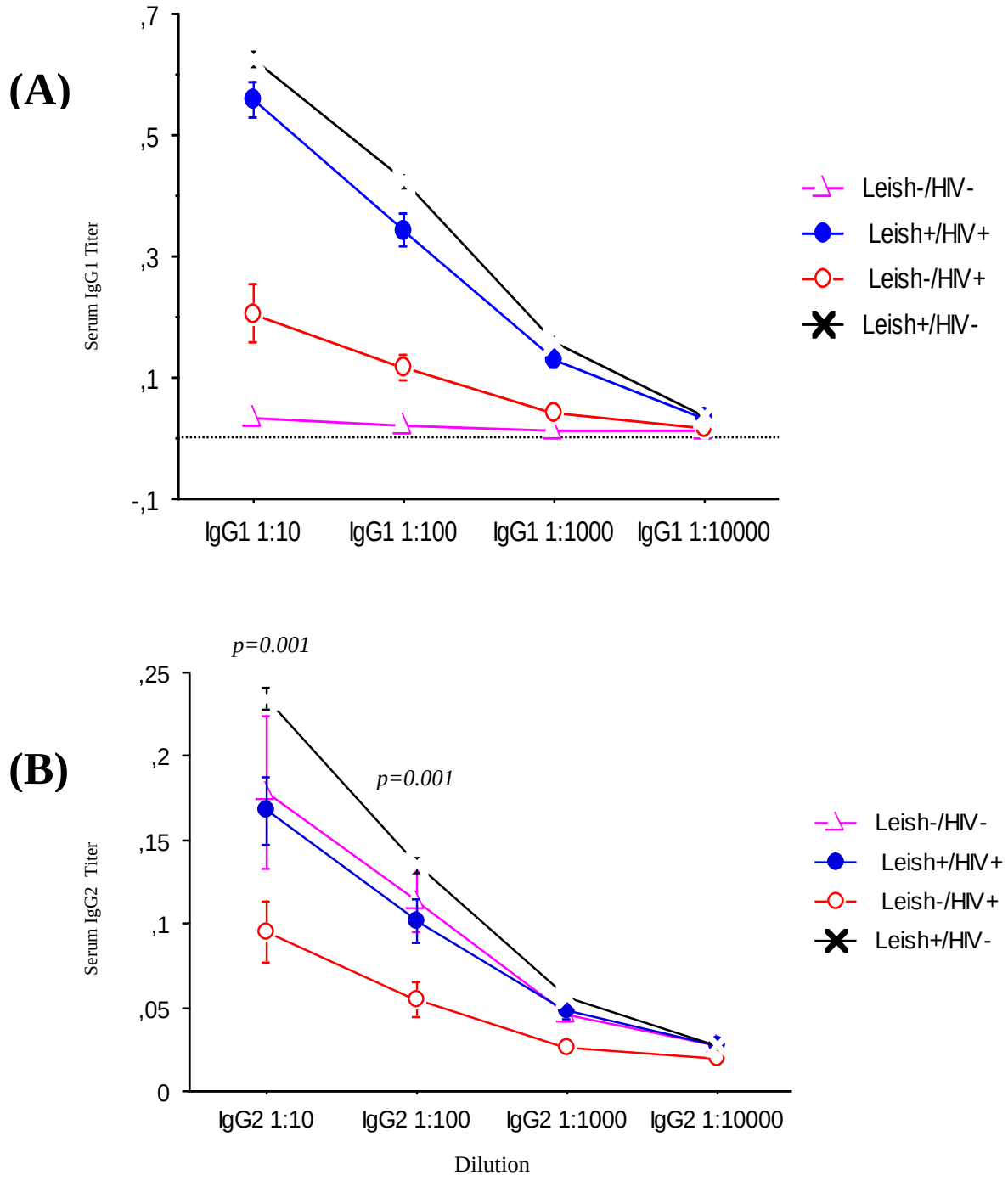

Figure 70: Serum antileishmanian IgG subtypes titers
(A):IgG1, (B): IgG2, Leish-HIV-: *Leishmania* and HIV double negative individuals (n=11), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=38), Leish-HIV+: patients with HIV only (n=16), Leish+HIV- : patients with leishmaniasis only (n=315), n is the number of samples analysed, data are mean ±SEM.

During the disease evolution, IgG1 was remarkably more secreted in the second trimester of the disease. Leish+HIV- patients with more marked symptoms and who needed antileishmanian therapy generally showed more IgG1. On the contrary Leish+HIV+ subjects presented low amount of this same IgG subtype (Figure 71).

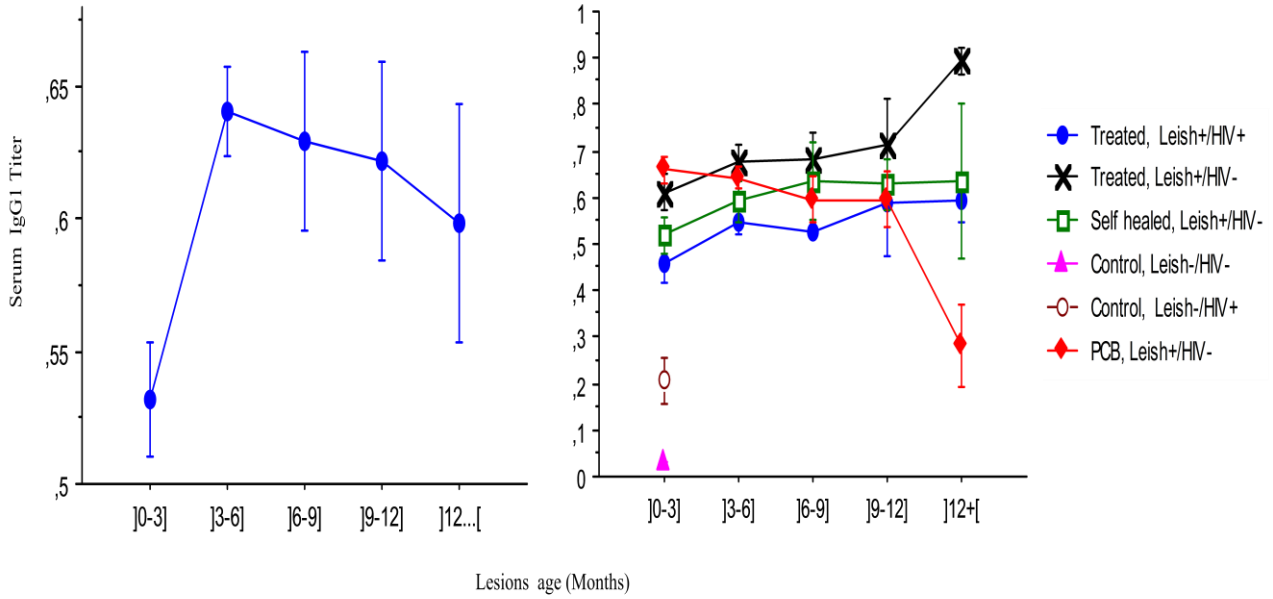

Figure 71: Changes occurred on serum IgG1 titer during CL evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV- : patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM, for each age range 24≤ n ≤171.

As far as the patient age is concerned, in the youth there was more IgG1 production (Figure 72). In general, Leish+HIV+ patients presented low IgG1 secretion.

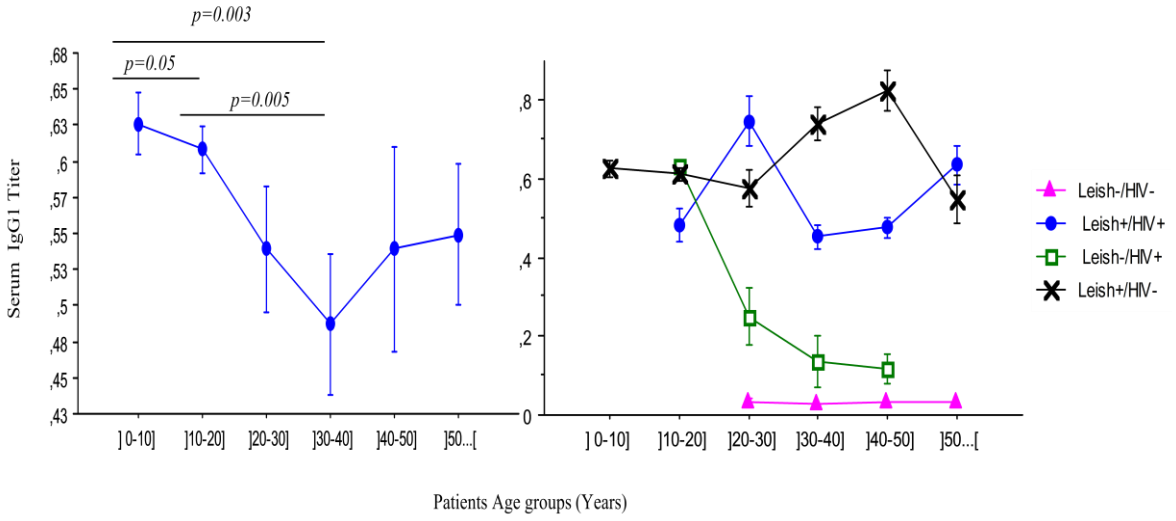

Figure 72: Serum IgG1 change in function of the patient age

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV- : patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, data are mean ±SEM.

In patients with old active lesions or those presenting very advanced healing process, IgG2 was significantly more secreted. During the resolution period, IgG2 titer reduced significantly. Anyway, Leish+HIV- patients who needed antileishmanian therapy seemed to show more serum IgG2. On the contrary Leish+HIV+ subjects presented low serum amount and a delayed up regulation of this same IgG subtype (Figure 73). Remarkedly, during CL evolution changes that occurred in IgG2 titer of Leish+HIV+ patients were globally observable on the total IgG of the same group.

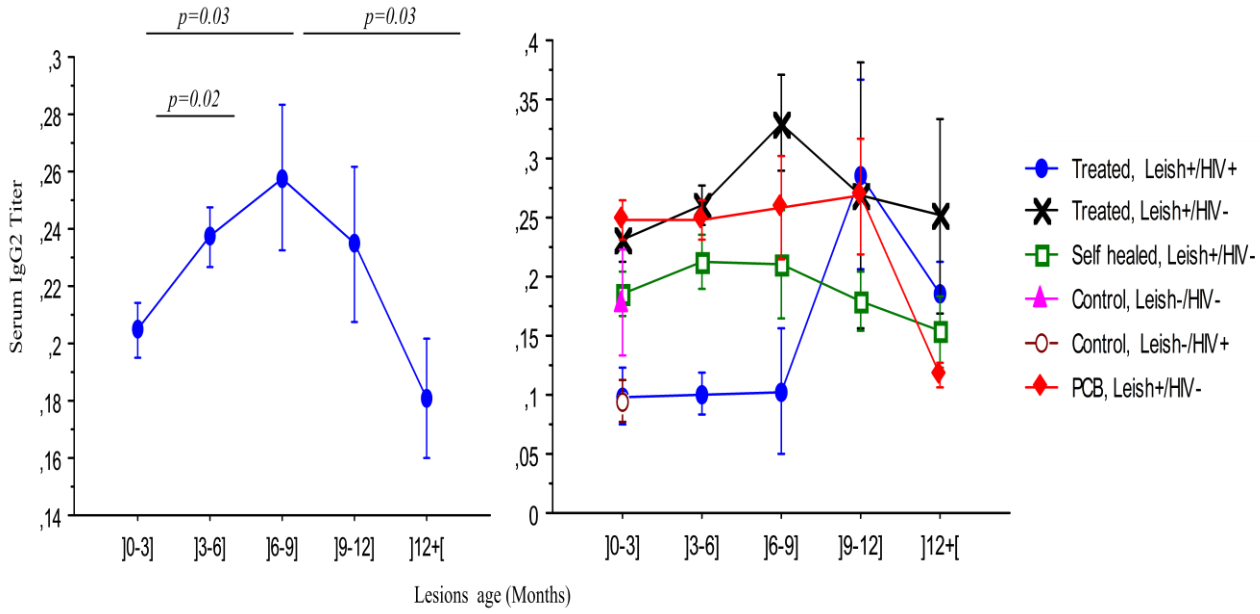

Figure 73: Changes occurred on serum IgG2 titer during leishmaniasis evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV- : patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM, for each age range 24≤ n ≤171.

The patient age did not significantly affect the serum IgG2 titer (Figure 74). But in each age range, even if co-infected patients generally presented high IgG2 titer as compared to the negative controls groups, they conversely showed low titer of this antibody subtype as compared to *Leishmania* mono-infected individuals.

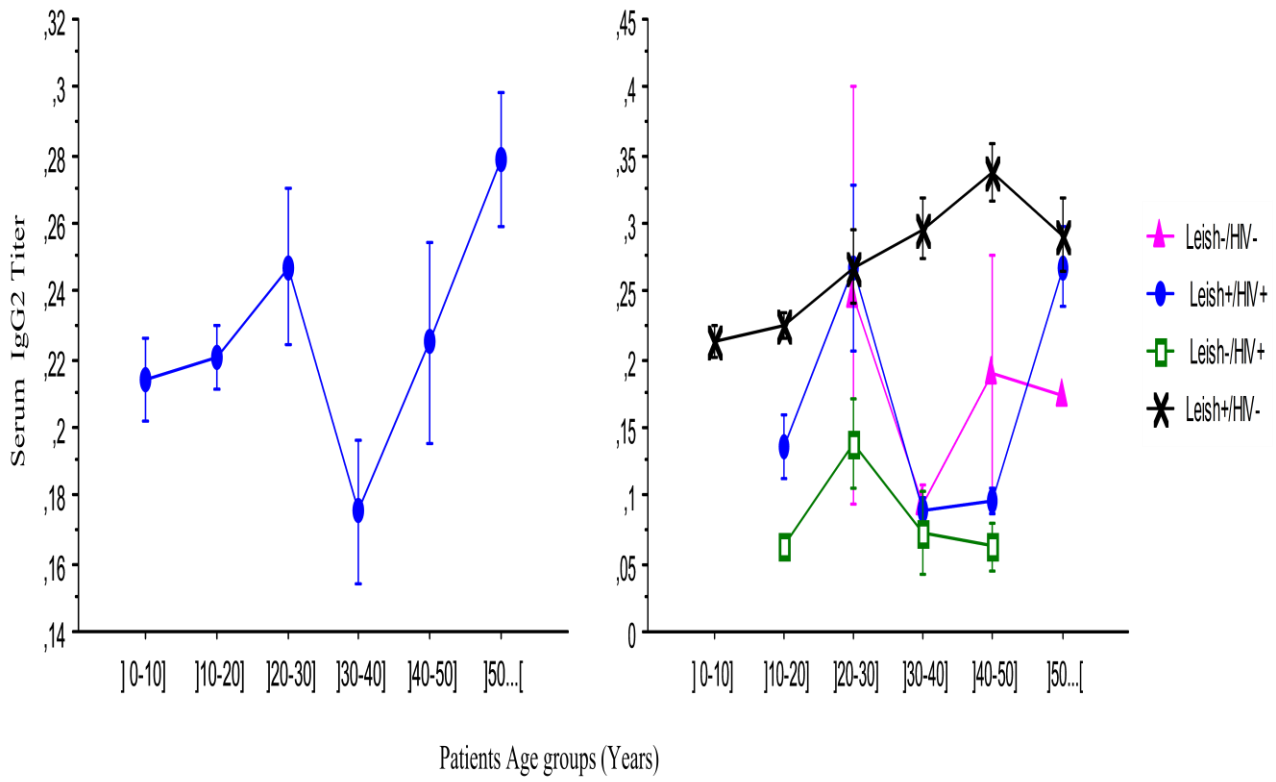

Figure 74: Age of the patient and changes in serum IgG2
Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV- : patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, data are mean ±SEM, for each age group, 20≤n≤70

iii. Correlations in total IgG and IgG subtypes

The total IgG production depended on the secretion of its different subtypes. Also, the production of these subtypes depended on each other (Figure 75). This can be observed through a significant correlation between total IgG and IgG1($r = +0.52$, $p< 0.0001$), total IgG and IgG2 ($r = +0.42$, $p<.0001$), as well as IgG1 and IgG2 ($r = +0.47$, $p< 0.0001$). The total IgG was more associated to IgG1 than IgG2.

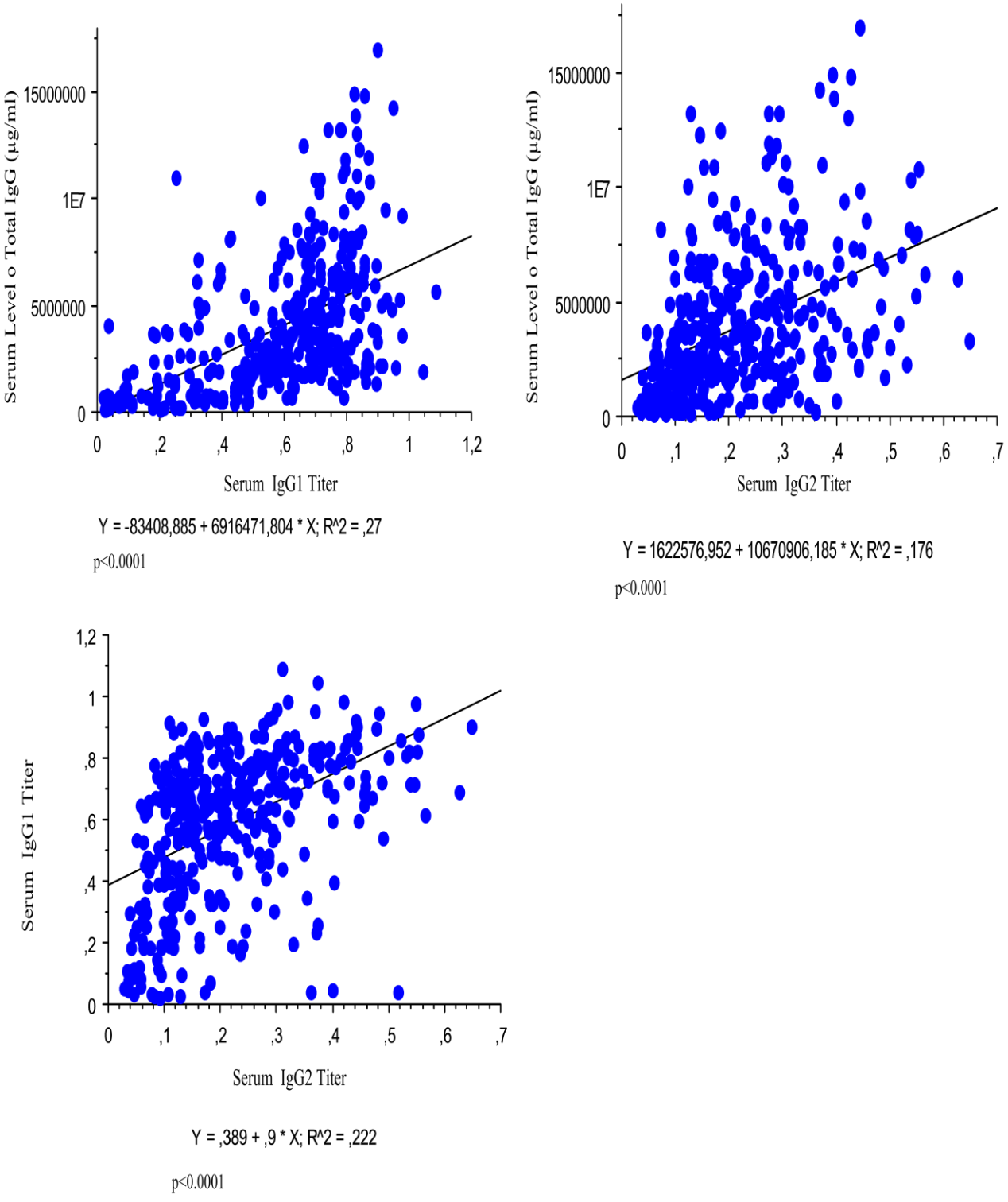

Figure 75: Representative regression curves showing the relationship between total IgG and IgG subtypes(IgG1 and IgG2)
Number of samples analysed n=353

III.1.8.2 Serum cytokines/chemokines profile of CL and/or HIV infected patiens

i. Overview of some cytokines upregulation in the serum

On a given serum sample, it is not all cytokines that were upregulated. As it is depicted on the Figure 76, TNF-α, IL-6 and IL-8 up regulation, for example, are very pronounced and can be easily observed.

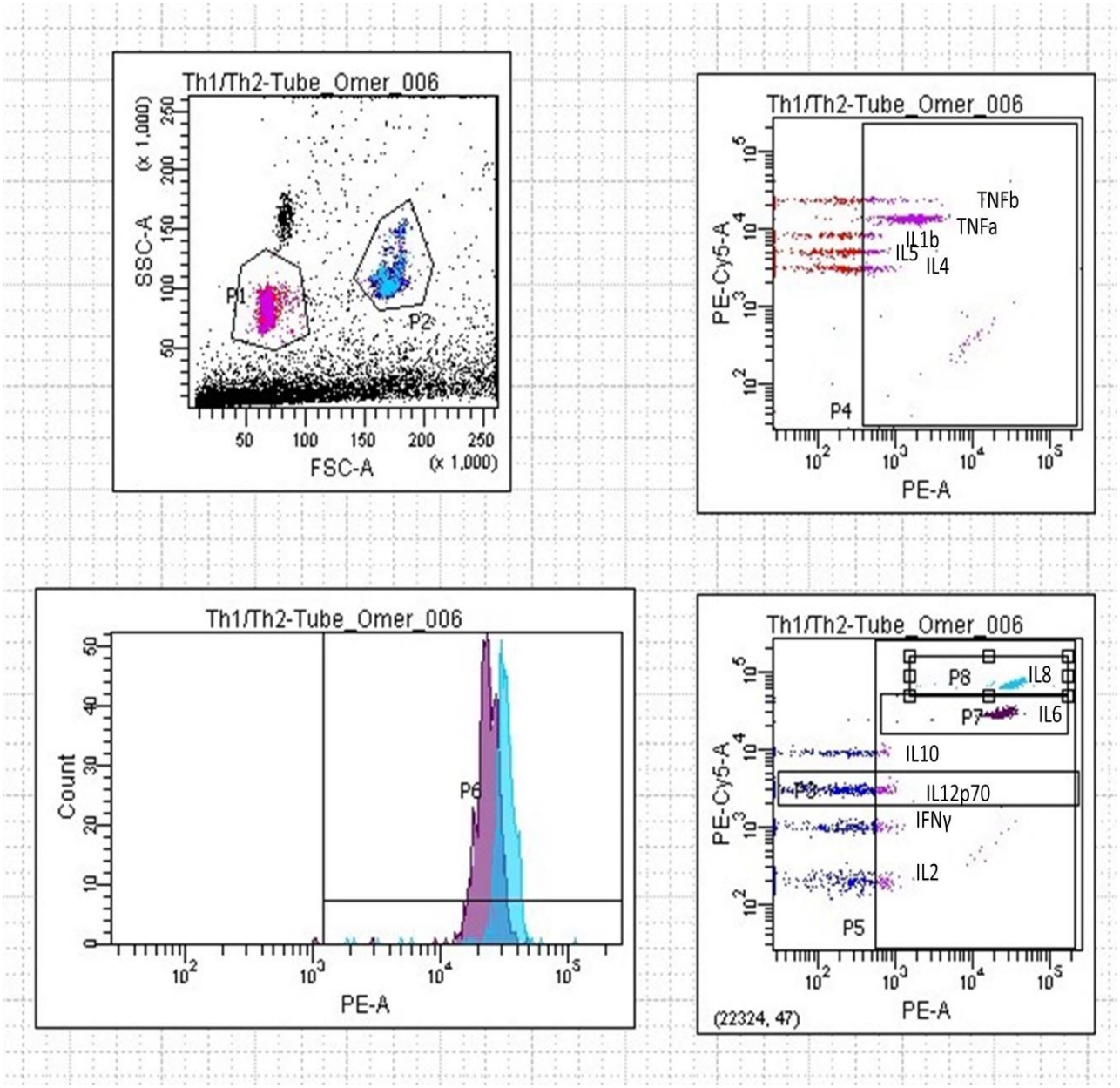

Figure 76: Illustration of the up regulation of some serum cytokines

ii. Th1cytokines

- **Serum Interferon-γ**

IFN-*γ* tended to be more up regulated in control groups as compared to leishmaniasis patients. HIV+ control group showed a high level of this cytokine as compared to others groups. No significant difference (*p>0.05*) between CL patients with different HIV serology (Figure 77)

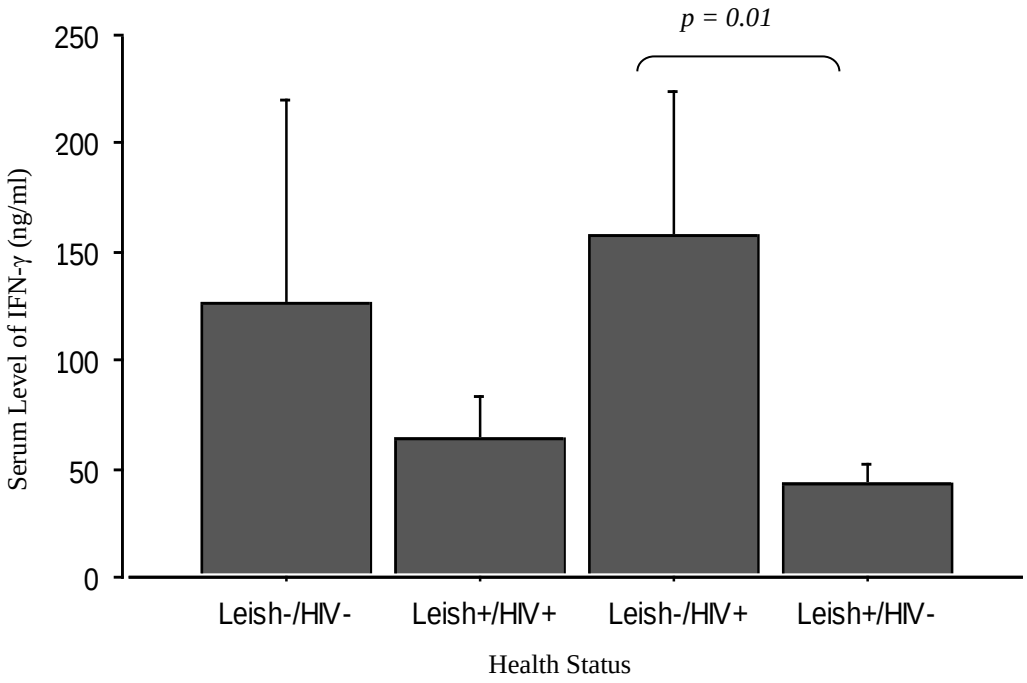

Figure 77: IFN-γ level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV- : patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

IFN-γ was always present in the blood of CL patients. No significant difference (*p > 0.05*) was observed in the serum level of this cytokine during CL evolution. Nevertheless, the production was more marked at the period during which the resolution process was the most advanced. At this moment, self healed individuals generally showed a tendency to produce more IFN-γ than patients who needed antileishmanian therapy to heal (Figure 78).

