Bioactive leishmanicidal alkaloid molecules from *Galipea longiflora Krause* with immunomodulatory activity

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“Happy are those who dream dreams and are ready to pay the price to make them come true”

Leon J. Sueños

A Walter, Adriana y Walter (jr), con quienes recorrí esta larga jornada
SUMMARY

According to the last report from WHO, leishmaniasis is endemic in 98 countries or territories, with more than 350 million people at risk. Published figures estimated an incidence of 2 million new cases per year (0.5 million of visceral leishmaniasis (VL) and 1.5 million of cutaneous leishmaniasis (CL). VL causes an estimated more than 50 000 deaths annually and 2 357 000 disability-adjusted life years lost, placing leishmaniasis ninth in a global analysis of infectious diseases.

Treatment of leishmaniasis has been based on the use of pentavalent antimonials but problems of toxicity and developing resistance have been reported. Traditional medicine and scientific studies have shown that the raw extract of Evanta (Galipea longiflora, Angostura longiflora (Krause) Kallunki) exhibits antileishmanial activity. We hypothesized that the healing observed when using this plant might not only be due to the direct action on the Leishmania parasite, but possibly to a parallel effect on the host immune response to the parasite. We first determined the toxicity of an alkaloid extract of Evanta (AEE) on eukaryotic cells in vitro and afterwards analyzed the effect of AEE on parasite growth. At 10 µg/ml we observed inhibition of Leishmania braziliensis promastigote growth while viability of eukaryotic cells was practically not affected. The whole extract was also found to be stronger than 2-phenylquinoline (2Ph), the most prominent alkaloid in AEE. AEE did not directly stimulate B or T cells or the mouse J774 macrophage cell line. However, AEE interfered with the activation of both mouse and human T cells, as revealed by a reduction of in vitro cellular proliferation and IFN-γ production. The effect was more evident when the cells were pretreated with AEE and subsequently stimulated with the polyclonal T-cell activators either Concanavalin A (ConA) or anti-CD3.

AEE treatment also reduced Leishmania-specific re-stimulation of lymphocytes both in vitro and in vivo as revealed by the reduced production of IFN-γ, IL-12 and TNF, signature cytokines of a Th1 immune response and also responsible of inflammatory reactions. More important, AEE treatment of mice hosting L. braziliensis modified the dynamics of the infection and showed that AEE is able to control both inflammation and parasite load. Additionally, the healing process was improved when AEE and the conventional drug meglumine antimoniate (SbV) were administered simultaneously.

Dendritic cells (DCs) play a pivotal role in T-cell stimulation and polarization of naïve T cells towards a Th1, Th2, Th17 or regulatory phenotype. Therefore, we investigated if AEE could alter the maturation/activation of DCs and if allostimulatory DCs properties were altered if activated in the presence of AEE or 2Ph. Neither AEE nor 2Ph altered the expression of activation markers on DCs, but the production of IL-12p40 and IL-23 was reduced. When we analyzed the allostimulatory capacity of AEE or 2Ph-treated DCs, we found that AEE or 2Ph did not affect the expression of various T-cell activation markers, but allogeneic CD4+ T-cells secreted lower levels of IFN-γ following co-culture with DCs treated either with AEE or 2Ph.

In conclusion the work presented in this thesis provides valuable insight into the effects of Evanta derived extract. The dual effect found for AEE, on the parasite and on the immune response, suggests that AEE may be useful in controlling the parasite burden and preventing over-production of inflammatory mediators, and subsequently avoiding tissue damage.
LIST OF PAPERS

This thesis doctoral is based on the following original papers (manuscripts), which will be referred to by their Roman numerals:


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AEE</td>
<td>Alkaloid extract of Evanta</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>CL</td>
<td>Cutaneous leishmaniasis</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Damage-associated molecular pattern</td>
</tr>
<tr>
<td>cDCs</td>
<td>Classical or monocyte derived DC</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>L-BMM</td>
<td><em>Leishmania</em> infected bone-marrow derived macrophages</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte- macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin 12</td>
</tr>
<tr>
<td>imDCs</td>
<td>Inmature DCs</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MCL</td>
<td>Mucocutaneous leishmaniasis</td>
</tr>
<tr>
<td>mDCs</td>
<td>Mature DCs</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility complex</td>
</tr>
<tr>
<td>NK</td>
<td>Natural-killer cell</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase-chain reaction</td>
</tr>
<tr>
<td>PKDL</td>
<td>Post Kala-Azar dermal leishmaniasis</td>
</tr>
<tr>
<td>SbV</td>
<td>meglumine antimoniate</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>VL</td>
<td>Visceral leishmaniasis</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER I

