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THE EFFECT OF IL-1RA AND IL-13 ON
CUTANEOUS LEISHMANIASIS IN BALB/C
MICE AS TO HYPERALGESIA AND THE
LEVELS OF IL-1 β AND IL-6

By

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A thesis submitted in partial
fulfillment of the requirements for
the degree of

MASTER OF SCIENCE

Molecular Biology

Lebanese American University

April 2005

Under the supervision of Dr. George Baroudy

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To Offer Degree

Master's of Science in Molecular Biology

Date of thesis defense: April 15, 2005

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Hamdan G. Hamdan

ACKNOWLEDGMENTS

I would like to express my deep appreciation and thanks to my supervisor Dr. George Baroudy for his help and support to accomplish this simple research.

All steps in this simple work could not have been achieved without directions, valuable comments and precious efforts of the Professor Nayef Saadé and Mr. Mark Karam. I am deeply grateful to both of them.

I would like to thank Dean of Arts and sciences Prof. Hashwa for his care and facilities to get all the materials for our work.

I am also cordially thanking Dean of Pharmacy Prof. Mroueh for his efforts and facilities from pharmacy school.

High gratitude to my instructors:

Dr. Roy Khalaf
Dr. Costantine Daher
Dr. Sima Tokajian

They have generously shared their extraordinary expertise and encouragement.

I am also so grateful to:

- Mr. Najib Abi Chedid for his generous help at many levels especially for preparing and providing us with the cultures of *Leishmania* throughout the whole project.
- Miss Helena Bou Farah for facilitating all technical issues and for her continuous support.
- Miss Rosaline Saba for her fruitful assistance in the ELISA part.
- Mr. Jean Karam for taking care of our experimental mice and for assisting us in many experiments.
- Mrs. Marie-Thérèse Mitri for her valuable help to provide us with references and articles.

Last but not least, I am grateful to:

- Prof. Garabedian
- Mr. Kamal Jaroudy
- Mr. Nafhat Fattouh
- Mrs. Rouba Haj-Sleiman
- Mr. Naaman Saadé
- Mr. Elias Mhanna
- Mrs. Lena Ahmarani
- Mr. Issam Nassar
- Mrs. Lara Khozami
- Mr. Marc Haber
- Mr. Rawad Hodiefy
- Mr. Omar Rahal
- Mr. Ziad Yassin
- Mr. Hossam Hawary
- Mr. Nael Alameh
- Mrs. Malak Essieli

And to all other friends for their efforts and help.

ABSTRACT

The Effect of IL-1ra and IL-13 on Cutaneous Leishmaniasis in BALB/c mice as to Hyperalgesia and the levels of IL-1 β and IL-6.

By

Hamdan Ghazi Hamdan

Recent researches demonstrated the presence of a sustained hyperalgesic state in all BALB/c mice (known to be susceptible to *Leishmania major*) inoculated with a high dose of *Leishmania major* (2.5×10^6 promastigotes per hind paw) as shown by the evident decrease in their pain thresholds. However, no neural involvement has been reported to date about role of IL-1ra and IL-13 on *Leishmania major*-induced inflammation as model. Thus, we aim in this project to prove the hypoalgesic effect of IL-1ra and IL-13 by reversing hyperalgesia in BALB/c mice induced by *Leishmania major* as a new model since other previous studies showed the anti-inflammatory effect of IL-1ra and IL-13 using different models (Saadé et al., 2000; Safieh-Garabedian et al., 1997a; Cunha et al., 2000).

This hyperalgesia should be accompanied with a significant increase in the levels of pro-inflammatory cytokines IL-1 β and IL-6 as is the case in the mice injected with a high dose of the parasite (Hirano, 1998; Kanaan et al., 2000). Both IL-1ra and IL-13 had the same effect on IL-1 β and reduced its levels which indicate its involvement in hyperalgesia directly. This confirms that both IL-1ra and IL-13 are hypoalgesic. Alternatively the two former cytokines had different effect on IL-6 which our results showed its indirect effect on hyperalgesia mechanism. IL-1ra decreased IL-6 levels while IL-13 increased it. It seems that IL-6 play as a mediator IL-6 to induce hyperalgesia by stimulation of other proinflammatory and anti-inflammatory cytokines (Dinarello, 1994, 1997).

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ABBREVIATIONS

ANOVA	Analysis of Variance
APC	Antigen Presenting Cells
cAMP	cyclic adenosine monophosphate
COX	Cyclic-oxegenase
CTL	Cytotoxic T Lymphocyte
DRG	Dorsal root ganglian
ELISA	Enzyme-linked immunosorbent assay
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte /macrophage-colony stimulating factor
HP	Hot Plate
Ig	Immunoglobulin
JAK	Janus-activated kinase
IL	Interleukin
INF- γ	Interferon- γ
i.p.	Intraperitoneal
I.U.	International Unit
kDa.	Kilodalton
L.C.	Langerhans Cells
LN	Lymph node
LPG	lipophosphoglycan
LPS	Lipopolysaccharide
μ l	Microliter

μm	Micrometer
MHC	major histocompatibility complex
moAB	Monoclonal Antibody
mRNA	Messenger Ribonucleic Acid
ng	nano gram
NGF	Nerve Growth Factor
NK	Natural Killer
NO	Nitric Oxide
PAN	Primary Afferent Nerves
PBS	Phosphate Buffer Saline
PGE ₂	Prostaglandin- E ₂
r.p.m	Round Per Minute
s	Second
SEM	Standard Error Mean
STAT	Signal transducers and activators of transcription pathway
TF	Tail Flick
TNF-α	Tumor Necrosis Factor-α
Th	T helper

*It's not suffering that diminishes man, but suffering
without meaning*

VICTOR FRANKL

*For all the happiness Mankind can gain is not
pleasure, But in rest from PAIN*

JOHN DRYDEN

After great PAIN, a formal feeling comes

EMILY DICKINSON

PAIN

*Is the motivation
For creation*

I dedicate my thesis to:

My beloved parents Ghazi and Elham Hamdan

*My dear sisters Sawsan, Salma, Maisaa, Jasmine
and Eliane Hamdan.*

To the memory of my grandfather Ibrahim Jabr

*Who have been a constant source of inspiration
to my career and life.*

Hamdan

CHAPTER I

INTRODUCTION

A. *Leishmania major*

1. *General Description:*

Leishmania major is an obligate intracellular parasitic protozoan of mononuclear phagocytes (Grimaldi Jr, 1982; Marzochi and Marzochi, 1994) that belongs to the kingdom Protista, phylum Sarcomastigophora, subphylum mastigophora, class Zoomastigophora, order Kinetoplastida, suborder Trypanosomatina, family Trypanosomidae, genus *Leishmania*.

The taxonomy of this genus is complex and often confusing since the differentiation is not based on the morphology of the organism but on the pathology and symptoms of the disease, site of infection, vector species and reservoir hosts. In addition, biochemical and serological factors are also used for characterization of the species complex. There are five principal species complexes, three "old world" and 2 "new world". There are two distinct types of diseases caused by the different species of *Leishmania*: visceral leishmaniasis (Kala Azar) and cutaneous leishmaniasis.

The visceral leishmaniasis is caused by *Leishmania donovani* (old world); it affects mainly males and children up to 10 years.

Cutaneous leishmaniasis: occurs in two distinct forms both in the old world and in the new world. These are described below.

New world:

- 1- *Leishmania mexicana*: causes a single painless lesion that heals within few months.
- 2- *Leishmania braziliensis*: causes single or multiple ulcers that seldom heal spontaneously.

Old world:

- 1- *Leishmania tropica*: causes a disease characterized by a dry cutaneous lesion which ulcerates but eventually heals leaving disfiguring scars (Ebrahimzadeh and Thomas., 1983).
- 2- *Leishmania major*: causes a disease characterized by a moist lesion (usually on the limbs) that ulcerates early. The lesions are slow to heal and they leave large disfiguring scars.

Leishmania major occurs in two distinct forms, the flagellated promastigote (which is also the infective stage) and the non-flagellated, amastigote, which is the intracellular stage, residing and multiplying within the macrophages of mammalian hosts (Molyneux and Ashford, 1983).

The amastigotes are spherical or ovoid in shape, about 2.5 to 5µm in diameter, and are contained within a parasitophagus vacuole in the macrophage. There is a prominent nucleus and kinetoplast with a non-emergent flagellum. The cytoplasm is vacuolated and contains lysosomes. The amastigotes contain exclusively the megasomes, which are thick, membraneous organelles containing digestive enzymes (Coombs et al., 1986).

Under the light microscope the promastigotes (14-20µm in size) have a long flagellum and a pointed posterior end, with the obvious presence of a nucleus and kinetoplast.

Moreover, the mitochondria are quite abundant in the promastigote. Both forms multiply by binary fission (Garnham, 1971).

2. *The life cycle of Leishmania:*

All *Leishmania* species are morphologically similar and show two major developmental stages through their life cycle: the amastigote that dwells inside the reticuloendothelial cells of the mammalian host, and the promastigote, that divides in the gut of a phlebotomine sandfly (Killick-Kendrick and Molineux et al., 1981) (Figure 1).

The life cycle begins when the vertebrate host is bitten by an infected insect. High densities of infecting parasites block the cardiac valve at the digestive tract of the sandfly and when insect takes the blood from the host, it ejects the valve's content, including the parasites. The insect's saliva contains chemical factors that increase the parasite infective power and exert a chemotactic effect upon reticuloendothelial cells, which are attracted to the place of inoculation. The parasites are then phagocytized by reticuloendothelial cells, and this promotes their reproduction and survival. As to dermatropic *Leishmania* species the lesion limited in the skin, but for viscerotropic *Leishmania* the parasite spreads from the initial skin lesion into other organs such as liver, spleen and bone marrow. The parasites profusely replicate inside the reticuloendothelial cells and eventually burst free from the infected macrophages, spreading the disease within the mammal host. It is still not obvious whether the hosts cells are actively involved in this incident, are physically disrupted by the infection, or undergo apoptosis (Chang et al., 2003; DosReis, 2000). As a new insect bites an infected vertebrate host, it takes infected macrophages, and the parasites transform into promastigotes, migrate into the midgut, become metacyclic

(infective parasites) during the next four to seven days, and migrate to the cardiac valve ready to be re-inoculated into a vertebrate host (Figure 1).

Due to physical and chemical changes among the hosts where *Leishmania* lives enforce thoroughly different stress conditions that must be avoided by the parasite's ability to adapt to diverse environments. In fact, during the development of the promastigote stage, its conversion into the metacyclic promastigote, its interaction with the host cell and the establishment of the infection, the parasite is frequently activating physiological responses which guarantee its survival. There are presently not enough studies on how the development of drug-resistance interacts with the physiological events that cause and regulate the parasite's differentiation through its life cycle (Ismaeel et al., 1998).

Figure 1. Life cycle of *Leishmania* (Ghosh and Bandyopadhyay, 2004).

B. Immunity

1. *Leishmania major* and macrophages interaction:

The most basic cellular mechanisms of resistance to infection are associated with the ability of phagocytes to engulf invading microorganisms; phagocytosis by macrophages exposes parasites to a respiratory burst and a hostile lysosomal compartment which in many cases is lethal to microorganisms. *Leishmania major* invades the macrophages in its vertebrate host (Titus *et al.*, 1994) (Figure 2). The ability of *Leishmania major* to survive inside macrophages when phagocytosed via many receptors, the major ones being the complement receptors type I and type III (Mosser and Rosenthal 1993). Complement dependent promastigote adhesion to the promastigote containing phagosome subsequently undergoes a maturation process involving the fusion with endocytic organelles including endosomes and lysosomes resulting in the mature phagolysosome (Antoine *et al.*, 1998). The mature phagolysosome is the major microbicidal site in macrophages due to the presence of lysosomal hydrolyzing enzymes, acidic pH, and toxic molecules such as nitric oxide (NO) and other reactive oxygen intermediates (Desjardin and Descoteaux 1997). Following amastigote establishment and multiplication, the infected macrophage eventually will lyse new amastigotes which are rapidly taken up by surrounding macrophages. Since amastigotes require the acidic environment for multiplication, they don't delay the maturation of the parasitized phagolysosome once they have been taken up by macrophages. This is due to the promastigotes surface molecule lipophosphoglycan (LPG) which appears to be the responsible for delaying phagolysosome maturation (Desjardin and Descoteaux 1997).

Figure 2. Interaction between infected macrophages and Th1

Following the initial process and survival of the innate immune response, the amastigotes must next survive the acquired immune response in order to establish a long term chronic infection. Macrophages play a central role in initiating the acquired immune response to infectious pathogens through presenting antigen and releasing regulatory cytokines to T cells. In order to resolve the *Leishmania major* infection, the acquired immune response must include the development of a Th1 immune response which is capable of producing INF- γ which subsequently activates macrophages microbicidal activity on the other hand development of Th2 lead to progressive of disease (Figure 2). Either Th1 or Th2 will step forward due to secretion of different cytokines which will determine the pathway will dominate (Reed and Scott., 1990). Which type will dominate is shown to depend principally relatively on the efficiencies of activation of the responding Th1 and Th2 cells.

2. *Jak-STAT signaling pathway:*

It represents one of most direct routes for intracellular signaling pathways to alter gene transcription. In case of viral infection interferons (it was initially discovered by studies on the effects of interferons) bind to receptors on noninfected neighboring cells. When activated by cytokine receptor activate a class of cytoplasmic tyrosine kinases called Janus kinases (Jaks) which phosphorylate and activate a set of latent gene regulatory proteins called STATs (signal transducers and activators of transcription) which move to the nucleus and stimulate the transcription of specific genes. Jak-STAT pathway is activated by more than 30 cytokines and hormones by binding to cytokine receptors (Alberts et al., 2002).