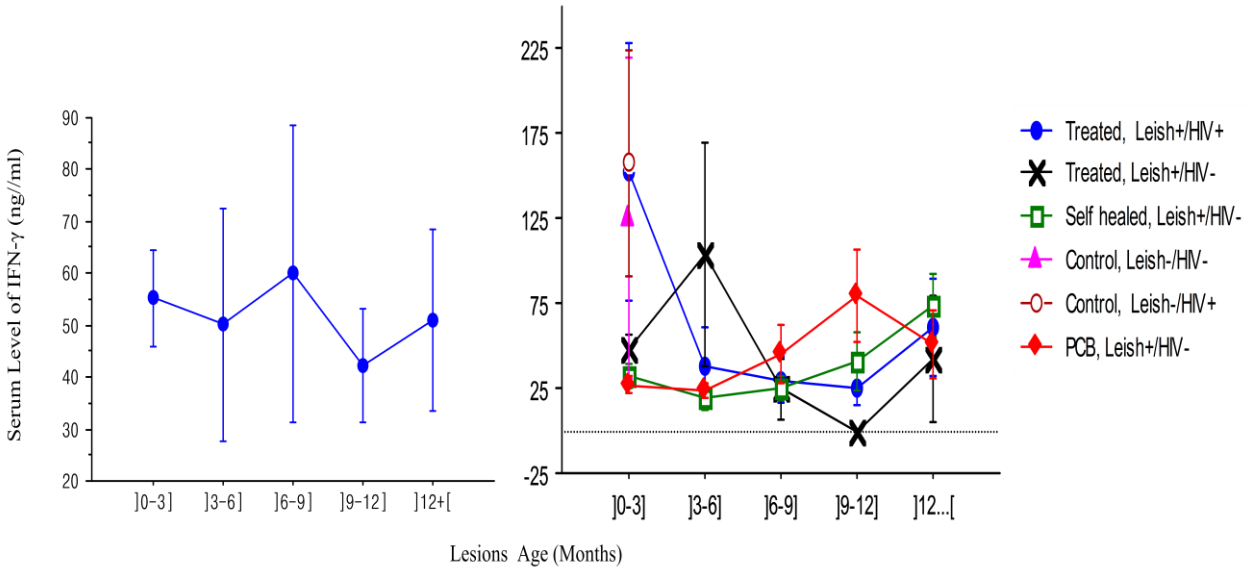

Figure 78: Changes occurred in the serum level of IFN-γ during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV- : patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

- **Serum Interleukine-1a**

In the serum of CL patients, IL-1α was scarcely produced (Figure 79). Remarkably, during the lesion evolution, the blood level of this cytokine remained very low in all the groups of patients (Figure 80).

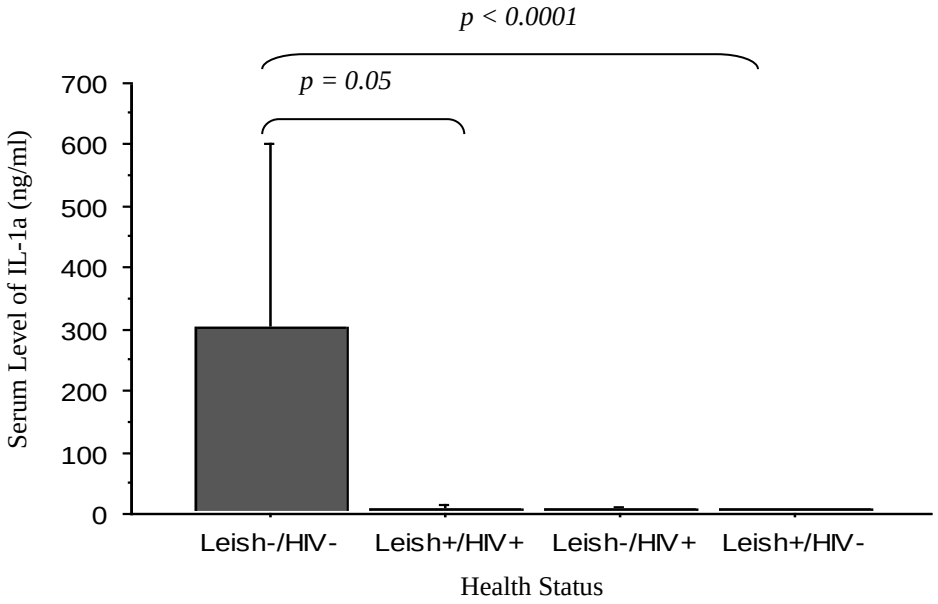

Figure 79: IL-1a level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV- : patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

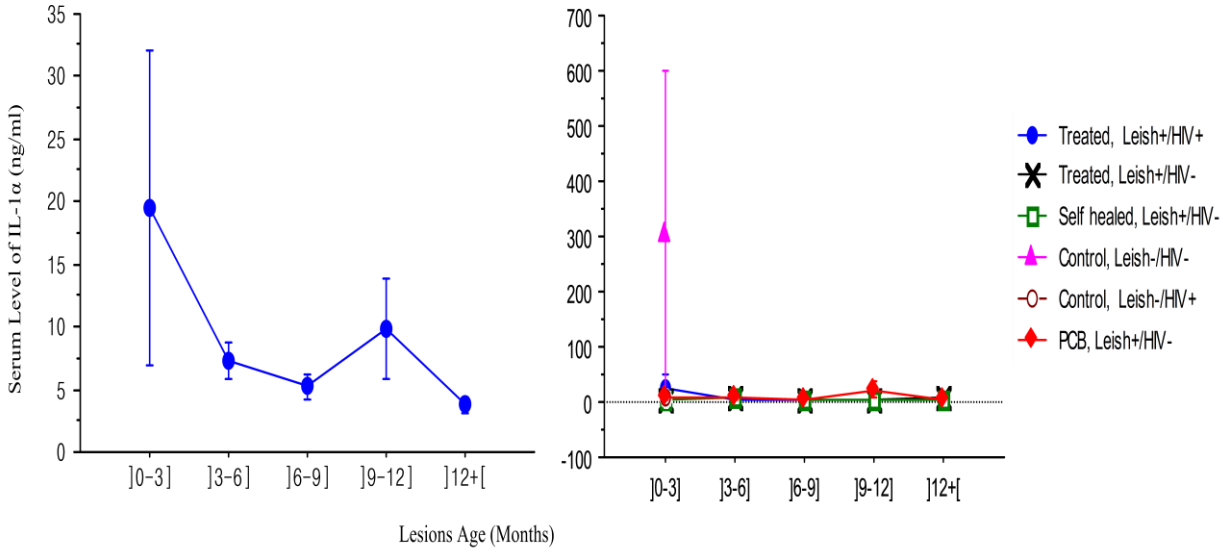

Figure 80: Changes occurred in the serum level of IL-1α during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV- : patients with leishmanaiasis only, Leish-HIV- : *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

- **Serum Interleukine-1b**

As it is depicted on the Figure 81, all groups of subjects generally up regulated IL-1b. However, CL individuals with a positive HIV serology showed the tendency to produce a less amount of this cytokine as compared to others.

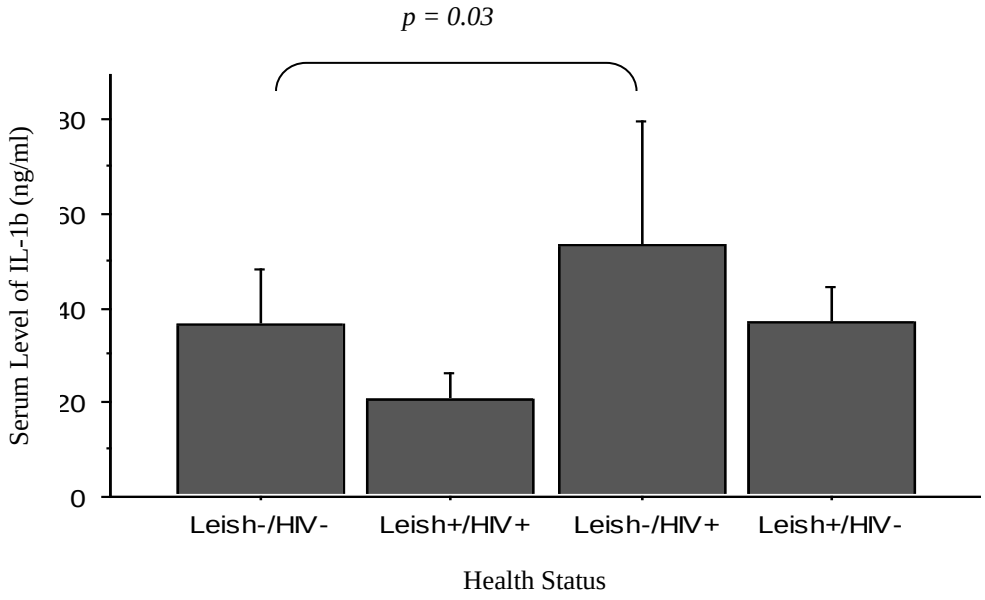

Figure 81: IL-1b level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV- : patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

The general tendency was that, at the early phase of CL, there was a low IL-1b production. In the second trimester of the disease progression, this production increased significantly ($p = 0.04$) and after this period was observed the drop of the cytokine level. It is remarkable that the spontaneous healing was related to the increased up regulation of IL-1b as it was noticed on none treated and placebo treated patients. Interestingly, during lesions evolution, immunodeficient patients presented low level of this pro-inflammatory cytokine as compared to individuals with a negative HIV serology (Figure 82).

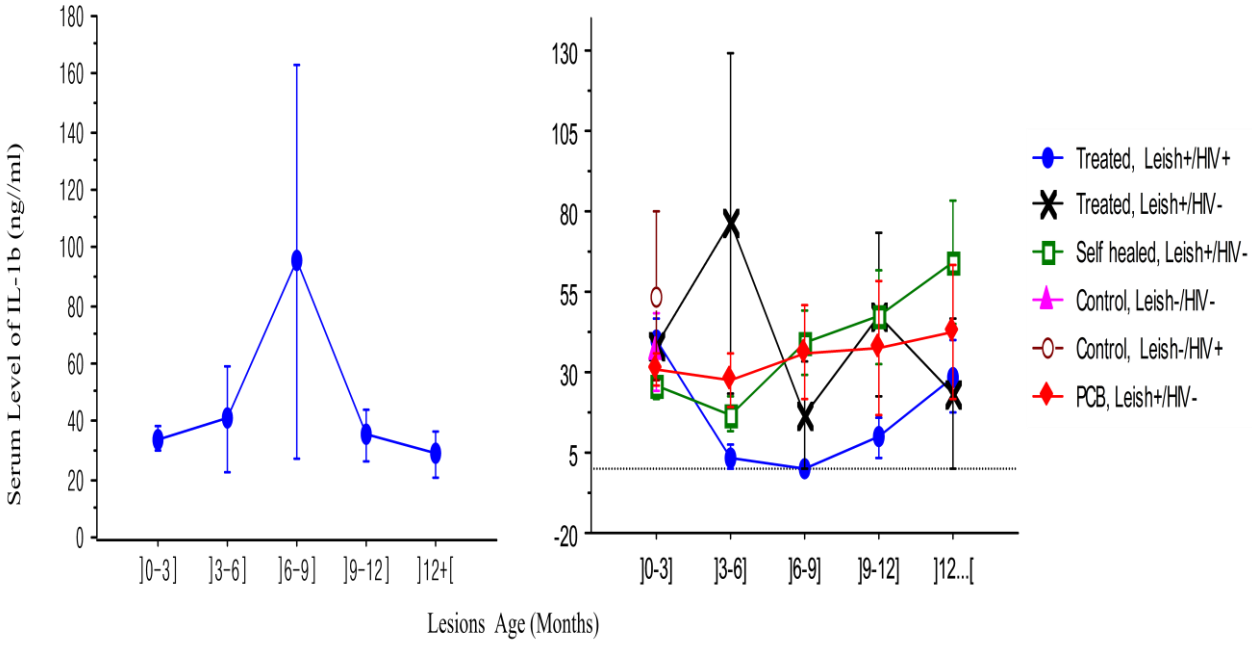

Figure 82: Changes occured in the serum level of IL-1b during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV- : patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

- **Serum Interleukine-2**

The Figure 83 which illustrates the IL-2 up regulation in the blood shows that CL patients produced a significant ($p = 0.0002$) low amount of this cytokine as compared to both Leish-HIV- and Leish-HIV+ controls. The HIV status did not influence the serum level of this cytokine in CL suffering subjects.

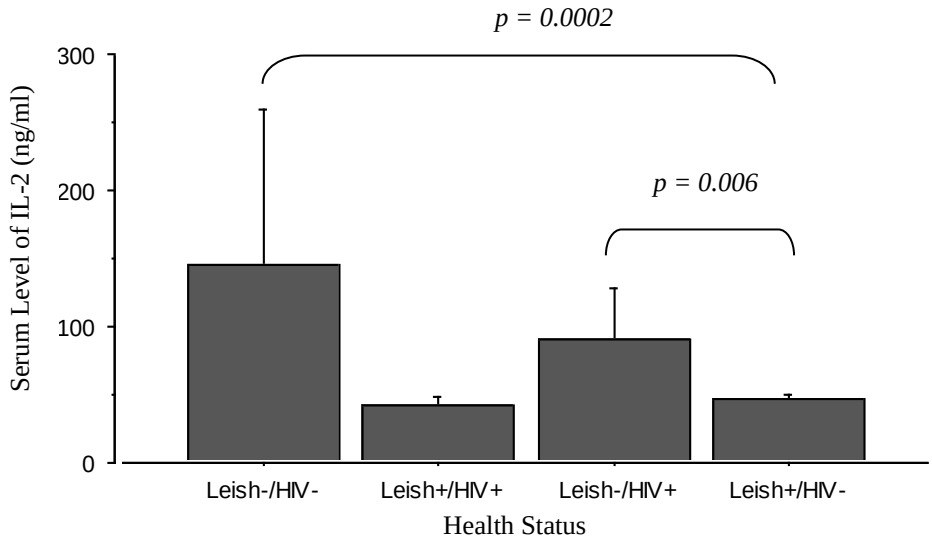

Figure 83: IL-2 level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV- : patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

When the serum IL-2 level was evaluated during the disease evolution (Figure 84), it came out that, the cytokine level tended to increase more in self-healed individuals while co-infected and treated *Leishmania* mono-infected subjects showed low level of IL-2 during the same period. In CL individuals, our analyses showed a significant (*p = 0.0006*) IL-2 up regulation in self healed patients as compared to those with persistent lesions who therefore received antileishmanian therapy. Patients with chronic lesions generally presented more serum IL-2 than those newly infected.

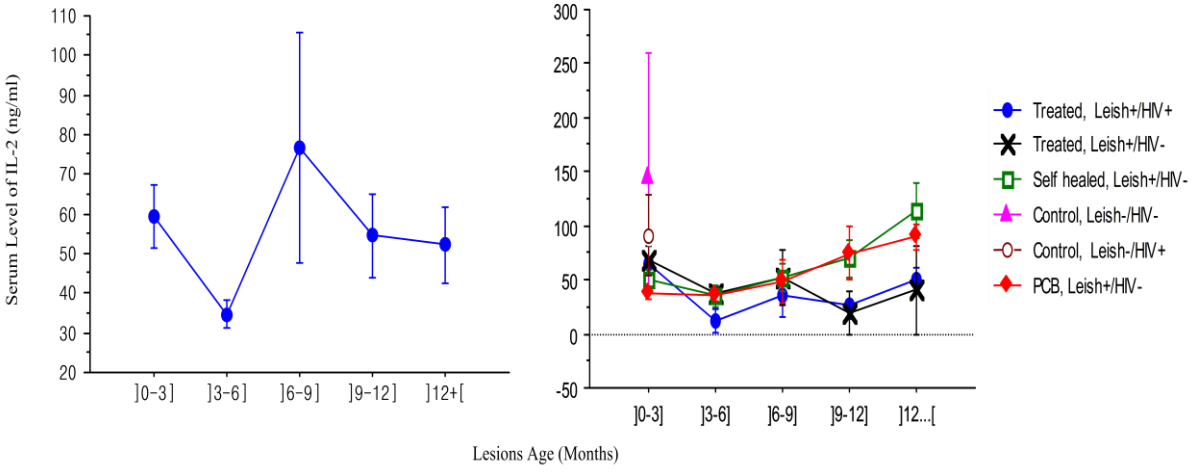

Figure 84: Changes occured in the serum level of IL-2 during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

- **Serum Interleukine-12p70**

As it is depicted on the Figure 85, IL-12p70 was less up regulated in the serum of individuals suffering from CL as compared to controls. Moreover, no significant difference was noticed between CL patients with different HIV serology.

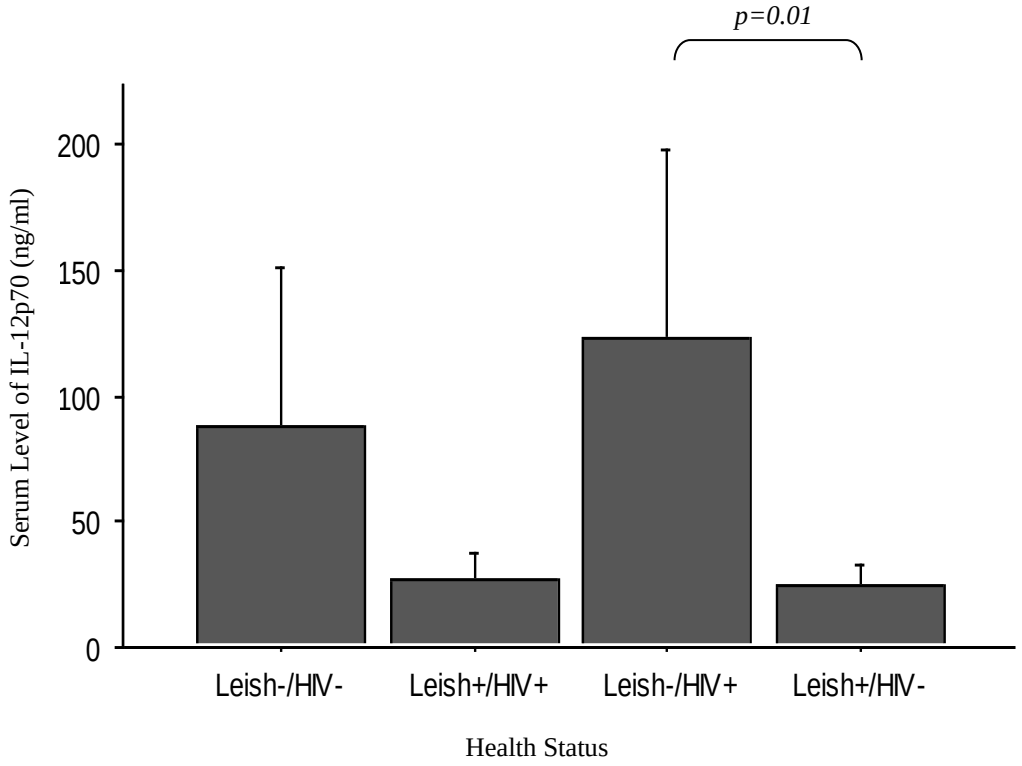

Figure 85: IL-12p70 level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV- : patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

A delayed in IL-12p70 production in the serum was observed in self-healed and placebo treated patients as compared to those who received antileishmanian drugs (Figure 86). Leish+HIV+ with early lesions showed a tendancy to present a high level of this cytokine; but co-infected patients with more chronic lesions showed a lower IL12p70 up regulation.

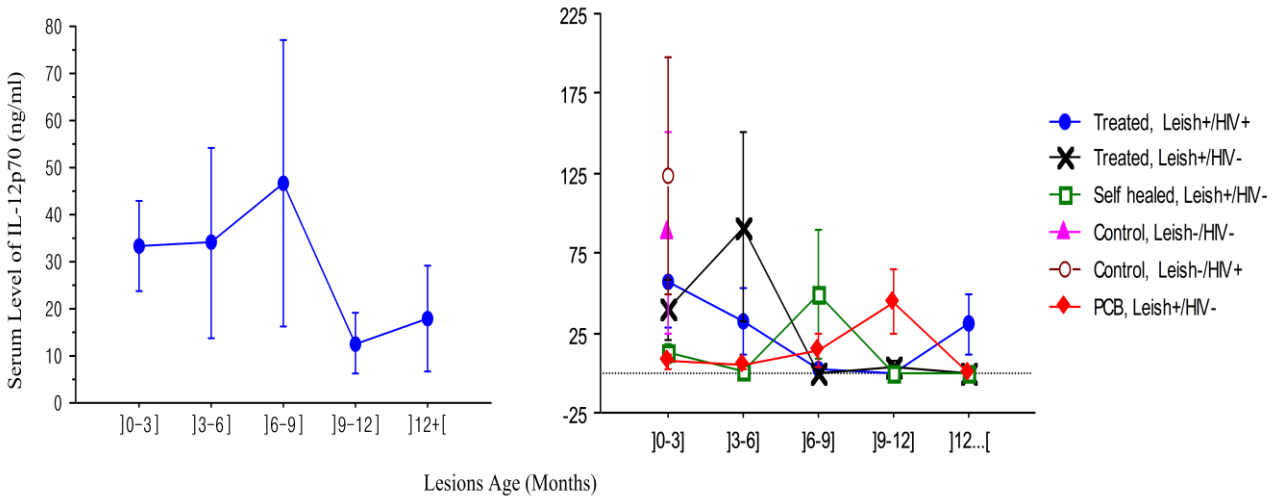

Figure 86: Changes occured in serum IL-12p70 level during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV- : patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

- **Serum Interleukine-18**

IL-18 was significantly (*p=0.02*) up regulated in the blood of Leish+HIV+ patients as compared to *Leishmania* mono-infected subjects (Figure 87).

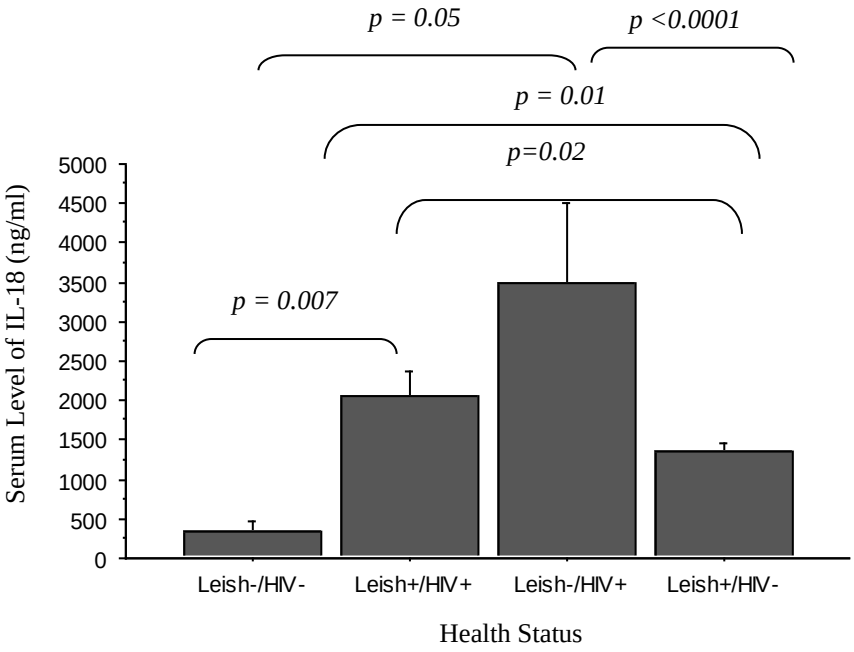

Figure 87: IL-18 level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV-: patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

In almost all the CL patients, it is in the third trimester of the lesion progression that was observed a very important production of IL-18 in the blood. Healing was marked by the drop in the amount of this cytokine (Figure 88).

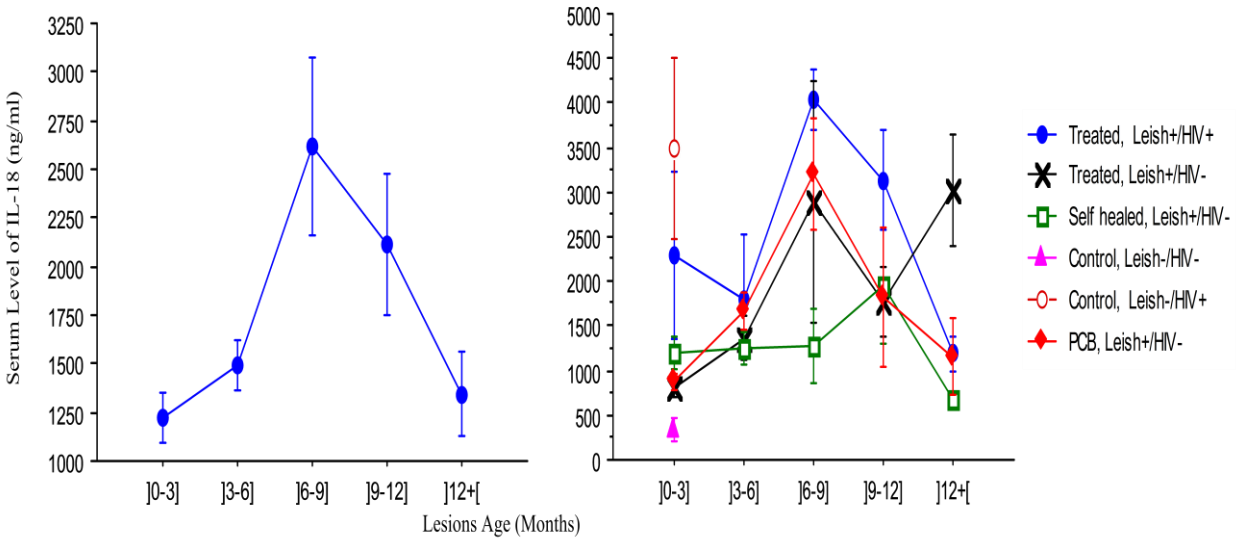

Figure 88: Changes occurred in the serum level of IL-18 during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

- **Serum Tumor necrozing factors**

If during this study TNF-α was up regulated in all the groups with a tendency of more production in Leish-HIV+ patients (Figure 89), TNF-β in the contrary was scarcely produced in all these groups (Figure 90).

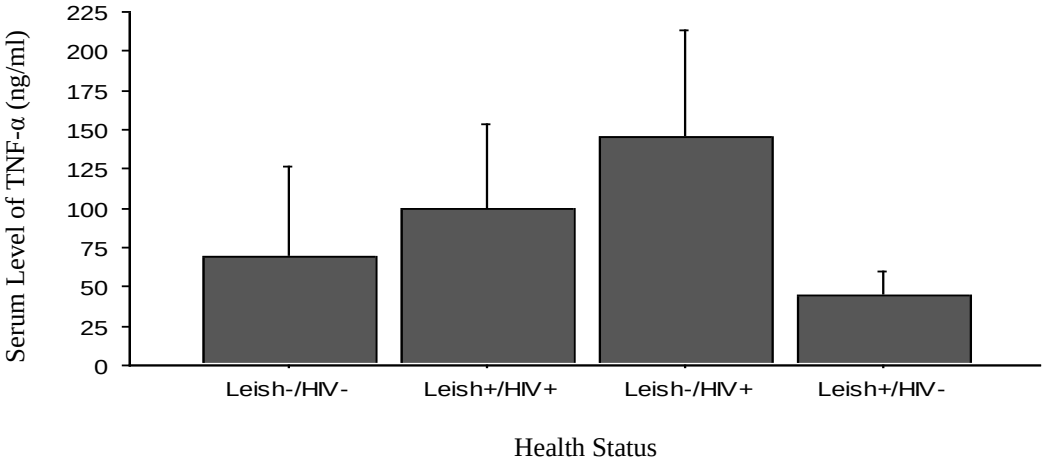

Figure 89: TNF- α level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV-: patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

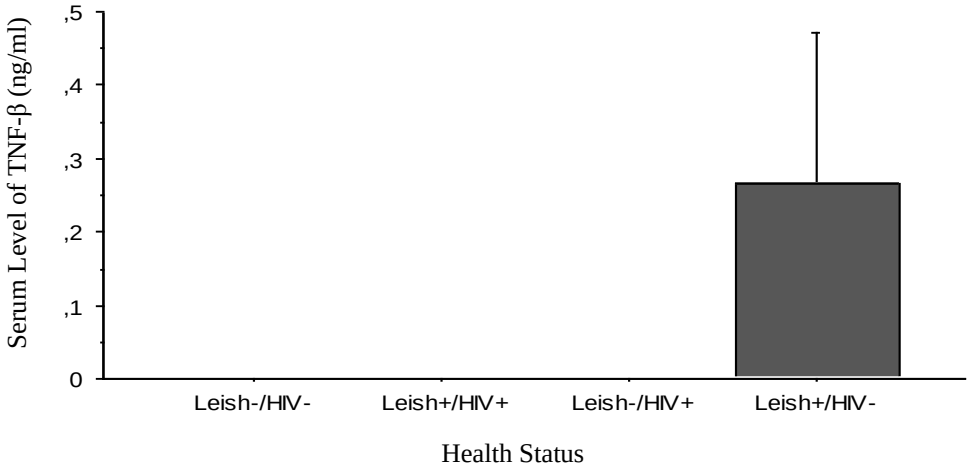

Figure 90: TNF-β level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV-: patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

Wether co-infected or mono-infected, self-healed or treated patients, there was no significant ($p>0.05$) difference in TNF-α production. Patients with more chronic

lesions did not presented any difference in term of the production TNF-α as compared to individuals newly affected (Figure 91).