1. Traditional Herbal Medicine

1.1 Introduction

Traditional herbal medicine is the most ancient form of healthcare known to mankind; herbs have been used in all cultures since history started to be documented. Herbal medicine sometimes known also as phytotherapy, phytomedicine, herbalism or botanical medicine is the use of herbs for their therapeutic or medicinal value. A medicinal herb is a plant or a part of a plant (leaf, stem, root, bark, etc.) that can be used for healing purposes.

The World Health Organization (WHO) estimates that around 4 billion people, 75-80% of the world population mainly in the developing countries, use herbal medicine for some aspect of primary health care. WHO indicates that of 119 plant-derived pharmaceutical medicines about 74% are used in modern medicine in ways that correlate directly with their traditional practices as plant medicines by native cultures (1-4). Now, pharmaceutical companies are leading research on plant materials collected from the rain forests for their potential medicinal value.

The use of plant materials as a source of medicines for a wide variety of human diseases have increased because of better cultural acceptability, better compatibility with the human body and lesser side effects, and also because of insufficient supply of drugs, unaffordable cost of treatments and development of resistance to currently used drugs for infectious diseases (1-4).

The major limitation of the incorporation of herbal medicine into modern medical practices is the lack of scientific and clinical data and better understanding of their efficacy and safety.
The research on traditional medicinal plants has to be focused on providing scientific evidence for the presence of active molecules and assessment of specific effects and toxicities. To ensure the efficacy, the quality and the safety of herbal products, standardization is of vital importance, so that the herbal medicines can be safely used to treat the diseases (2-5).

1.2 Some historical facts

The use of plants as medicines precedes written human history; archaeological data indicate that humans were using medicinal plants since the Paleolithic era, around 60,000 years ago.

The study of herbs started in ancient times, according to written records available, with the Sumerian civilization in 4000 B.C., who created stone tablets with lists of hundreds of medicinal plants. Ayurveda medicine in India has used herbs as early as 1900 B.C. Sanskrit writings such as the Rig Veda from around 1500 B.C., are some of the earliest available documents detailing the medical knowledge that formed the basis of the Ayurveda system. The ancient Egyptians, in 1536 B.C., wrote the Ebers Papyrus (6), with information on over 850 medicinal plants. The first Chinese herbal document, the Pen Tsao, written by the Chinese emperor Shennong, lists 365 medicinal plants and their use, which includes Ephedra (ephrine to modern medicine) and chaulmoogra (plant of the genus Hydnocarpus, one of the first effective treatments for leprosy). De herbis et curis and Therapeutics are the writings from Greek and Roman medicinal practice that provided the pattern for later western medicine (7).

During the Early Middle Ages, Benedictine monasteries were the primary source of medical knowledge in Europe and England. However, most of their efforts were focused on translating
and copying ancient Greco-Roman and Arabic works, rather than creating new information (7).

Modern medicine has been effective in the treatment of many diseases, resulting in an increase in the life span of the population; however, negative factors such as side effects and high cost, among others, have led to the adaptation of herbal medicine as a valuable alternative.

Currently, many drugs used in modern medicine derive from traditional medicine, e.g. opium, aspirin, atropine, quinine, etc. There are about 120 active molecules obtained from higher plants and used by modern medicine today and many of them are used in a way that is very close to their traditional use (8).

The study of natural products with potential use in medicine as a goal must be done in association/collaboration with field ethnobotanists in order to develop reasonable laboratory hypotheses and for a more productive work (9,10).