3. *Th1/Th2:*

In response to antigenic challenge, an animal generally responds with either a humoral or cell-mediated response known as delayed type hypersensitivity (DTH), but not both (Parish, 1972; Katsura, 1977; Scott, 1989; Müller et al., 1989). Two types of helper cell clones, called Th1 and Th2, have been defined on the basis of their pattern of cytokine secretion (Romagnani, 1991; Street and Mosmann, 1991) (Figure 3). These two types of helper cells appear to have dramatically different functions. Th1 cells, because of the factors that they produce, help in the induction of DTH responses via macrophage activation and generation of cytotoxic T lymphocytes (CTL). Th2 cells cannot induce DTH but rather induce B cells to make and secrete antibodies. Th1 cells can also provide B cell help, but Th2 cells are much more efficient (Mosmann and Coffman, 1989). Although both Th1 and Th2 cells secrete a large number of factors, only Th1 cells secrete

interleukin-2 (IL-2), tumor necrosis factor-beta (TNF- β) and interferon- γ (INF- γ), molecules involved in inducing macrophages activation in DTH responses and CTL proliferation (Cher and Mosmann, 1987; Stout and Bottomly, 1989). The correlation of Th1 and Th2 responses is due to cytokines expressed by each subset, which eventually determine the fate of the parasite within the host cell (macrophage) (Figure 3).

Figure 3. A simplified model of the possible interactions between polarised Th1 and Th2 responses

The murine model of infection with *Leishmania major* provided the first correlation in vivo between (1) the development of protective immunity and an expansion of Th1 CD4⁺ T cells in resistant mice and (2) the expression of progressive disease and the development of a CD4⁺ Th2 cell response in susceptible mice (Locksley et al., 1987; Scott et al., 1988; Heinzel et al., 1989; Mosmann and Coffman, 1989). Disease healing is mediated by Th1 cells and characterized by a strong DTH and poor antibody response,

whereas nonhealing infection which is mediated by Th2 cells and is accompanied by a strong antibody response without DTH activity against extracellular pathogens (Abbas et al., 1996; Heinzel et al., 1989; Leiw, 1989).

Cytokines can cross-regulate each other production, and this affects the activities of Th1 and Th2 cells (Titus et al, 1994).

In general, an efficient clearance of intracellular pathogens is based on innate cell activation, while antibody responses are best suited for extracellular infections. The decision of naive $CD4^+$ T cells to become Th1 or Th2 has important consequences in the success of an immune response and the progression of diseases. It has been hypothesized that T helper pathway (either Th1 or Th2) in mice infected with *Leishmania major* is determined by the cytokines produced in the first week of infection. Th1 cells produce primarily interferon-gamma ($IFN-\gamma$) and tumor necrosis factor α ($TNF \alpha$), which are required for cell-mediated inflammatory reactions that are known to activate host defenses against intracellular pathogens; Th2 cells secrete interleukin (IL)-4, IL-5, IL-10 and IL-13, which mediate B cell activation and antibody production (Swain et al., 1990; Murphy et al., 2000; Cherwinski et al., 1987).

The selective differentiation of precursor $CD4^+$ T cells into effector Th1 and Th2 cells is influenced by a variety of extracellular factors, such as the cytokine environment, the dose of antigen and the source of co-stimulation (Constant and Bottomly, 1997). Among these, the most effective polarizing factor is the cytokine environment. The presence of IL-4 during activation drives the differentiation of precursor $CD4^+$ T cells into Th2 cells, whereas the presence of IL-12, IL-18 and $IFN-\gamma$ promotes differentiation into Th1 cells

(Hsieh et al., 1993; Le Gros et al., 1990; Nakanishi et al., 2001; Seder et al., 1992; Swain et al., 1990).

4. *Susceptible Versus Resistant Mice:*

Upon experimental infection with *Leishmania major*, distinct features of the spectrum of clinical manifestations seen in patients with cutaneous leishmaniasis can be reproduced in inbred mice of different genetic backgrounds (Behin et al., 1979; Mitchell et al., 1981). Mice from the majority of inbred strains (C3H/HeN, CBA, C57BL/6, 129Sv/Ev, C57BL/6 and DBA) develop locally cutaneous lesions, which spontaneously resolve (heals spontaneously after a few weeks). These mice do not develop lesions after a second inoculation of *Leishmania major* and belong to the resistant phenotype. Mice from a few strains (BALB/c and SWR) develop (Th2 response) severe and uncontrolled lesions without becoming immune to reinfection and are representative of the susceptible phenotype (Mitchell et al., 1981). Local infection is not effectively controlled by the immune response of these mice and the disease disseminates to involve visceral organs with eventually fatal outcome. The nonhealer phenotype is controlled by multiple genes suggesting that different genetic factors contribute to susceptibility by different mechanisms (Demant et al., 1996 and Lipoldova et al., 2000). This murine model of infection has been used to characterize the immune responses developing in both resistant and susceptible mice. The critical difference is that lesions of resistant mice heal and lesions of susceptible mice do not heal but tend to increase in size and to ulcerate. Therefore, it is more appropriate to distinguish between healer and nonhealer mouse strains (Preston et al., 1976). The resolution of lesions is accompanied by the

development of complete resistance to reinfection. MHC class II-restricted CD4⁺ T cells are of critical importance for the development of clinical disease (Reiner et al., 1994 and Locksley et al., 1993). Whereas MHC class I-restricted CD8⁺ T cells are of minor importance during primary infection (Wang et al., 1993).

5. *Cytokines and their role in susceptibility and resistance to leishmania major:*

Cytokines are small low-molecular-weight proteins (typically 5-30 kDa); some are glycoproteins while others are synthesized as larger precursors, which are cleaved to give the active molecule. Cohen et al., (1974) who proposed the term "Cytokins" which has become the commonly accepted name for this group of proteins. Later it was proposed the term "Interleukin" (Aarden et al., 1979). They are produced by their cells and act upon receptors on their target cells. They are produced by diverse cells and have a variety of regulatory roles. The life times of cytokines are usually short and their destruction is usually a regulated process. They have an important role in regulating immune response to foreign antigens on invading organisms through development of cellular and humoral immunity, cellular proliferation and differentiation and inflammatory responses, therefore in many diseases and disturbance of these processes there is changed regulation of cytokine production and function (Borish et al., 1996). The cytokines act upon by binding, in an antigen-nonspecific manner to specific receptors on the membrane of target cells initiating a signal transduction pathway that controls gene expression in those cells evoking particular biologic activities (Henderson and Poole, 1994).

a. Interleukin-1 (IL-1):

Interleukin-1 (IL-1) is a pleiotropic cytokine that acts in the context of inflammation, cell growth and tissue repair. Furthermore, IL-1 is emerging as a critical molecular link between the immune and neuroendocrine systems, playing a key role in processes such as homeostatic regulation, pain perception and appetite (Bianchi et al., 1998 and Inoué, 2001).

It represents one of the most pleiotropic proinflammatory cytokines. It acts alone, but more importantly, it induces the generation and secretion of a wide variety of proinflammatory cytokines and products which amplify and sustain the response (Dinarello, 1994). The IL-1 family consists of three types of ligands i.e. two proinflammatory agonists (IL-1 α and IL-1 β) and one endogenous receptor antagonist (IL-1 receptor antagonist (IL-1ra)). IL-1ra is a structural variant of IL-1 that inhibits the proinflammatory effects of IL-1 by functioning as a competitive inhibitor of IL-1 binding (Arend, 2001). It also shown that IL-1 is required for normal regulation of Th1/Th2 responses and may negatively regulate IL-4 expression (Satoskar et al., 1998).

IL-1 signaling is mediated by a complex system, including the cytokines IL-1 α and IL-1 β , and the IL-1 receptor antagonist (IL-1ra), which binds IL-1 receptors and blocks the effects of IL-1 (Bresnahan and Cunnane, 1998). However IL-1 α and IL-1 β differ in their active subcellular compartments. IL-1 β is active in its secreted form (17.5 kDa), whereas its cytosolic precursor is inactive; IL-1 α is mainly active as an intracellular precursor (31 kDa) and as a membrane-associated form (23 kDa), but only marginally active in the secreted form (17.5 kDa).

The role of IL-1 in leishmaniasis seems to be dependent on the parasite species causing the disease and the host response. Macrophages from susceptible BALB/c mice infected with *Leishmania major* produce more IL-1 mRNA than do resistant mice; it is one of the most important cytokines produced by the macrophages which induces T-cell activation (Harms et al., 1991). IL-1 and TNF- α induce further secretion of the proinflammatory cytokines (Durum et al., 1993).

a . 1. Interleukin-1 receptors (IL-1R):

There are two types of IL-1 receptors (Figure 4), type I IL-1R (IL-1RI) have a long cytoplasmic tail and it is responsible for induction of intracellular signal transductions after binding with IL-1 (which appears to mediate all of the known biological functions of IL-1) (O'Neill and Dinarello, 2000; Sims et al., 1988). Type II IL-1R (IL-1RII) has a short cytoplasmic domain and is inactive towards inflammatory response. IL-1RII on the cell surface, membrane form IL-1RII, working as decoy receptors, leads to a net decrease in IL-1 available to bind to IL-1RI so it inhibits the responses to IL-1 which increases the affinity of IL-1 to its receptor and is required for signaling (Born et al., 2000). Besides, IL-1RII is released from the cell surface in a soluble form (sIL-1RII) that binds to IL-1 and prevents this cytokine from reaching the cell surface (Mantovani et al., 2001). So the type II IL-1 receptor (IL-1RII) is a negative regulator of the IL-1 system and acts as a decoy receptor (Colotta et al., 1993; McMahan et al., 1991). The additional IL-1 receptor accessory protein (IL-1RacP) is necessary for IL-1 signal transduction (Greenfeder et al., 1995) (Figure 4).

IL-1RI and IL-1RII have different affinities for IL-1ra. IL-1RI binds to IL-1ra with higher affinity than IL-1 α and β . In contrast, IL-1RII binds to IL-1 β 10-20 fold better than IL-1ra or IL-1 α (Arend et al., 1994). Notably, signaling through the functional IL-1R also leads to IL-1 β release (Dinarello et al., 1987 and Warner et al., 1987).

Figure 4. IL-1 β binds to the membrane-bound IL-1RI, resulting in a conformational change to the receptor. The IL-1/IL-1RI complex then forms a heterodimer complex with the IL-1 receptor accessory protein (IL-1RAcP). The intracellular domains of the receptor proteins have Toll domains, which are important for signal transduction. This complex recruits the IL-1RI-associated kinase (IRAK), leading to signal transduction and gene activation, sIL-1RI = soluble IL-1 receptor type I; sIL-1RII = soluble IL-1 receptor type II; IL-1RAcP = IL-1R accessory protein; IRAK = IL-1RI-associated kinase. (Dinarello and Moldawer, 2001).

a. 2. *Interleukin-1 alpha (IL-1 α):*

IL-1 α is rarely found in the circulation or extracellular biological fluids and only in cases of serious illness when it is thought to have originated from lysed cells (Wakabayashi et al., 1991), and cleavage by extracellular proteases (Kobayashi et al., 1988).

The exact role of IL-1 α in the differentiation of T cells is uncertain. Some studies with T-cell clones suggested that IL-1 α favors the expansion of Th2 cells (Litchman et al., 1988; Weaver et al., 1988). But studies using in vitro priming system indicated that IL-1 α was not necessary to generate a Th2 response (Seder et al., 1994).

It has been reported that infected macrophages produce high levels of IL-1 α , and that as a result, infecting macrophages with *Leishmania major* favors the outgrowth of Th2 cells (Chakkalath et al., 1994 and Cillari et al., 1989). This is consistent with in Milena et al., (1997) in which susceptible BALB/c cells produced significantly more IL-1 α in the primary in vitro response (Milena et al., 1997). However, treating mice with an anti-IL-1 α receptor antibody decreases lesion size in mice infected with *Leishmania major* but does not affect either the immune response to or the rate of clearance of *Leishmania major* by the treated animals (Theodos et al., 1994).

In contrast to these results recent studies showed that treatment BALB/c mice with IL-1 α during T cell priming induced Th1-development that attenuated disease progression in susceptible BALB/c mice, which demonstrate that this effect was dependent on, and in the treatment employed as effective as, IL-12 (Stebut et al., 2003). Subsequently demonstrate that IL-1 α (and TNF- α) are required for maximal IL-12-driven Th1 development in BALB/c, but not C57BL/6, T cells (Shibuya et al., 1999). Other studies showed that the IL-1 α -treated BALB/c mice had slightly increased amounts of IL-12 in

draining lymph nodes suggesting that IL-1 α mediated up-regulation of IL-12 production could contribute to Th1 development (Stebut et al., 2003).

a. 3. Interleukin-1 beta (IL-1 β):

Pro-IL-1 β remains primarily cytosolic and is not completely active until it is cleaved by proteolytic enzymes and transported out of the cell (Tocci, 1997) (Figure 5).