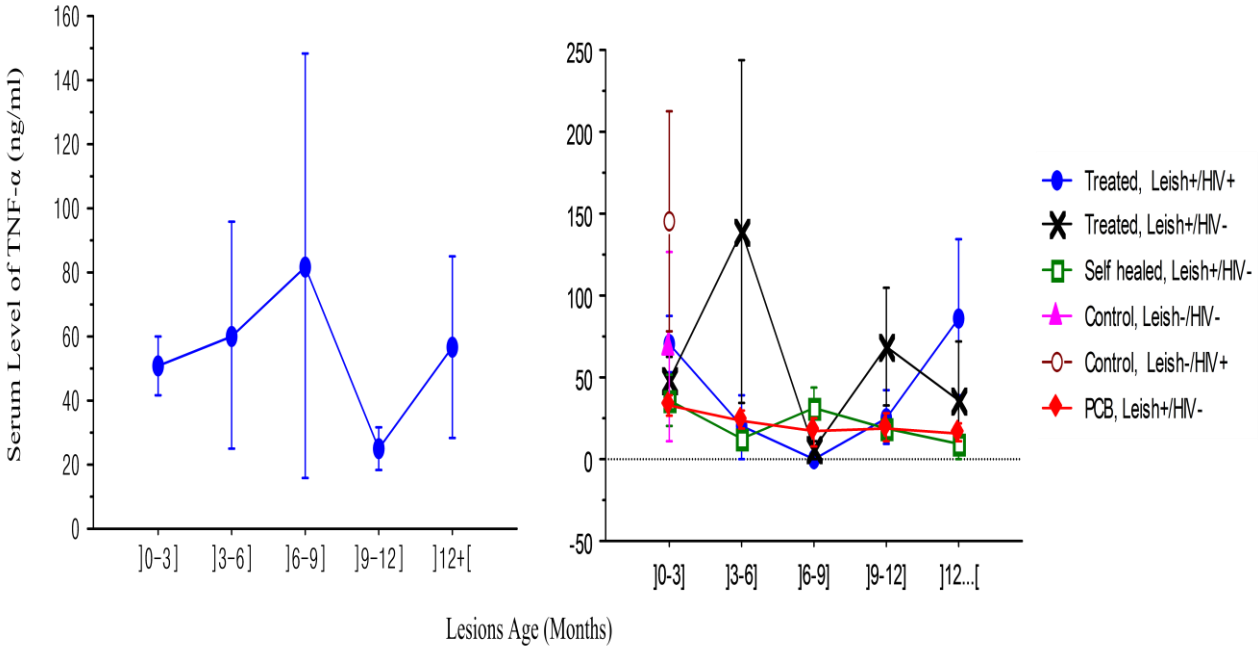

Figure 91: Changes occured in serum TNF-α level during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

iii. Th2/Treg cytokines

- **Serum Interleukine-4**

Whether co-infected or mono-infected, CL patients presented low level of IL-4 in their serum. Nevertheless, Leish+HIV+ patients showed a tendency to produce a high amount of this cytokine as compared to only *Leishmania* infected individuals (Figure 92).

The serum level of IL-4 was low in self-healed patients and those who received only a placebo. However, at the early phase of the disease (first and second trimesters), this cytokine level was high in the serum of immunodepressed and patients who needed the antileishmanian therapy for lesions resolution. During lesions healing

process, IL-4 production reduced remarkably in immunodepressed patients (Figure 93).

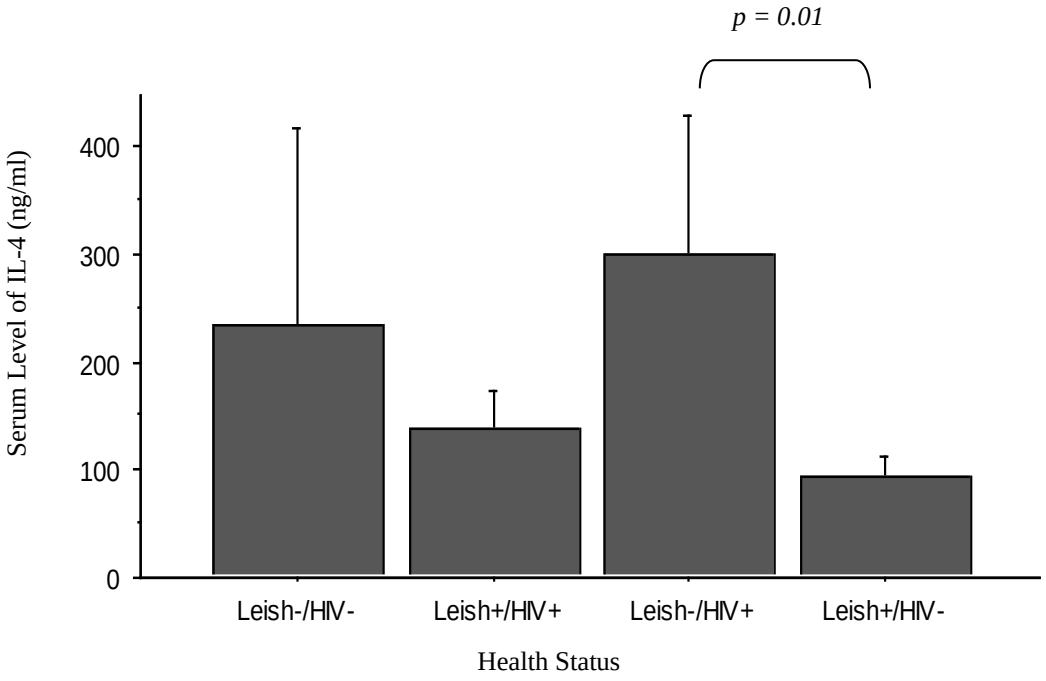

Figure 92: IL-4 level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV- : patients with leishmanaisis only (n=308), n is the number of samples analysed, data are mean ±SEM.

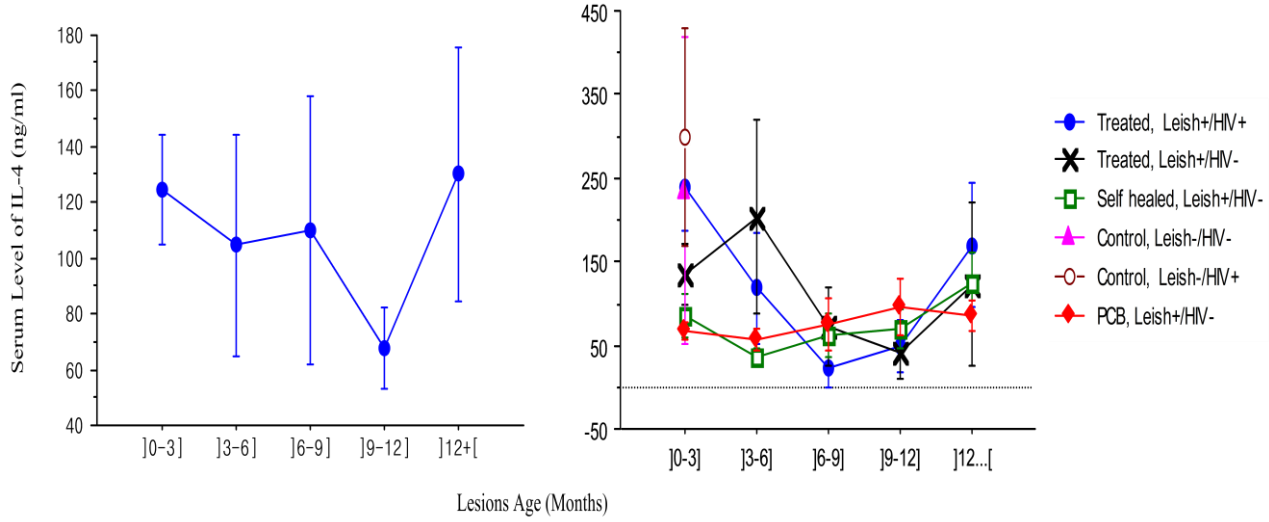

Figure 93: Changes occurred in the serum level of IL-4 during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV- : patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

- **Serum Interleukine-5**

The study of IL-5 showed that the serum level of this cytokine was significantly low in CL patients as compared to control groups (Figure 94).

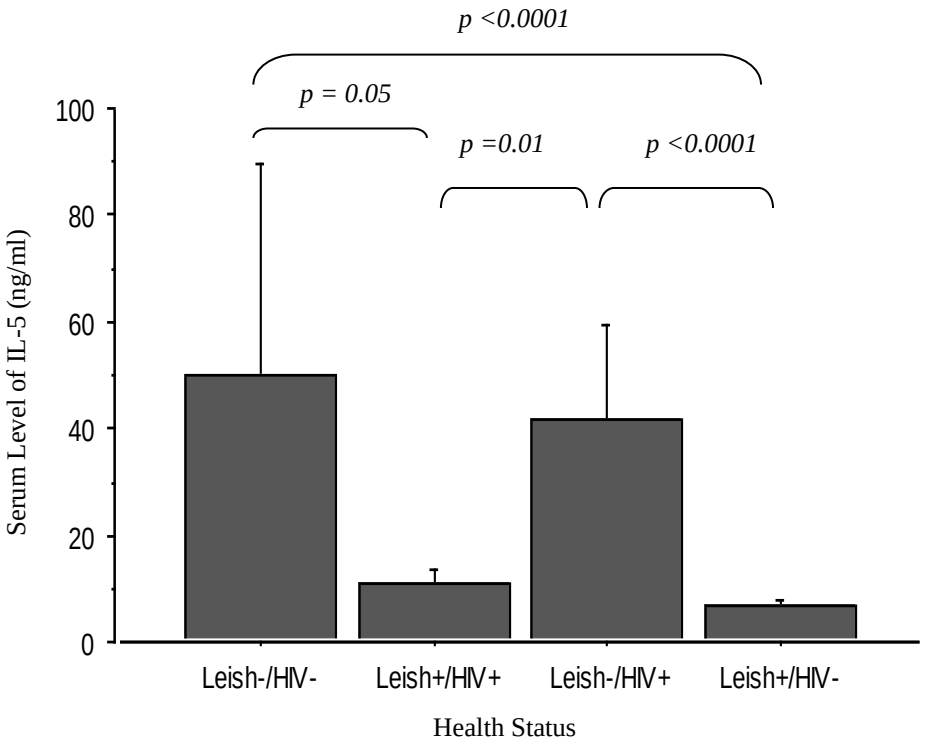

Figure 94: IL-5 level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV-: patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

Our statistical analysis showed a significant ($p = 0.002$) IL-5 up regulation in self-healed patients as compared to those with persistent lesions whether with a positive HIV serology or not. It was also noticed that in early lesions the IL-5 production was significantly ($p = 0.02$) high as compared to the production in the second trimester. We remarked that in the second and third trimester of lesions evolution co-infected individuals presented low level of this cytokine as compared to others groups (Figure 95). After this period of intensive manifestation of CL, the serum level of IL-5 generally increased with a very significant ($p = 0.005$) amount one year later.

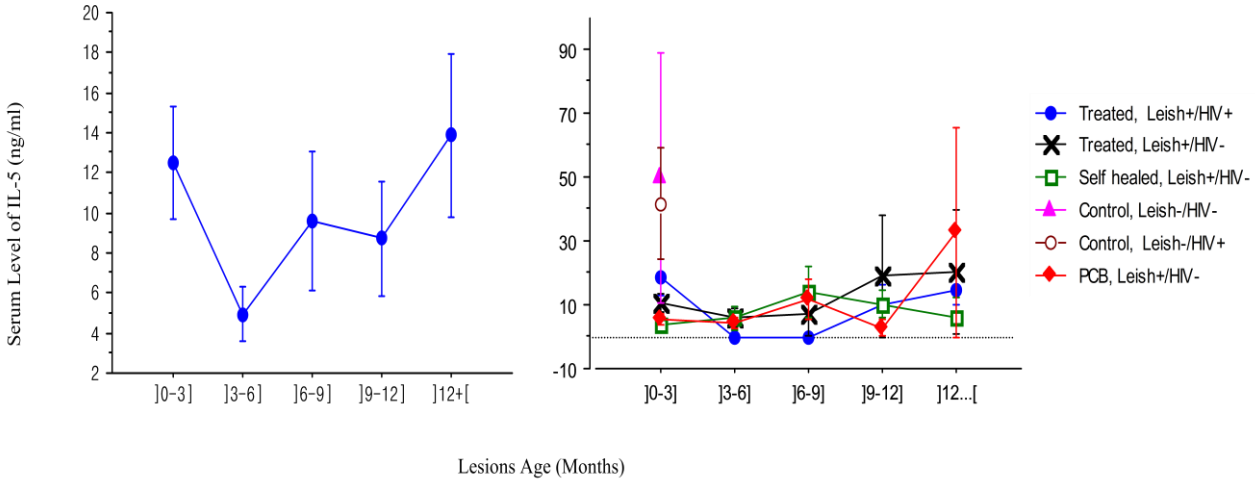

Figure 95: Changes occured in serum IL-5 level during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

- **Serum Interleukine-8**

Leish+HIV+ patients presented very low amount ($p=0.009$) of IL-8 in their serum as compared to CL patients with a negative HIV serology (Figure 96). A significant ($p = 0.05$) IL-8 production was also observed in self-healed patients as compared to those with persistent lesions who therefore received antileishmanian therapy.

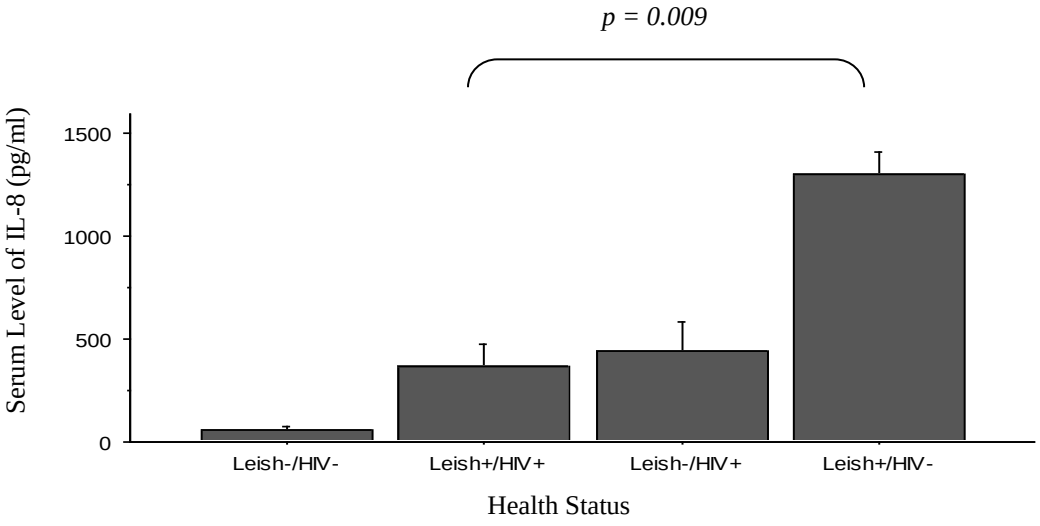

Figure 96: IL-8 level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV-: patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

As it is shown on the Figure 97, in the second trimester of the lesion evolution CL patients significantly ($p = 0.03$) produced more IL-8 than in the first trimester. Anyway, immunodepressed individuals always showed a low level as compared to self healed patients. Patients with more chronic lesions presented a significant ($p < 0.05$) low amount of this same cytokine.

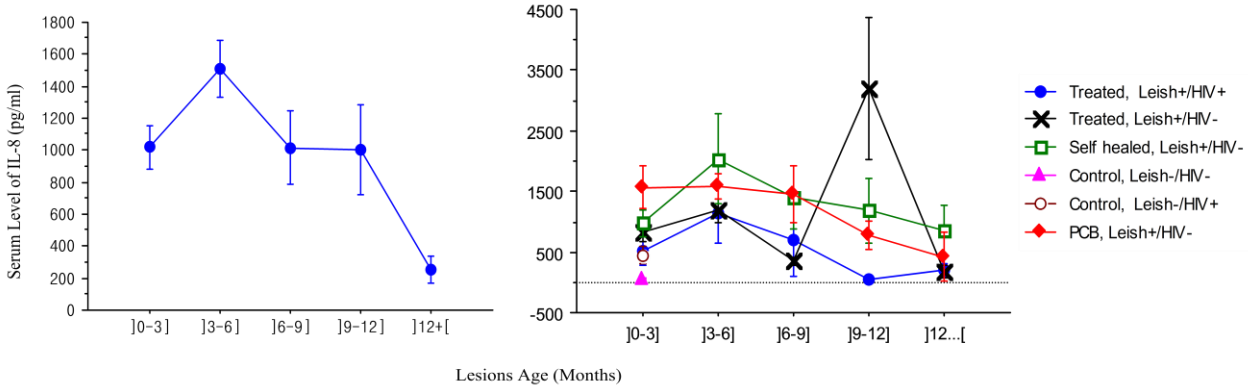

Figure 97: Changes occured in serum IL-8 level during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

- **Serum Interleukine-10**

As it is depicted on the Figure 98, CL patients produced fewer blood IL-10 than the negative control. Amongst CL patients, Leish+HIV+ subjects tended to produce a less amount as compared to Leish+HIV- individuals.

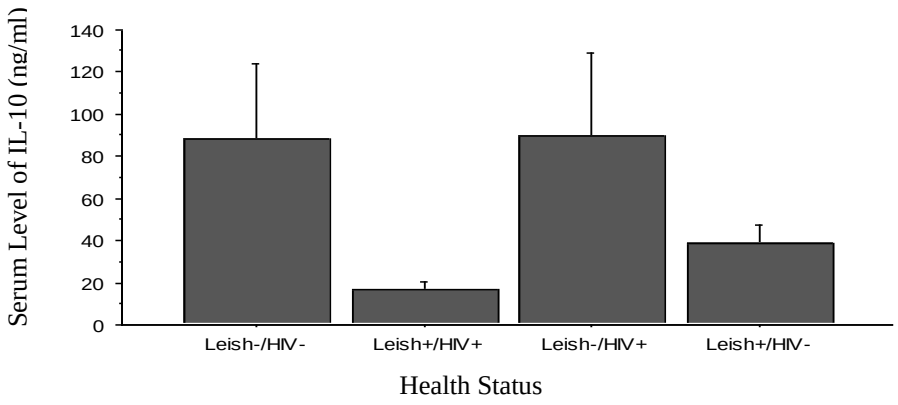

Figure 98: IL-10 level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV-: patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

During CL evolution, more susceptible patients and mainly Leish+HIV+ subjects showed lower production of IL-10 all the time (Figure 99). Remarkably, self-healed patients as well as patients who needed antileishmanian drugs showed a high IL-10 upregulation.

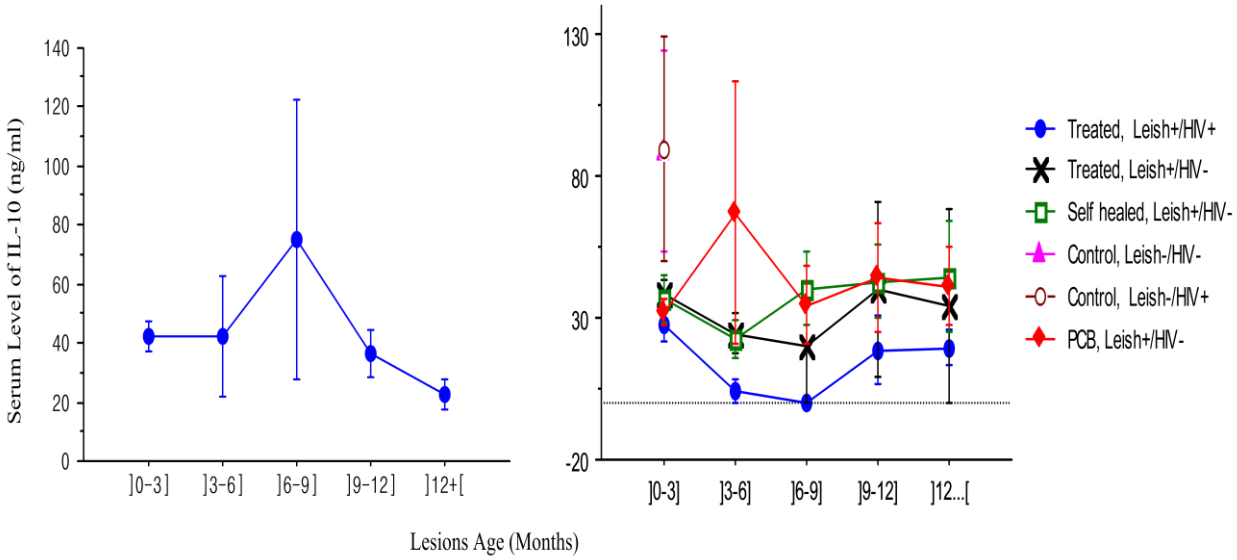

Figure 99: Changes occured in serum IL-10 level during lesions evolution and influence of the therapy and HIV status
Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

III.1.8.4 Correlations in some blood Th1/Th2 cytokines

High positive ($r \geq 0.7$) and significant ($p < 0.0001$) correlations were observed between IFN-γ, IL-1b, IL-4, IL-12p70 and TNF-α while weak positive correlations were observed between some other cytokines namely IL-8 and IL-6 ($r = +0.3$, $p < 0.0001$), IFN-γ and IL-10 ($r = +0.2$, $p < 0.0001$). In some cases, no significant correlation was observed. This general tendency was maintained and even reinforced in Leish+HIV+ patients.

Interestingly, we noticed that self-healed patients who neither received the leishmanicidal drugs nor the placebo showed a correlation between some blood Th1 and Th2 cytokines: IFN-γ/IL-4 ($r = +0.5$, $p < 0.0001$), IFN-γ/IL-5 ($r = +0.4$, $p < 0.0001$), IFN-γ/IL-10 ($r = +0.6$, $p<0.0001$), IL-2/ IL-4 ($r = +0.4$, $p = 0.001$), IL-2/IL-5 ($r = +0.4$, $p < 0.0001$) IL-2/IL-10 ($r = +0.6$, $p < 0.0001$) TNF-α/IL-4 (*0.8,*

p<0.0001)TNF-α /IL-10 (*r = +0.7, p < 0.0001*). On the other hand, in *Leishmania*-HIV co-infected patients, all these correlations were more pronounced e.g: IFN-γ/IL-4 (*r = +0.8, p < 0.0001*), IFN-γ/IL-10 (*r = +0.8, p < 0.0001*), IL-2/ IL-4 (*r = +0.8, p <0.0001*), IL-2/ IL-10 (*r = +0.9, p <0.0001*).

We then studied the association between IFN-γ, the main Th1 cytokine involved in the macrophage activation and the induction of the intracellular parasite killing by this cell, and others cytokines up regulated in the blood. On Table VI, it is shown that IL-1b, IL-4, IL-12p70 and TNF-α production were highly associated to IFN-γ up regulation.

Tableau VI: Mathematical association between INF-γ up-regulation and production of some serum cytokines

	IFN-γ (X)
IL-1b (Y)	Y = 0.652X + 2.914 ($r^2 = 0.759, p < 0.0001$)
IL-2 (Y)	Y = 0.143X + 42.712 ($r^2 = 0.102, p < 0.0001$)
IL-4 (Y)	Y = 1.654 X + 25.952 ($r^2 = 0.781, p < 0.0001$)
IL-5 (Y)	Y = 0.07X + 6.032 ($r^2 = 0.193, p < 0.0001$)
IL-12p70 (Y)	Y = 0.857X – 13.54 ($r^2 = 0.843, p < 0.0001$)
TNF-α (Y)	Y = 1.37X – 19.672 ($r^2 = 0.869, p < 0.0001$)

n=375

iv. Th17 axis cytokines

- **Serum Interleukine-6**

In general, it was noticed that, IL-6 production by Leish+HIV- subjects was higher than that of others individuals including co-infected patients who rather produced a very low amount of this cytokine. Leish-HIV- individuals did not show any up regulation of this cytokine (Figure 100).

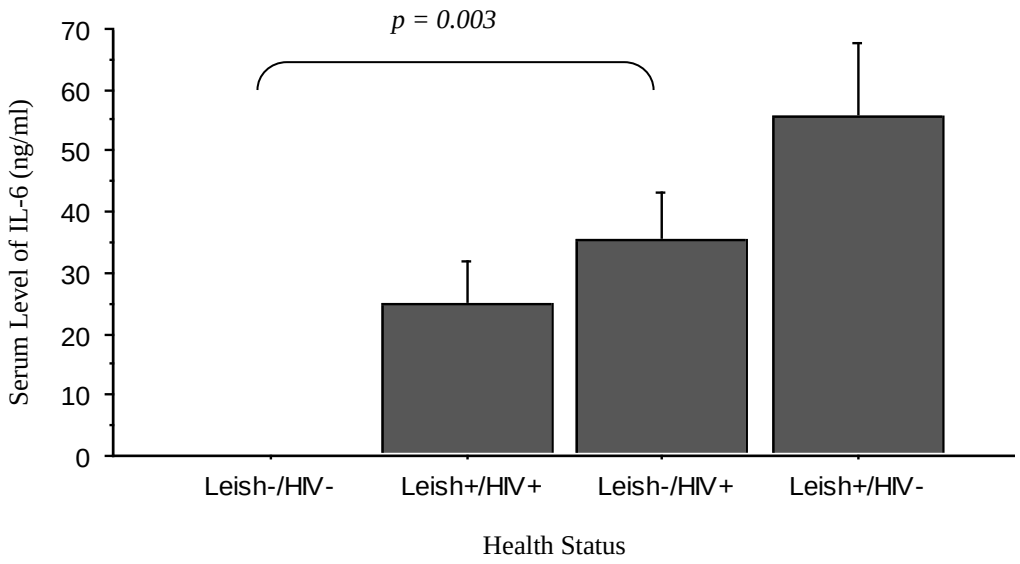

Figure 100: IL-6 level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV-: patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

In general, during the lesion evolution, the serum level of IL-6 tended to reduce in all the groups (Figure 101).