1.3 Biological background

All plants synthesize chemical substances as part of their normal metabolic activities. These phytochemicals are of two types: primary metabolites, used by the plant for its basic functions (e.g. sugars, fats), and secondary metabolites for specific functions, for example, used as a defense or to attract insects for pollination. It is this secondary type of phytochemicals which has potential therapeutic actions in humans. Plants synthesize a variety of secondary metabolites but most are derivatives of a few biochemical structures: polyphenols, glucosides, terpenes and alkaloids.
Alkaloids are a class of chemical compounds containing a nitrogen ring and can be purified from crude extracts by acid-base extraction (Fig.1). Among known alkaloids are caffeine and nicotine (stimulants); morphine (analgesic); berberine (antibacterial); vincristine (anticancer) and quinine (antimalarial) (11).

Fig. 1. Basic structure of quinoline alkaloids

2. The Amazonas and Evanta

2.1 The Amazon rainforest

The Amazon rainforest covers a vast area of Ecuador, Peru, Bolivia and Brazil, around the Equator. It is a dense, warm, wet forest that treasures an incredible biodiversity. The Amazon rainforest in South America is the world's greatest natural and the most powerful and bioactively diverse natural phenomenon on the planet (12). Rainforest plants are rich in secondary metabolites, particularly alkaloids. The National Cancer Institute in USA identified 3000 plants that are active against cancers. Seventy percent of these plants are found in the rainforest. Twenty-five percent of the active ingredients in today's cancer-fighting drugs come from organisms found in the rainforest. Vincristine, extracted from the rainforest plant periwinkle (Catharanthus roseus), is one of the world's most powerful anticancer drug. It has dramatically increased the survival rate for acute childhood leukemia since its discovery (13,14).
In 1983, there were no pharmaceutical manufacturers in USA involved in research programs to discover new drugs from plants. Today, over 100 pharmaceutical companies are engaged in plant research projects for possible drugs to treat different diseases (13,15).

2.2 Galipea longiflora Krause (Evanta)

*G. longiflora* (*Angostura longiflora Krause*) is a tree of the family Rutaceae found in the Amazon area of South America. This genus has around 40 species distributed from Guatemala to Bolivia and in the south region of Brazil. The tree is 12 m high, has strong smell and is characterized by the common occurrence of spines and winged petioles and alternate or opposite, simple or palmately or reduced to spine leaves. In Bolivia this tree is found in the tropical woods of the last Andean counterforts in the areas of Beni and La Paz and is used as antiparasitic medicinal plant. *G. longiflora* is known widely by its vernacular name Evanta. The information about the traditional uses of *G. longiflora* is mainly from the ethnicities Tacana, Mosetene (La Paz) and Tsimane (Beni). An ethnobotanic survey and workshop with representatives of 16 communities has been published which includes a list of 140 Amazonian plant species and its medicinal uses. This field work has been published as a book with more than 30 co-authors and was developed in coordination with the Indigenous council of the Tacana Communities (Consejo Indígena de Los Pueblos Tacana, CIPTA). The identification of the specie and the voucher samples of *G.longiflora* are found in the “Herbario Nacional” of La Paz (AS49, zona de Caicahuara y SD17, Santa Rosa de Maravillas) (16-18).

The traditional and frequent use of Evanta is in the treatment of diarrhea caused by intestinal parasites and as fortifying agent for children and adult people. For the treatment of
leishmaniasis, fresh or dehydrated bark is smashed and applied directly on the ulcers; in addition, the infusion of the plant is drunk (19).

During the period 1985-91, a group of French-Bolivian researchers confirmed the antiparasitic/anti-\textit{Leishmania} activity of the extracts obtained from Evanta. A total of 12 quinolinic alkaloids were isolated and identified from the leaves, bark and roots. Some of the alkaloids isolated from Evanta showed to be new structures and due to the efficacy demonstrated against the parasite \textit{Leishmania} and also on \textit{in vivo} models of infection, they were patented (Chimaninas A, B, C y D, US4209519/15/04/93) (20-23).

In 2006 \textit{G. longiflora} was selected as one of the most promising plants of more than 800 crude extracts and pure substances evaluated for its antiparasitic activity using \textit{in vitro} methods (24). Further studies have been done on the toxico-kinetic behavior and on acute and chronic toxicity in murine model using crude extracts, total alkaloids and pure substances obtained from the bark of Evanta (25,26).