As to the infection with *Leishmania major*, it was shown that the level of IL-1 β in the injected paw of BALB/c mice increases significantly at day 3 following the injection of the parasites to return back to the normal level at the end of the first week post-injection. Then, this level increases again at the end of the second week post-injection and is maintained throughout the observation period (7 week) (Kanaan et al., 2000). This is in accordance with the observation that the macrophages from susceptible mice secrete large amount of IL-1 in response to *Leishmania major* infection, while these from resistant mice produce significantly lower levels of IL-1 (Cillari et al., 1989). Treating mice with an anti-IL-1 receptor inhibits the development of lesions in cutaneous leishmaniasis, but has little, if any effect, on the parasite number in the lesions as well as the cytokines that the animals produce in response to infection (Theodos et al., 1994). Although IL-1 is not required for mounting an immune response and antigen-dependent proliferation, it appears to be required for normal regulation of Th1/Th2 response (Satoskar et al., 1998) since IL-1 acts as a co-factor of the activation of Th2 cells (Kurt-Jones et al., 1987; Litchman et al., 1988) by stimulating the *Leishmania major* infected macrophages to present antigens to Th2 cells and IL-4 synthesis (Chakkalath and Titus, 1994). Furthermore, IL-1 β reduces the Langerhans cells (LC) mediated migration of

Leishmania major to regional lymph nodes since the depletion of IL-1 β enhances the parasite-induced LC migration, whereas treatment with recombinant IL-1 β reduces the rate of infected LC in the lymph nodes (Arnoldi and Moll, 1998). Consequently, IL-1 seems to exacerbate the leishmanial lesion in infected BALB/c mice especially by the induction of prostaglandins synthesis (Ajdary et al., 1997).

a . 4. Interleukin-1 receptor antagonist (IL-1ra):

IL-1ra is a member of the IL-1 family and shares 26% sequence homology with IL-1 β , it is synthesised with a leader sequence, processed, and then secreted from the cell (Figure 6). It is produced both constitutively and during inflammation IL-1ra binds to IL-1 receptors; however, it does not activate IL-1 receptor type I, as it does not allow IL-1 receptor accessory protein to form the heterodimer complex necessary for signal transduction. Because IL-1ra has no agonist activity, it is referred to as a pure receptor antagonist (Arend, 1997; 1998).

The IL-1ra is a specific antagonist of IL-1, in that it binds the IL-1 receptor without transmitting any signal into the cell (Hannum et al., 1990) (Figure 6). IL-1 receptor antagonist (IL-1ra) binds to IL-1 receptors; however, it does not elicit a cellular response (Dinarello, 1996). The secreted isoform of the IL-1ra efficiently competes with IL-1 for occupancy of the functional receptor, while having a substantially lower affinity for the decoy receptor (McIntyre et al., 1991).

Figure 5. IL-1Ra competes with IL-1 β for the IL-1RI. If IL-1Ra binds to the receptor, the binding site for the accessory protein is blocked, thus cell signalling and activation is prevented. , sIL-1RI = soluble IL-1 receptor type I; sIL-1RII = soluble IL-1 receptor type II; IL-1RAcP = IL-1R accessory protein; IRAK = IL-1RI-associated kinase (Dinarello, 1996).

Figure 6. IL-1 signaling: IL-1 binds IL-1RI and recruits IL-1RAcP. This heterotrimeric complex initiates signal transduction. IL-1Ra binds to the IL-1RI with similar affinity as IL-1 but prevents IL-1RAcP binding thus preventing signal transduction. IL-1RI = IL-1 receptor type I; IL-1Ra = IL-1 receptor antagonist IL-1RAcP = IL-1 receptor accessory protein (Dinarello, 1996).

IL-1ra is an acute phase protein synthesized rapidly in liver in response to infection and endotoxin (Gabay et al., 1991). It limits cytokine-mediated hyperalgesic responses to Carrageenin (Pool et al., 1995).

b. Interleukin-4 (IL-4):

IL-4 produced by Th2 cells, acts as an autocrine growth factor for Th2 cells, it promotes the proliferation and differentiation of activated B-cells, the expression of class II MHC antigens (Fishman and Peretson, 1994). The production of IL-4 is associated with susceptibility to *Leishmania major* infection in BALB/c mice (Liew and Donnell 1993, Reed and Scott 1993, Bogdan and Rollinghoff 1993, Titus et al., 1994, Reiner and Locksley 1995, Heinzl et al., 1989 and Launois et al., 1997).

Treating BALB/c mice with a neutralizing anti-IL-4 antibody allows the animals to cure their infection by promoting the outgrowth of Th1 cells (Chatelain et al., 1992; Saddick et al., 1990). Both Matthews et al. and Mohrs et al., showed that the same mouse strain was resistant (Mohrs et al., 1999 and Matthews et al., 2000) and IL-4 receptor- α -deficient animals were more resistant to *Leishmania major* than IL-4-deficient mice (Noben-Trauth et al., 1999). Some reports used IL-4-deficient mice have presented contradictory evidence for the importance of IL-4 in the disease process. Noben-Trauth et al., (1996) reported that IL-4-deficient mice, on a BALB/c background, remained susceptible to *Leishmania major*. Genetic mapping studies have identified that the IL-4 gene present within a genomic region related to susceptibility in mouse (Roberts et al., 1993; Beebe et al., 1997). This region also includes the IL-13 gene, and it is noteworthy that the IL-13 protein can also utilize the IL-4ra (Zurawski et al., 1995).

c. Interleukin-6 (IL-6):

IL-6 is a pleiotropic cytokine which is produced by a number of cell types including macrophages, fibroblasts, macrophages, dendritic cells, T and B lymphocytes, endothelial cells, glial cells and keratinocytes in response to a variety of external stimuli (e.g. IL-1, TNF) and it is also produced by CD4⁺ Th2 (Hirano, 1996; Van Snick 1990) and promotes Th2 differentiation by inducing the expression of IL-4 gene during activation of CD4⁺ T cells (Diehl and Rincon 2002). It plays a role in B-cell stimulation and in regulation of acute-phase responses and inflammation. Although coordinately induced from activated macrophages with IL-1 and tumor necrosis factor alpha (TNF- α), IL-6 suppresses the production of the previous two cytokines from macrophages, whereas IL-1 and TNF- α induce further secretion of the proinflammatory cytokines (Durum et al., 1993). While Rincon and Flavell (1997b) reported that it acts as a key component in the link between innate immunity and Th2 responses, and as inducer of IL-1 and macrophages/NK (natural killer cells) response to intracellular pathogens. It's involved in the regulation of the immune and acute phase responses of inflammation (Elenkov et al., 2000; Gijbels and Billiau 1992; Kishimoto, 1989).

It has been suggested that IL-6 favors the outgrowth of Th2 cells (Rincon et al., 1997a). Several cell types, including macrophages, dendritic cells, and T cells, produce IL-6. Originally thought of as a B-cell growth factor, IL-6 is now known to have effects on several components of the immune system (Akira et al., 1993, Taga et al., 1997). Some studies showed that IL-6 deficiency appeared to generally lower the expression of both Th1 and Th2 cytokines but not their overall balance of expression. As a result, BALB.B IL-6^{-/-} mice remained susceptible to infection with *Leishmania major* (Titus et al., 2001).

Resistant-background C57BL/6 IL-6^{+/-} and IL-6^{-/-} controlled infection with *Leishmania major* IL-6, therefore, is not required for healing and resolution of infection with *Leishmania major*. The IL-6^{-/-} and IL-6^{+/-} animals all had elevated levels of IFN- γ mRNA and lower levels of IL-4 mRNA compared to susceptible BALB/c mice. IL-6, therefore, is not required for strong Th1 responses against *Leishmania major* (Moskowitz et al., 1997).

Titus et al., (2001) found that susceptible mice produced between two to fourfold more IL-6 than cells from resistant mice. Because this result suggested that IL-6 secretion correlates with susceptibility to infection with *Leishmania major*, they hypothesized that IL-6^{-/-} BALB.B mice would be more resistant to infection with *Leishmania major* than susceptible wild-type BALB.B mice (Kopf et al., 1994) but contrary to their hypothesis, the lesion size in IL-6^{-/-} BALB.B mice was not significantly different from that seen in wild-type susceptible BALB.B mice, small differences (both positive and negative) between the susceptibilities of IL-6^{-/-} mice and control BALB.B mice to infection with *Leishmania major* (Titus et al., 2001).

Interestingly, even in the absence of any polarizing cytokine, IL-6 directed the differentiation of the CD4⁺ cells to a Th2 phenotype, since the cells differentiated in the presence of IL-6 produce high amounts of IL-4, but not IFN γ , after re-stimulation. IL-6 did not modify the differentiation of the Th2 cells directed by IL-4. IL-6, however, impaired Th1 differentiation. IL-6 required producing IL-4, but this IL-4 production was impaired when the IL-6 pathway was blocked by the addition of anti-IL-6 antibody (Rincon et al., 1997a; Diehl and Rincon 2002).

High IL-4 production was re-established in cells that were differentiated in the presence of IL-6^{-/-} APC together with an exogenous source of IL-6. Thus, IL-6 production by APC during activation of CD4⁺ T cells plays a major role in determining the type of cytokines that effector CD4⁺ T cells will secrete (Diehl and Rincon 2002).

d. Interleukin-12 (IL-12):

IL-12 is a heterodimeric cytokine comprised of p40 and p35 subunits that together form the biologically active p70 molecule (Gately et al., 1998). IL-12(p70) functions via binding to a heterodimeric receptor (IL-12R β 1 and IL-12R β 2) that is expressed on the surface of IL-12-responsive cells. It is produced by macrophages and enhances cytotoxicity and interferon-gamma (INF- γ) production by T and natural killer (NK) cells (Kobayashi et al., 1989; Stern et al., 1990).

IL-12R β 2-deficient mice of a genetically resistant background such as C57BL/6 are susceptible to *Leishmania major* infection and develop a parasite specific Th2 immune response. Mice deficient for the IL-12-specific chain of the IL-12 receptor (IL-12R β 2) provide evidence that IL-12 signaling is critical to development of a parasite specific Th1 response and resistance to *Leishmania major* infection in the experimental murine leishmaniasis system (Chakir et al., 2003).

It has been implicated as a critical cytokine in the development of Th1 cells both in vitro and in vivo (Manetti et al., 1993; Afonso et al., 1994). Some studies reported that treatment of highly susceptible BALB/c mice with IL-12 during early *Leishmania major* infection results in increased levels of IFN- γ production and converts these mice to a resistant phenotype (Heinzel et al., 1993; Afonso et al., 1994; Sypek et al., 1993). Other

studies showed that IL-12 is indispensable and essential for protective immunity against *Leishmania major*. Since IL-12-deficient mice derived from a strain genetically resistant to infection with *Leishmania major* were shown to be susceptible to it, developing a strong Th2 response after injection of a high dose of parasites and there is no rescue pathway appears to compensate for the lack of IL-12 (Mattner et al., 1996) and suppression or ablation of IL-12 can inhibit parasite clearance and result in the development of susceptibility to infection. Neutralization of IL-12 induces a susceptible phenotype (Scharton-Kersten et al., 1995; Mattner et al., 1996). In vitro studies have demonstrated that Th2 commitment precedes from a rapid loss of IL-12 responsiveness due to the down-regulation of the IL-12 receptor beta-2 chain (IL-12R β 2) in BALB/c T cells (Guler et al., 1997).

In contrast to the above studies others reported that treating resistant mice with an anti-IL-12 antibody does not necessarily prevent the mice from healing (Heinzel et al., 1995; Scharton-Kersten et al., 1995; Sypek et al., 1993). However, whether low or high levels of IL-12 are made, BALB/c, C57, and C3H mice produce equivalent amounts of the cytokine following infection with *Leishmania major* (Reiner et al., 1994; Scharton-Kersten et al., 1995; Milena et al., 1997).

e. Interleukin-13 (IL-13):

Interleukin-13, the most recently identified cytokine to join the T helper 2 cytokine family is distantly related to IL-4. It shares a number of structural characteristics with IL-4 and belongs to the same α -helix protein family (de Saint et al., 1998). Furthermore, recent reports have highlighted the complex and integrated roles for IL-13 and IL-4 in the

development of Th2 cell responses. IL-13 shares ~30% homology with IL-4, and appears to have certain overlapping biological activities (Zurawski and de Vries, 1994). Interestingly, the genes encoding IL-4 and IL-13 are closely linked on human chromosome 5 and mouse chromosome 11 (Frazer et al., 1997; McKenzie et al., 1998, 1999).

IL-4 and IL-13 induce a variety of immunomodulatory functions on a wide range of cell types, including macrophages, NK cells, fibroblasts, eosinophils, airway smooth muscle cells, and endothelial cells, highlighting their pleiotropic activities. Both cytokines also have important immunosuppressive and anti-inflammatory activities on macrophages and other cells, including inhibition of pro-inflammatory cytokines (IL-1, IL-6, IL-10, IL-12, TNF- α , GM-CSF, G-CSF as reviewed in (Brombacher, 2000).

The overlapping biological functions of IL-4 and IL-13 on some cell types are due to at least, one shared component of otherwise distinct receptors; it is noteworthy that the IL-13 protein can also utilize the IL-4R α , is evident that both IL-4 and IL-13 can cross-compete for the IL-4R α , but that only IL-4 binds directly to this receptor chain (Zurawski et al., 1993), indicating their origin from a possible gene duplication event during evolution. IL-4 and IL-13 production is restricted to activated T lymphocytes, especially Th2 cells, mast cells and basophiles (Zurawski et al., 1995).

The evidence for this came from competition studies between IL-4 and IL-13 and the ability of IL-4 antagonists to inhibit both IL-4 and IL-13-induced responses on the same cell type (Kruse et al., 1992 and Aversa et al., 1993).

Two IL-13 binding chains have been identified, IL-13R α 1 and IL-13R α 2, and these are members of the class I cytokine receptor family. The main structural difference between

the two IL-13 receptors is that the IL-13R α 1 chain has a longer intracellular domain than IL-13R α 2. IL-13R α 1 is a low affinity IL-13 binding chain that requires the recruitment of IL-4R α into a receptor complex for the formation of a high affinity IL-13 receptor and the initiation of signal transduction so IL-13 binds to its own primary binding chain (IL-13R α 1) to which IL-4 does not bind, and recruits IL-4R α into a receptor complex, resulting in an increase in binding affinity and the initiation of signal transduction (Aman et al., 1996; Hilton et al., 1996). IL-13R α 2 binds IL-13 with a higher affinity than IL-13R α 1, but its function as a signaling molecule remains uncertain. Indeed, it has been suggested that IL-13R α 2 may function as an inhibitor of IL-13 activity. It is the IL-13R α 2 chain that has been engineered to act as a soluble antagonist of IL-13 function (Donaldson et al., 1998). This complexity of receptor usage and the potential diversity of signaling pathways combine with the temporal and spatial expression of the individual ligands to create a diversity of possible responses.