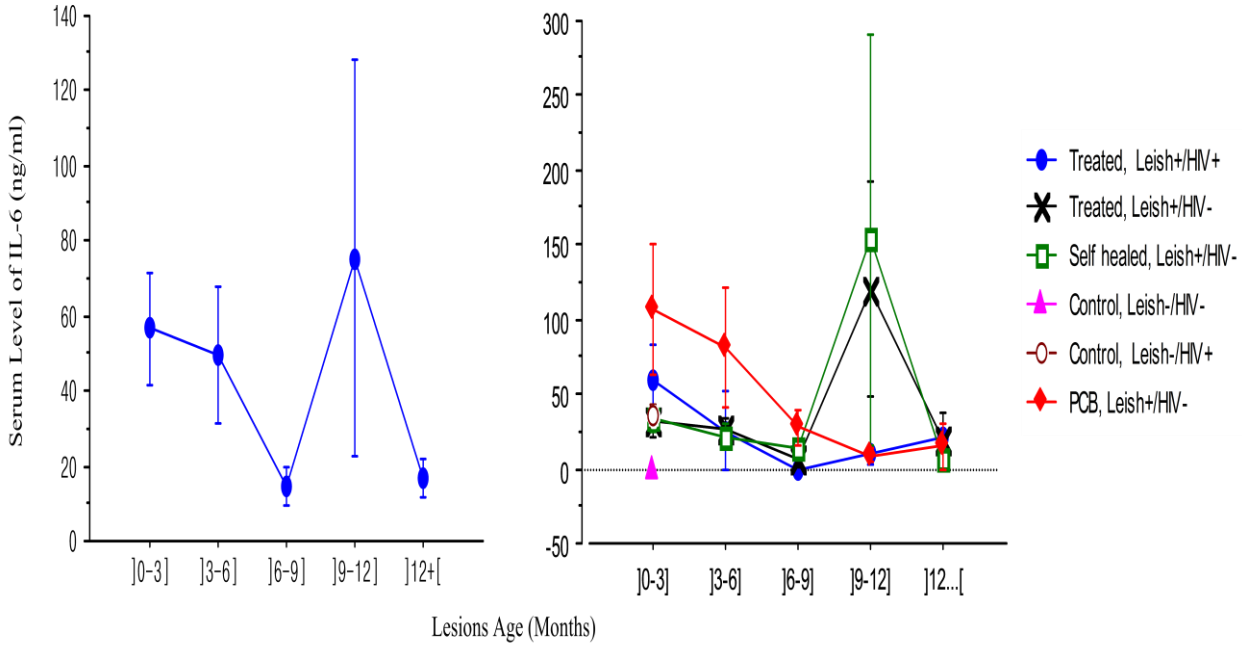

Figure 101: Changes occured in serum IL-6 level during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

➢ **Serum Interleukine-17A**

IL-17A was not significantly produced in the blood of CL patients whatever the HIV serology status (Figure 102).

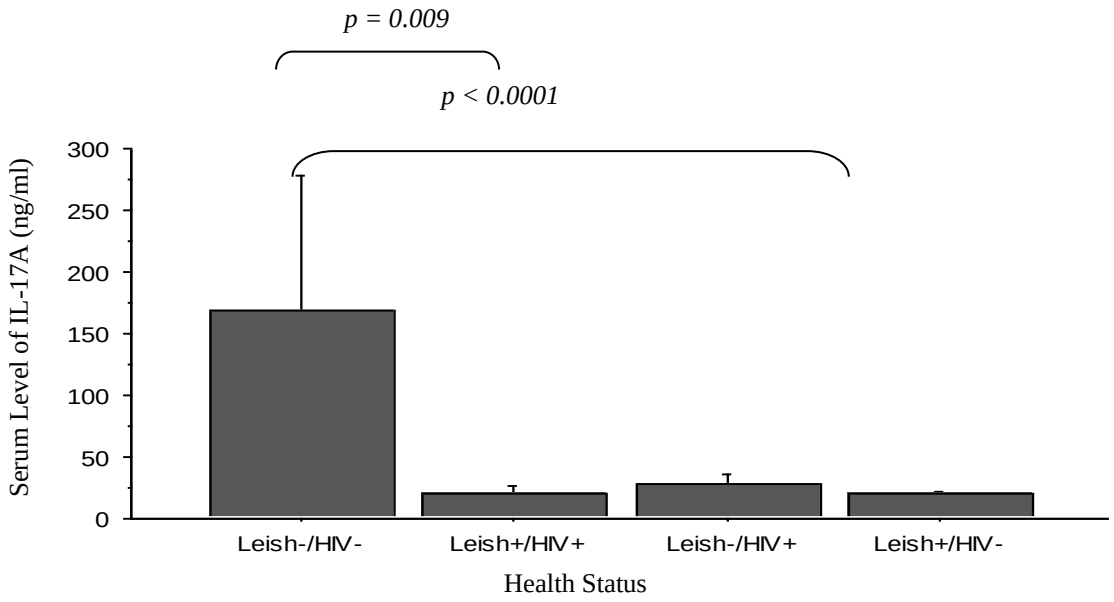

Figure 102: IL-17A level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV-: patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

Change in the serum level of IL-17A was not really visible during the lesion evolution (Figure 103).

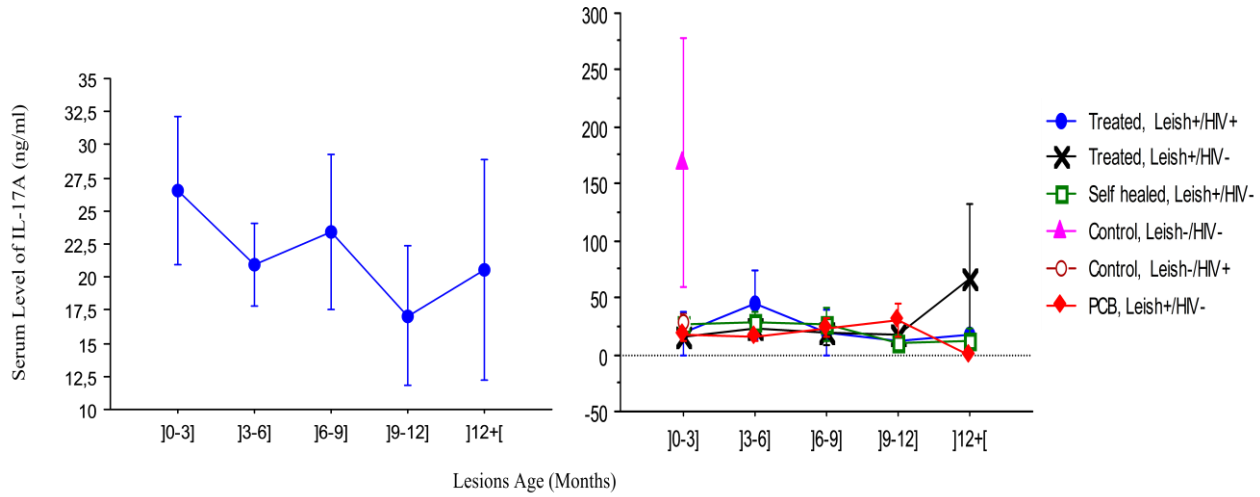

Figure 103: Changes occured in serum IL-17A level during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

- **Serum Interleukine-22**

The blood level of IL-22 was markedly very low on persons with only CL (*p=0.0003*). this is depicted on Figure 104.

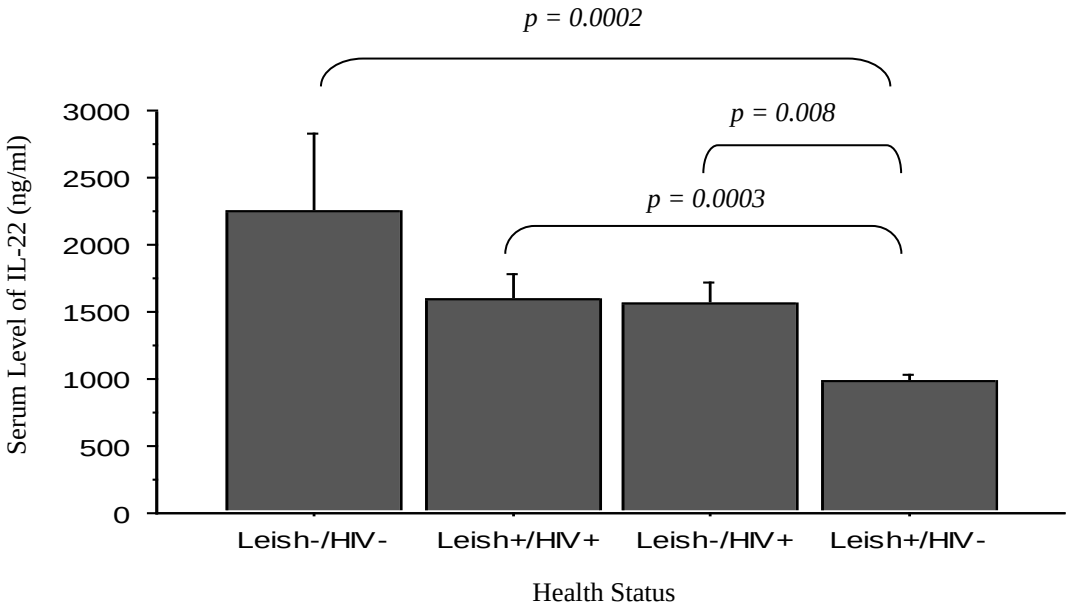

Figure 104: IL-22 level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV-: patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

As it can be observed on Figure 105, IL-22 blood level was in general higher in Leish+HIV+ patients during leishmaniasis evolution as compared to its level in Leishmania monoinfected individuals.

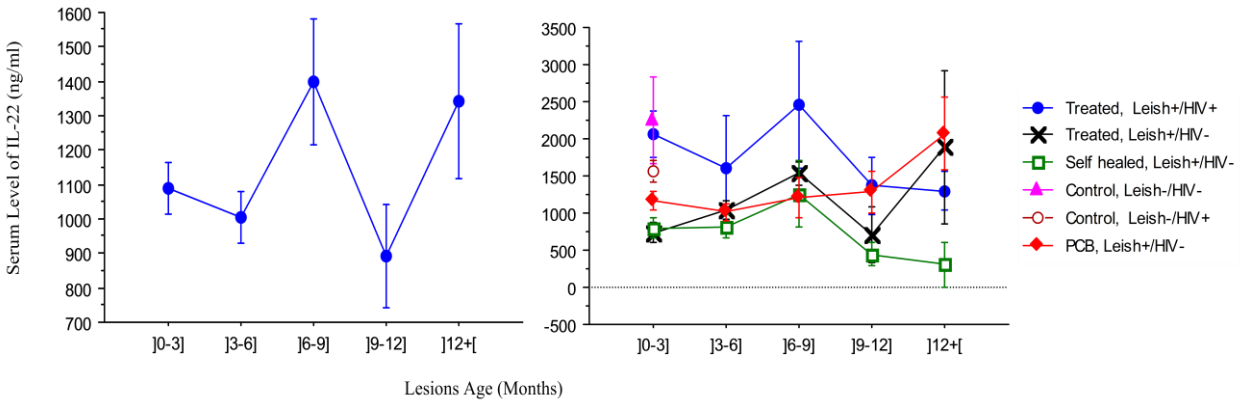

Figure 105: Changes occured in serum IL-22 level during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV- : patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

➢ **Serum Interleukine-23p19**

As it is discriminated in Figure 106, the IL-23p19 serum level was significantly (*p=0.0001*) high in leishmaniasis subjects infected by HIV as compared to patients with CL only. In the course of lesions evolution, the level of this cytokine tended to be in general maintained in the blood (Figure 107).

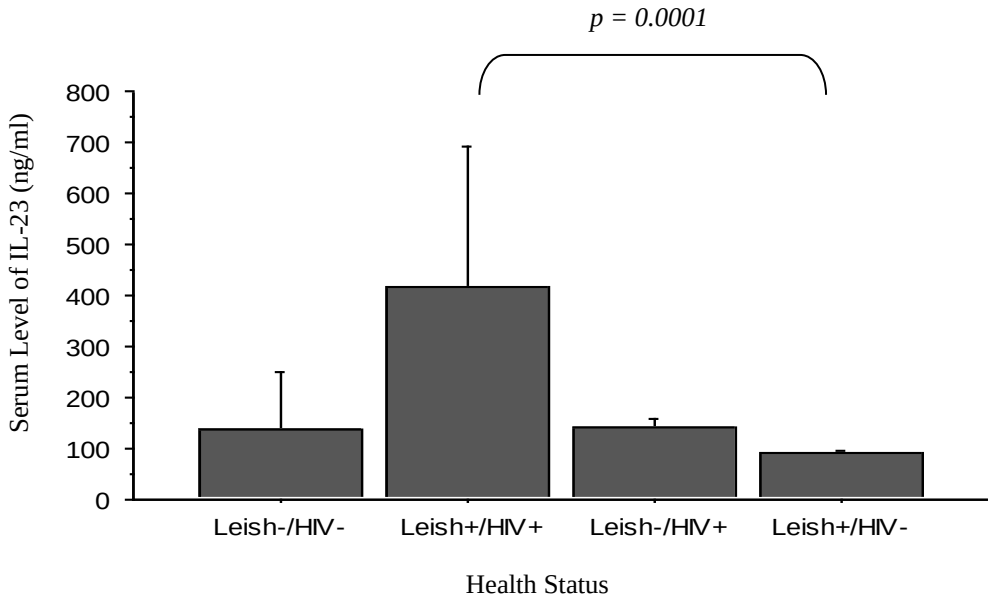

Figure 106: IL-23p19 level in the serum of patients presenting cutaneous leishmaniasis and/HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV-: patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

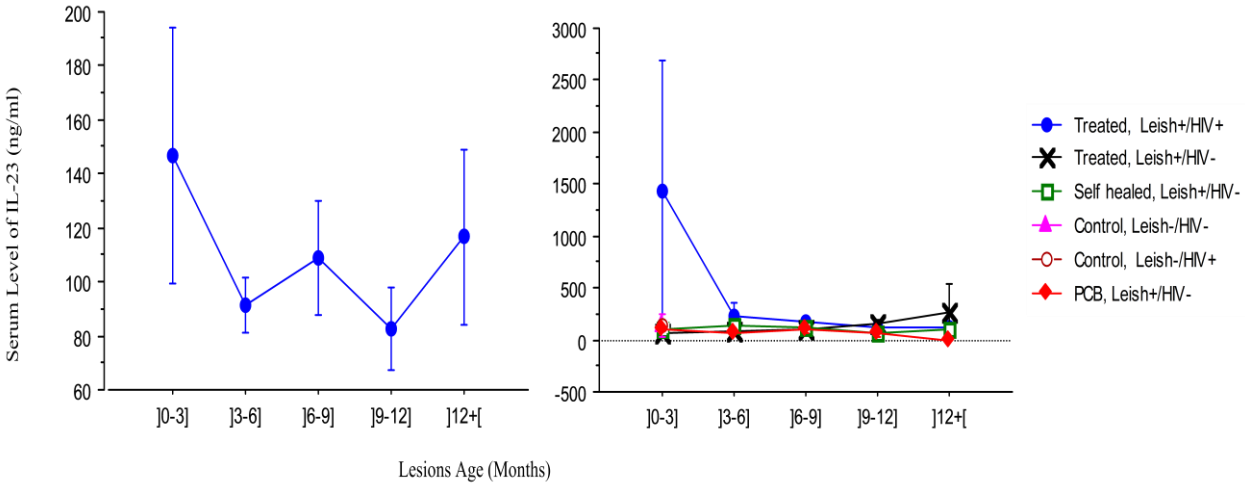

Figure 107: Changes occured in serum IL-23 level during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

v. Chemokines

- **Monokine induced by IFN-γ**

HIV+ patients presented more monokine induced by IFN-γ (MIG) in their blood as compared to HIV-. Thus, *Leishmania*/HIV co-infected individuals showed a significantly up regulation of MIG in their blood as compared to patients with CL only (Figure 108).

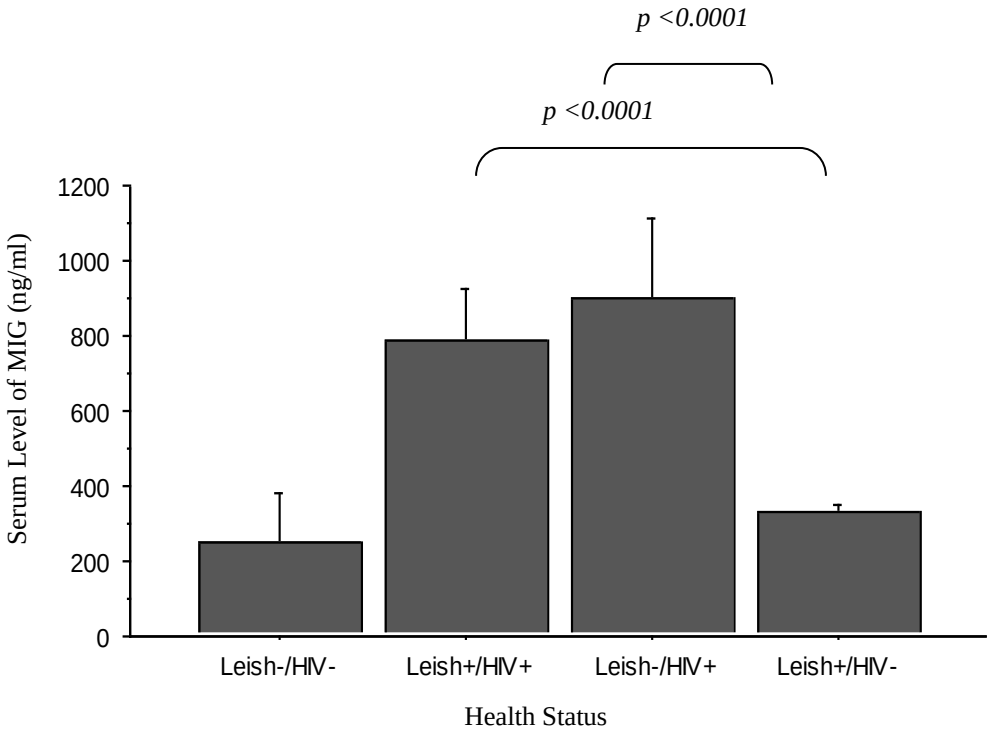

Figure 108: MIG level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV-: patients with leishmanaisis only (n=308), n is the number of samples analysed, data are mean ±SEM.

In the course of lesion evolution, HIV+ subjects maintained a high level of MIG in the blood while patients with CL only showed a tendency to decrease their MIG after 6 to 9 months (Figure 109).

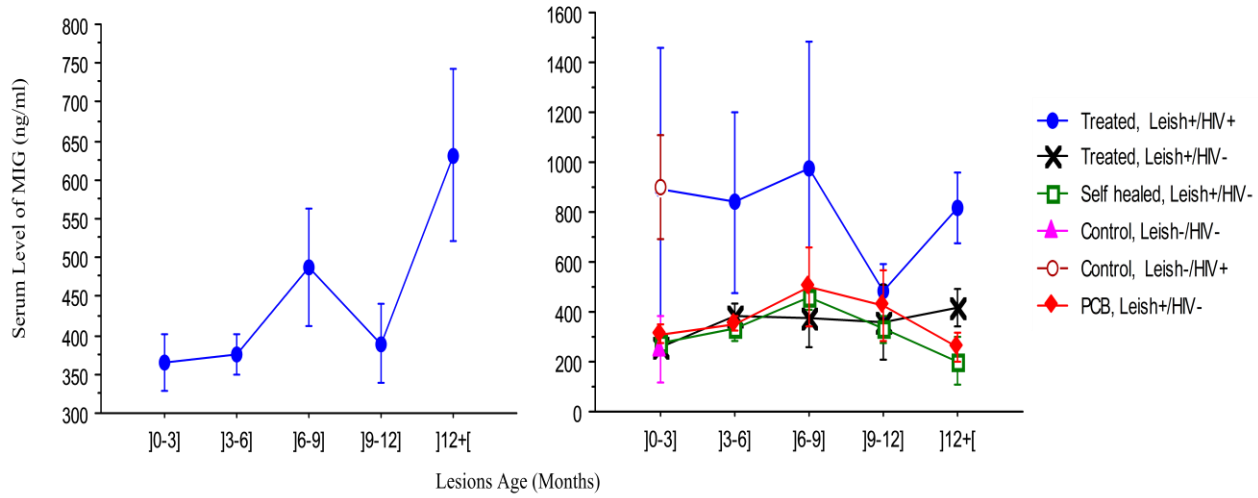

Figure 109: Changes occured in serum MIG level during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

- **Macrophage inflammatory protein-1α**

As it is illustrated in Figure 110, immunodepressed patients were unable to up regulate more macrophage inflammatory protein-1α (MIP-1α) in their blood.

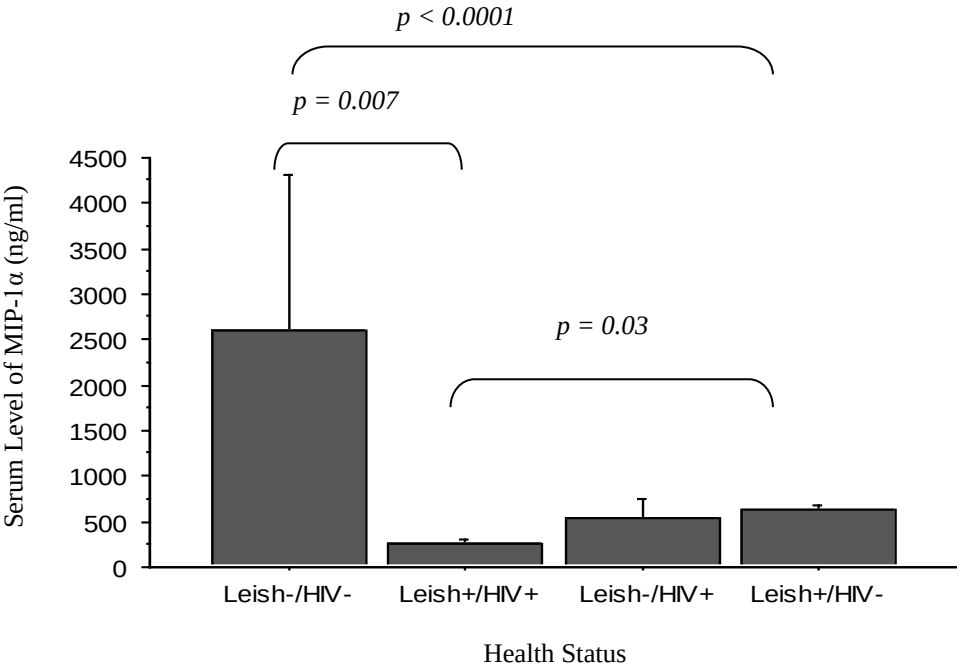

Figure 110: MIP-1α level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV-: patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

As it is shown on the Figure 111, in the second trimester of the lesion evolution CL patients produced more MIP-1α than in the first trimester. This production droped later during the healing process.

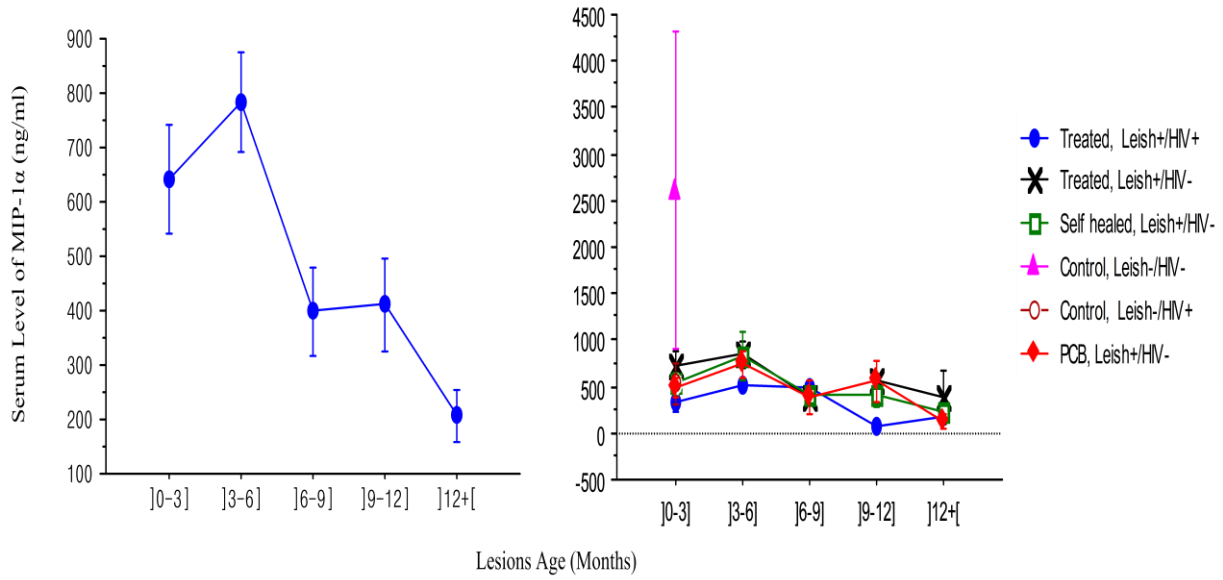

Figure 111: Changes occured in serum MIP-1α level during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

- **Macrophage inflammatory protein-1β**

Macrophage inflammatory protein-1β (MIP-1β) was poorly produced by Leish+HIV+ patients. This can be observed in Figure 112 showing a significant (*p=0.003*) high production of this chemokine in the blood of patients with leishmaniasis only compared to co-infected.

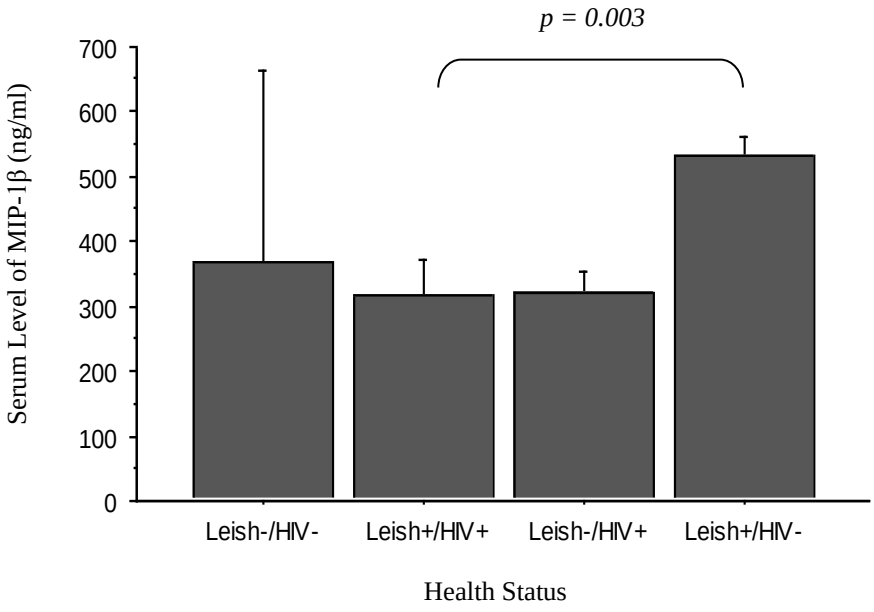

Figure 112: MIP-1β level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection
Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV- : patients with leishmanisis only (n=308), n is the number of samples analysed, data are mean ±SEM.

As it is shown on the Figure 113, in the second trimester of the lesion evolution CL patients produced more MIP-1β than in the first trimester. In general, during lesions evolution, immunodepressed individuals showed a low level as compared to self healed patients.