In the present study the crude total alkaloid extract of Evanta (AEE) was used. The major alkaloid component of this extract is 2-phenylquinoline (2Ph). In the following figure the alkaloids present in the crude extract are shown (21,25,26).
[MS+1]= 206 (C15 H11 N)   [MS+1]= 200 (C14 H17 N)   [MS+1]= 236 (C16 H13 N O)

[MS+1]= 172 (C12 H13 N)   [MS+1]= 278 (C18 H15 N O2)

[MS+1]= 230 (C15 H19 N O)   [MS+1]= 308 (C19 H17 N O3)

[MS+1]= 228 (C15 H17 N O)

Fig.2. Alkaloids present in AEE.

3. Leishmaniasis

3.1 Global burden

According to the last report from WHO, leishmaniasis is endemic in 98 countries or territories, with more than 350 million people at risk. Published figures estimated an incidence of 2 million new cases per year (0.5 million of visceral leishmaniasis (VL) and 1.5 million of cutaneous leishmaniasis (CL). VL causes an estimated of more than 50 000 deaths
annually, a rate surpassed among parasitic diseases only by malaria, and 2 357 000 disability-adjusted life years lost, placing leishmaniasis ninth in a global analysis of infectious diseases (27).

### 3.2 Etiology of leishmaniasis

Leishmaniasis is caused by the obligate intracellular parasite *Leishmania* spp and belongs to the kinetoplastid family. The parasitic protozoa are transmitted by female sand flies belonging to the genus *Phlebotomus* or *Lutzomyia*.

*Leishmania* exists in two basic forms: amastigote and promastigote. The amastigote is the intracellular form present in the vertebrate host, it is a non-motile form and it divides by longitudinal binary fission at 37 °C. Amastigotes are 3-6 µm in length and 1.5-3.0 µm in width. Even though amastigotes seem to lack a flagellum, it is simply that the flagellum does not protrude beyond the body surface and it cannot be seen by light microscopy (Fig. 3a). Amastigotes are taken up from the blood of an infected host when the female sand fly bites, and in the sand fly gut they develop into promastigotes where they multiply.

Promastigotes are 15-30 µm in body length and 5 µm in width; it is extracellular, has a flagellum, is motile, and grows and divides by longitudinal binary fission at 27 °C in the sand fly. Promastigotes can be grown *in vitro* at 25 °C (Fig. 3b).

Fig. 3. A macrophage containing *Leishmania* amastigotes (a) and *Leishmania* promastigotes (b).
3.3 Taxonomy

The parasites of the genus *Leishmania* are among the most diverse of human pathogens, both in terms of geographical distribution and the variety of clinical syndromes that they cause (24). Over 20 species and subspecies of *Leishmania* infect humans, each causing a broad spectrum of clinical manifestations.

The genus *Leishmania* has been classified into two subgenera: *Leishmania*, present in both the old and the new worlds, and *Viannia*, restricted to the new world. The classification of *Leishmania* is shown in further detail in Fig. 4 (27).

![Taxonomy of Leishmania parasite.](image)

3.4 Life cycle

Leishmaniasis is transmitted by the bite of a female phlebotomine sand fly. The sand fly injects the infective stage, metacyclic promastigotes, during a blood meal. Metacyclic promastigotes that reach the puncture wound are phagocytozed by macrophages and transform into amastigotes. Amastigotes multiply in infected cells and affect different tissues, depending on the *Leishmania* species. This starts the clinical manifestations of leishmaniasis.
Sand flies become infected during blood meals on an infected host when they ingest macrophages infected with amastigotes. In the midgut of the sand fly, the parasites differentiate into promastigotes, which multiply, differentiate into metacyclic promastigotes and migrate to the proboscis (28,29) (Fig. 5).

![Leishmania life cycle](image)


### 3.5 Clinical forms

The range of manifestations of leishmaniasis is wide and presents in four different clinical forms: CL, MCL, VL and post Kala-Azar dermal leishmaniasis (PKDL).