Although both IL-4 and IL-13 can cross compete for IL-4R α when it forms part of the IL-4R α -IL-13R α 1 complex, only IL-4 binds directly to IL-4R α (Zurawski et al., 1993). As a consequence, the IL-4R α -IL-13R α 1 complex was also identified as a functional IL-4 receptor (Callard et al., 1996).

Matthews et al., (2000) demonstrated for the first time that IL-13 is an important component for the generation and maintenance of immune responses to *Leishmania major* infection. The over expression of IL-13 resulted in C57BL/6 mice becoming susceptible to *Leishmania major*, and significantly, this was independent of IL-4 expression. Conversely, IL-13-deficient BALB/c mice were resistant to *Leishmania major* and they noted that BALB/c mice doubly deficient for IL-4 and IL-13 were

significantly more resistant to *Leishmania major* infection than either of the single cytokine-deficient animals or even the resistant C57BL/6 mouse strain. IL-13 acts independently of IL-4 and thus identifies IL-13 as a major component of the immune response to *Leishmania major*.

IL-13 has been shown to inhibit the production of IL-12 by macrophages and to inhibit *Leishmania major* parasite killing in vitro (Oswald et al., 1992 and Doherty et al., 1993). It is probable that by over-expressing or ablating IL-13, the levels of IL-12 production will be modified. Thus, over-expression of IL-13 in transgenic animals may be acting to prevent the onset of a curative Th1 response following infection. Equally, IL-13 may be acting directly on the macrophage by inhibiting parasite killing mechanisms. Thus, these alternative pathways are not mutually exclusive and would represent an integrated immune response to *Leishmania major* infection. In addition, it is also possible that in the case of the IL-13-transgenic mice, the observed decrease in IL-12 production may be due to the increased parasite load suppressing normal macrophage function (Matthews *et al.*, 2000).

C. Inflammation:

Inflammation is a physiologic response of a host to its invasion by a foreign body or a pathogen. The cardinal symptoms of inflammation are redness (erythema), swelling (edema), heat (fever) and pain. Usually, those symptoms are accompanied by the loss of function of the inflamed area. The increase in vascular diameter (vasodilation) at the site of infection leads to an increase on blood volume and therefore to heat and redness.

Furthermore, the increase in vascular permeability leads to edema and contributes to the swelling and redness of the area (Dray, 1994).

1. *Good pain, bad pain:*

Pain can be persistent or chronic, persistent pain can be subdivided into two classes: acute pain and neuropathic pain. The first results from the presence of a noxious stimulus that produces actual tissue damage and activation of nociceptors in the injured tissue while the second results from direct injury to nerves in the peripheral or central nervous system. Acute pain is biologically useful because it signals injury or disease and subsides as healing progresses. In contrast chronic pain is unlikely to resolve or pain that's lasts longer than the usual healing time and serves no useful biologic roles since it limits normal functions of human.

Acute nociception refers to our ability to detect and react to potentially damaging stimuli, which are usually mechanical or thermal in nature. Under circumstances of tissue injury, stimuli that would not normally be considered painful become capable of eliciting intense pain.

Figure 5. Effects of tissue injury on pain sensitization. The curve on the right shows normal pain response as a function of stimulus intensity. After injury, the curve shifts to the left: noxious stimuli become more painful (hyperalgesia), and innocuous stimuli are experienced as pain (allodynia). (Gottschalk and Smith 2001).

This hypersensitivity to stimulation of injured tissue and surrounding uninjured tissue has been termed “hyperalgesia” (Figure 5). An example of classical hyperalgesia in humans is the unpleasant pricking pain evoked by a blunt probe applied adjacent to injured tissue. In other situations, a stimulus normally perceived as innocuous, such as a brush of the skin, becomes intensely painful: this phenomenon is termed “allodynia” (Warfield and Bajwa, 2004; Lewin et al., 2004; Markenson, 1996).

2. *Nociceptors:*

The propagation of pain is initiated with the activation of physiological receptors, called nociceptors, widely found in the skin, mucosa, membranes, deep fascias, connective tissues of visceral organs, ligaments and articular capsules, periosteum, muscles, tendons, and arterial vessels. The receptors correspond to free nervous endings and represent the more distal part of a first-order afferent neuron consisting of small-diameter fibers, with little or unmyelinated, of the A-Delta (rapid) (Fitzgerald and Lynn, 1977) or C type (slow) (Bessou and Perl, 1969), respectively.

The nociceptors found in the skin originate from small nervous stems that, when approaching the epidermis, lose their myelin, ramifying into extensive plexuses. Two types of free nervous endings exist: the ramified ones originating from 1 or 2 myelinated fibers forming intraepithelial terminations and the nonencapsulated glomerular bodies, deriving from a single unmyelinated fiber and organized in a densely spiral manner below the epidermis or the mucosa. In other organs, this organization may vary because the type of propagated stimulation, the form of propagation, and the quality of the painful

sensation depend on the receptor nervous fiber complex and the innervated organ (Millan et al., 1999, Willis and Westlund., 1997).

Normally, the painful sensation results from specific activation of the nociceptors by mechanical, thermal, or chemical stimulus, and not by the hyperactivity of other sensory modality receptors. They present higher thresholds than the other receptors and respond progressively according to the intensity of the stimulus. However, the sensitization of the nociceptors causes reduction of the thresholds and, in some cases, spontaneous activity (Mense, 1983; Millan et al., 1999; Willis and Westlund., 1997).

3. *Peripheral afferent fibers:*

Peripheral afferent fibers First-order afferent fibers are classified into three types in terms of structure, diameter, and conduction velocity. In the presence of a noxious stimulus, the primary nociceptive afferents show differentiated patterns of propagation.

a. C-Fibers:

C-type fibers are unmyelinated, ranging in diameter from (0.4 to 1.2) μm and have a velocity of conduction 0.5–2.0 m/s. They propagate information in a slower way, at times secondary to the action of the A-Delta afferents. Their prolonged potentials undergo summation along time and induce the manifestations of dull pain.

b. A- δ (Delta) Fibers:

A-Delta fibers are barely myelinated, ranging in diameter from (2.0 to 6.0) μm and have a velocity of conduction 12–30 m/s. They propagate modally specific information, with

marked intensity and short latency. They promote a quick sensation of first phase or acute pain, triggering withdrawal actions

c. A- β (Beta) Fibers:

The A-Beta fibers are myelinated, with a diameter of more than (10 μm) and a velocity of conduction 30–100 m/s, and do not propagate noxious potentials in normal situations; however, they are fundamental in the painful circuitry because they participate in the mechanisms of segmental suppression (Willis and Westlund., 1997).

4. Factors involved in hyperalgesia:

Synaptic transmission between nociceptors and dorsal horn neurons is mediated by chemical neurotransmitter released by A δ and C fibers as well as nonnociceptive afferents is the amino acid glutamate.

Upon repeated application of noxious mechanical stimuli, nearby nociceptors that were previously unresponsive to mechanical stimuli now become responsive a phenomenon called sensitization. The sensitization of nociceptors after injury or inflammation results from the release of variety of chemicals by the damaged cells and tissues in the vicinity of the injury. These are some of important substances involved in sensitization and pain:

a) Prostaglandins:

It's a metabolite of arachidonic acid and is generated by the enzyme cyclooxygenase released from damaged cells. The major action of prostaglandin is sensitization of primary afferent nociceptors (PANs) to noxious stimuli (e.g., chemical, heat, mechanical) (Chahl and Lggo, 1977; Birrell et al., 1991), probably via activation of cyclic adenosine

monophosphate (cAMP) (Taiwo and Levine, 1991) and sensitize nerve afferents to bradykinin (Coleman et al., 1994). Prostaglandins are not known to activate cutaneous afferents directly (Chahl and Lggo, 1977). On the other hand it activates nociceptors directly during inflammatory conditions (Birrell et al., 1991).

b) Bradykinin:

It is released following tissue injury and is present in inflammation. It acts directly on PAN and cause pain (Whalley et al., 1994). Bradykinin cannot account alone for the hyperalgesia seen in inflammation, it acts synergistically with prostaglandin and cause release of arachidonic acid and conversion to prostaglandin E₂ (PGE₂), subsequently, PGE₂ directly stimulates PANs or sensitize them to noxious stimuli (Chahl and Lggo, 1977).

c) Protons:

Inflammation or tissue injury causes a decrease in pH of the extracellular space secondary to local hypoxia, resulting in activation, excitation and sensitization of PANs. Protons, which lower pH, selectively activate nociceptors and sensitize them to noxious stimuli (Steen et al., 1992).

d) Substance P:

Located in the dorsal root ganglion neurons, substance P is transported to the periphery and released after PAN activation (Harmar and Keen, 1982; White and Helme, 1985). It

susceptibility to infection in resistant mice (Scharton-Kersten et al., 1995; Mattner et al., 1996; Belosovic et al., 1989).

g) Cytokines:

Such as (IL-1 which induces PGE₂ secretion in nonneuronal cells which activates PAN and IL-6 which induce release of PGE from mononuclear cells, which activate PAN), tumor necrosis factor and nerve growth factor. Two types of cytokines classified accordingly to their role in inflammation:

1. Proinflammatory Cytokines:

They are involved in initiation and maintenance of inflammation and are produced by, and act upon, a variety of cell types including monocytes, macrophages and lymphocytes (Henderson and Poole, 1994). The role of proinflammatory cytokines in cell-mediated immunity in general and in leishmaniasis specifically has not been fully defined. Considerable recent evidence, based on several inflammatory models, is in favor of the hypothesis that the hyperalgesia is a part of the illness-induced syndrome produced by the proinflammatory cytokines (Watkins et al., 1994). These cytokines (IL-1 β and TNF- α) have been shown to produce hyperalgesia either directly or through a cascade of events leading to it (Lewin and Mendell, 1993; Dray, 1994). Furthermore, the increased plasma levels of these cytokines may lead either to a direct irritation of nociceptors (free nerve endings) or to indirect activation of nerve centers responsible for the illness-induced hyperalgesia (Dray, 1994; Watkins et al., 1994). Many experimental studies in recent years have provided evidence that proinflammatory cytokines induce or increase inflammatory as well as neuropathic pain. Blockade of proinflammatory cytokines or

administration of anti-inflammatory cytokines in most cases reduced neuropathic hyperalgesia in animal models (Sommer, 2001).

a. Interleukin-1 (IL-1):

Interleukin-1 is a proinflammatory cytokine, produced by many types of cells, including immune cells in the periphery, as well as glia and neuron cells within the central nervous system (Dinarello, 1997a), it induces prostaglandin PGE₂ which activates PAN (Dayer et al., 1986). IL-1 shares many biological properties with other proinflammatory cytokines, particularly with IL-18; both cytokines belong to the same gene family and share structural and functional similarities with each other (Dinarello, 1999) and with TNF- α (Moldawer, 1994). In addition to its role in regulation of the inflammatory response, IL-1 plays a major role in immune-to-brain communication, in modulation of neural and neuroendocrine systems, and in behavioral changes during illness. Induction of IL-1 results in: (1) changes in several neurotransmitter systems, including the monoamines, acetylcholine and glutamate; (2) neuroendocrine alterations, including activation of the hypothalamus–pituitary–adrenal axis (3) physiological changes, including fever, loss of body-weight, activation of the sympathetic nervous system, and suppression of peripheral immune response; (4) behavioral alternations, including anorexia, altered sleep patterns, fatigue, and reduction of locomotors, exploratory, social, and sexual behaviors (Besedovsky and del-Rey, 1996; Maier and Watkins, 1998; Rothwell and Luheshi, 2000; Yirmiya et al., 2000).

The present findings indicate that mice with impaired IL-1 signaling display lower thermal and mechanical pain sensitivity, compared to their wild-type controls. IL-1rKO

mice do not express the IL-1 receptor type I and do not respond to either IL-1 α or IL-1 β (Labow et al., 1997), IL-1rAcPKO mice do not express the IL-1 receptor accessory protein; consequently they lack IL-1 signaling and show no responses to IL-1 (Cullinan et al., 1998).

a. 1. Interleukin-1 beta (IL-1 β):

Interleukin-1 β is a cytokine which has been observed to produce nociceptive effects. When administered peripherally (Ferreira et al., 1988; Maier et al., 1993; Watkins et al., 1994; Safieh-Garabedian et al., 1995) or intracerebroventricularly (Oka et al., 1995; Watkins et al., 1994), IL-1 β has been observed to produce a profound thermal hyperalgesia. Interleukin-1 β has also been implicated in the mediation of thymulin induced hyperalgesia in peripheral tissues (Safieh-Garabedian et al., 1997b). Finally, it has been hypothesized that IL-1 β may also serve as a mediator that sensitizes nociceptive neurons during inflammation (Jean et al., 1995). IL-1 β has been shown to play a central role in the generation of mechanical hyperalgesia (Ferreira et al., 1988). The peripheral pro-nociceptive action of IL-1 β may be mediated by a complex signaling cascade and secondary production of nitric oxide, bradykinin or prostaglandins (Poole et al., 1999).

IL-1 β has also been implicated in modulation of pain sensitivity. Exogenous administration of IL-1 β usually produces hyperalgesia. Initial studies found that IL-1 β produces hyperalgesia, following either peripheral or central administration (Ferreira et al., 1988; Oka et al., 1993; Watkins et al., 1994). In peripheral nerves, IL-1 β has been found in (DRG) neurons and in Schwann cells. In addition, expression of IL-1 receptor type I (IL-1RI) mRNA was detected in dorsal root ganglion neurons, suggesting a

possible autocrine or paracrine action of IL-1 β on sensory processing (Copray et al., 2001). IL-1 β also plays an important pain-modulator role within the spinal cord. Intrathecal administration of IL-1 β induces mechanical allodynia and thermal hyperalgesia (Tadano et al., 1999; Reeve et al., 2000; Falchi et al., 2001). It enhances the release of substance P in the spinal cord (Malcangio et al., 1996) and induces COX 2 expression (Newton et al., 1997).