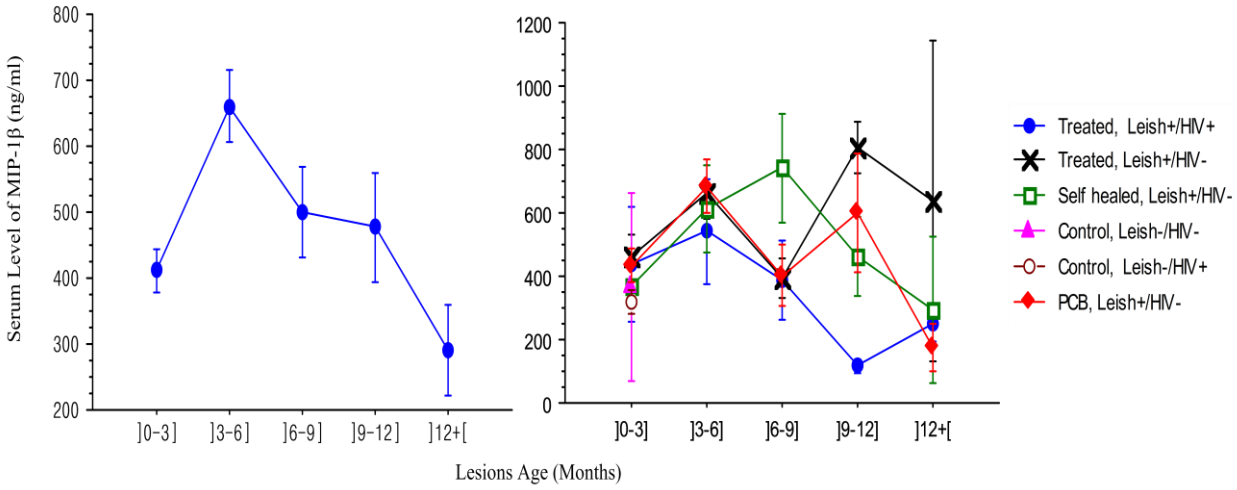

Figure 113: Changes in serum MIP-1β level during lesions evolution and influence of the therapy
Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

➢ **Monocyte chemoattractant protein-1**

The study of blood monocyte chemoattractant protein-1 (MCP-1) did not show a real difference between *Leishmania*/HIV co-infected subjects and those with a single leishmaniasis infection (Figure 114).

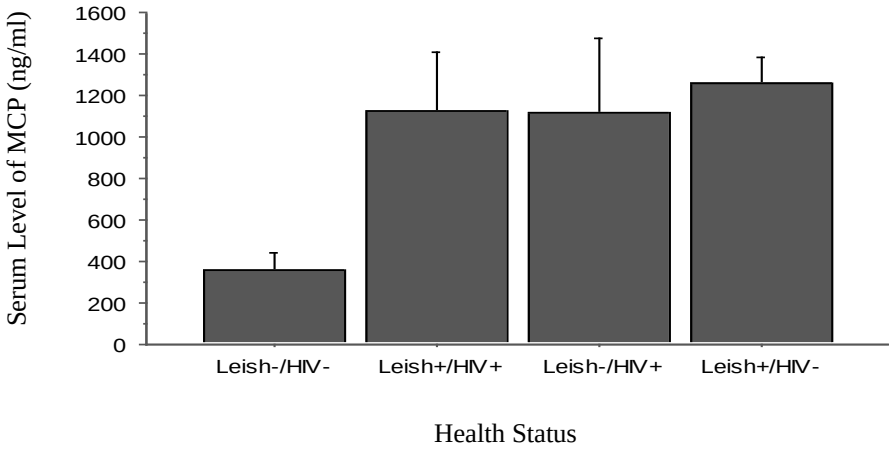

Figure 114: MCP-1 level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV- : patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

In the second trimester of the lesion evolution CL patients generally produced a significant (*p=0.0006*) amount of MCP-1 as compared with the first trimester. In old lesions (more than six months) patients also showed a low level of this chemokine (Figure 115).

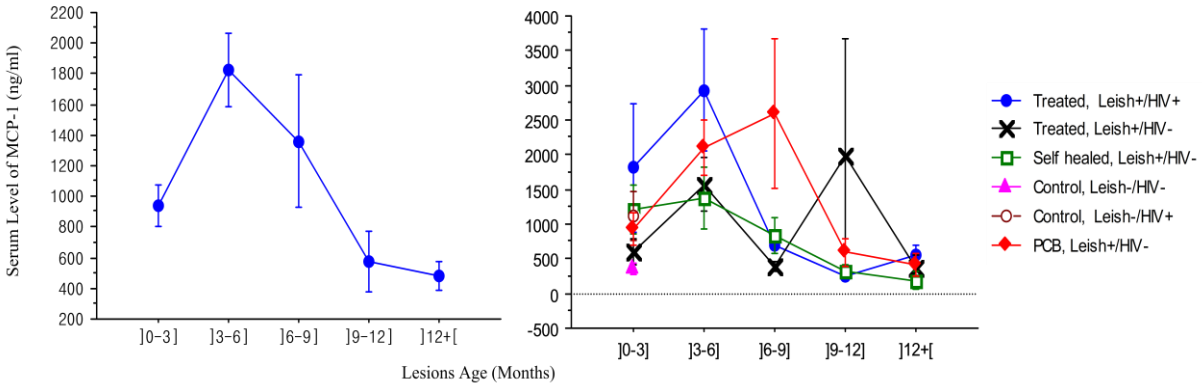

Figure 115: Changes occured in serum MCP-1 level during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

vi. Granulocyte-colony stimulating factor

As it is shown in Figure 116, the serum level of granulocyte-colony stimulating factor (G-CSF) was not significantly ($p>0.05$) influenced by the HIV serology status of a CL patient. During leishmaniasis evolution, G-CSF level in the blood was not significantly altered even after one year (Figure 117).

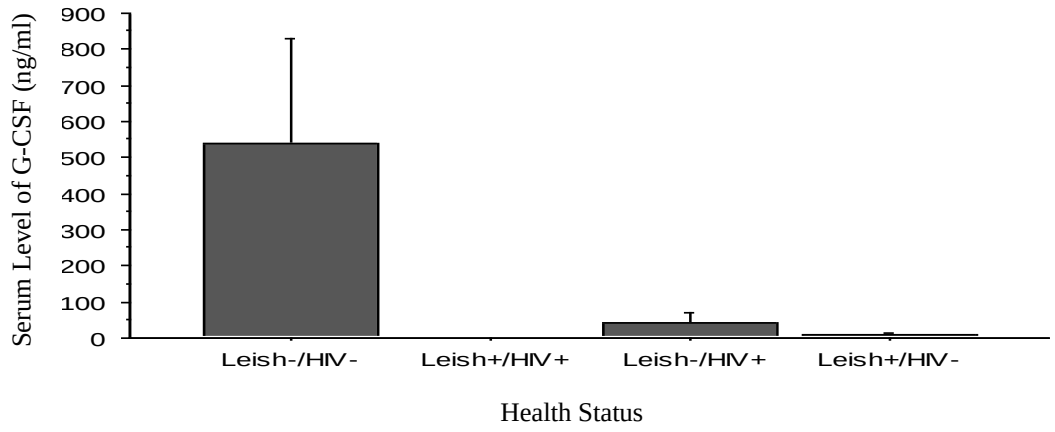

Figure 116: G-CSF level in the serum of patients presenting cutaneous leishmaniasis and/or HIV infection

Leish-HIV-: *Leishmania* and HIV double negative individuals (n=8), Leish+HIV+: *Leishmania*-HIV co-infected patients (n=28), Leish-HIV+: patients with HIV only (n=16), Leish+HIV-: patients with leishmaniasis only (n=308), n is the number of samples analysed, data are mean ±SEM.

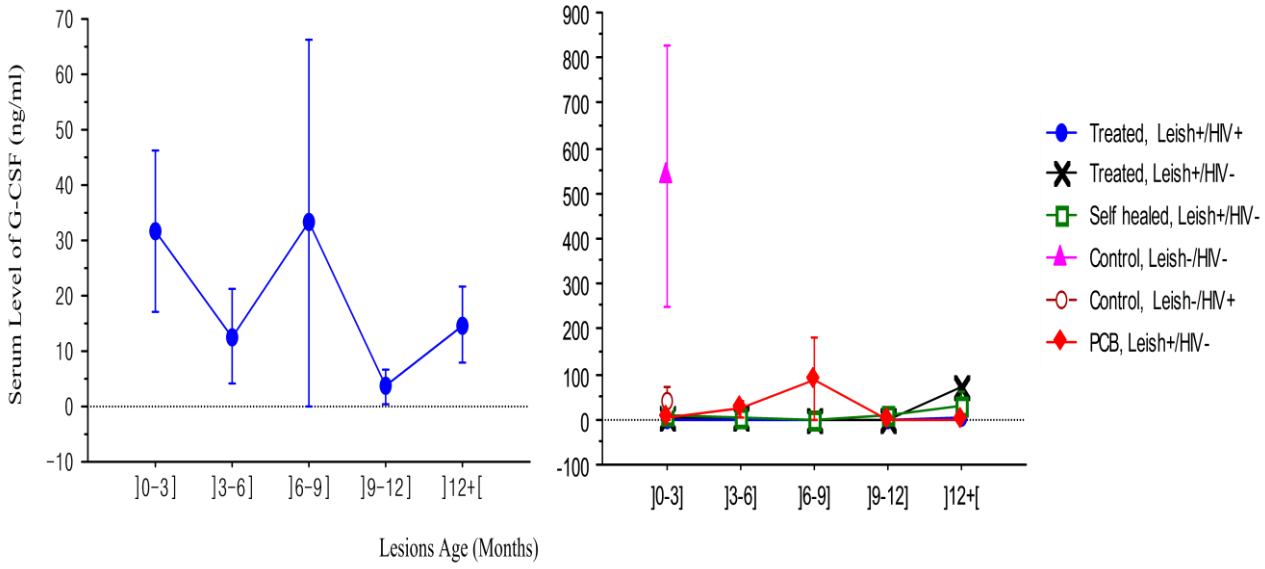

Figure 117: Changes occured in serum G-CSF level during lesions evolution and influence of the therapy and HIV status

Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish+HIV-: patients with leishmaniasis only, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish-HIV+: patients with HIV only, PCB: Placebo, data are mean ±SEM.

Regarding the systemic cytokines profil, we observed that there is no clear cut on the Th1/Th2 paradigm in human CL. As a cutaneous disease, CL needs local actors to be resolved. This is why it was necessary to go deeper in this study by investigating on local cytokines involved in this resolution.

III.1.9 Assessment of the local cytokines profil of cutaneous leishmaniasis patients

III.1.9.1 Expression of local cytokines at the cutaneous leishmaniasis lesion sites

The figure 118 shows some cells which infiltrated leishmaniasis lesions and which expressed local cytokines at these sites (in these cases IFN-γ and TGF-β). In the figure, positive cells expressing a given cytokine are red. Their number varied from one cytokine to another.

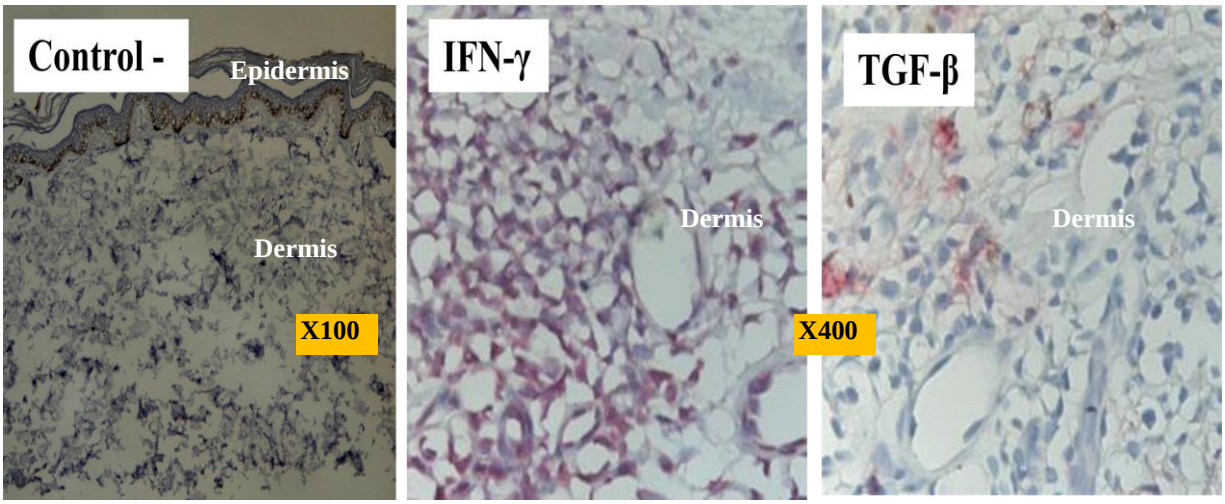

Figure 118: Samples of immune cells expressing cytokines at the lesion site
The negative control is the healthy skin, positive cells expressing cytokines are red.

III.1.9.2 Comparative representation of local cytokines at cutaneous leishmaniasis lesion sites

i. Local interferon-γ

Cells which expressed local IFN-γ were present in both epidermis and dermis of CL patients. The HIV status significantly influenced this local expression so that co-infected individuals showed a poor amount of cells of this category (Figure 119).

In the dermis, during CL evolution, IFN-γ+ cells were highly and significantly (*p=0.007*) expressed in the second semester of the disease as compared to the beginning. After this, the amount tended to reduce in the dermis of all the CL patients. From the ninth months after the onset of CL the number of this cell type was significantly (*p=0.01*) very low in the dermis (Figure 120).

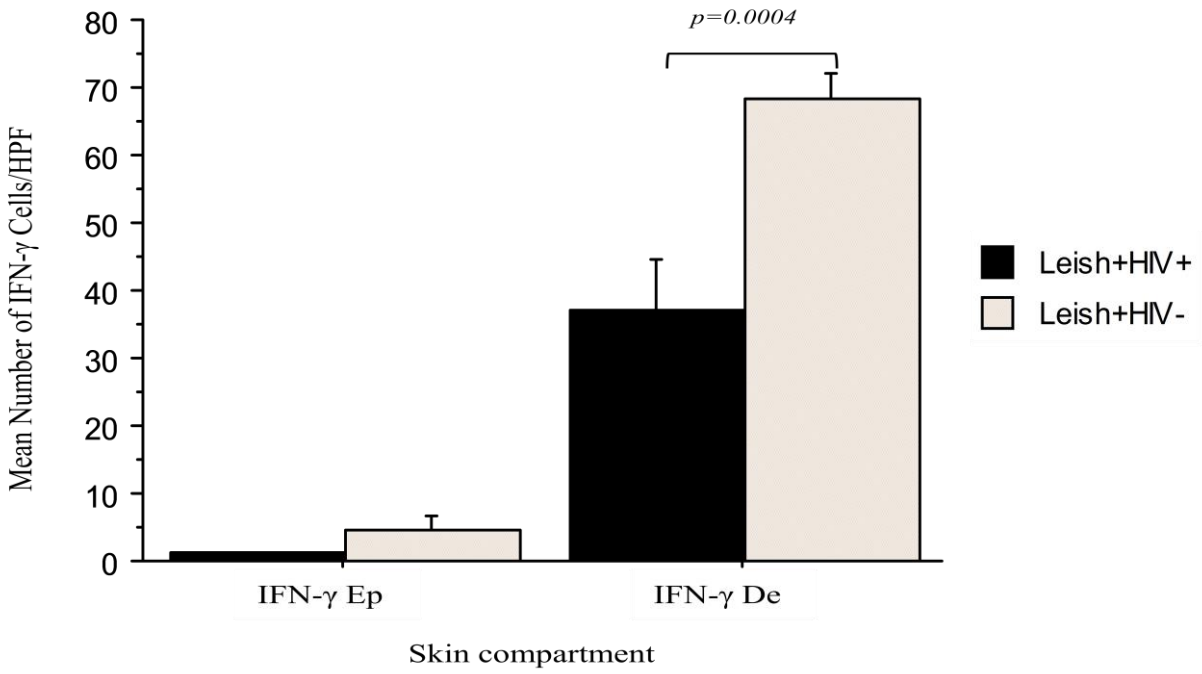

Figure 119: Local IFN-γ + Cells count in the skin of patients with cutaneous leishmaniasis and/or HIV infection

Leish+HIV+: *Leishmania*-HIV co-infected patients (number of total biopsies n=21), Leish+HIV-: patients with leishmaniasis only (number of total biopsies n=119), data are mean ±SEM.

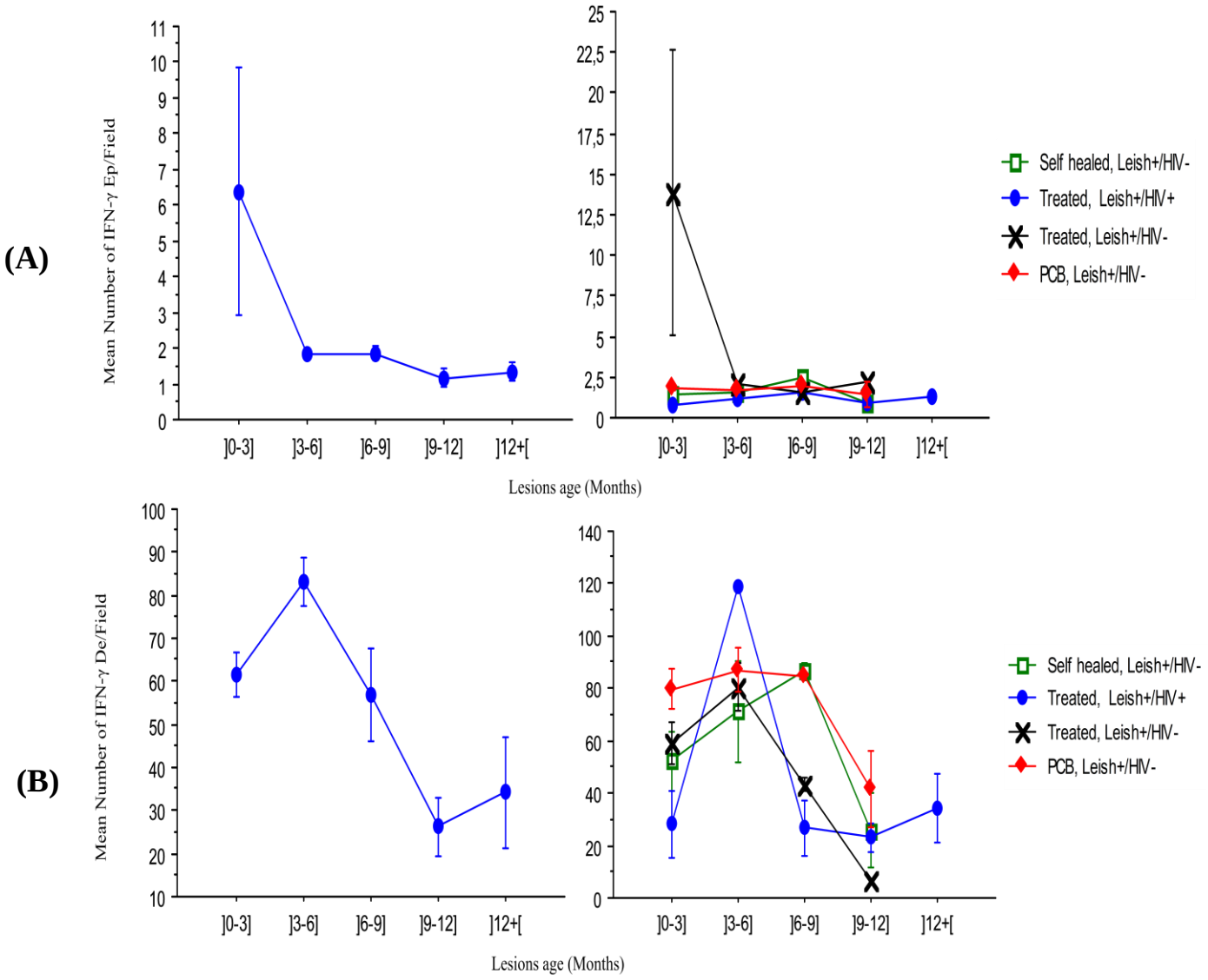

Figure 120: Changes in local IFN-γ+ cells count during lesions evolution and influence of the therapy

(A): Epidermis, (B): Dermis, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish-HIV+: patients with HIV only, Leish+HIV-: patients with leishmaniasis only, PCB: Placebo, data are mean ±SEM, for each age range $7 \leq n \leq 69$.

ii. Local interleukine-5

In both epidermis and dermis, patients with CL only tended to show a lower expression of local IL-5 when compared to Leish+HIV+ individuals (Figure 121).

During the CL evolution, the number of cells expressing the IL-5 cytokine fluctuated (Figure 122).

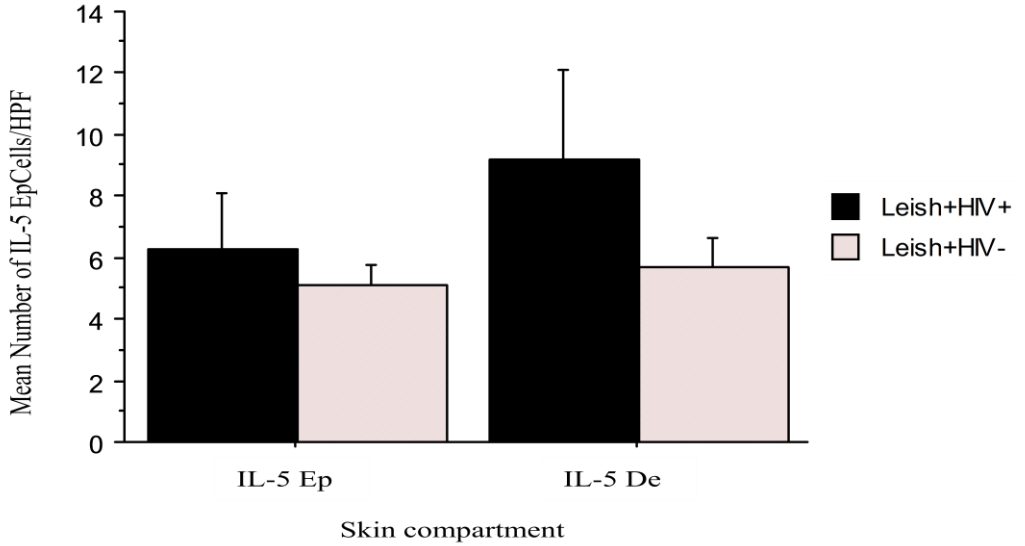

Figure 121: Local IL-5+ Cells count in the skin of patients with cutaneous leishmaniasis and/or HIV infection

Leish+HIV+: *Leishmania*-HIV co-infected patients (number of total biopsies n=21), Leish+HIV-: patients with leishmaniasis only (number of total biopsies n=119), data are mean ±SEM.

(A)

(B)

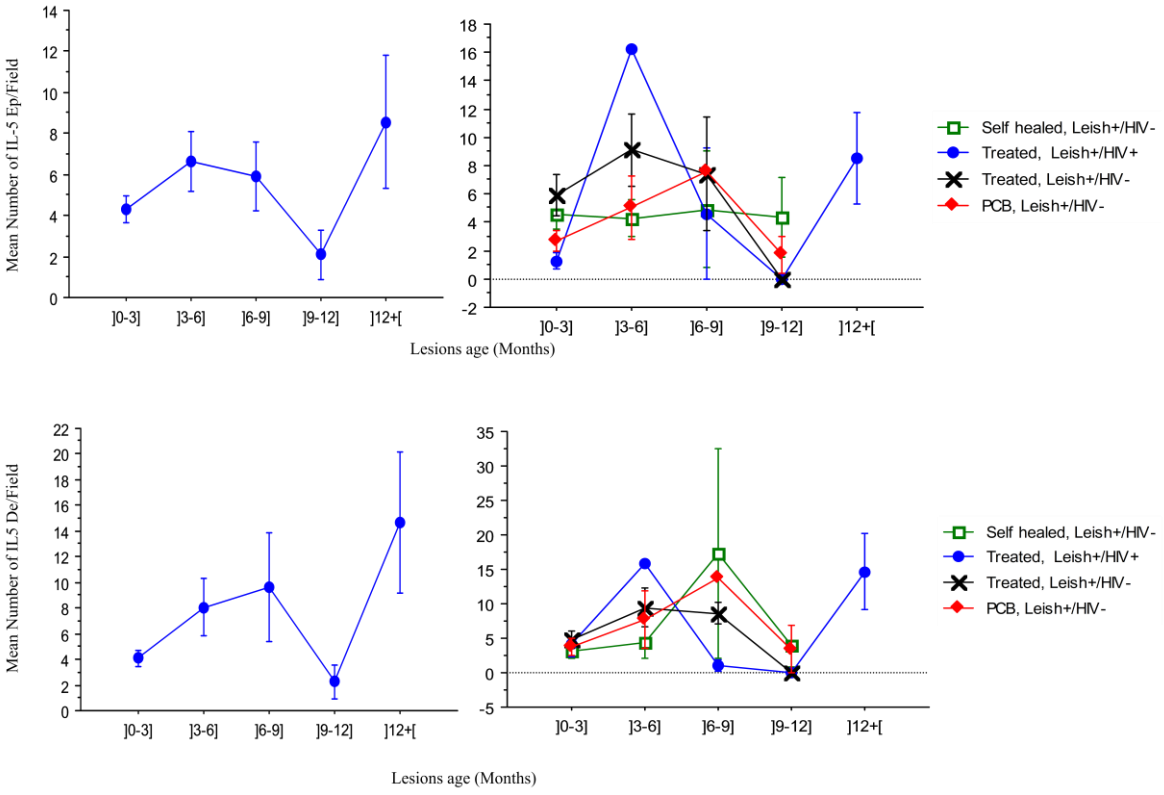

Figure 122: Changes in local IL-5+ cells count during lesions evolution and influence of the therapy and HIV status

(A): Epidermis, (B): Dermis, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish-HIV+: patients with HIV only, Leish+HIV-: patients with leishmaniasis only, PCB: Placebo, data are mean ±SEM, for each age range $7 \leq n \leq 69$.

iii. Local interleukine-6

The HIV serology status had a real influence on the expression of local IL-6. In fact, in both epidermis and dermis, *Leishmania*-HIV co-infected patients showed a significant low expression of this cytokine as compared to subjects with CL only (Figure 123).