CL is mainly caused by *Leishmania major* and *Leishmania tropica* in the old world and *Leishmania braziliensis* and *Leishmania mexicana* in the new world. But CL may be caused by other *Leishmania* species such as *Leishmania guyanensis, Leishmania naiffi, Leishmania*
The species involved depend on the geographical distribution, and the disease may appear as simple or diffuse ulcerations on skin, mainly in the face, causing sometimes disfiguration of the patient. The disease may regress spontaneously or evolve, thus requiring treatment (27,30-32).

*L. braziliensis* is also the principal causative agent for MCL, though additional species have been described (*L. amazonensis, L. panamensis* and *L. guyanensis*). MCL is characterized by chronic ulcers on the skin, mouth and nose, with destruction of underlying tissue (e.g. nasal cartilage). Tissue destruction with disfigurement can be very severe (27,31,32).

*Leishmania* parasites can also produce more severe, life-threatening VL caused by the *Leishmania donovani* in the Indian subcontinent, Asia and East Africa or *Leishmania infantum* in Europe, North Africa and Latin America and *Leishmania infantum/chagasi* in Europe and Latin America (27,28).

### 3.6 Current treatment available

#### 3.6.1 Pentavalent antimonials

Two pentavalent antimonials are available: meglumine antimoniate and sodium stibogluconate. They are chemically similar, and their toxicity and efficacy are related to their antimonial (SbV) content. Pentavalent antimonials are usually administered parenterally (intramuscularly) but can be administered intralesionally for the treatment of CL (33,34).

Pentavalent antimonials have been in use against leishmaniasis for more than six decades. Although they have an obvious effect on the parasite, their molecular and cellular
mechanisms of action are not yet well understood. While there are two forms, SbV and SbIII (trivalent antimony), it is not clear yet which is the active form (35). Initial studies suggested that sodium stibogluconate SbV inhibits macromolecular biosynthesis in amastigotes (36), possibly via perturbation of energy metabolism due to inhibition of glycolysis and fatty acid beta-oxidation (37). However, the specific targets in these pathways have not been identified. In humans, pentavalent antimonials can produce severe side effects, such as cardiotoxicity and hepatotoxicity, when administered systemically (38).

3.6.2 Amphotericin B

Amphotericin B is a polyene antibiotic, administered intravenously. The primary site of action of amphotericin B on L. donovani promastigotes appears to be membrane sterols that result in a loss of the permeability barrier to small metabolites (39).

Among the side effects, nephrotoxicity is common, leading to frequent interruption of treatment in some patients. Other uncommon but serious toxic effects are hypokalemia and myocarditis. Treatment should always be given in a hospital to allow continuous monitoring of patients.

3.6.3 Paromomycin

Paromomycin (aminosidine) is an aminoglycoside antibiotic, usually administered by intravenous infusion. Paromomycin inhibits protein synthesis and modifies membrane fluidity and permeability of the parasite (40). Other studies have reported that exposure of L. donovani promastigotes and amastigotes to paromomycin, decreased their mitochondrial potential, indicating that mitochondria are the targets of this drug (41).
Treatment with paromomycin produces reversible ototoxicity in 2% of the patients, renal toxicity is rare and some patients may develop hepatotoxicity when administered parenterally.

3.6.4 Miltefosine

This alkyl phospholipid (hexadecylphosphocholine) was originally developed as an oral anticancer drug but was shown to have antileishmanial activity. It has been demonstrated that miltefosine induces apoptosis-like death in *L. donovani* (42).

Miltefosine commonly induces gastrointestinal side effects, such as anorexia, nausea, vomiting (38%) and diarrhea (20%). Most episodes are brief and resolve as treatment is continued. Occasionally, the side-effects can be severe and require interruption of treatment. Miltefosine is potentially teratogenic and should not be administrated to pregnant women.
CHAPTER II

1. The immune system

Humans, as other mammals, live in an environment that is inhabited by a vast range of microorganisms, pathogenic and non-pathogenic, and has huge numbers of substances that are a potential threat to their survival. The immune system has as a primary function to protect the host against microbial invasion, but it also has to maintain homeostasis.