2. *Anti-Inflammatory Cytokines:*

They down regulate inflammatory processes such as IL-1ra, IL-4, IL-10 and IL-13. They are supposed to be involved in modulating immune and inflammatory events, allowing resolution of the inflammation and a return of the damaged tissue to its normal state (Henderson and Poole, 1994).

a. *Interleukin-1 receptor antagonist (IL-1ra):*

Inflammatory hyperalgesia is prevented by experimental administration of endogenous IL-1ra, and neutralizing antibodies to IL-1 receptors reduce pain associated behavior in mice with experimental neuropathy (Cunha et al., 2000).

IL-1 was also found to play an important role in mediating the hyperalgesic effects of various inflammatory stimuli. For example, treatment with IL-1 neutralizing antibodies or with IL-1ra were found to attenuate or block the hyperalgesia produced by endotoxin (Maier et al., 1993), complete Freund's adjuvant (Safieh-Garabedian et al., 1995), nerve injury (Sommer et al., 1999) and carrageenin or bradykinin (Poole et al., 1995; Cunha et al., 2000). Whereas blockade of IL-1 at the spinal cord level can prevent hyperalgesia

induced by various inflammatory stimuli, including subcutaneous peripheral inflammation (Watkins et al., 1997; Samad et al., 2001).

IL-1ra injection produced an almost complete reversal of the endotoxin (ET) induced hyperalgesia (both thermal and mechanical), while hyperalgesia assessed by hotplate (HP) and tail flick (TF) tests was partially reduced (Safieh-Garabedian et al., 1997a). Experiments in various animal models suggest that IL-1 is an important contributor to neuropathic pain. This lead to a suggestion that IL-1ra may serve an analgesic function under the appropriate conditions. Using a rodent L5 spinal nerve transaction model, it was shown that although administration of IL-1Ra alone failed to reduce pain, it improved the ability of soluble TNF receptors to do so. This was associated with reduced expression of IL-6, but not IL-1, in the spinal cord (Sweitzer et al., 2001). Patients with fibromyalgia, a condition associated with chronic pain, have higher levels of circulating IL-1ra. In general, the majority of studies have shown that exogenously injected IL-1 β is pro-nociceptive in animals but not clearly in man. This question can only be answered when and IL-1 receptor antagonist is used clinically, but the animals studies suggest that IL-1 may be an important pain mediator in humans.

b. Interleukin-6 (IL-6):

The pleiotropic cytokine IL-6 is markedly upregulated during various pathologic situations generally associated with increased pain and hyperalgesia (Sommer, 2001). It induces release of prostaglandin E (PGE) from mononuclear cells, which activate primary afferent nociceptors (PAN) (Cunha et al., 1992). Increased IL-6 serum levels have been detected in patients with neuropathies, malignant tumors, musculoskeletal disorders, burn

injury, autoimmune and chronic inflammatory conditions. All these disorders have in common a tenderness sensation and hypersensitivity of the affected tissues. Most experimental studies report pro-inflammatory and pro-nociceptive roles for IL-6. Intraplantar, intracerebroventricular or intrathecal injection of IL-6 induces hyperalgesia or allodynia in rats. IL-6^{-/-} mice present with reduced thermal hyperalgesia after carrageenan inflammation or nerve injury. Antisera neutralizing endogenous IL-6 inhibit LPS-induced hyperalgesia (Jongh et al., 2003; Sommer, 2001). It also induces allodynia and hyperalgesia via actions in the dorsal horn (De Leo et al., 1996) and the intraplantar injection of IL-6 has been shown to produce hyperalgesia (Cunha et al., 1992). Dinarello (1994, 1997b) reported that IL-6 has more anti-inflammatory actions than proinflammatory ones. Two studies have examined the effect of exogenously administered IL-6 on nociception with some- what conflicting results Oka et al. (1995) found that intracerebroventricular injection of IL-6 induced thermal hyperalgesia in naive rats whereas intracisternal IL-6 had anti-nociceptive effects in an acute orofacial pain model, yet hyperalgesic effects in an inflammatory orofacial pain model (Choi et al., 2003). Recent study by Flatters et al. (2004) reported that exogenous spinal IL-6 (100–500 ng) had no significant effect on electrically evoked neuronal responses in naive rats. In contrast, following neuropathy, spinal IL-6 produced a dose-related inhibition of the electrically evoked C-fiber. In addition, spinal IL-6 markedly inhibited mechanical neuronal responses in neuropathic rats. Higher doses of spinal IL-6 also inhibited, to a lesser degree thereby suggest that IL-6 is a potential modulator of neuropathic pain.

c. Tumor Necrosis Factor- α (TNF- α):

There have been correlations between tissue levels of TNF- α and pain and hyperalgesia in a variety of painful diseases (Barnes et al., 1992; Lindenlaub and Sommer, 2003; Shafer et al., 1994; Tak et al., 1997). It induces release of IL-1 and IL-6 (Lindsay and Harmar, 1989). TNF- α is widely considered the prototypic proinflammatory cytokine due to its principal role in initiating the cascade of activation of other cytokines and growth factors in the inflammatory response. After injury or during inflammation, TNF- α is synthesized and released by a multitude of cell types. It has also been demonstrated that Schwann cells can produce TNF- α after injury, suggesting a possible role in neuropathic pain (Sommer et al., 1998).

TNF- α induces production of IL-1 β , IL-6 and IL-8. This cytokine cascade finally results in the activation of cyclooxygenase-2 dependent prostanoid release and the release of catecholamines from sympathetic fibers (Poole et al., 1999).

d. Interleukin-13 (IL-13):

IL-13 has a suppressive effect on IL-1 synthesis and induces B lymphocyte expansion and immunoglobulin production (Vannier et al., 1992; Hart et al., 1989). Concerning the effect of IL-13 on the pain thresholds, Lorenzetti et al., (2001) suggested that IL-13, released by lymphocytes, limits the inflammatory hyperalgesia (due to E.coli endotoxin, LPS injection) by inhibiting the production of TNF- α , IL-1 β and PGE₂. Similarly, the pretreatment with IL-13 prevented, in a dose dependent manner, the hyperalgesia induced by UVB. This is in correlation with the concomitant down-regulation of the increased levels of TNF- α and IL-1 β by UVB (Saadé et al., 2000).

D. The Purpose of this Study:

Our laboratory is involved in studying the effect of anti-proinflammatory (IL-13, IL-6 and IL-1ra) and proinflammatory cytokine (IL-1 β) on hyperalgesia induced by BALB/c mice using *Leishmania major* as a model. This is due to the preferential development of Th2 response in the susceptible mice (such as BALB/c).

Moreover, the subcutaneous injection of high doses of *Leishmania major* (2.5×10^6 per paw) in the hind paw of susceptible mice leads to an obvious hyperalgesia even before the appearance of the lesion (Kanaan et al., 2000) which appears 3 – 4 weeks after the injection (Corry et al., 1994).

Thus, we will try to:

Follow the time course of the pain threshold (using the tail flick and the hot plate tests) in the mice injected with high doses of *Leishmania major*.

Compare the two time courses in both models in order to relate them to hyperalgesia.

IL-13:

On the other hand, IL-13 is a key factor in determining the susceptibility of mice to *Leishmania major* (Brombacher, 2000; Mathews et al., 2000). On the other hand, this cytokine was shown to have hypoalgesic effect (Saadé et al., 2000). Thus, it is possible to:

- Determine the time course of IL-1 β and IL-6 levels in mice infected with high dose *Leishmania major* and treated with IL-13.
- Relate them to the time course of the degree of hyperalgesia.

IL-1ra:

IL-1ra is one IL-1 family play an anti-proinflammatory by blocking the action of IL-1 β which induces pain and other proinflammatory cytokines.

Here we look forward to find out the effect of IL-1ra on BALB/c (susceptible to infection of *Leishmania major*) infected with high and low dose *Leishmania major* (in the Paw of mouse) if it increase the threshold of pain or decrease it using the tail flick and hot plate test, and find out if it alter the susceptibility of course of infection due *Leishmania major* depending on the following:

- Determine the time course of IL-1 β and IL-6 levels in mice infected with high dose *Leishmania major* and treated with IL-1ra.
- Relate them to the time course of the degree of hyperalgesia.

CHAPTER II

MATERIAL AND METHODS

A. Experimental Animals

Adult female BALB/c mice (*Mus musculus*) (weight 20-50g) were used in all the experiments performed in this study. The animals were housed under optimal conditions of light and temperature (12 photoperiod; $22 \pm 3^\circ\text{C}$) and received solid food and water ad libitum. During the period of the experiments, the mice were kept, in groups of six, in clear plastic cages with solid floors covered with 3-6 cm of saw dust. All experimental procedures were carried out with strict adherence to the ethical guidelines for the study of experimental pain in conscious animals (Zimmerman, 1983).

B. Parasite Culture and Preparations

Leishmania major (MHOM/SU/73/5 ASKH) parasites (provided by the London School of Hygiene and Tropical Medicine) were grown at $22 \pm 1^\circ\text{C}$ to $25 \pm 1^\circ\text{C}$ in a standard monophasic liquid broth medium and subcultured bi-weekly. The broth medium is made up of 10g/l beef extract, 10g/l peptone A, and 5g/l NaCl. The medium was then autoclaved and its pH adjusted to 7.2-7.4. After it cools down to 50°C we aseptically add 20% Fetal Calf Serum (FCS) (Sigma) and a solution of 100 IU/ml of streptomycin and penicillin. the liquid medium used to injected *Leishmania major* composed of 4g NaCl, 0.2g CaCl_2 , 0.2g KCl and 0.2g glucose in 500 ml distilled water (Lock's solution) supplemented with 10% sterile FCS. The medium containing the promastigote forms of the parasite were centrifuged for 10 min at (2500 rpm) and the precipitated parasites were suspended in 1ml Lock's medium. The parasites count was determined by a

hemocytometer (Neubauer chamber) and readjusted to have 2.5×10^6 parasites per 50 μ l Lock's medium.

C. Experimental Groups:

The reported results are based on six major sets of experiments each involving different groups of mice (6 mice/group).

In the first set of experiments, three groups were subjected to different pain tests three days before any treatment, then one group received intraplantar injection (i.pl.) injection high dose of *Leishmania major* (2.5×10^6 promastigotes/50 μ l Lock's medium/left hind paw), The second group received i.pl injection of Lock's medium (50 μ l /left hind paw). The third group is a naïve (no injection or treatment). The former two groups were used as control. All groups were subjected to pain tests three times weekly following the injections for a period ranging between 24 to 35 days.

In the second sets of experiments, four groups of mice were subjected to pain tests three days before any treatment, first group received i.pl. injection high dose of *Leishmania major* (2.5×10^6 promastigotes/50 μ l Lock's medium/left hind paw) but without treatment, second group received daily intra-peritoneal (i.p.) injections for six consecutive days of interleukin-13 (15 ng/mouse) and third group injected with high dose and treated with interleukin-1 receptor antagonist for 15 days (15 ng/animal). The fourth group consisted of naïve mice (neither infected nor treated) and was used as control. All groups were subjected to pain tests three times weekly following the injections for a period ranging between 24 to 55 days.

In the third sets of experiments, which consisted of five groups of mice received i.pl. injections of *Leishmania major* (2.5×10^6 /50 μ l overlay) and were sacrificed at the

following time intervals (one group per time interval) 2, 4, 6, 13 and 21 days post-infection. The tissues were collected and processed for the assessment of IL-1 β and IL-6 levels.

In the fourth sets of experiments, which consisted of five groups of mice received i.pl. injections of *Leishmania major* ($2.5 \times 10^6/50\mu\text{l}$ overlay) in addition to daily i.p. injection of IL-1ra for three weeks except weekends (Saturday and Sunday), and were sacrificed at the following time intervals (one group per time interval) 2, 4, 6, 13 and 21 days post-infection. The tissues were collected and processed for the determination of the levels of IL-1 β and IL-6.

In the fifth sets of experiments, which consisted of five groups of mice received i.pl. injections of *Leishmania major* ($2.5 \times 10^6/50\mu\text{l}$ overlay) in addition to daily i.p. injection of (IL-13) for six days, and were sacrificed at the following time intervals (one group per time interval) 2, 4, 6, 13 and 21 days post-infection. The tissues were collected and processed for the determination of the levels of IL-1 β and IL-6.

In the sixth sets of experiments, consists of five groups of mice were naïve and were sacrificed at the following time intervals (one group per time interval) 2, 4, 6, 13 and 21 days post-infection. The tissues were collected and processed for the determination of the levels of (IL-1 β and IL-6) to be used as a control.

D. Behavioural Measurements

The tail flick (TF) and the hot plate (HP) tests were used to assess the thermal pain thresholds.

1. *The Tail Flick Test*

The tails of the mice were immersed into a beaker of distilled water, the temperature of which being adjusted at $T = 50^{\circ}\text{C}$ ($\pm 0.5^{\circ}$). The time interval between the immersion of the tail and the tail flicking reaction was recorded. Each animal was subjected to this test three times with a three minutes interval between consecutive measurements and the average was recorded (Kanaan et al., 1996).

2. *The Hot Plate Test*

Each animal was placed on a heated pad with the temperature being adjusted at $T = 51^{\circ}\text{C}$ ($\pm 0.5^{\circ}$). The latency of paw licking or jumping was taken as an index of pain threshold (Kanaan et al., 1996).