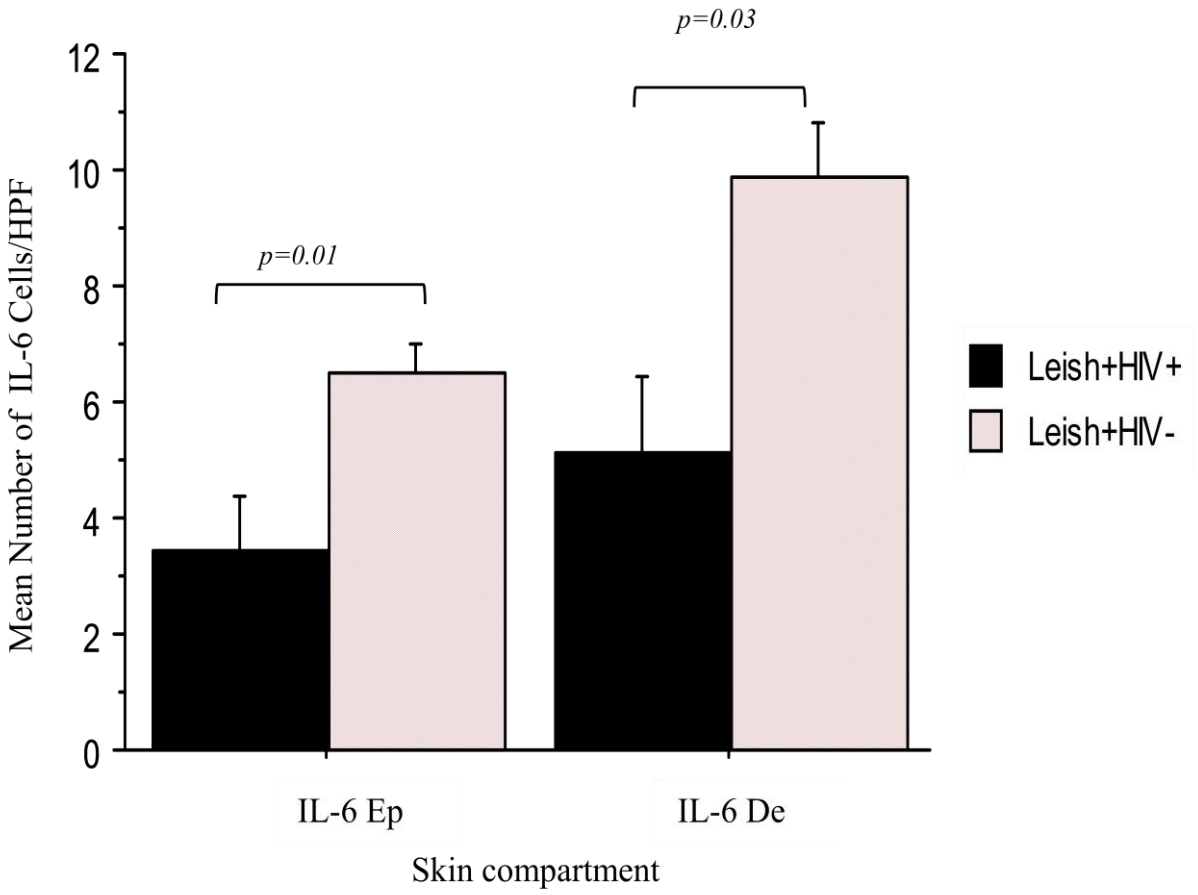

Figure 123: Local IL-6 + Cells count in the skin of patients with cutaneous leishmaniasis and/or HIV infection
Leish+HIV+: *Leishmania*-HIV co-infected patients (number of total biopsies n=21), Leish+HIV-: patients with leishmaniasis only (number of total biopsies n=119), data are mean ±SEM.

Be it in the epidermis and in the dermis, the number of IL-6+ cells was low in very old (e.g. 9 months) lesions. It is in the second trimester of the lesion evolution that was registered the highest amount of dermal IL-6+ cells (Figure 124).

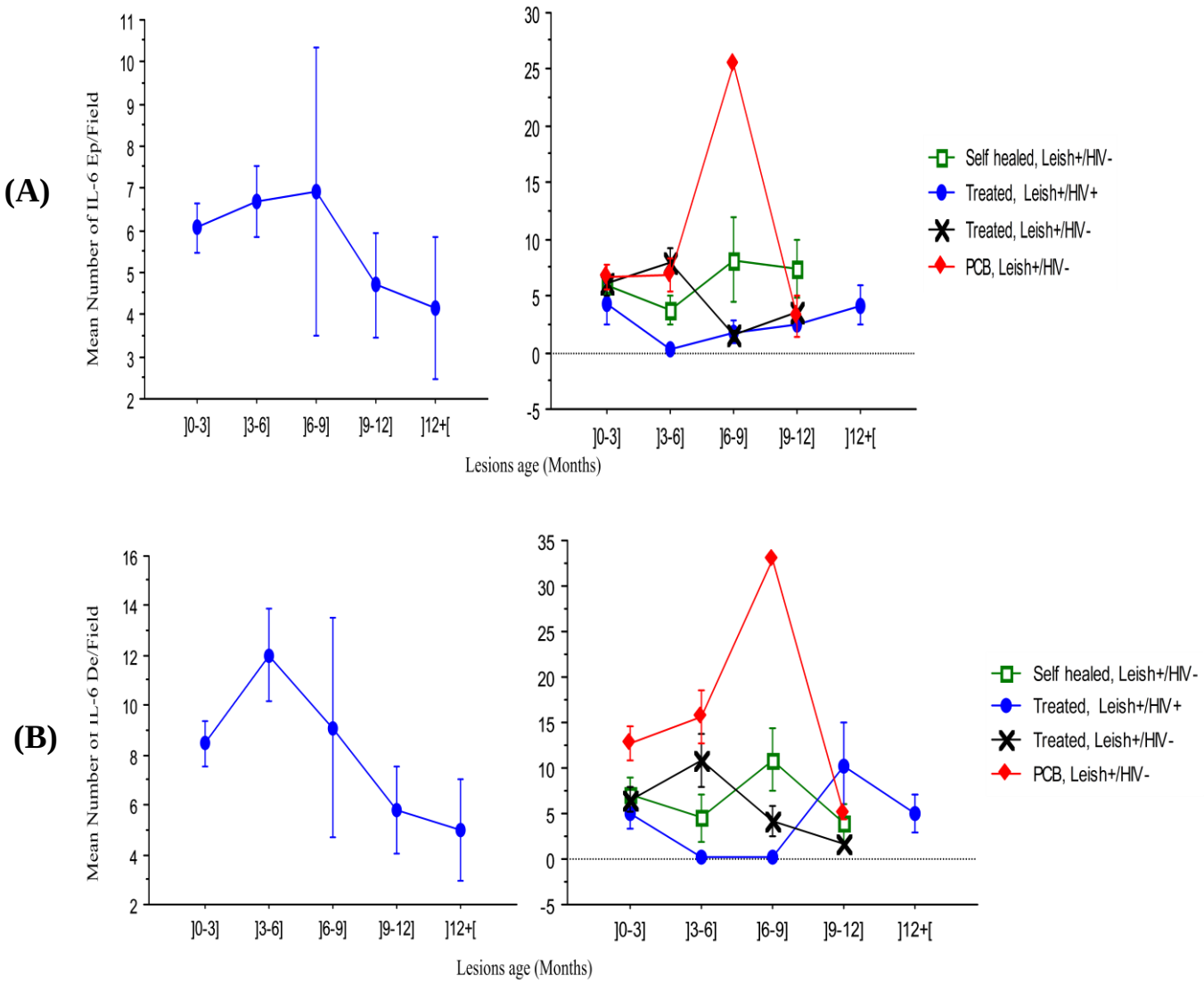

Figure 124: Changes in local IL-6+ cells count during lesions evolution and influence of the therapy and HIV status

(A): Epidermis, (B): Dermis, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish-HIV+: patients with HIV only, Leish+HIV-: patients with leishmaniasis only, PCB: Placebo, data are mean ±SEM, for each age range $7 \leq n \leq 69$.

iv. Local transforming growyh factor-1β

TGF-1β was very expressed in both epidermis and dermis. Nevertheless, there was no significant ($p>0.05$) difference in the expression of this cytokine between Leish+HIV+ and Leish+HIV- individuals (Figure 125).

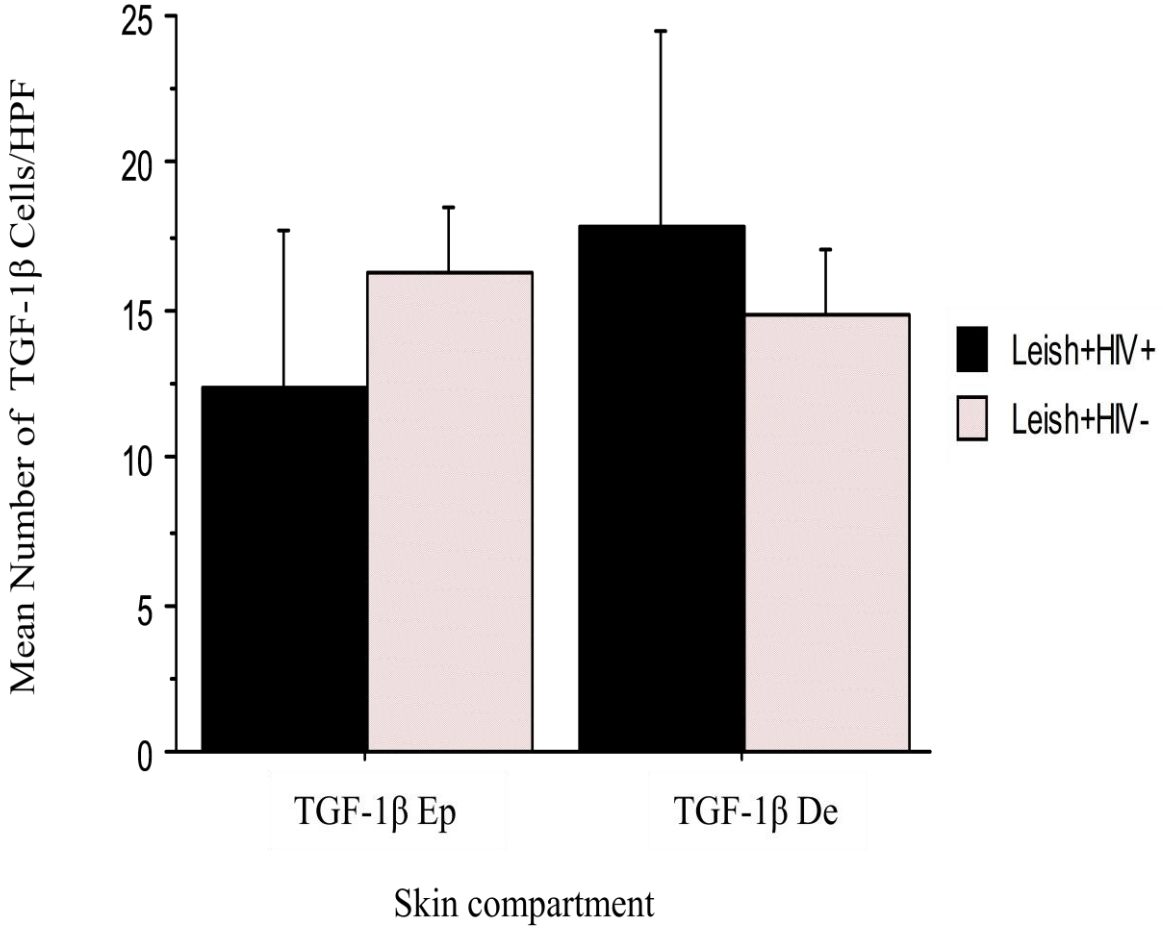

Figure 125: Local TGF-1β+ Cells count in the skin of patients with cutaneous leishmaniasis and/or HIV infection
Leish+HIV+: *Leishmania*-HIV co-infected patients (number of total biopsies n=21), Leish+HIV-: patients with leishmaniasis only (number of total biopsies n=119), data are mean ±SEM.

The Figure 126 shows the change occurred in the TGF-1β+ cells amount during CL evolution. The second semester of the lesion was characterized by more TGF-1β+ cells as compared to other periods. In old lesions, the number of these cells decreased more in the epidermis

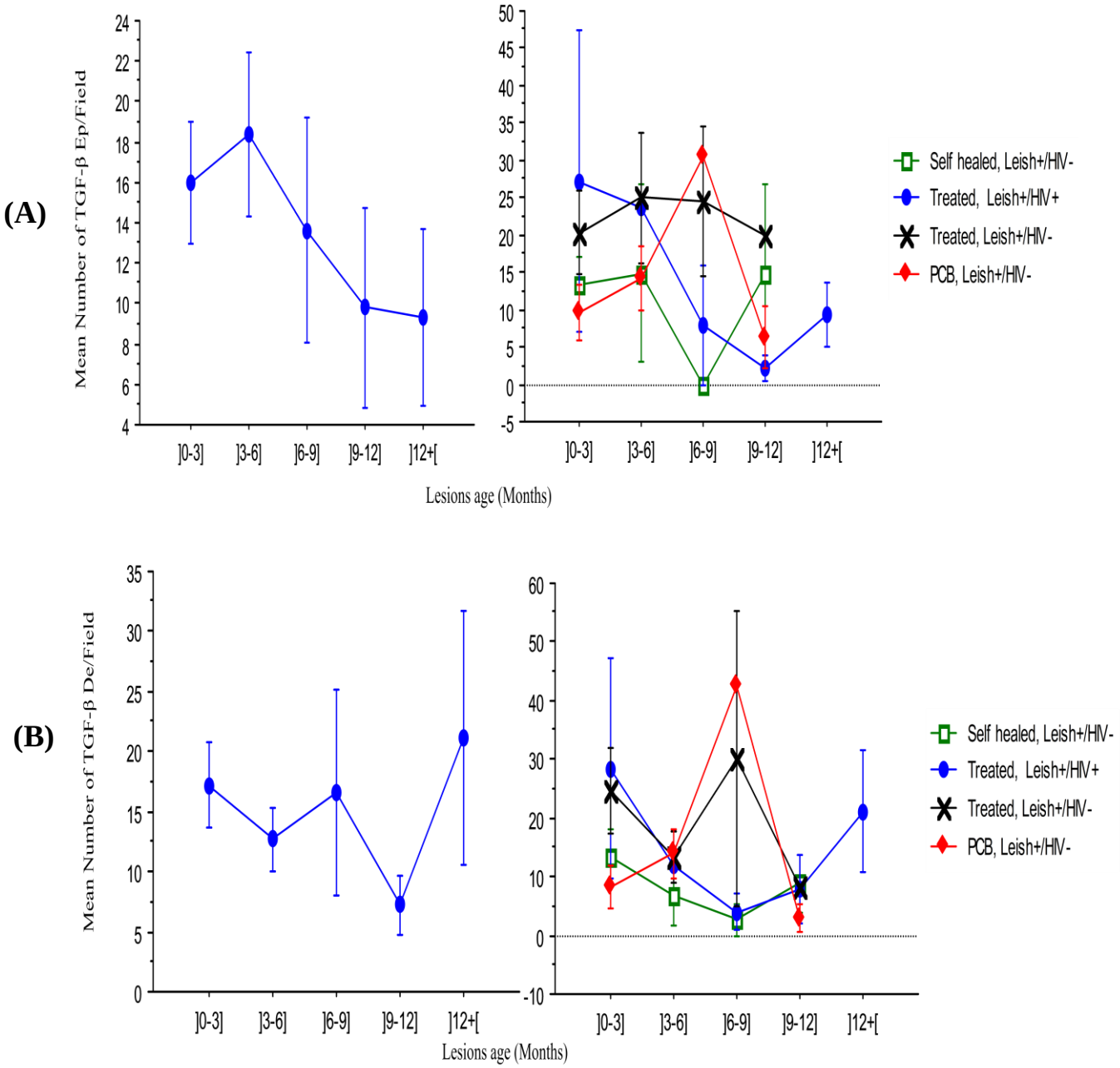

Figure 126: Changes in local TGF-1β+ cells count during lesions evolution and influence of the therapy and HIV status

(A): Epidermis, (B): Dermis, Leish-HIV-: *Leishmania* and HIV double negative individuals, Leish+HIV+: *Leishmania*-HIV co-infected patients, Leish-HIV+: patients with HIV only, Leish+HIV-: patients with leishmaniasis only, PCB: Placebo, data are mean ±SEM, for each age range $7 \leq n \leq 69$.

III.1.9.3 Correlation between local immune cells and local cytokines

There was a high and significant ($r = +0.6$, $p<0.0001$) correlation between cells expressing IL-5 and CD4+ cells but not with Foxp3+ cells which rather showed a high

and significant (*r = +0.5, p<0.0001*) correlation with IFN-γ. Activated Th1 cells (CXCR3+) also showed a correlation with this cytokine (*r = +0.5, p<0.0001*) (Table VII).

Tableau VII : Correlation between local immune cells and local cytokines

	Immune Cells								
Cytokines	**CD1a**	**CD4**	**CD8**	**CD20**	**CD54**	**CD68**	**CD83**	**CXCR3**	**Foxp3**
IFN-γ	r=+0.3 p=0.01	r=+0.2 p=0.01	r=+0.2 p=0.002	r=+0.4 p<0.0001	ns	r=+0.4 p<0.0001	ns	r=+0.3 p=0.0001	r=+0.5 p<0.0001
IL-5	r=+0.2 p=0.03	r=+0.6 p<0.0001	ns	ns	ns	ns	ns	ns	ns
IL-6	ns	r=+0.3 p<0.0001	ns	r=+0.4 p<0.0001	r=+0.2 p=0.003	r=+0.3 p=0.0005	ns	r=+0.2 p=0.003	r=+0.4 p<0.0001
TGF-1β	r=+0.2 p=0.01	r=+0.2 p=0.03	r=+0.2 p=0.05	r=+0.2 p=0.02	ns	r=+0.3 p=0.002	ns	r=+0.4 p<0.0001	r=+0.4 p<0.0001

ns= non significant p>0.05, n=140

III.1.10 Therapy regimens and blood cytokines production

The impact of the therapy regimens on cytokines production was evaluated one month after the beginning of the treatment. In all cytokines analyzed, there was no significant difference (*p > 0.05*) noticed between the Allopurinol regimen and the Amphotericin B/ Metronidazol regimen. Multivitamin treated patients expressed more IL-6 (*p = 0.05*) and more IL-8 (*p = 0.0002*) than AB+M treated subjects.These differences were not observed between non treated and treated individuals.

III.2 DISCUSSION

In most cutaneous leishmaniasis (CL) endemic countries, diagnosis is based mainly on clinical and epidemiological criteria (van der Meider *et al*., 2007). The presence of single or multiple nodular/ulcerative skin lesion(s) can be considered indicative of CL (Molina *et al*., 2003). Based on these criteria and on parasitological diagnosis, in the Cameroon Mokolo CL focus, we registered 146 patients presenting active lesions and 261 persons bearing suspected scars from a total of 32 466 subjects screened .

The prevalence of active lesions (0.4%) reported in the current study is less than 1.3% previously mentioned by Dondji in 1997. Regardless the fact that in Mokolo focus CL rate may be in reduction, this difference could be accounted for by the sampling method. Earlier studies conducted mass sampling rather than the door-to-door survey carried out in the present study. Consequently, in the previous study, only informed people and those able to travel to the meeting site were surveyed. The door-to-door sampling had access to all those living the geographical area surveyed and therefore provided a better picture of the disease. Another reason of this lower prevalence in our study could derive from the use of mosquito bednets. Mokolo residents have recently received from Cameroon government free pyrethrenoid-impregnated mosquito nets to prevent malaria transmission. It is known that the bed net usage reduces the numbers of mosquito bites and decreases the transmission of several insect borne diseases such as leishmaniasis (Das *et al.*, 2007; Vanlerberghe *et al*., 2010). Curtains impregnated with insecticides also provide a high degree of protection against indoor transmission of CL (Kroeger *et al*., 2002). In essence, our data confirmed that Mokolo remains an active endemic focus of CL to date.

Looking at the age of infected subjects, our findings showed that in Mokolo CL is more prevalent in individuals aged ≤ 20 years. Some authors like Padovese and co-wokers (2009) previously obtained similar results in their study area in Ethiopia. Although the behavioural study was not conducted, the low prevalence in aged individuals might be due not only to their behavioural pattern but also to their immune protection since a long last immunity is generally observed in *Leishmania* infected

persons as reported by Cruz and co-workers (1999). Regarding the gender of CL subjects, males of this same age bracket were more affected than females. Ashord and Jarry (1999) earlier mentioned that in youth, males are more concerned by leishmaniasis than females. Even if Alexander and his team (2010) reported that in mice, males are more susceptible to *Leishmania* than females in term of the parasite burden, we think that the difference may be more attributable to the behavioural pattern of boys who generally wear clothes that cannot cover many parts of their body, and who also have tendency to play outdoor thus exposing themselves to sand fly bites more than girls. This is in line with the idea of Deniau and Houin (1999) who reported that apart from the Indian focus, there is not real difference between sexes as concern leishmaniasis. These authors also justified such a difference in India by the high exposure of boys rather than their high sensitivity to *Leishmania* parasite. However, a tendency of more females bearing CL lesions in the whole population censed was noted and this was probably due to their higher representation in the sample (52.4% females vs. 47.6% males).

We also investigated the occupation of our patients and found that schoolchildren were the highly affected (49.7%) followed by farmers (23.5%). The same tendency was obtained in northern Ethiopia with 45% and 33 % respectively (Padovese *et al.*, 2009). As leishmaniasis is an insect-borne disease rather than the contagious one, the school milieu cannot be considered as a high contamination one. The high risk of schoolchildren may due to the fact that they generally belong to the more vulnerable age group i.e those who have the tendency to play without clothing as it is mentioned above.

Regarding the body site of the lesion, our patients presented more lesions on their limbs followed by the head (face). These are parts of the body more exposed to insects' bites. In the same focus, years ago, Djibrilla and collaborators (1979) and later Dondji (1997) registered the same trends. However, in Ethiopia, even if the same body parts are known to be the most affected, the head comes before arms and legs (Padovese *et al.*, 2009).

In our study area, the highest number of CL cases was remarkably recorded between August and December. This is a couple of months after the beginning of the rainy season. During this period, climatic conditions are favourable to the development

and the swarm of phlebotomine sand flies (Djibrilla *et al.,* 1979). Phlebotomine sand flies are usually active by night when the temperature is sufficiently high (19–20 ºC) and when the wind speed is approximately 1 m/s. The number of their generations is related to the duration of the hot season. Life expectancy of adult flies depends on the hygrometry and the temperature. With low temperature and high humidity, adult sand flies live longer (Léger & Depaquit, 1999). Dondji *et al.* (2000) previously recorded *Phlebotomus duboscqi* asthe suspected vector of CL in the Mokolo focus, during this season. Since the incubation period of CL can last from one to four months, or exceptionally from a couple of days to more than one year (Dedet, 1999c; CFSPH, 2009), the gap between the beginning of favourable conditions for the vector development and the outburst of new cases can therefore be easily understood.

We investigated on the HIV status of all the participants. Among CL-infected individuals, 4.8% were co-infected with HIV. Both HIV serotypes (HIV-1 and HIV-2) co-occured in CL patients of our study geographical zone. It is reported that, today leishmaniasis is emerging as the third most frequent infection associated with AIDS in various parts of the world, particularly in *Leishmania*-endemic countries (Pourahmad *et al*., 2009). The current data on *Leishmani*a/HIV co-infection available shows that 90% of cases are from southern Europe (France, Italy, Spain, and Portugal). However, these figures may be misleading, because a large proportion of cases in many countries of Africa or Asia might be underreported or undiagnosed due to lack of diagnostic facilities and poor reporting system (WHO, 2007). In Sub-Sahara Africa, cases of leishmaniasis associated with HIV infection have been reported from Senegal, Guinea Bissau, Mali, Cameroon, Ghana (Lartey *et al*., 2006), Senegal (Ndiaye *et al*., 1996), Ivory Coast (Kouassi *et al*., 2005) and Burkina Faso (Niamba *et al*., 2007). In Central African Sub-Region, leishmaniasis is unknown in many countries; cases have been documented from Cameroon and Chad. An imported case of CL from Chad was described in Central African Republic in 2003 by Kassa-Kelembho and co-workers. But a case of VL was previously described in this country in 60s (Cagnard et Lindec, 1969). Another cases were also mentioned in Congo in 60s (Prevot *et al.*, 1968) and Cameroon (Kaptué *et al*., 1992). To our knowledge, no CL case associated with HIV infection is documented in this African Sub-Region. Our study is the first report of HIV infections in CL subjects in Cameroon and Central Africa as a whole.

Padovese and collaborators (2009) working on cutaneous and mucocutaneous leishmaniases in northern Ethiopia reported 5.6% cases of co-infection with HIV. Among leishmaniasis infected individuals recorded during our investigation, 4.8% presented a HIV positive serology. In both studies, it is observable that around 5% of CL patients were HIV co-infected. This is fewer than in visceral leishmaniasis cases, since Pourahmad and co-workers (2009) mentioned that 25–70% of adult patients with visceral leishmaniasi are HIV positive. Also, 1.5–9% of patients with AIDS in south-western Europe suffer from newly acquired or re-activated visceral leishmaniasis (Puig & Pradinaud, 2003; Paredes *et al.*, 2003).

Leishmania major is a causative agent of cutaneous leishmaniasis (Badolato *et al.*, 1996). Nevertheless some other species are responsible for CL namely *L. tropica*, *L. mexicana, L. amazonensis, L. guyanensis, L. panamensis* (Patel & Shah, 2008). Species incriminated in CL in Mokolo focus has been previously identified by Dondji and collaborators (1998) as *L. major* Zymodem MON26. From both CL and *Leishmania*/HIV co-infected individuals registered in the present study, we also identified all isolates as *L. major*. In the year 2003, Pratlong *et al*. isolated *L. major* MON25 from localised cutaneous leishmaniasis patients with HIV infection in France. However, in northern Cameroon, *L. donovani* (Same-Ekobo, 1999) is suspected in the visceral leishmaniasis endemic focus of Kousseri. *L. major* is essentially zoonotic, but it can be responsible for anthroponotic leishmaniasis as well; it is present in many countries including Cameroon (Dondji *et al.*, 1998). The suspected vector in the Mokolo CL endemic focus is *Phlebotomus duboscqi* (Dondji *et al*, 2000; Same-Ekobo, 1999; Dondji, 2001).

It is reported that cutaneous leishmaniasis, on the exposed parts of the body, produces large numbers of skin ulcers, as many as 200 in some cases. In general, half of these lesions caused by *L. major* or *L. Mexicana* heal in 3 months, those caused by *L. tropica* take about 10 months and those due to *L. braziliensis* persist much longer (Patel & Shah, 2008). During the present study, patients presented a maximum of 20 lesions and the total surface ranged from 94.99 to 25 036.02 mm^2 per patient. In some cases, these lesions persisted more than one year. According to the CFSPH (2009), many CL lesions remain localized, but in some cases, the parasites may spread via the lymphatics and produce secondary lesions on the skin, or occasionally the mucosa, of

other parts of the body. Regional lymphadenopathy sometimes occurs. The presence of lymphangititis in some patients as well as an ectopic localization (armpit) of a lesion on one of our Leish+HIV+ patient is in line with this assertion.

Working on rats, Ballas & Davidson (2001) observed a delayed wound healing in aged rats and suggested that the age-associated healing delay in the rat may not be related to the appearance or abundance of distinct myofibroblast or apoptotic cell populations. Proteolysis may have a significant role in delayed wound healing in aged animals. In the present study, we demonstrated that healing process was not significantly influenced by the patient age, but in the same age range it is rather influenced by the HIV status.

There are reports on leishmanial infection preceding HIV in *Leishmania*-HIV co-infections, suggesting reactivation of subclinical leishmanial infections by HIV (Gradoni *et al.,* 1993). The converse also occurs with *Leishmania* parasites capable of increasing HIV-1 replication (Berhe *et al.*, 1999; Wolday *et al*., 1999; Olivier *et al*., 2003). Leishmaniasis recidivans (lupoid leishmaniasis), another rare form, is characterized by the development of new lesions around the edges of a healed skin lesion. It is most often caused by *L. tropica* or *L. braziliensis*, and it does not heal without treatment (CFSPH, 2009). In the present study, we reported leishmaniasis recrudescence after healing in some HIV infected subjects. It is believed that, following recovery from CL, a small number of viable parasites persist in the host (Botelho *et al*., 1998; Nateghi *et al*., 2010) and under immunosuppressive conditions; reactivation of leishmaniasis occurs in AIDS patients (Nateghi *et al.*, 2010). In our study, immunodepressed patients presented then many atypical clinical features. This result confirms that the clinical manifestations of leishmaniasis in the HIV-infected cover a particularly wide spectrum. Clinical forms that would be atypical in the immunocompetent are quite common in the immunocompromised (López-Vélez, 2003).