The immune function has been theoretically divided into innate and adaptive immunity, which both have to work together. Innate immunity is the first line of defense, characterized by rapid response to a large but limited number of stimuli. Its components include physical, chemical and biological barriers, specialized cells and soluble molecules. These components are present in all individuals, regardless of previous contact with the antigen. Among the mechanisms of the innate immunity are phagocytosis, activation of the complement system, activation of natural-killer (NK) cells, secretion of preformed molecules, such as antimicrobial agents (e.g. defensines), proteins of the complement system, newly synthesized molecules, such as acute phase proteins and release of cytokines and chemokines. Innate immunity is activated by specific stimuli; the well-known pathogen associated molecular patterns (PAMPs), and endogenous damage-associated molecular patterns (DAMPs). PAMPs and DAMPs interact with receptors on the surface of the innate immune cells, the pattern recognition receptors (PRR), such as Toll-like receptors (TLRs), receptors that are already encoded in the germline.
The second line of defense constitutes the adaptive (or acquired) immune response that is characterized by a late response. The mechanisms of the adaptive responses are activation of T and B cells, production of cytokines and antibodies. The main characteristics of the adaptive responses are: high specificity of recognition for any foreign molecule, memory (long-lived cells that persist and can be restimulated rapidly after encounter with the same specific antigen) and tolerance to self components. Adaptive responses are activated by interaction with the antigen-specific receptors expressed on the surface of T and B-cells and encoded by genes that are assembled by somatic rearrangement of germ-line-gene elements to form a T-cell receptor (TCR) and immunoglobulin B-cell receptor (BCR) genes.

2. The immune response in leishmaniasis

2.1 The battle of *Leishmania* with host defense mechanisms-the complement system

The complement system is an efficient mechanism of the innate immune response that helps to clear pathogens from the organism by disrupting the membrane of the target cell, and consists of a tightly regulated network of more than 30 proteins (43). Apart from the lysis of target cells, the central effect of complement activation, opsonization of pathogens and cellular activation (chemotaxis) are also important. The complement system can be activated through different pathways that are summarized in Fig. 6. Most of the complement components circulate as soluble proteins in the blood and other body fluids in an inactive form while others are present as membrane-associated proteins.
When an infected female sand fly inoculates metacyclic promastigotes into the skin dermis of the host during blood feeding, the promastigotes interact immediately with serum components. Metacyclic promastigotes of *Leishmania* have been shown to activate complement components of both the lectin and the alternative pathways. Opsonization of *Leishmania* metacyclic promastigotes with complement is fast and lysis via the membrane attack complex (C5b–C9 complex) (MAC) begins 60 s after serum contact. Complement activation results in efficient killing of 90% of all inoculated parasites within 3 min (44,45). *Leishmania* parasites must evade complement-mediated lysis until they are engulfed by a
macrophage. The resistance to the complement lysis depends on the morphological stage of the parasite, *L. major* procyclic promastigotes, for example, cannot resist complement action, while the metacyclic form can fully avoid complement lysis (46). This difference in complement resistance has been shown to depend upon lipophosphoglycan (LPG), the main surface molecule on the promastigote parasite surface. The LPG structure differs between *Leishmania* species and also on the morphological stage of the parasite (47). LPG found on the surface of metacyclic promastigotes is longer and that seems to be the reason why CAM can not attach, so lysis is eluded. However, *L. donovani* promastigotes prevent C5 convertase formation by adhering the inactive C3bi subunit on their surfaces (48). Another important molecule that contributes to parasite virulence is the surface glycoprotein gp63. This is a metalloprotease, also called leishmanolysin, that is abundant on the membrane surface of all *Leishmania* species. Gp63 may contribute to parasite virulence by controlling complement fixation while taking advantage of the opsonic properties of complement (49,50).

**2.2 Phagocytosis, an important defense mechanism against infection, but *Leishmania* parasite takes advantage of it**

The major phagocytic cells are neutrophils, macrophages, monocytes and dendritic cells (DCs). Phagocytic cells use different Fc receptors and complement receptors to improve uptake of particles that have been marked by the adaptive and innate immune responses for elimination (51). These cells engulf microbes and localize them in intracellular vacuoles (phagosomes) where they are exposed to toxic effector molecules when lysosomes fuse with the phagosomes. Among the toxic molecules are radical oxygen intermediates (ROI) (superoxide, hydroxyl radicals) and radical nitrogen intermediates (RNI) such as nitric oxide (NO), and degradative enzymes. Phagolysosomes have an acidic pH and their content of
hydrolases and degradative enzymes are capable of breaking down the internal components of the phagocytized particle.