E. Data Analysis

For the behavioural tests, the values obtained from individual animals in each experimental group were averaged for each time interval in each test and plotted against time. The degree of significance of differences between the values obtained for each time interval in the different groups was compared to the control values using one-way ANOVA followed by Bonferoni post-hoc test.

F. Immunoassay

1. Tissue Processing

The skin of both right (non-injected) and left (injected) hind paws of the infected mice, was removed under deep anaesthesia with pentobarbital (50mg/kg). The removed parts of skin were stored at a temperature of -82°C. Tissues were removed after 2, 4, 6, 13, 21 days post infection of *Leishmania major* or both treated with (IL-1ra and IL-13) and none treated mice. Both paws (treated and none treated) were individually homogenized for 1 minute at a speed of 20,000 in 1.2 ml of phosphate buffered saline (PBS) added to it as a buffer for homogenization. For 100ml PBS it contains: 0.818g. NaCl, 0.02g KCl, 0.02g KH₂PO₄, 0.115g Na₂HPO₄, adding 100 ml double distilled water and regulating the pH in a range between 7.2 - 7.4.

A buffer for homogenization is prepared for the 100 ml of PBS by adding: 2.3376g of NaCl, 0.5g of bovine serum albumin (BSA), 50µl of tween 20 and 2 tablets protease inhibitor. The homogenized samples were centrifuged at 13000 g for 60 minutes at 4°C; supernatants were removed and stored in pyrogen/endotoxin-free tubes at -82°C until further usage.

2. IL-1β Assay

A mouse IL-1β (mIL-1β) kit is provided by The BioSource International, Inc. mIL-1β kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for mIL-1β has been coated onto the wells of the microtiter strips provided. 50µl of control specimens and samples are added to their corresponding

wells, followed by the addition of a 50µl of biotinylated anti-IL-1β (Biotin Conjugate) into each well except the chromogen blanks. During the first incubation (for one hour and a half at 37°C), the mIL-1β antigen binds simultaneously to the immobilized (capture) antibody on one site, and to the solution phase biotinylated antibody on a second site, after removal of excess second antibody by washing four times, a 100µl of Streptavidin-Peroxidase (enzyme) is added into each well except the chromogen blanks. This binds to the biotinylated antibody to complete the four member sandwich. After a second incubation (for 30 minutes at room temperature) and followed by washing four times to remove all unbounded enzyme, 100µl of stabilized chromogen (which is acted upon by the bound enzyme to produce color) is added and incubated for 30 minutes at room temperature and in the dark, followed by adding stop solution (100µl). The intensity of this colored product is directly proportional to the concentration of mIL-1β present in the samples. The optical density was read by a plate reader using 450 nm filter.

3. *IL-6 Assay*

A mouse IL-6 (mIL-6) kit is also provided by the BioSource International, Inc. mIL-6 kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for mIL-6 has been coated onto the wells of the microtiter strips provided. 100µl of standard diluent's buffer added to zero wells (Wells reserved for chromogen blank should be left empty) then 100µl of samples are added to the other empty wells followed by incubation for 2 hours at room temperature. During the first incubation, the mIL-6 antigen binds simultaneously to the immobilized (capture) antibody on one site, after washing four times a 100µl of biotinylated anti-IL-6 (biotin

Conjugate) solution into each well except the chromogen blanks followed by incubation for 30 minutes at room temperature then washing wells four times. 100 μ l of Streptavidin-HRP is added into each well except the chromogen blanks followed by incubation for 30 minutes at room temperature. This binds to the biotinylated antibody to complete the four member sandwich. After a third incubation and washing four times to remove all unbounded enzyme, a 100 μ l of stabilized chromogen (which is acted upon by the bound enzyme to produce color) is added and incubated for 30 minutes at room temperature and in the dark, followed by adding stop solution (100 μ l). The intensity of this colored product is directly proportional to the concentration of mIL-1 β present in the samples. The optical density was read by a plate reader using 450 nm filter.

CHAPTER III

RESULTS

A. *Leishmania major* -induced Hyperalgesia**1. *The effect of Lock's medium injection***

This assay was performed to ensure that the transport medium used in subsequent experiments did not have a detrimental effect on experimental outcome. The injection of 50 μ L of Lock's medium per left hind paw had no effect on either the hot plate test or the tail flick test for pain thresholds (Figures 1A and 1B).

2. *The effect of high dose of Leishmania major*

The hot plate and tail flick test were used to assess the extent of hyperalgesia produced by the intraplantar injection of *Leishmania major* (2.5×10^6 promastigotes/50 μ L Lock's medium/left hind paw). As shown in Figure 2A, the injection of *Leishmania major* produced a significant decrease in the HP latency. It dropped significantly at day one post-infection from (17.00 ± 0.16 s) in the control mice to (10.61 ± 0.95 s) ($P = 0.0002$) reaching a peak at day 10 post-infection when the HP latency was (8.43 ± 0.32 s) as compared to (16.9 ± 0.26 s) in the control mice with a P value less than 0.001 (Figure 2A).

The TF latencies dropped significantly at day 1 post-infection (3.72 ± 0.12 s) as compared to the control (5.037 ± 0.086 s) ($P = 0.0004$) and reached a peak at day 10 post-infection when the TF latency dropped from (5.17 ± 0.036 s) in the control mice to (3.09

$\pm 0.027s$) ($P < 0.001$) sustaining this level throughout the experiment as shown (Figure 2B).

A

B

Figure 1. Time course of the effect of intraperitoneal injection of Lock's medium on the thermal hyperalgesia of mice as assessed by (A) the hot plate test and (B) the tail flick test. Mice were injected with 50 μ l of Lock's medium per left hind paw. They were tested at daily intervals for hot plate and tail flick latency. Each result is the mean of 5 mice $\pm$ standard error mean (SEM). The degree of significance was calculated with reference to naïve using one-way ANOVA followed by the Bonferroni post-hoc test.

A

B

Figure 2. The time course of the effect of intraperitoneal injection of *Leishmania major* (high dose) on the thermal hyperalgesia of mice as assessed by (A) the hot plate test and (B) the tail flick test. Mice were injected with 2.5×10^6 promastigotes per 50μ per left hind paw. They were tested at daily intervals for hot plate and tail flick latency. Each result is the mean of 5 mice $\pm$ standard error mean (SEM). The degree of significance was calculated with reference to naïve using one-way ANOVA followed by the Bonferroni post-hoc test.

B. Effects of interleukin -13 On Pain Thresholds

This assay was performed to assess the effect of daily injection of interleukin-13 (IL-13) during the first six days of infection on the pain thresholds of mice injected with high dose of *Leishmania major*.

1. Effect of interleukin-13 on the pain thresholds in high dose *Leishmania major*-infected mice

Treatment with daily intraperitoneal (i.p.) injections of IL-13 (15ng i.p./mouse) for six consecutive days produced a significant reduction of high dose *Leishmania major*-induced hyperalgesia during the period of treatment. The HP latencies increased to ($14.29 \pm 0.239s$) at day 6 as compared to *Leishmania major* alone ($7.88 \pm 0.21s$) ($P < 0.001$) and as compared to the control which was (15.01 ± 0.075) ($P > 0.05$) (Figure 3A). At day 8 post-infection, the levels started to decrease to reach a plateau close to the levels of the IL-13-non-treated infected mice. For example, at day 20 the HP latency of the mice treated previously with IL-13 was ($6.90 \pm 0.28s$) as compared to ($6.86 \pm 0.25s$) ($P > 0.05$) for the mice injected only with high dose of *Leishmania major* and to ($15.33 \pm 0.13s$) ($P < 0.001$) in the naïve mice (Figure 3A). As to the tail flick latency, the daily treatment with 15ng of IL-13 per mouse increased from ($3.76 \pm 0.18s$) to ($5.09 \pm 0.11s$) ($P < 0.001$) at day 1 post-infection knowing that the naïve mice had a TF latency of ($5.037 \pm 0.086s$) (Figure 3B). This effect was sustained until day 8 (the injection of IL-13 stopped at day 6) when the TF latencies decreased to levels very close to those in mice which had not been treated with IL-13 (Figure 3B). This was significantly less than that seen in control mice ($3.14 \pm 0.022s$) as compared to ($5.10 \pm 0.059s$) at day 24 post-infection, the P value

being less than 0.001) while the difference is not significant ($P > 0.05$) with the TF latency of the mice injected with a high dose of the parasite ($3.12 \pm 0.039s$) (Figure 3B).

A

B

Figure 3. The time course of the effect of the daily intraperitoneal injection of IL-13 on *Leishmania major* (high dose) induced hyperalgesia as assessed by (A) the hot plate test and (B) the tail flick test. Mice were injected with 2.5×10^6 promastigotes per 50μ per left hind paw and with 15 ng of IL-13 for 6 consecutive days. They were tested at daily intervals for hot plate and tail flick latency. Each result is the mean of 5 mice $\pm$ standard error mean (SEM). The degree of significance was calculated with reference to naïve and mice infected with high dose of the parasite using one-way ANOVA followed by the Bonferroni post-hoc test.

C. Effects of interleukin -1 receptor antagonist On Pain Thresholds

This assay was performed to assess the effect of daily injection of interleukin-1 receptor antagonist (IL-1ra) during the 13 days of infection on the pain thresholds of mice injected with high dose of *Leishmania major*.

1. Effect of interleukin-1receptor antagonist on the pain thresholds in high dose *Leishmania major*-infected mice

Treatment with daily intraperitoneal injections of IL-1ra (15 ng i.p./mouse) was for 15 days except weekends (Saturday and Sunday). It produced a significant reduction of high dose *Leishmania major*-induced hyperalgesia during the period of treatment except on Mondays when pain tests taken (both hot plate and tail flick) pain is observed (Figure 4A&B). The HP latencies increased to ($15.3 \pm 0.27s$) at day 7 as compared to *Leishmania major* alone ($10.51 \pm 0.26s$) ($P < 0.001$) and as compared to the control which was 15.81 ± 0.165 ($P > 0.05$) this effect was the same on day 8 and 9 (Figure 4A). And at day 14 the HP latencies increased to (14.65 ± 0.2) as compared to *Leishmania major* alone ($10.138 \pm 0.186s$) ($P < 0.001$) and as compared to the control which was ($15.82 \pm 0.186s$) ($P > 0.05$) and this effect was the same on day 15 and 16. At day 5 the HP latencies of treated mice with IL-1ra decreased to ($12.71 \pm 0.24s$) as compared to *Leishmania major* alone ($11.245 \pm 0.29s$) ($P > 0.05$) and as compared to the control ($16.03 \pm 0.276s$) ($P > 0.05$). at day 12 HP latency of treated mice decreased to ($13.055 \pm 0.142s$) and become close to the group infected only with *Leishmania major* ($10.21 \pm 0.266s$) and as compared to the control mice ($15.83 \pm 0.155s$) ($P > 0.05$) (Figure 4A). At day 21 post-infection, the levels started to decrease to reach a plateau close to the levels of the IL-1ra-non-treated infected mice. For example, at day 21 the HP latency of the

mice treated previously with IL-1ra was ($10.16 \pm 0.291s$) as compared to ($10.138 \pm 0.186s$) ($P > 0.05$) for the mice injected only with high dose of *Leishmania major* and to ($15.33 \pm 0.13s$) ($P < 0.001$) in the naïve mice (Figure 4A).

As to the tail flick latency on day 7 ($5.38 \pm 0.214s$) knowing that the naïve mice had a TF latency of ($5.91 \pm 0.048s$) ($P > 0.05$) while that of mice infected only with *Leishmania major* ($3.15 \pm 0.126s$) ($P < 0.001$) this effect was the same on day 8 and 9 (Figure 4B). On day 14 the TF latency of treated mice ($5.42 \pm 0.2s$) as compared to the infected mice only ($3.18 \pm 0.017s$) ($P > 0.05$) and as compared to the control mice ($6.04 \pm 0.097s$) ($P > 0.05$) and this effect was same for day 15 and 16 (Figure 4B).

At day 5 the TP latencies of treated mice with IL-1ra decreased to ($3.83 \pm 0.241s$) as compared to *Leishmania major* alone ($3.16 \pm 0.126s$) ($P > 0.05$) and as compared to the control ($5.95 \pm 0.131s$) ($P > 0.05$). at day 12 HP latency of treated mice decreased to ($3.055 \pm 0.104s$) and become close to the group infected only with *Leishmania major* ($3.14 \pm 0.0266s$) and as compared to the control mice ($5.93 \pm 0.097s$) ($P > 0.05$) (Figure 4B). At day 21 post-infection, the levels started to decrease to reach a plateau close to the levels of the IL-1ra-non-treated infected mice. For example, at day 21 the HP latency of the mice treated previously with IL-1ra was ($2.93 \pm 0.035s$) as compared to ($2.94 \pm 0.073s$) ($P > 0.05$) for the mice injected only with high dose of *Leishmania major* and to ($5.78 \pm 0.077s$) ($P < 0.001$) in the naïve mice (Figure 4B).

A

B

Figure 4. The time course of the effect of the daily intraperitoneal injection of IL-1ra on *Leishmania major* (high dose) induced hyperalgesia as assessed by (A) the hot plate test and (B) the tail flick test. Mice were injected with 2.5×10^6 promastigotes per 50μ per left hind paw and with 15 ng of IL-1ra for 15 days except weekends. They were tested at daily intervals for hot plate and tail flick latency. Each result is the mean of 5 mice $\pm$ standard error mean (SEM). The degree of significance was calculated with reference to naïve and mice infected with high dose of the parasite using one-way ANOVA followed by the Bonferroni post-hoc test.