Early diagnosis and effective treatment of leishmaniasis would aid in the clinical management and eventual outcome of the disease (van der Meider *et al*., 2008). On a Burkina Faso patient with disseminated CL associated with HIV1 infection, skin lesions failed to respond to full treatment courses of Amphotericin B, sodium Stibogluconate, and liposomal Amphotericine B but were successfully treated

with Miltefosine, an alkylphosphocholine analogue (Schraner *et al*., 2005). During our investigations, we observed that the Amphotericin B / Metronidazol regimen or the Allopurinol regimen provided satisfactory outcomes in the therapy of CL in both Leish+/HIV- and Leish+/HIV+ subjects but with a longer delay for the co-infected patients. Interestingly, out of 7 Leish+/HIV+ subjects, only one was under antiretroviral therapy. From this therapy outcome, it is then remarkable that in HIV+ patients, a suit treatment of diseases such as leishmaniasis itself is of great interest to improve the patient health status.

As far as concern the treatment used by people before their first visit, we observed that in Mokolo, the majority of patients did not take any drug or they used non conventional antileishmanian drugs. On the other hand during our door-to-door survey we met people who naturally did not intent to reach the hospital while they presented suspected CL lesions. Leishmaniasis is known as a neglected tropical disease (Wacker & Lachat, 2006, Hotez *et al.,* 2009). We therefore remarked that the disease is not only neglected by pharmaceutical industries and medical personnel but also by the patient him/herself. Anyway, those who tried to combat the disease using empirical methods showed the improvement of their condition.

Dependently on some parameters like the patient age, the HIV status etc. our patients presented different clinical features as well as variable therapeutic outcomes. This is why we further explored their cellular and humoral reactions because it is reported that in murine models infected with *L. major* both the innate and acquired immune responses are necessary for parasite clearance (Hernández-Ruiz *et al.*, 2010).
It is documented that *Leishmania* lesions are characterized by the recruitment of anti-inflammatory cells such as neutrophils, eosinophils, blood monocytes (Badolato *et al*., 1996), lymphocytes and mast cells (Saha *et al*., 2004). The initial “silent” phase of the disease, lasting 4–5 weeks in mice, favors establishment of the peak load of parasites in the dermis in the absence of lesion formation or any overt histopathologic changes in the site. The second phase corresponds to the development of a lesion associated with an acute infiltration of neutrophils, macrophages, and eosinophils into the dermis and is coincident with the killing of parasites in the site (Belkaid *et al*., 2000). It is also known that *Leishmania* and HIV create a synergy for destruction of cell-mediated immunity and susceptibility toward opportunistic pathogens (Ezra *et al*., 2010). Like

HIV, *Leishmania* infection causes the depletion of T-helper cells; therefore, co-infection increases the risk and speed of immunosuppression (Pourahmad *et al*., 2009).

In the skin biopsies from our CL patients, H&E and Giemsa staining clearly showed a huge infiltration of anti-inflammatory cells at CL lesion sites. We further characterized some of these cells.

Dendritic cells (DC) are reported to be present in almost all tissues and they internalize antigens from environment with high efficiency (Ghosh & Bandyopadhyay, 2004). They are source of complements such as C3, C5, and C9 (Reis *et al*., 2006). In the skin, Langerhans cells and dermal dendritic cells are actively involved in the surveillance of their environment. These cells use phagocytosis, macropinocytosis, micropinocytosis, and receptor-mediated endocytosis to sample their environment and capture many types of exogenous molecules and microorganisms (Marovich *et al*., 2000). Dendritic cells are pacemakers of the immune response (Satthaporn & Eremin, 2001) and they are members of the innate immune system, which are deployed to determine whether a host response is needed and, if so, what kind of response (Lipscomb & Masten, 2002).They are then known as immunomodulators (Moll & Berberich, 2001). DC are professional antigens presenting cells (APC) (Henri *et al*., 2002) which are specialized in Ag capture and migration to secondary lymphoid organs and T-cells priming. It is reported that DC can induce primary immune responses thus permitting establishment of immunologic memory (Lipscom & Masten, 2002; Ghosh & Bandyopadhyay, 2004). They are known to activate both CD4 and CD8 T cells (Ruiz & Becker, 2007) and even B cells (Banchereau & Steinman, 1998; Satthaporn & Eremin, 2001). In leishmaniasis, it is suggested that the ability of *Leishmania* amastigotes to infect human DC involves the participation of multireceptor-ligand interactions namely antibodies/FcR, complements components/CR, and proteoglycan/heparin-binding protein (Bosetto & Giorgio, 2007). In mice, *L. major* activates draining lymph node and bone marrow DC through toll like receptor type 9 (TLR9) (Fackher *et al.*, 2009). DC is able to uptake *L. major* amastigotes but not promastigotes (Woelbing *et al.*, 2006). A contradictory report showed that mouse bone marrow DC (Prina *et al.*, 2003) and splenic DC can uptake a small number of promastigotes which early undergo degradation (Ghosh & Bandyopadhyay, 2004). After the internalization of parasites, DC undergo changes in

surface phenotype suggesting maturation (Marovich *et al*., 2000). This activation is characterized by upregulation of both MHC classes I and II (Ghosh & Bandyopadhyay, 2004; Maurer *et al*., 2009) and increased expression of co-stimulatory molecules such as CD40, CD54, CD80, CD86 (Ghosh & Bandyopadhyay, 2004). Maturing DC up regulate a number of activation markers, such as MHC class II, as well as the costimulatory molecules CD40, CD54, and CD86. We investigated the role of CD54+cells in human cutaneous leishmaniasis. CD54 (ICAM-1) markers are expressed on DC and activated endothelial cells (Virella & Goust, 2001). In decreased expression of DC maturation markers such as CD83 and CD54 indicated dysfunctional APC. CD54+ markers are also present on extravasated positive T memory cells (Meymandi *et al.*, 2009) and moderately on activated B memory cells (Jeoung *et al*., 2004) as well as on fibroblasts, neutrophils and eosinophils (Takashi *et al*., 2001). CD83 is preformed inside monocytes, macrophages and dendritic cells, but it is only stably expressed on activated dendritic cells (Cao *et al.*, 2005). Our results did not show a clear difference in the number of local CD54+ cells infiltrated lesions between immunocompetent and immunodepressed individuals. But the number of cells expressing CD83 marker was significant low in this last group of patients indicating dysfunctional APC.

In the present study, immunodepressed individuals presented a very low amount of DC activated DC suggesting their inability to control the parasite load and the lesion resolution. Co-infected should be also unable to well initiate the humoral response towards *Leishmania* parasite since DC link innate and adaptative immune responses (Stäger *et al*., 2006). The capacity of *Leishmania* and HIV-1 to target DC through a common cell-surface molecule, namely DC-SIGN (dendritic cell specific ICAM-3-grabbing non-integrin), points to a possible dangerous liaison between these two pathogens. *Leishmania* and HIV-1 interact dynamically with DC and exploit this cell for their reciprocal benefit (Garg *et al*., 2007).

We observed a significant increase in the skin CD1a+ cells count at the lesion site. In agreement with previous studies (Geiger *et al*., 2009), these cells were more present in the epidermis than in the dermis. It was so in both co-infected and mono-infected subjects. On the other hand, a high positive correlation was observed between dDC and clinical features suggesting that these cells are actively involved in the lesion

resolution. However, we suggest their local intervention at the lesion site to be more investigated. In fact it is reported that spleen DC display high spontaneous motility (Jebbari *et al*., 2002) but this mobility is inhibited by products from *L. major*. Also, the emigration of LC from the skin is markedly reduced by purified LPG suggesting that *Leishmania* parasites have the ability to interfere with transportation of Ag to or within lymphoid organs by DC (Ghosh & Bandyopadhyay, 2004). To corroborate this idea, Iezzi and collaborators (2006) demonstrated that lymph node DC rather than skin-derived DC initiate specific T cell responses after *L. major* infection. In the same line, Ritter and co-workers (2009) later demonstrated that LC is not involved in the Ag presentation in lymph node and these authors thought that LC could be involved in the conditioning of tissue for inflammatory response. Bomfim and collaborators (2007) thought rather that in lymphoid organs neutrophils might be involved in the cross-priming of CD8+ T cells by DC that have ingested *Leishmania* inside neutrophils as shown for *Listeria monocytogenes*. All these observation show that the *Leishmania* parasite inside DC impairs their function (Favali *et al*., 2007). But in human CL, these cells have to be deeply investigated for their local role at the parasite inoculation site.

The role of macrophage is known in the leishmaniasis. These cells play a triple role since they are host cells, antigen-presenting cells (APC) that activate specific T cells, and effector cells whose leishmanicidal efficacy depends on the presence of activating cytokines such as IFN-γ and TNF-α (Ruiz & Becker, 2007). The amastigotes attachment to MΦ occurs through FcR and CR3 (Woelbing *et al.*, 2006; Bosetto & Giorgio, 2007). Our investigations showed a low number of MΦ in more vulnerable individuals namely Leish+/HIV+ patients suggesting their inability to control the parasite load. In few macrophages present at the lesion site of this group of patients, regardless the fact that *Leishmania* amastigotes should be more resistant, there was a lack of local cytokines such as IFN-γ to activate cells for killing process.

In the blood as well as in the skin, CD4+ cells count was very low in co-infected patients. Conversely the number of CD8+ cells was generally not affected or tended to be higher in this group of condition. Corollary, the CD4/CD8 ratio was very low in these subjects. Patients with persistent lesions also showed a high CD8+ cells amount. It was previously reported that both CD4+ and CD8+ T cells are important for the immune response against *Leishmania* human CL (Antonelli *et al.*, 2005).

Nevertheless, some authors showed that the participation of CD8+ T cells immune response against *Leishmania* does not affect the parasite (Ruiz & Becker, 2007) and consequently does not guarantee the clearance of the infection (Da-Cruz *et al*., 2010). Some previous researchers showed that CD8+ cells might contribute to the development of ulceration mainly on patients with CL (Bomfim *et al*., 2007; Faria *et al*., 2009). Our present study is in line with those of all the authors who showed the detrimental role of CD8+ cells in human CL healing process. Nevertheless, we cannot discard observation from Antonelli and co-workers since we observed a significant infiltration of CD8+ cells in self-healed patients. It is known that CD8+T cells are required for optimal IFN-γ production by CD4+T cells in experimental CL (Herath *et al*., 2003). In the immune depressed patients the CD4/CD8 ratio might be a cause of their condition since a higher CD4/CD8 ratio contribute to chronic wound healing (Jones *et al*., 2004, Da-Cruz *et al*., 2010). In addition, two types of CD8+ T cells have been proposed namely Tc1 and Tc2 (Ruiz & Becker, 2007). Th1/Tc1 response with IFN-γ production leads to CL lesions resolution (von Stebut, 2007a; Geiger *et al*., 2009). If we assume CD8+ cells to contribute to lesions progression, it may be due to the Tc2 type. We can attempt to propose the mechanism used in this circumstance. The cytotoxicity role of CD8+ T cells is well documented. They exert this role through two complementary pathways namely perforine/granzyme and Fas/FasL pathways (Rusell & Ley, 2002; Ruiz & Becker, 2007). CD8+ granzyme A+ was shown to contribute to human CL progression (Faria *et al*., 2009). Fas/FasL level is reported to increase in the blood of patients suffering from VL associated with HIV infection (Eidsmo *et al*., 2002). On the other hand, CD8+ T cells are reported to kill autologous infected macrophages (Eidsmo *et al*., 2002; Ruiz & Becker, 2007). So the macrophage depletion in the skin of *Leishmania*-HIV co-infected as observed in the present study can be attributable not only to the cell burst due to over multiplication of amastigotes inside the cell but also to the CD8+ T cells-induced apoptosis. Ruiz and Becker (2007) suggested that *Leishmania* antigens present in the parasitophorous vacuole of macrophage could cross the membrane with the help of a protein known as sec61, and once inside the cytosol, they are degraded by proteasome. Peptides are then transported to the lumen of the reticulum endoplasmic and bound to the MHC class I

molecules to be transported through the Golgi apparatus to the cell surface where they are presented to CD8 cells which can trigger the apotosis of the host cell.

During our investigations, we also analyzed the role of mast cells. These cells are associated with chronic inflammatory diseases (Maurer *et al.,* 2003; Amato *et al*., 2008; Tuon *et al*., 2008). Their physiological role in wound healing has been demonstrated in vitro and in vivo (Bayat *et al*., 2008). In these conditions, they promote fibroblast growth and stimulate production of the extracellular matrix, accelerating the scar formation (Tuon *et al.,* 2008). They contribute both as initiators as well as effectors of innate immunity. MC promote cell migration through liberation of chemotactic factors such as MIP-1α that recruit polymorphonuclear granulocytes, macrophages, dendritic cells as well as T cells, and facilitate the influx of cells and inflammatory mediators into infected tissues through the liberation of potent vasoactive components that increase local blood flow and vascular permeability (Villaseñor-Cardoso *et al*., 2008).

Mast cells (MC) are reported to be critically involved in the elicitation of protective immune responses to bacteria, intestinal parasites, and other infectious pathogens (Maurer *et al*., 2003). In some diseases like asthma, MC degranulation contributes to the exacerbation of the pathology (Hanninen & Vliagoftis, 2005). However, their role in cutaneous leishmaniasis is controversial. It is reported that MC can be activated in vitro and in vivo by *Leishmania* and *Leishmania* products (Amato *et al*., 2008; Romão *et al*., 2009). In the first step of leishmaniasis, unspecific antibodies bind to the parasite surface, which will attach on MC by Fc receptors, promoting degranulation with cytokines release (TNF-α, IFN-γ, IL-4, IL-10, IL-12 and IL-6) (Amato *et al*., 2008). Maurer and co-workers (2006) demonstrated, in mice, the protective role of mast cells against *L. major* infection. On the contrary Romão and collaborators (2009) showed that several innate immunity mediators, including some released by mast cells, play roles in the outcome of the disease. They argued that when mast cells are degranulated before challenge with *L. major*, susceptible mice become more resistant to infection, as measured by decrease of lesion size and lower parasite loads. Mast cell degranulation shifts Th2 response to Th1 in these susceptible mice independently of IL-4. These cells contribute to susceptibility of BALB/c mice to *L. major*. These same authors reported that MC degranulation reduces IL-4 production

and enhances mRNA expression for IFN-γ, inducible nitric oxide, in response to infection. As per Villaseñor-Cardoso and collaborators (2008), early histamine release favours Th2 response. Previously, Saha and co-workers (2004) thought that mast cell cytokines play a pro-parasitic role in susceptible but an antiparasitic role in resistant mice. These authors mentioned that IgE- sensitized MC release TNF-α, a host protective cytokine, or IL-4, a disease-promoting cytokine among variety of other mediators like histamine. This regulatory role of MC depending on the genetic host background was suggested by the Villaseñor-Cardoso's team (2004). As we can observe, all these authors worked on mice models. A report from Tuon and collaborators (2008) on human CL caused by *L. brazilensis* and that caused by *L. amazonensis* revealed that MC count was higher in patient with early healing after treatment, and there was a positive association of disease's duration and MC count. At the end these authors suggested that in CL, healing can be associated with mast cells, which can be associated with age, species and disease duration.

We can now attempt to speculate on the physiological role of MC in human CL. Obviously, MC cannot be considered as bystander cells during the disease evolution. We agree that they are involved in chronic lesions since we observed a positive association with MC and the lesion age. The MC infiltration was more noticed in lesions in the third trimester of lesions evolution. Their presence in the lesion site may be important for lesion resolution only if after their infiltration they subsequently undergo degranulation. This degranulation may be the means by which MC locally control the disease and hasten the healing process. Our results are in line with these hypotheses. In our study we found that, in human CL, the extensive degranulation can be obtained in both immunocompetent and immunodeppressed patients. But in immunocompetent patients this degranulation is more marked. On the other hand, a negative correlation was observed between MC degranulation and the number of active lesions, the total surface of lesions, the healing duration respectively. This merely means that the higher the MC degranulation, the lower the number of active lesions, the smaller the lesion size and the shorter the healing duration and vice versa. Interestingly, more vulnerable subjects namely those with persisted lesions, Leish+HIV+ patients showed low MC degranulation. Demo and collaborators (1999) mentioned that an antigen-mediated crosslinking of MC surface IgE receptors result in

degranulation and the release of histamine, TNF-α, serotonine, tryptase, chymase and decarboxypeptidase. On the other hand, Bayat and co-workers (2008) reported that the biologically active substances released by MC stimulate tissue repair including neovascularization. They added that tryptase in cooperation with heparin stimulates migration and division of vascular endothelial cells. These same authors mentioned that histamin (using the H2 receptor) and IL-4 released by MC increase fibroblast migration and proliferation and then wound healing. Therefore, we think that if MC are not involved in the parasite clearance, these cells at least contribute to restore the tissue at the lesion site and so to close the lesion.

Both leishmaniasis and HIV infection exert cumulative deficiency of the cellular immune response since both agents damage similar immune resources. HIV related CD4+ T cell depletion implies a lack of T cells to recognize *Leishmania* antigens and to stimulate B-lymphocytes. This leads to an oligo B-cell response, which explains the elevated frequency of false negative *Leishmania* serology results in co-infected patients (Parades *et al.,* 2003). In Leish+/HIV+ patients, humoral specific response to *Leishmania* turns out to be partial, weak or absent, due to the fact that the cellular immunity is affected after infection by HIV, causing a decrease of total lymphocytes in the production of antibody (Cruz *et al*., 2006). In the present study, we showed a very low infiltration of B cells in the skin of immunodepressed individuals. The B cell deficiency can explain the low production of antileishmanian IgG observed in the blood of this group of patients. Herwaldt (1999) previously reported that antibody to *Leishmania* may not be detectable, particularly if the HIV infection preceded the leishmanial infection. However, parasites may be abundant, even in atypical sites and cells, which facilitate parasitological diagnosis.

Antibodies (Ab) may facilitate parasite uptake by MΦ and DC and then modulate the fate of intracellular parasites and the function of APC (Ji *et al*., 2005). The intensity of the Ab response appears to reflect both parasites load and the chronicity of infection (Ozbilge *et al*., 2006). In immunocompetent subjects we showed a positive correlation between total IgG and clinical features. We therefore agreed that by the ELISA test, blood antibody level can be used to diagnose leishmaniasis severity. Nevertheless, in immunodepressed patients this diagnosis is not

obvious at all since we showed rather that their clinical features do not reflect the blood antibody level.

In dogs, IgG2 subclass was reported to be more produced than IgG1 (Vercammen *et al*., 2002) and a ratio IgG1/IgG2 ≥ 1 would characterize the sera of visceral leishmaniasis infected animals evolving towards the overt disease while ratio IgG1/IgG2 ≤ 1 would characterize the sera response of vaccinated protected dogs (Mohammadi *et al*., 2006). In humans, IgG1 and IgG3 are predominantly upregulated (Ozbilge *et al*., 2006). Our findings are in agreement with this previous report in humans since IgG1 titer was always higher than IgG2 titer independently on the immune status and so, the ratio should be IgG1/IgG2 > 1. Investigations in murine models of *Leishmania major* and *L. donovani* infections clearly demonstrated a Th2/IL-4/IgG1 relationship with disease progression and a Th1/IFN-γ/IgG2a relationship with resistance and protective immunity (Anam *et al*., 1996). In humans, this trend was not clear-cut during our investigation. As IgG1 and IgG3 (but not IgG2 and IgG4) have receptors on macrophages and neutrophiles (Virella, 2001b), due to its high blood level we think that IgG1 can be also more involved in the parasite uptake by these cells.

Further, we wanted to understand more about the role of serum cytokines in human CL. In murine leishmaniasis, the action of Th1 (IFN-γ, IL-2, TNF-α) results in cure while the action of Th2 cytokines (IL-4, IL-5, IL-10) results in progression of the disease (Patel & Shah, 2008). The serum IFN-γ level tended to be high in Leish+/HIV+ patient as compared to Leish+/HIV-. However, this tendency was observed mainly in the early stage of the disease. It is reported that *Leishmania* promastigotes can directly stimulate natural killer (NK) cells which have been implicated in the natural protection and healing of leishmaniasis by their ability to secrete the macrophage activating cytokine interferon. Some studies demonstrated that early production of interleukin (IL)-12 triggers IFN secretion by NK cells (Ma & Montaner, 2000; Nylén *et al*., 2003). Olivier and co-workers (2003) reported that a Th1 response characterized by IFN-γ and Il-2 production is present in the early stages of HIV infection and the progression to AIDS is associated with the switch to Th2 marked by IL-4 and IL-10 upregulation.

Earlier studies demonstrated that IL-12 is a potent inducer of IFN- γ from a variety of cell types (Stobie *et al*., 2000). IFN-γ production potently enhances the ability of monocytes and neutrophiles to produce IL-12 (Ma & Montaner, 2000). If we are in agreement with these last authors that IL-12 production during an immune reaction is tightly modulated by positive (and negative) feedback, the inhibitor role of TFN-α on IL12p70 production as they reported in the same article is questioned in case of human CL since we noticed a high positive correlation between these two cytokines. TNF-α participates in the induction of NO production and macrophage activation leading to the elimination of intracellular pathogen (Liew *et al*., 1990; von Stebut *et al*., 2002) and IL-12 plays a central role in both the induction and magnitude of primary Th1 response *in vitro* and *in vivo*. *L. major* harboring DC produce large amounts of IL-12p70 in CD40 ligand-dependent manner (Marovich *et al*., 2000; von Stebut *et al*., 2002). IL-12 is required in the memory Th1 pathway to mediate a successful biologic outcome (Stobie *et al*., 2000). The synergistic role of TNF and IL-12 in the macrophage activation is documented (Liew *et al*., 1990; Ezra *et al*., 2010).

IL-8 is a cytokine chemotactic for neutrophiles (Badolato *et al*., 1996) and it increases the expression of ICAM-1 (Bayat *et al*., 2008). Infection of monocytes with *L. major* induces IL-8 but not the proinflammatory cytokines IL-1β and TNF-α. This suggests that the expression of chemokines may contribute to the cellular recruitment in *Leishmania* lesions (Badolato *et al*., 1996). Infection with *Leishmania* promastigotes leads to neutrophiles accumulation via the production of a chemotactic factor by the parasites, and this effect is amplified by the induction of IL-8 production (van Zandbergen *et al*., 2002). Cytokine members of the CXC class, such as IL-8, and platelet factor-4, act mainly on neutrophils. In the present study, immunodeficient individuals secreted very low amount of this chemoattractant cytokine.

IL-6 is a pleiotropic cytokine which is produced by macrophages, lymphocytes, and many nonhematopoietic cells. It plays a role in B-cell stimulation and in regulation of acute-phase responses and inflammation. IL-6, therefore, is not required for strong Th1 responses against *L. major* (Moskowitz *et al.*, 1997). Our co-infected patients presented low level of IL-6 and this can be a consequence of the macrophages depletion.

Kane and Mosser (2001) demonstrated that IL-10 produced by *L. major* infected macrophages prevented macrophage activation and diminished their production of IL-12 and TNF-α. Thus, these authors noticed the detrimental role of IL-10 in the disease. It is reported that IL-10 is a key cytokine produced by various effector skin cells, e.g. Treg, Th2 cells, MC, and keratinocytes. It is an immunomodulatory cytokine: this cytokine can influence Th1/Th2 differentiation by inducing Th2-dominated immunity, antigen-presenting cell functions, and antigen-presenting cell-mediated T cell activation. Interestingly, treatment with anti-IL-10 in chronic *L. major* infections resulted in complete resolution of the lesions and is associated with sterile cure. However, IL-$10^{-/-}$ mice are unable to mount a protective memory Th1 response, suggesting that full elimination of antigen from the organism is counterproductive for the maintenance and survival of effector memory T cells. Thus, IL-10 and its dual functions are important for the control of skin immunity (Maurer *et al*., 2009). Klein and Enders (2007) reported that Treg cells (CD4+ CD25+) produce IL-10 and TGF-beta, lymphokines that are both strong immunosuppressants inhibiting Th1 and Th2 cells. During our investigations in human CL, we observed that more susceptible patients produced a fewer amount of IL-10. This is in agreement with Nateghi and collaborators work (2010) in which the authors showed that cells from self healed CL patients produced a high amount of IL-10. Rostami and co-workers (2010) also observed that CD4+/CD8+ T cells collected from spontaneously cured CL patients produced a high amount of IL-10 and IFN-γ. This corroborate that in human, IL-10 has an important role for the control of skin immunity and the resolution process of CL.

Humans infected with HIV do not show evidence of a response that is predominantly of the Th1 and Th2 type (Olivier *et al.*, 2003). In cutaneous leishmaniasis precisely, Th1 cytokines predominate over Th2 cytokines while in visceral and mucocutaneous leishmaniasis, there is increase in Th2 cytokines. Other Th2 cytokines like IL-13 and transforming growth factor-beta (TGF-β) have been reported to be produced in VL (Patel & Shah, 2008). The Th1/Th2 paradigm in leishmaniasis has been largely established in mice. It has been demonstrated that one *Leishmania* species (*L. major*) can induce Th1 and Th2 responses in resistant and susceptible mice respectively (von Stebut 2007b; Cangussú *et al*., 2009). On the other

hand, two different species *L. major* and *L. amazonensis* induced respectively Th1 and Th2 responses in the same mouse strain (de Souza *et al*., 2000).

In human, Cangussú and collaborators (2009) reported that CL is usually self-limiting and the lesions heal spontaneously. This outcome is associated with a polarized Th1 response, control of the disease and development of resistance. Nevertheless, these same authors mentioned that some patients develop Th2 response and consequently need treatment. Our findings showed that patients with only CL or *Leishmania major*-HIV co-infected subjects revealed a high positive correlation between some blood Th1 and Th2 cytokines. Self-healed patients, who were characterized by less severe clinical features, also presented the same trend suggesting that in human CL, the same parasite specie, namely *L. major* can induce a mixed Th1/Th2 response independently on the lesion severity and the HIV serology.

In human beings, it is reported that Th1 lymphocytes produce IFN-γ and TNF-β but not IL-4 and IL-5. Th2 lymphocytes produce IL-4, IL-5, IL-9, and IL-25 but not IFN-γ or TNF-β. Both classes produce GM-CSF, TNF-α, IL-2, IL-3, IL-10, and IL-13 (Borish & Steinke, 2003). The inhibitory role of IL-4 on IFN-γ and TNF-α as reported by many authors (Liew *et al*., 1990) was not evident in this study since IL-4 production was highly associated to IFN-γ up regulation. IL-4 production by differentiated cells is largely cytokine-autonomous, whereas IFN-γ production is highly cytokine regulated. Thus, T cells primed in the presence of IL-4 develop into IFN-γ producers if IFN-γ is included in the priming culture and if the cells are challenged in the presence of IL-12 (Hu-Li *et al*., 1997).

Finally, we observed that in the blood, Th1 and Th2 cytokines paradigm is less clear-cut. This can explain why Herwaldt (1999) mentioned that T-cell and cytokine responses in infected human beings are more complex and less polarized than they are in mice, and the immune responses differ among the leishmanial syndromes and species. However, locally, the vulnerability (e.g. HIV+) of the CL patient depend more on the Th2 profile. In fact, the number of local IFN-γ producing cell was very low in immunodepressed patients. In line with our data, impaired immune responses of HIV+ individuals in infections with e.g. malaria (Diallo *et al*., 2004), tuberculosis (Soumare *et al*., 2008) have been mentioned

The treatment regimen did not show a real impact of the HIV serology on the blood cytokine upregulation. Amphotericine B complexes with 24-substituted sterols, such as ergosterol in cell membrane, thus causing pores which alter ion balance (Croft & Coombs, 2003). This medicine might also stimulate the production of MΦ and increase their phagocytosis capacity (Dedet, 1999b). Allopurinol incorporates into parasite RNA with lethal effects (Dedet, 1999b; Markle & Makhoul, 2004).