Several macrophage subsets with distinct functions have been described. Two main subsets are the classically activated macrophages (M1 macrophages), which mediate defense and inflammatory reactions and the alternatively activated macrophages (M2 macrophages), which have anti-inflammatory function and regulate wound healing (52).

*Leishmania* parasites are obligate intracellular protozoan microorganisms, contributing to their own phagocytosis by favoring the opsonization with C3bi interacting with the macrophage complement receptor 3 (CR3), rather than with C3b interacting with CR1. When the parasite attaches to the macrophage using CR3 instead of CR1, it eludes oxidative burst during phagocytosis (53).

Once inside of the macrophage, *Leishmania* promastigotes acquire different tactics to evade toxic lysosomal compounds found in phagolysosomes. It has been shown that *Leishmania* parasites delay normal phagosome formation/maturation allowing time for the parasite to develop into the more acid hydrolase resistant amastigote form (54). The delay in phagosome maturation results in delay of maturation by failure to recruit lysosomes that contain cytotoxic chemicals. This mechanism depends on the promastigote cell surface molecule LPG that prevents the formation or disrupts lipid microdomains on the phagosome membrane by modifying the molecular structure of lipid bilayers (55-57). Once the promastigote has entered the phagosome, LPG inserts into lipid rafts and inhibits phagosome–lysosome fusion. *L. donovani* accumulates a ring of F-actin around the phagosome providing a physical barrier preventing interaction with late endosomes. It is believed that the enlarged F-actin ring
surrounding the phagosome stops the fusion of membrane markers with the phagosome. LPG also plays an active role by inhibiting lysosomal enzymes, too. Additional metacyclic promastigote-derived virulence factors may cross the phagosome membrane and thereby reach their cytosolic targets (55). (Fig. 7).

When the phagolysosome is formed, the acidic enzymes present in the lysosomes, get in contact with *Leishmania* parasites, but the parasites can maintain its intracellular pH near to neutral due to a proton pump that is found on its surface. The acid phosphatases also found on the surface of *Leishmania* parasites inactivate the oxidative activity of superoxide and hydroxyl radicals produced by macrophages (58-60). It has been shown that some *Leishmania* species possess mechanisms to resist the action of ROI or RNI. One of the mechanisms is the recognized ROI-scavenging function of phosphoglycans and the expression of superoxide dismutase, catalase and/or peroxiredoxins most of which are shown to promote the persistence of *Leishmania* within macrophages (61-64). Phagolysosomes, with their microbicidal mechanisms, represent an aggressive environment that *Leishmania* parasites

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**Fig. 7.** Mechanisms of evasion of *Leishmania* parasite (Nat Rev Microbiol. 2011 Jul 11;9(8):604-15). Reproduced with permission from Nature Publishing Group.
must subvert and finally evade. Amastigotes, then, replicate (up to 1,000-fold), and eventually the macrophage is lysed and the infectious amastigotes are released into the milieu, where they infect neighboring macrophages (60).

2.3 Participation of neutrophils and monocytes

The tissue damage produced by the mosquito bite induces the release of endothelial chemotactic cytokines that recruit neutrophils, eosinophils and macrophages and initiates a strong inflammatory reaction. In addition, promastigotes induce macrophages to secrete CCL2 (MCP-1) and CXCL1 (IL-8, in humans) that act as chemoattractants for monocytes and neutrophils, respectively. Development of clinically evident lesions occurs coincident with the influx of these inflammatory cells (65,66). In Fig. 8 the monocyte-to-macrohage recruitment and initiation of the inflammatory response processes are shown.
Fig. 8. Initiation of inflammatory response in *Leishmania* infection. During infection and tissue stress, monocyte recruitment has a key role in providing the damaged tissues with adequate numbers of macrophages. *Leishmania major* parasites that have infiltrated the skin after a sandfly bite elicit a local macrophage response. Extravasation of the monocytes is followed by differentiation into macrophages that phagocytose the parasites and present their antigens to T cells. Interferon-γ (IFNγ) production from T cells drives an M1 response that contains parasite growth. TH1, T helper 1. (Nat Rev Immunol. 2011 Oct 14;11(11):723-37). Reprinted with permission from Nature Publishing Group.
2.4 The central role of DCs

DCs are the most potent antigen-presenting cells (APCs), capturing, processing and presenting antigens to initiate an acquired immune response or induce tolerance. DCs are considered to be the link between innate and adaptive immunity because they are recruited and activated by effectors of innate immunity and can stimulate the adaptive immune response by presenting antigens to T cells, both CD4\(^+\) and CD8\(^+\), and B cells (67).