D. Re-injection of IL-1ra at week 43 postinfection of *Leishmania major*:

Re-injection of IL-1ra (15 ng i.p./mouse) for six consecutive days for 4 mice infected with *Leishmania major* before week 43 produced a significant reduction of high dose *Leishmania major*-induced hyperalgesia during the period of treatment. The HP latencies from 8.825 ± 0.96 increased to $(14.37 \pm 0.45\text{s})$ at day 1 post-injection of IL-1ra as compared to *Leishmania major* alone ($9.13 \pm 0.26\text{s}$) ($P < 0.001$) and as compared to the control which was (15.68 ± 0.26) ($P > 0.05$) (Figure 5A). At day 2, 3 and 4 post-injection of IL-1ra the latency of mice re-treated with IL-1ra was close to the naïve group and P value was greater than 0.001, while it was extremely significant with the group infected only with *Leishmania major* ($P < 0.05$). At day 7 the latency of treated group decreased back to become close to its previous value ($P > 0.05$) before treatment with IL-1ra and become close to the mice infected only with *Leishmania major* ($P > 0.05$) when injection stopped at day 5 (Figure 5A).

As to the tail flick latency, the daily treatment for six consecutive days with 15ng of IL-1ra per mouse increased from $(2.76 \pm 0.1\text{s})$ to $(5.18 \pm 0.33\text{s})$ ($P < 0.001$) at day 1 post-infection knowing that the naïve mice had a TF latency of $(5.84 \pm 0.047\text{s})$ and the infected with *Leishmania major* alone was (2.674 ± 0.13) (Figure 5B). This effect was sustained until day 4 during the period of injection of IL-1ra. At day 7 the latency decreased again to become close to its previous value ($P > 0.05$) and close to mice infected only with *Leishmania major* when injection stopped at day 5 (Figure 5B).

A

B

Figure 5. The time course of the effect of the intraperitoneal re-injection of IL-1ra for 6 consecutive days on mice post-infected with *Leishmania major* (high dose) after 43 weeks induced hyperalgesia as assessed by (A) the hot plate test and (B) the tail flick test. Mice were injected before 43 weeks with 2.5×10^6 promastigotes per 50μ per left hind paw and with 15 ng of IL-1ra for 15 days except weekends. They were tested at daily intervals for hot plate and tail flick latency. Each result is the mean of 5 mice $\pm$ standard error mean (SEM). The degree of significance was calculated with reference to naïve and mice infected with high dose of the parasite using one-way ANOVA followed by the Bonferroni post-hoc test.

E. *Effect of Leishmania major infection on the levels of IL-1 β*

This assay was performed to assess the effect of the infection with high dose of *Leishmania major* on the levels of IL-1 β in the paws of BALB/c mice.

The intraplantar injection of high dose of *Leishmania major* (2.5×10^6 promastigotes/50 μ l Lock's medium/left hind paw) caused a significant change in the levels of IL-1 β in the injected hind paw starting day 2 post-infection increasing from (147.06 ± 12.59) pg/hind paw in the naïve mice to (406.88 ± 29.39) pg/hind paw in the injected mice ($P < 0.001$). However, there was no significant difference between the level of IL-1 β in the paws of naïve mice (147.06 ± 12.59 pg/hind paw) and its level in the right (non-injected) paw of the infected mice (170.60 ± 32.42 pg/hind paw) the P value being greater than 0.05 (Figure 6). The levels of IL-1 β reach a peak at day 4 post-infection when it became 434.64 ± 36.39 pg/hind paw in the injected paw which is extremely higher than the level in the naïve mice ($P < 0.001$). Furthermore, the difference between the levels of IL-1 β in the paws of naïve mice (147.06 ± 12.59 pg/hind paw) and those of the non-injected paws of the infected mice (365.92 ± 50.17 pg/hind paw) became significant, the P value being less than 0.01 (Figure 6). The difference between the levels of IL-1 β in the injected paws and those in the paws of naïve mice remain significant throughout the period of the experiment despite a small decrease at day 13 post-infection (304.41 ± 38.36 pg/hind paw) increasing again at day 21 post-infection to become 328.73 ± 11.65 pg/hind paw ($P < 0.001$). On the other hand, the levels of IL-1 β in the non-injected paws started to decrease starting day 6 post-infection to become very close of the levels in the naïve mice at day 21 post-infection (146.73 ± 9.92 pg/hind paw) ($P > 0.05$) (Figure 6).

Figure 6. The effect of the injection of high dose (2.5×10^6 promastigotes/50 μ l Lock's medium/left hind paw *Leishmania major* on the levels of IL-1 β in the left (injected) paw and the right (non-injected) paw at days 2,4,6,13 and 21 post infection. the result is the mean of 5 mice $\pm$ standard error mean (SEM). The degree of significance was calculated with reference to naïve and mice infected with high dose of the parasite using one-way ANOVA followed by the Bonferroni post-hoc test.

F. Effect of *Leishmania major* infection on the levels of IL-6

This assay was performed to assess the effect of the infection with high dose of *Leishmania major* on the levels of IL-6 in the paws of BALB/c mice. The intraplantar injection of high dose of *Leishmania major* (2.5×10^6 promastigotes/50 μ l Lock's medium/left hind paw) caused a significant increase in the levels of IL-6 at day 2 post-infection only in the injected hind paw of the mice (85.45 ± 15.5 pg/hind paw) ($P < 0.01$) as compared to the level of IL-6 in the paws of naïve mice (29.06 ± 1.84 pg/hind paw). However, no significant change in the level of IL-6 was observed in the non-injected paws of the mice ($P > 0.05$) (Figure 7). Also a mild change in the level of this cytokine

was observed at day 4 post-infection in both the injected paw (47.42 ± 5.75 pg/hind paw) and the non-injected paw (48.7 ± 7.42 pg/hind paw) as compared to the control value, the P value being greater than 0.05 in both cases (Figure 7). At day 6 post-infection, there was a mild increase in the level of IL-6 in the injected paws (43.087 ± 5.51 pg/hind paw), as compared to the control value the ($P < 0.05$) while the increase in the levels of IL-6 in the non-injected paws (37.09 ± 9.17 pg/hind paw) was insignificant ($P > 0.05$) (Figure 7). As also shown in figure 9A, the levels of IL-6 further decreased at day 13 post-infection to become (39.23 ± 8.24) pg/hind paw and (26.15 ± 2.90) pg/hind paw in the injected and the non-injected paws respectively, the P value being 0.211 (insignificant difference) as compared to naïve mice. At day 21 post-infection, the levels of IL-6 in both injected paw (35.50 ± 2.90 pg/hind paw) and non-injected paw (25.52 ± 1.79 pg/hind paw) became very close to the naïve levels ($P > 0.05$); however, the levels of IL-6 in the latter was mildly lower than its levels in the former case, the P value being less than 0.05 (Figure 7).

Figure 7. The effect of the injection of high dose (2.5×10^6 promastigotes/50 μ l Lock's medium/left hind paw) *Leishmania major* on the levels of IL-6 in the left (injected) paw and the right (non-injected) paw at days 2,4,6,13 and 21 post infection. Each result is the mean of 5 mice $\pm$ standard error mean (SEM). The degree of significance was calculated with reference to naïve and mice infected with high dose of the parasite using one-way ANOVA followed by the Bonferroni post-hoc test.

G. Effect of IL-1ra on the levels of IL-1 β in *Leishmania major*-induced inflammation

This assay was performed to assess the effect of the daily intraperitoneal injection of 15ng of IL-1ra for the first consecutive 15 days except weekends on the levels of IL-1 β in the paws of mice infected with high dose of *Leishmania major*.

Treatment with daily injections of IL-1ra (15 ng i.p./mouse) for fifteen consecutive days except weekends produced at day 2 post-infection, a significant reduction in the levels of IL-1 β in the injected paws of mice infected with high dose *Leishmania major* and treated with IL-1 β (191.044 ± 39.88 pg/hind paw) as compared to the levels of IL-1 β in the paws of mice infected only with high dose of the parasite (406.88 ± 29.39 pg/hind paw), the P value being less than 0.001. However, the difference between the levels of IL-1 β in the

former case and that of the control value (147.06 ± 12.59 pg/hind paw) was insignificant ($P > 0.05$) (Figure 8). The effect of IL-1ra remained obvious throughout the whole period of injection when the difference between the levels of IL-1 β in the injected paws of mice infected with high dose *Leishmania major* and treated with IL-1ra and the levels of IL-1 β in the paws of mice infected only with high dose of the parasite was significant. For example, at day 4 post-infection, the injection of IL-1ra reduced the level of IL-1 β from (434.64 ± 36.39 pg/hind paw) in the mice infected only with high dose of the parasite to (183.82 ± 37.58 pg/hind paw) in the mice treated with IL-1ra ($P < 0.001$). At this same date, there was no significant difference between the latter group and the control value, the P value being greater than 0.05. The maximum effect of IL-1ra on the levels of IL-1 β was reached at day 6 post-infection when the levels of IL-1 β in the injected paws of mice infected with high dose *Leishmania major* and treated with IL-1ra was (163.73 ± 33.9 pg/hind paw) as compared to the levels of IL-1 β in the paws of mice infected only with high dose of the parasite (366.20 ± 29.29 pg/hind paw) ($P < 0.001$). Similar results were also observed at day 13 post-infection but the difference between the levels of IL-1 β in the injected paws of IL-1ra-treated mice (205.21 ± 14.99 pg/hind paw) and in those of mice infected only with *Leishmania major* (304.41 ± 38.36 pg/hind paw) became less significant ($P < 0.05$) (Figure 8). One week after stopping the injection of IL-1ra (at day 21 post-infection), the levels of IL-1 β in the injected paws of IL-1ra-treated mice increased to become (326.30 ± 23.41 pg/hind paw) as compared to (328.73 ± 11.65 pg/hind paw) in the paws of mice infected only with *Leishmania major* ($P > 0.05$). However, the difference between the former group and the control value became extremely significant ($P < 0.001$) (Figure 8).

Figure 8. The effect of the injection of IL-1ra on BALB/c mice infected with high dose (2.5×10^6 promastigotes/50 μ l Lock's medium/left hind paw) *Leishmania major* on the levels of IL-1 β in the left (injected) paw and the right (non-injected) paw at days 2,4,6,13 and 21 post infection. Each result is the mean of 5 mice $\pm$ standard error mean (SEM). The degree of significance was calculated with reference to naïve and mice infected with high dose of the parasite using one-way ANOVA followed by the Bonferroni post-hoc test

H. *Effect of IL-1ra on the levels of IL-6 in Leishmania major-induced inflammation*

This assay was performed to assess the effect of the daily intraperitoneal injection of 15 ng of IL-1ra for the first consecutive 15 days except weekends on the levels of IL-6 in the paws of mice infected with high dose of *Leishmania major*.

Treatment with daily injections of IL-1ra (15 ng i.p./mouse) for fifteen consecutive days except weekends produced at day 2 post-infection, a significant decrease in the levels of IL-6 in the injected paws of mice infected with high dose *Leishmania major* and treated with IL-1ra (47.82 ± 6.26 pg/hind paw) as compared to the levels of IL-6 in the paws of mice infected only with high dose of the parasite (85.45 ± 15.50 pg/hind paw), the P value remains being less than 0.01. However, the difference between the levels of IL-6 in the former case and that of the control value (29.069 ± 1.84 pg/hind paw) was insignificant ($P > 0.05$) (Figure 9). At day 4 post-infection, when the difference between the levels of IL-6 in the paws of IL-1ra non-treated mice and the control value became mildly significant ($P < 0.05$), the IL-6 levels in the paws of IL-1ra treated mice showed a significant increase as compared to the control value reaching (51.61 ± 5.20 pg/hind paw) ($P < 0.05$), this result being very close of the levels of IL-6 in the paws of mice infected only with high dose of the parasite ($P > 0.05$). Similar results were observed at day 6 post-infection, when the difference between the IL-6 level in the injected paws of IL-1ra treated mice (60.67 ± 5.83 pg/hind paw) and the control value became very significant ($P < 0.01$), but the difference between the former result and the levels of IL-6 in the paws of IL-1ra non-treated mice became insignificant ($P > 0.05$) (Figure 9). At day 13 post-infection, when the difference between the levels of IL-6 in the

injected paws of mice infected with *Leishmania major* alone became and the control value became insignificant ($P > 0.05$), the difference between this latter group and the levels of IL-6 in the injected paws of IL-1ra treated mice (47.45 ± 3.00 pg/hind paw) was extremely significant ($P < 0.001$). However, at day 21 post-infection, the levels of IL-6 in the injected paws of IL-1ra treated mice became (55.79 ± 3.71 pg/hind paw), which is higher than the levels of IL-6 in the paws of mice infected only with the parasite (35.50 ± 2.90 pg/hind paw) ($P < 0.01$) extremely higher than the levels of this cytokine in the paws of naïve mice ($P < 0.001$) (Figure 9).

Figure 9. The effect of the injection of IL-1ra on Balb mice infected with high dose (2.5×10^6 promastigotes/50 μ l Lock's medium/left hind paw) *Leishmania major* on the levels of IL-6 in the left (injected) paw and the right (non-injected) paw at days 2, 4, 6, 13 and 21 post infection. Each result is the mean of 5 mice $\pm$ standard error mean (SEM). The degree of significance was calculated with reference to naïve and mice infected with high dose of the parasite using one-way ANOVA followed by the Bonferroni post-hoc test

I. Effect of IL-13 on the levels of IL-1 β in *Leishmania major*-induced inflammation

This assay was performed to assess the effect of the daily intraperitoneal injection of 15 ng of IL-13 for the first consecutive 6 days on the levels of IL-1 β in the paws of mice infected with high dose *Leishmania major*.