CONCLUSION AND PERSPECTIVES

It came out of our present investigations that cutaneous leishmaniasis in Cameroon is still a reality and Mokolo remains an active endemic focus. It remains a neglected tropical disease in the country. In Mokolo focus, more than one person out of 100 is a victim of CL and 5 individuals out of 1 000 present active lesion(s). In 20 subjects with active lesions, one shows a positive HIV serology; women being at high risk.Schoolchildren of the age range 10 to 20 years are the most affected. Many cases are registered a couple of months after the beginning of the rainy season. CL causative, or one of the causative, agent in Mokolo even in HIV infected patients is *Leishmania major.* Clinical signs such as the number, the total surface of active lesions as well as the time of lesion resolution are more marked in some patients namely co-infected patients.

In the skin, there is a severe depletion in the number of antigen presenting cells such as dendritic CD1a+ cells, macrophages-CD68+; as well as the low infiltration lymphocytes T-CD4+ and B-CD20+ in *Leishmania*-HIV co-infected individuals. Mast cells degranulation is very low in this same group of patients. Anti-leishmanian IgG and IgG1 and 2 isotypes are up regulated in all CL patients and can be used to diagnose the disease and evaluate its severity, but in individuals with a positive HIV serology this is not always obvious. In the blood, Th1/Th2 seems to be ambiguous, but in the local immune response, the severity of CL symptoms due to the immunedepression is related to the shift toTh2. The local immune reaction is more efficient to clear parasite and hasten the healing process in CL.

Finally, our immunological studies suggest severe alterations in the protective immune response initiated by antigen presenting cells and mediated by IFN-γ producing T cells. Both humoral and cellular responses are affected in immunodepressed patients.

This is the first report of *Leishmania major*/HIV co-infection in Cameroon and Central Africa. A detailed understanding of the immunological responses in patients with more severe disease presentation or those who scarcely respond to usual antileishmanian therapy, the HIV status should be assessed. Another clinical implication is the fact that immunocompromized patients, especially those with e.g. altered T cell responses due to immunosuppressive therapy and/or cancer, diabetes, may show similar alterations in their anti-*Leishmania* immune response.

Leishmania/HIV co-infected individuals is important for the development of optimized therapeutic regimens for this severely affected group. Our findings provide important data for the development and implementation of successful control programs against CL and HIV co-infection in this geographical area. These investigations are of great public health importance as both CL and HIV infection co-occur in the region and successful control programs against HIV should integrate opportunistic infections such as leishmaniasis.

In the future we plan:

- the extension of the study geographical area to other localities of the northern Cameroon and the research of others eventual leishmaniasis foci in the country ;

- the integration of the visceral leishmaniasis into the study ;

- the research and characterization of others eventual leishmaniasis causative agents in the country and their involvement in *Leishmania*/HIV co-infection ;

- the study of other factors namely the influence of other transmissible (malaria, shistosomiasis etc.) or non transmissible (diabetes etc.) diseases on the outcome of cutaneous leishmaniasis on human or on murine models ;

- the study of the role of IgG3 and IgG4 isotypes on human CL associated with HIV infection ;

- the assessment on the therapy used (e.g. plants extracts) by local population;

- the launch of an educational and training initiative to increase the awareness and the level of information and disease management in both the medical and scientific community in Cameroon.

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http://fr.wikipedia.org/wiki/Mayo-Tsanaga

http//fr.wikipedia.org/wiki/Mokolo

APPENDIX

Appendix 1

REPUBLIQUE DU CAMEROUN
Paix – Travail – Patrie

MINISTERE DE LA SANTE PUBLIQUE

SECRETARIAT GENERAL

DIVISION DE LA RECHERCHE
OPERATIONNELLE EN SANTE
Tel 223 45 18

REPUBLIC OF CAMEROON
Peace – Work - Fatherland

MINISTRY OF PUBLIC HEALTH

SECRETARIAT GENERAL

DIVISION OF HEALTH
OPERATIONS RESEARCH
Email: minsanterecherche@yahoo.fr

N° 1130 - 44 /AAR/MSP/SG/DROS/CRC Yaoundé, le 07 FEV 2007

LE MINISTRE

A

Monsieur le Docteur Blaise DONDJI,
Department of Biological Sciences,
Faculty of Natural Sciences - University of Ngaoundéré
B.P 454 Ngaoundéré, Cameroun.

Objet : Autorisation Administrative de Recherche

Monsieur,

Après examen du projet de recherche «Cutaneous Leishmaniasis in Northern Cameroon : Immunological Studies of Leishmania and Leishmania/HIV Co-infection in the Mokolo Focus », bénéficiant du soutien financier de la Fondation WOLSKWAGEN de Hanovre en Allemagne que vous avez introduit en tant qu'investigateur principal de l'équipe de recherche par ailleurs composée de Pr. Albert SAME EKOBO de la FMSB de l'Université de Douala, Dr Esther VON STEBUT du Département de Dermatologie de Gutenberg University – Germany et du Pr. Marcus MAURER du Département de Dermatologie et d'Allergie de Charité – Berlin Schumannstrasse – Germany,

J'ai l'honneur de vous signifier l'autorisation administrative à démarrer cette recherche. Vous voudrez bien noter que la Division de la Recherche Opérationnelle en Santé est chargée du suivi de ce projet et devra être tenue informée sur une base semestrielle de vos activités de recherche prévues pour une durée de cinq ans (2007-2011) et des conclusions de cette recherche. Le Ministère se réserve par ailleurs le droit d'effectuer des missions de suivi évaluation administrative et éthique de la mise en œuvre du projet.

Veuillez agréer, Monsieur l'expression de ma parfaite considération.

Copies :
- DROS
- DLM
- Doyen FMSB
- DPSP/EN
- CSSD de MOKOLO

Médecin Olanguena Aivogo

Appendix 2

COMITÉ NATIONAL D'ETHIQUE
NATIONAL ETHICS COMMITTEE

- N° d'enregistrement : FWA IRB00001954 BP 1937, Yaoundé, Tel: (237)-220 9075
- Arrêté N° 079 /A/MSP/DS du 22 OCTOBRE 1987 portant création et organisation d'un Comité d'Ethique sur la recherche impliquant les êtres humains

Yaoundé, le 19 Avril 2006

CLEARANCE ETHIQUE

Le Comité National d'Ethique a examiné ce jour le projet de recherche intitulé : ***"Leishmaniose cutanée au nord Cameroun : études immunologiques sur Leishmania et co-infection Leishmania/VIH au foyer endémique de Mokolo "*** introduit par ***le Dr Blaise DONDJI*** et collaborateurs.

Le sujet est digne d'intérêt. Il comporte un risque minime pour le participant (Prélèvement du sang veineux, éventuellement prélèvement cutané).

La notice d'informations destinée aux participants et le formulaire de consentement éclairé manquant dans le document initial ont été fournis.

Pour toutes ces raisons, le Comité National d'Ethique ne formule aucune objection à la réalisation de cette étude.

En foi de quoi la présente Clearance Ethique est délivrée pour servir et valoir ce que de droit.

LE PRESIDENT,

Pr. KAPTUE Lazare

Appendix 3

HOPITAL DE DISTRICT DE MOKOLO
PROJET LEISHMANIOSE AU NORD CAMEROUN
B.P.143 MOKOLO

NOTICE D'INFORMATION POUR LES PARTICIPANTS

TITRE DU PROJET DE RECHERCHE : Leishmaniose cutanée au nord Cameroun : Etudes immunologiques sur *Leishmania* et co-infection *Leishmania*/VIH au foyer endémique de Mokolo

Cette recherche évaluera la prévalence de la leishmaniose cutanée et la co-infection *Leishmania*/VIH dans la région de Mokolo et étudiera les aspects immunologiques qui sous-tendent ces deux conditions.

Les sujets recrutés pour cette étude seront sollicités par l'équipe de recherche une à deux fois pendant six mois à compter de la date du premier contact. Au cours de cette enquête, deux biopsies et du sang (10-20 ml) seront prélevés chez les participants pour le diagnostic de la leishmaniose cutanée ou « *Mblegoyave* », **le test du SIDA** et des études immunologiques. Avant la prise de la biopsie, le sujet sera anesthésié localement pour limiter la douleur. Les conditions maximales d'asepsie seront observées au cours de la prise de la biopsie et du sang. Les sujets dont les lésions seront diagnostiquées comme dues à la leishmaniose cutanée seront traités si les lésions persistent après un mois. Quant aux individus testés positifs au VIH, ils seront référés au Comité National de Lutte contre le SIDA à travers le Groupe Technique Provincial de l'Extrême-Nord pour suivi. L'actuel projet contribuera financièrement au traitement des sujets séropositifs au VIH pendant cinq(5) ans, période après laquelle ils seront reversés au Comité National de Lutte contre le SIDA à travers le Groupe Technique Provincial de l'Extrême-Nord. Toutefois la participation à cette enquête ne donne droit à aucune rémunération financière.

Vous êtes informés que vos noms seront codés et seuls le Docteur Blaise DONDJI, investigateur principal de cette enquête, le Professeur Albert SAME EKOBO, le Directeur de l'Hôpital de District de Mokolo, le Chef du Groupe Technique Provincial de l'Extrême-Nord du Comité National de Lutte contre le SIDA auront accès à vos informations personnelles.

Les données obtenues et faisant l'objet de communication scientifique seront présentées en agrégats et aucune identification personnelle des individus participant au projet ne sera mentionnée.

En cas de besoin d'informations supplémentaires ou d'une complication quelconque liée à cette enquête, nous vous prions de contacter soit le Docteur Blaise DONDJI, soit le Professeur Albert SAME EKOBO, le Directeur de l'Hôpital de District de Mokolo, le Chef du Groupe Technique Provincial de l'Extrême-Nord du Comité National de Lutte contre le SIDA.

Nous tenons à vous rappeler que votre participation à cette étude est volontaire et que vous ne courez aucun risque si vous cessez d'y participer.

Appendix 4

FORMULAIRE DE CONSENTEMENT ECLAIRE*

TITRE DU PROJET DE RECHERCHE : Leishmaniose cutanée au nord Cameroun : Etudes immunologiques sur *Leishmania* et co-infection *Leishmania*/VIH au foyer endémique de Mokolo

INVESTIGATEUR PRINCIPAL: Dr Blaise DONDJI
AUTORISATION DU COMITE D'ETHIQUE: Clearance Ethique du 19 avril 2006

Monsieur/Madame,

L'équipe du projet de recherche susmentionné vous invite à participer à ce projet. Cette recherche évaluera la prévalence de la leishmaniose cutanée et la co-infection *Leishmania* /HIV dans la région de Mokolo et étudiera les aspects immunologiques qui soutendent ces deux conditions.

Procédure de la recherche: prise de biopsies et du sang (10-20 ml) pour le diagnostic de la leishmaniose cutanée ou « *Mblegoyave* », **le test du SIDA** et des études immunologiques avec anesthésie locale et asepsie maximale.

Risques/Inconfort éventuels: Douleur lors de la prise du sang

Bénéfices: Traitement des malades de leishmaniose cutanée, connaissance du statut sérologique pour le VIH, suivi des séropositifs au VIH à la charge du projet pendant cinq (5) ans.

Compensation: Pas de rémunération financière liée à votre participation à cette recherche.

Confidentialité: Les noms seront codés et seuls le Docteur Blaise DONDJI, le Professeur Albert SAME EKOBO, le Directeur de l'Hôpital de District de Mokolo, le Chef du Groupe Technique Provincial de l'Extrême-Nord du Comité National de Lutte contre le SIDA auront accès à vos informations personnelles

Nature de la participation: Volontaire

Personne à contacter: Dr Blaise DONDJI, le Professeur Albert SAME EKOBO, le Directeur de l'Hôpital de District de Mokolo, le Chef du Groupe Technique Provincial de l'Extrême-Nord du Comité National de Lutte contre le SIDA et le Comité National d'Ethique.

Nom du participant :

Date, Signature du participant ou de son représentant légal

Nom du Membre de l'Equipe de Recherche :

Date, Signature du Membre de l'Equipe de Recherche

Témoin au consentement pour les participants incapables de lire ou d'écrire

Date, Signature du Membre du témoin au consentement

** Ce document sera signé en trois(3) exemplaires, dont un sera remis au participant, le 2e sera gardé dans le dossier de l'investigateur principal et l'autre dans le dossier médical du participant*

Appendix 5

HOPITAL DE DISTRICT DE MOKOLO
PROJET LEISHMANIOSE AU NORD CAMEROUN
***Leishmania* et co-infection *Leishmania*/VIH**
B.P. 143 MOKOLO-CAMEROUN

BULLETIN DES RESULTATS DU TEST SEROLOGIQUE DU VIH

Nom et Prénom(s) ……Age……Sexe……Poids……

Lieu de résidence……Profession…….....Statut matrimonial……

Examens	Résultats		Test réalisé par	Date
Test de VIH	**Test 1** ***VIH1+VIH2***			
	Test2	***VIH1***		
		VIH2.		
Taux de CD	***TCD4*** ***TCD8*** **TCD3** ***TCD4/TCD8***			

Fait à Mokolo, le ……..……par……

Appendix 6

HOPITAL DE DISTRICT DE MOKOLO
PROJET LEISHMANIOSE AU NORD CAMEROUN
B.P. 143 MOKOLO-CAMEROUN

QUESTIONNAIRE D'ENQUETE DE TERRAIN

- **Identification et Localisation du ménage**

  Canton/Village :……
  Quartier :……...
  Numéro du ménage : PL……..
  Nom du Chef de ménage ou de son représentant……...

- **Questions**

1) Combien de personnes habitent habituellement ce ménage ?.....

   Nombre par tranche d'âge et par sexe

Age (ans) / Sexe	]0-10]	]10-20]	]20-30]	]30-40]	]40-50]	]50-60]	>60
Masculin							
Féminin							

2) Y-a-t-il dans cette concession une(des) personne(s) ayant une plaie / bouton de type leishmaniose (Mblegoyave, Mbleplesh, Mwele, Jéova, Gourleng) ou toute autre plaie/bouton dont elle ignore l'origine ? OUI ☐ NON ☐ (Cochez)

3) Y-a-t-il dans cette concession une(des) personne(s) ayant déjà eu la leishmaniose (Mblegoyave, Mbleplesh, Mwele, Jéova, Gourleng)? OUI ☐ NON ☐ (Cochez)

4) Si OUI, pouvons-nous rencontrer cette(ces) personne(s) aujourd'hui ou plus tard (date)? OUI ☐ NON ☐ (Cochez)

5) Si OUI, préciser le nombre de personnes ayant des lésions ou cicatrices suspectes…….
   (*Dans le tableau ci-dessus, préciser entre parenthèse le nombre de sujets suspects avec mention ''L''pour lésion active ou ''C'' pour cicatrice typique*)

6) Pouvez-vous nous dire ce qui cause la leishmaniose ?.....(Réponse)

7) Dormez-vous dans une moustiquaire ? OUI ☐ NON ☐ (Cochez)

8) Si OUI combien de personnes utilisent-elles de moustiquaire ?..... (Nombre)

9) Utilisez-vous un insectifuge ou un insectifuge ?
   NON ☐ SPORADIQUEMENT ☐ REGULIEREMENT ☐ (Cochez)

Date, Nom et signature de l'enquêteur

PROJET LEISHMANIOSE AU NORD CAMEROUN
Leishmania et co-infection *Leishmania* HIV
B.P.143 MOKOLO-CAMEROUN

Appendix 7

FICHE DE SUIVI DES SUJETS LEISHMANIENS ET/OU SEROPOSITIFS POUR LE HIV

N°	RENSEIGNEMENTS GENERAUX SUR LE SUJET								DATE	DONNEES SUR LA LEISHMANIOSE				DONNEES SUR SEROLOGIE HIV			
	Nom(s) et Prénom(s)	Sexe	Age	Niveau d'étude	Profession	Statut matrimonial	Lieu de résidence	Voyage des 12 derniers mois		Signes cliniques et Sites *	Durée avant visite	Traitement oui/non **	Tps de guérison	Dernier test du HIV : Date ou pas	ARV oui /non	Transfusion sanguine ? Date ?	Résultats du test actuel

*: Lésion (L), Nodule (N), Cicatrice (C), signe précédé chacun du nombre d'atteintes ; en indice, leurs diamètres respectifs (en mm) ; suivi du site. Exemple :($1C_{40}$)Fr+ ($2C_{8,10}$ /$1L_{12}$)Av+($1L_5$)Ja' se lit: une cicatrice de 40 mm au front plus deux cicatrices de diamètres respectifs 8 et 10 mm et une lésion de diamètre 12 mm toutes à l'avant-bras droite, plus une lésion de diamètre 5 mm à la jambe gauche

**: si oui, préciser le produit

Appendix 8

MATERIALS, REAGENTS AND DRUGS USED

DEVICES AND BIG MATERIALS

Agitator RH Basic Kika Latechnik

BECTON DICKINSON FACS Count

Camera Olympus

Centrifuge Thermo Electron Corporation CL30R

CoverplateTM System

Cytometer Becton, Dickinson and Company LSRII

Distillator AutodistillTM Freshman-4

Heater

Incubator HORO

Knick pH-meter 761 Calimatic

LEICA RM 2245 Microplate reader Elx808™ Fa BioTek

Microscope Nikon

Motor bike, HONDA100

New Brunswick Scientific Ultra Low Temperature Freezer

Refrigerator Natural Tropical

Thermo Shandon Histocentre2

Timer Quantum

Tissue Tek® Cryo -Consol

Tissue-Tek® VIPTM5 Jr

Scale: Sartorius Basic

Vortex-Genie 2

SOME SMALL MATERIALS

Centrifuge tube Centri StarTM Cap

Compress

Cotton

Cryogloves

Cryogenic vials NALGEN

Disposal gloves TG Medical TM

Ependorf tubes

Micropipettes (5, 10, 50,100 µl)

Pipette 10 ml, 50 ml

Pipette tips

Perforated adhesive plaster Cathy Yougo

Slides and cover slides

Surgical blades

Syringe Cathy Yougo®

REAGENTS AND TESTS

Abbott Determine®-HIV1/2 IIlinois, USA, Goa, India

Acetic acid 96% Merck

Alcohol 70% Nedalco

Alcohol isopropylicus (2-propanol) 100% Hedinger

Antibody Diluent Dako EnVisionTM FLEX DM830

Anti human IgG (γ-chain spez.), biotinylated, Biozol 2040-08

Anti human IgG1 Fc Biotin, Invitrogen MH1515

Anti human IgG2 Fd Biotin, Invitrogen MH1522

Bioline SD HIV1/2 3.0, Standard Diagnostics Inc., Kyonggi-do, South Korea

BD FACS Count TM CD4/CD3 and CD8/CD3 Reagents

BD FACS Count TM System Fixative solution

CD83, clone 1H4B; NovocastraTM

Chloridric acid Titri Pur® Merk c(HCl) 1 N

CXCR3, clone 1C6; Pharmingen

Dako RealTM AP Substrate Buffer, Dako, Denmark

Dako RealTM Chromogen Red 1, Dako, Denmark

Dako RealTM Chromogen Red 2, Dako, Denmark

Dako RealTM Chromogen Red 3, Dako, Denmark

Dako RealTM Levamisole, Dako, Denmark

Dako RealTM Link, Biotinylated Secondary Antibobies, Dako, Denmark

Disodium hydrogen phosphate, $2H_2O$ (Na_2HPO4, $2H_2O$) 0.1 M Serva

FIENBIOCHEMECA. HEIDELBERG

Eosin Y (Yellowish) Merck

Ethanol 96% Martin &Werner Mundo OHG

FlowCytomix Human Th1/Th2 11plex Kit (IFN-γ, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, TNF-α, TNF-β) BMS810FF, Bender MedSystem®

Formalin

Formol Officinal Soluté aqueux à 35% MEDIPHAR EXPRESS, Douala-Bassa, Cameroun

Foxp3, clone 236A/E7, Abcam

Giemsa'azur eosine methylene blue solution Merck KGaA

Hydrochloric acid Merck c(HCl) 2N

Hydrogen peroxide 30% Merck

IFN-γ, clone IFN-γ (H-145): sc-8308; Santa Cruz Biotechnology

IL-6, clone 1936; R&D Systems.

Maxisorb Immunoplattes, flat bottom, Nunc 442404

Mayer's hemalum solution Merck

Monoclonal Mouse Anti-CD1a, clone 010, Dako

Monoclonal Mouse Anti-Human CD8, Clone C8/144B DK-2600 GlosTrup, Denmark

NaCl Roth and NaCl Merck

NaH_2PO_4 $1H_2O$ Roth

Paraffin weiss DAB Pionier 5658H

Phosphoric Acid, Sigma

Potassium dihydrogen phosphate (KH_2PO_4) 0.1M Serva FIENBIOCHEMECA. HEIDELBERG

Retrocheck HIV® (1+2), Qualpro Diagnostics, Goa, India

Roti®-Histokitt Carl Roth GmbH+Co.KG

RPMI

Steril water Ecotainer® Aqua B. Braun

Streptavidin HRP R&D System 555214

TMB Substrate Reagent Set (A+B), BD Bioscience 555214

Tris Pufferan® p.a. Carl Roth GmbH+Co.KG ($C_4H_{11}NO_3$)

Tween 20, Sigma

Sodium hypochlorite solution LaCroix Bleach, Colgate-Palmolive, Cameroon

Xylol (Xylene-mixture of isomers) Applichen

Labeled Streptavidin-Biotin (LSAB) Dako, Denmark

Lyophilized Monoclonal Mouse Antibody CD4, Clone 1F6, Code NCL-CD4-1F6, NovocastraTM , United Kingdom

Lyophilized Monoclonal Mouse Antibody CD54 (ICAM-1), Clone 23G12, Code NCL-CD54-307, NovocastraTM, United Kingdom

Lyophilized Monoclonal Mouse Antibody CD20, Clone 7D1, Code NCL-CD20-7D1, NovocastraTM, United Kingdom

Monoclonal Mouse Anti- CD1a, Clone 010, Code M3571, Dako, Denmark

Monoclonal Mouse Anti-Human CD8, Clone C8/144B, Dako, Denmark

Monoclonal Mouse Anti-Human CD68, Clone PG-M1, Dako, Denmark

FLEX Monoclonal Mouse Anti-Human Mast Cell Tryptase, clone AA1, Dako, Denmark

Link, Biotinylated Secondary Antibodies (AB2), Dako A REALTM K5005, Denmark

Streptavidin alkaline phosphatase (AP) Dako B, Dako REALTM K5005, Denmark

Target Retrieval Solution High pH (50X), Dako EnVisionTM FLEX DM828, Denmark

TGF-β, clone TGF-β(V):sc-146; Santa Cruz Biotechnology.

DRUGS

Allopurinol (Zylloric 300) Glaxo Smithkline

Amphotericin B (Fungizone) Bristol-Myers Squibb Srl 03012 Anagni (FR), Italie

Biomultivitamin (A, B1, B2, B3, C, D3) HEMKISH PHARMACEUTICALS & CHEMECALS Pvt.Ltd

Cotrimoxazol STRIDES ARCOLAB LIMITED Bangalore-India

Lidocaine Injection BP 2% ROTEX MEDICA TRITTAU, Germany

Metronidazol PHARMAQUICK S.A Cotonou, Bénin

Povidone iodine 10% Solution BP PURNA PHARMACEUTICALS NV, Belgium

Triomune (Lamivudine, Stavudine and Nevirapine) Cipla

Appendix 9

LIST OF ARTCLES AND COMMUNICATIONS

1- **Ngouateu O.B.**, Weller K., Bröhl K., Kamtchouing P., Same-Ekobo A., Dondji B., Maurer M., von Stebut E. (2015). Impaired T cell-dependent protection against *Leishmania major* infection in HIV-positive patients is associated with worsened disease outcome. *Experimental Dermatology*. **24**: 296–319.

2- **Ngouateu O.B.**, Kollo P., Kamtchouing P., Ravel C., Derreure J., Same-Ekobo A., Maurer M., von Stebut E., Dondji B. (2014). Epidemiological investigations and clinical features of cutaneous leishmaniasis associated with HIV infection in Northern Cameroon. Third Conference of the African Epidemiological Association, First Conference of the Cameroon Society of Epidemiology 4th–6th June 2014, Mont Febe Hotel Yaounde. *African Journal of Epidemiology* **2**(1): 10.

3- **Ngouateu O.B.**, Kollo P., Ravel C., Derreure J., Kamtchouing P., Same-Ekobo A., von Stebut E., Maurer M., Dondji B. (2012). Clinical features and epidemiology of cutaneous leishmaniasis and *Leishmania major*/HIV co-infection in Cameroon: Results of a large cross-sectional study. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **106**: 137–142.

4- **Ngouateu O.B.**, Dondji B., Maurer M., von Stebut E. (2011). Epidemiological and immunological investigation on cutaneous leishmaniasis (CL) and *Leishmania*/HIV co-infection in Cameroon. 38th Annual Meeting of German Society of Dermatology, 17–19 February 2011, Tubingen, Germany. *Experimental Dermatology* **20**:172.

5- **Ngouateu O.B.**, Kollo P., Ravel C., Derreure J., Same-Ekobo A., Kamtchouing P., Maurer M., von Stebut E., Dondji B. (2011). Epidemiological, clinical and histopathological investigations on cutaneous leishmaniasis associated with HIV infection in northern Cameroon. 60th Annual Meeting of The American Society of Tropical Medicine and Hygiene, December 4–8, 2011, Philadelphia, PA, USA

6- **Ngouateu O.B.**, Kamtchouing P., Same-Ekobo A., Dondji B., Maurer M., von Stebut E. (2011). Impairment of protective immunity against *L. major* infection in

HIV-coinfected patients is associated with worsened disease outcome. 7th European Congress on Tropical Medicine and International Health. Barcelona, Spain. *Tropical Medicine and International Health.*

7- **Ngouateu O.B.**, Kollo P., Ravel C., Dereure J., Same-Ekobo A., Kamtchouing P., Maurer M., von Stebut E., Dondji B. (2011). Cutaneous leishmaniasis associated with HIV infection in Cameroon: clinical features, epidemiological and histopathological investigations in Mokolo endemic focus. 18th Annual Conference of the Cameroon Society of Biosciences 1-3 December 2011 Douala , Cameroon.

8- **Ngouateu K.O.B.**, Same-Ekobo A., Kamtchouing P., Dondji B., Maurer M., von Stebut E. 2010). Epidemiological and immunological investigations on cutaneous leishmaniasis (CL) and *Leishmania*/HIV co-infection in the Mokolo focus, Far North Region, Cameroon. Grantees meeting organized by the Volkswagen Foundation, Hannover, Germany, and the European Foundation Initiative for the African Research into Neglected Diseases within the Africa Initiatives "Communicable Diseases in Sub-Saharan Africa-From the Africa Bench to the Field" and "Neglected Tropical Diseases" 3–6 September 2010, Hamburg, Germany.

9- **Ngouateu O.B.**, von Stebut E., Maurer M., Same-Ekobo A., Kamtchouing P., Dondji B. (2009). Epidemiological investigations on cutaneous leishmaniasis and *Leishmania*/HIV co-infection in the Mokolo focus, Far North Province, Cameroon. 36 Jahrestagung der Arbeitsgemeinschaft Dermatologische Forschung, 5–7. Mãrz 2009, Heidelberg. *Experimental Dermatology* **18**: 302.

Appendix 10

FULL ARTICLES PUBLISHED FROM THE THESIS