Treatment with daily injections of IL-13 (15 ng i.p./mouse) for six consecutive days produced, at day 2 post-infection, a significant reduction in the levels of IL-1 β in the injected paws of mice infected with high dose *Leishmania major* and treated with IL-13 (164.90 $\pm$ 18.58 pg/hind paw) as compared to the levels of IL-1 β in the paws of mice infected only with high dose of the parasite (406.88 $\pm$ 29.39 pg/hind paw), the P value being less than 0.001. However, the difference between the levels of IL-1 β in the former case and that of the control value (147.06 $\pm$ 12.59 pg/hind paw) was insignificant (P>0.05) (figure 10). The effect of IL-13 remained obvious throughout the whole period of the experiment when the difference between the levels of IL-1 β in the injected paws of mice infected with high dose *Leishmania major* and treated with IL-13 and the levels of IL-1 β in the paws of mice infected only with high dose of the parasite was extremely significant (P < 0.001). Also, the difference between the levels of IL-1 β in the former case and that of the control value remained insignificant (P > 0.05) throughout the experiment period. The maximum effect of IL-13 on the levels of IL-1 β was reached at day 13 post-infection when the levels of IL-1 β in the injected paws of mice infected with high dose *Leishmania major* and treated with IL-13 was (110.12 $\pm$ 21.35 pg/hind paw) as compared to the levels of IL-1 β in the paws of mice infected only with high dose of the

parasite (304.405 ± 38.36 pg/hind paw) ($P < 0.001$). Similar results were also observed at day 21 post-infection (Figure 10).

Figure 10. Effect of the daily intra-peritoneal injection of IL-13 on the level of IL-1 β in the paws of mice infected with high dose (2.5×10^6 promastigotes/50 μ l Lock's medium/left hind paw) *Leishmania major*. Mice were injected with 15 ng of IL-13 (i.p.) for 6 consecutive days and the levels of IL-1 β were assessed at days 2,4,6,13 and 21 post-infection. Each result is the mean of 5 mice $\pm$ standard error mean (SEM). The degree of significance was calculated with reference to naïve and mice infected with high and low dose of the parasite using one-way ANOVA followed by the Bonferroni post-hoc test.

J. Effect of -IL-13 on the levels of IL-6 in *Leishmania major*-induced inflammation

This assay was performed to assess the effect of the daily intraperitoneal injection of 15 ng of IL-13 for the first consecutive 6 days on the levels of IL-6 in the paws of mice infected with high dose and low dose *Leishmania major*.

Treatment with daily injections of IL-13 (15 ng i.p./mouse) for six consecutive days produced, at day 2 post-infection, no reduction in the levels of IL-6 in the injected paws of mice infected with high dose *Leishmania major* and treated with IL-13 (73.74 ± 3.31 pg/hind paw) as compared to the levels of IL-6 in the paws of mice infected only with high dose of the parasite (85.45 ± 15.50 pg/hind paw), the P value remains being greater than 0.05. However, the difference between the levels of IL-13 in the former case and that of the control value (29.069 ± 1.84 pg/hind paw) was extremely significant ($P < 0.001$) (Figure 11). At day 4 post-infection, when the difference between the levels of IL-6 in the paws of IL-13 non-treated mice and the control value became mildly significant ($P < 0.05$), the IL-6 levels in the paws of IL-13 treated mice showed an extremely significant increase as compared to the control value reaching (83.98 ± 7.64 pg/hind paw) ($P < 0.001$). Similar results were observed at day 6 post-infection, when the difference between the IL-6 level in the injected paws of IL-13 treated mice (62.52 ± 7.26 pg/hind paw) and the control value remained significant ($P < 0.01$), but the difference between the former result and the levels of IL-6 in the paws of IL-13 non-treated mice became insignificant ($P > 0.05$) (Figure 11). However, no significant difference in the levels of IL-6 was observed at day 13 post-infection among the three experimental groups, the P value being 0.3704 (Figure 11), the levels of IL-6 in the injected paws of mice infected

with high dose *Leishmania major* and treated with IL-13 increased again at day 21 post-infection to become (42.36 ± 3.81 pg/hind paw), which is quite higher than the levels of IL-6 in the paws of naïve mice (29.069 ± 1.84 pg/hind paw) ($P < 0.05$) but not significantly higher than the levels of this cytokine in the paws of IL-13 non-treated mice (35.50 ± 2.90 pg/hind paw) ($P > 0.05$) (Figure 11).

Figure 11. Effect of the daily intraperitoneal injection of IL-13 on the level of IL-6 in the paws of mice infected with high dose (2.5×10^6 promastigotes/50 μ l Lock's medium/left hind paw) *Leishmania major*. Mice were injected with 15 ng of IL-13 (i.p.) for 6 consecutive days and the levels of IL-6 were assessed at days 2, 4, 6, 13 and 21 post-infection. Each result is the mean of 5 mice $\pm$ standard error mean (SEM). The degree of significance was calculated with reference to naïve and mice infected with high and low dose of the parasite using one-way ANOVA followed by the Bonferroni post-hoc test.

CHAPTER IV

DISCUSSION

High dose *Leishmania major* (2.5×10^6) injected in left hinds of mice induced a hyperalgesia at day 1 post-infection. Hyperalgesia induced by *Leishmania major* parasites since lock's media injection did not induce pain, the P value of lock's media group and naïve group (none infected) was greater than 0.05. Beside the lesions started to develop in the infected group later at week 30 postinfection were typical to *Leishmania major* (Appendix 1).

IL-1 β levels increased significantly in those mice infected with high dose *Leishmania major* during the period of experiment in comparison with the naïve group where P value was less than 0.001 (extremely significant) which was consistent with previous studies done by Kanaan et al., (2000). The former immunoassay results were consistent with pain test results which showed a hyperalgesia in mice group infected with *Leishmania major* only during the period of experiment which indicates that IL-1 β is directly involved in hyperalgesia.

IL-6 levels increased significantly in those mice infected with high dose *Leishmania major* only at day 2 postinfection ($P < 0.001$) then decrease to become close to naïve group till the end of experiment where P value became greater than 0.05. The previous immunoassay results indicate that IL-6 might not be involved in hyperalgesia directly. Sudden rise in IL-6 levels in high dose group at day 2 postinfection suggest a mediation

role for IL-6 to induce hyperalgesia by stimulation of other proinflammatory and anti-inflammatory cytokines (Dinarello, 1994, 1997).

Treatment infected mice with IL-1ra reversed the hyperalgesia induced by *Leishmania major* during the period of injection, pain latencies both in HP and TF increased to become insignificant with naïve group ($P > 0.05$) and extremely significant with high dose *Leishmania major* alone where P value was less than 0.001.

Pain latencies decreased after IL-1ra injection was stopped to be insignificant ($P > 0.05$) with mice infected with *Leishmania major* only. As to immunoassay results, levels of IL-1 β in those infected mice and treated with IL-1ra showed a significant decrease due to treatment with IL-1ra in comparison to high dose *Leishmania major* alone, on the other hand treated mice showed insignificance in IL-1 β levels with those of naïve group ($P > 0.05$) and the pain latencies of treated and naïve groups was so close.

When treatment with IL-1ra was stopped, IL-1 β levels increased significantly compared to the naïve group ($P < 0.001$) and to be insignificant and close with infected mice only ($P > 0.05$).

Both immunoassay and pain tests are consistent and indicate that IL-1 β is directly involved in hyperalgesia and IL-1ra is hypoalgesic antagonist.

Treatment infected mice with IL-1ra decreased IL-6 levels at day 2 post-infection and sustained at a low level during the period of treatment and insignificant with the high dose only ($P > 0.05$) even though no pain was observed in the treated group in that period which indicates that IL-6 is not involved in hyperalgesia directly, beside after treatment

with IL-1ra was stopped IL-6 increased suddenly and significantly compared to both naïve and high dose alone groups at day 21 and 44 post-infection where P value was less than (0.001). This sudden increase in IL-6 levels might suggest a role in accelerating the severity of Leishmaniasis disease by stimulation of other Th2 cytokines that suppress Th1 pathway.

Treatment infected mice with IL-13 reversed the hyperalgesia induced by *Leishmania major* during the period of injection, pain latencies both in HP and TF increased to become insignificant and close with naïve group ($P > 0.05$) and extremely significant with high dose *Leishmania major* alone where P value was less than 0.001.

Pain latencies decreased after IL-13 injection was stopped to be insignificant ($P > 0.05$) with mice infected with *Leishmania major* only. As to immunoassay results, levels of IL-1 β in those infected mice and treated with IL-13 showed a significant decrease due to treatment with IL-13 in comparison to high dose *Leishmania major* alone, on the other hand treated mice showed insignificance in IL-1 β levels with those of naïve group ($P > 0.05$).

Treatment infected mice with IL-13 caused a significant reduction in the levels of IL-1 β even after treatment with IL-13 was stopped in comparison with high dose *Leishmania major* where P value was less than 0.001 (extremely significant), alternatively the difference between treated and naïve group in levels of IL-1 β was insignificant ($P > 0.05$) throughout the experiment period, although a pain was observed during that period which suggest other cytokines might be involved in inducing that pain like TNF- α , NGF and PGE₂.

Both immunoassay and pain tests are consistent and indicate that IL-1 β is directly involved in hyperalgesia and IL-13 is hypoalgesic.

Infected mice treated with IL-13 showed high levels of IL-6 during the period of treatment and were significant with those of high dose *Leishmania major* alone (except day 2 postinfection) and naïve groups during the period of treatment. When injection stopped, levels of IL-6 decreased to become close and insignificant with high dose and naïve groups.

During the period of treatment with IL-13, levels of IL-6 sustained high although no hyperalgesia was observed in that period. This further indicates that IL-6 is not involved in hyperalgesia in direct pathway which is consistent with the results got from treatment with IL-1ra.

Further studies on IL-1ra and its role in course of infection must be preceded. The leishmaniasis in left hinds was more severe than mice infected only with *Leishmania major*. Lesions of hinds developed faster in mice treated with IL-1ra than non treated and infected mice; beside the paw size was thicker in the mice treated with IL-1ra than the non treated case which is a sign that IL-1ra might be involved in Th2 pathway and might stimulate production cytokines from Th2 family that might dominate over Th1 pathway and suppress it. Most of the mice treated with IL-1ra died after 35 weeks postinfection and lost their paw before death due to the severe progress of the disease (Appendix 2), while most of mice infected with *Leishmania major* survived more than 100 weeks postinfection.

Re-injection with IL-1ra at week 43 post-infection reduced the pain thresholds significantly which suggest further studies to find out cascade pathways for some factors involved in hyperalgesia such as IL-1 β , TNF- α , NGF and PGE₂ and to assess their levels in infected mice only and the treated-infected mice at different days post-infection. In addition the paw thickness of the treated group decreased due to re-treatment of IL-1ra which might propose further studies to testify if IL-1ra in reducing the inflammation in that damaged area and how can be used in treatment chronic diseases.

The relatively high levels of IL-6 in IL-1ra-treated mice suggest that IL-6 prefers anti-inflammatory pathway to induce Th2 pathway specially its sudden rise at day 21 and day 44 after treatment with IL-1ra was stopped. It seems that IL-6 play a two faces on Th1/Th2 pathway differentiation and stimulates Th2 and inhibits Th1 pathway (Rincon and Diehl, 2002). It has been suggested that IL-6 favors the outgrowth of Th2 cells (Rincon et al., 1997) but is not required for healing and resolution of infection with *Leishmania major*. IL-6 directed the differentiation of the CD4⁺ cells to a Th2 phenotype, since the cells differentiated in the presence of IL-6 produce high amounts of IL-4, but not IFN- γ , after restimulation with IL-6 (Rincon and Flavell, 1997) (Appendix 3).

On the other hand IL-13 might activate production of IL-6 which might participate in dominating Th2 pathway over Th1 and accelerate the progress of leishmaniasis disease. Beside the first week of infection is critical to determine which pathway with differentiate either Th1 or Th2 and the choice to which one will proceed is determined by

several factors and the cytokines produced due to infection is one the most important factors (Locksley et al., 1993). The elevation of IL-6 level in the treated mice with IL-13 during the first 6 days might induce further more cytokines in the Th2 family thereby make worse the disease of leishmaniasis (Appendix 3).

The overexpression of IL-13 resulted in C57BL/6 resistant mice to become susceptible to *Leishmania major*, and significantly, this was independent of IL-4 expression. Conversely, IL-13-deficient BALB/c mice were resistant to *Leishmania major* and they noted that BALB/c mice doubly deficient for IL-4 and IL-13 were significantly more resistant to *Leishmania major* infection than either of the single cytokine-deficient animals or even the resistant C57BL/6 mouse strain. IL-13 acts independently of IL-4 and thus identifies IL-13 as a major component of the immune response to *Leishmania major* (Matthews et al., 2000) which suggest that IL-13 treatment for six consecutive days accelerated the leishmaniasis disease specially the first week postinfection is critical in deciding either Th1 or Th2 will dominate (Locksley et al., 1993) (Appendix 3).

As a conclusion, both IL-13 and IL-1ra are hypoalgesic; both reversed hyperalgesia induced by *Leishmania major* and reduced levels of IL-1 β that is involved in hyperalgesia, beside they had different effects on levels of IL-6 since IL-1ra reduced its levels while IL-13 increased IL-6 levels. Both IL-1ra and IL-13 might change the course of infection by help of IL-6 and accelerate the disease and aggravated leishmaniasis and caused death in short period in contrast to high dose *Leishmania major* alone group.

Appendixes

Appendix 1. Indicates the effect of high dose (2.5×10^6) *Leishmania major* alone infected in left paw (red color) compared to non injected right paw (green color) of mice at week 35 postinfection.

Appendix 2. Indicates the effect of treated mice with IL-1ra and infected with high dose (2.5×10^6) *Leishmania major* in left paw (red color) compared to non injected right paw (green color) of mice at week 35 postinfection.

Appendix 3. Indicates the effect of treated mice with IL-13 and infected with high dose (2.5×10^6) *Leishmania major* in left paw (red color) compared to non injected right paw (green color) of mice at week 35 postinfection

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