DCs are a heterogeneous cell population with different subsets identified. In mice and humans there are two types of DCs: classical or myeloid DCs (cDCs) and plasmacytoid DCs (pDCs), both containing distinct subsets. To date, many different classifications of the cDCs exist, e.g. based on location and/or expression of surface markers, and they are often not the same for mice and humans. One such classification is the division of DCs into immature DCs (imDCs) and mature DCs (mDCs).

ImDCs reside in tissues where they patrol their surroundings for harmful signals. They express many receptors on their surface that aid in phagocytosis of antigens, such as Fc receptors, complement receptors and PRRs. Activation through PRRs leads to stimulation of the imDCs where they undergo a process called maturation. They lose their phagocytizing capacity and upregulate the surface expression of MHC class I and class II peptide complexes and co-stimulatory molecules (CD40, CD54, CD80 and CD86), and start to secrete proinflammatory cytokines (Fig. 9) (71,72). Mature DCs (mDCs) also upregulate chemokine receptors enabling migration into draining lymph nodes. All these phenotypic changes in DCs allow them to prime T-cell mediated immune response (72).
ImDCs are present in the tissue where they act as “sentinels” and capture any antigen they encounter. DCs recognize microbes, and secrete cytokines. Cytokines secreted by DCs in turn activate effector cells of innate immunity such as eosinophils, macrophages and NK cells. Activation/maturation triggers DCs migration towards secondary lymphoid organs. These migratory mDCs display antigens in the context of classical MHC class I and class II or non-classical CD1 molecules, which allow selection antigen-specific T lymphocytes. Activated T lymphocytes reach the injured tissue, where they eliminate microbes and/or microbe-infected cells. B-cells, activated by DCs and T-cells differentiate into plasma cells that produce antibodies against the initial pathogen. (Immunity. 2010 Oct 29;33(4):464-78). Reprinted with permission from Elsevier.

Activated DC produce IL-12, especially important because its expression during infection regulates innate responses and determines the type and duration of adaptive immune response mainly attributed to its involvement in the development of Th1 cells. IL-12 promotes the differentiation of naïve CD4\(^+\) T-cells into T helper 1 (Th1) cells that produce IFN-\(\gamma\) and aid in cell-mediated immunity. IL-12 induces IFN-\(\gamma\) production by NK cells, T-cells, DCs, and macrophages (73).
Approximately six weeks post *Leishmania* infection, the number of cDC increases at the site of infection, as does the percentage of infected DCs. DCs are attracted through mechanisms of the innate immune response, e.g. mast cell-derived mediators, cytokine and chemokine release by neutrophils (74,75).

The imDCs phagocytose the amastigote form of the parasite mainly through the Fc receptor (FcR) I and FcRIII. In the case of *L. major*, imDCs are activated and they upregulate MHC class I and II expression as well as co-stimulatory molecules (76). At the same time infected DCs release proinflammatory cytokines including IL-12.

Many studies have shown that IL-12 is necessary not only in the initial defense of *Leishmania* infections, but also to maintain an established response against this parasite (77-80).

In addition, it has been suggested that IL-12 is indispensable in the development of a protective immune response and that in the absence of IL-12, susceptibility to *L. major* is due to the failure to induce a Th1 response but not because of the development of a Th2 response (81). Conversely, recent studies have demonstrated that under certain circumstances Th1 development can be achieved in the absence of IL-12 (82).

Besides IL-12, there are other cytokines that contribute to induce a Th1 response, such as IL-23, IL-27 and IL-1β, which are also secreted in the early stages of infection. IL-18 is another proinflammatory cytokine that can help in evoking Th1 immune responses particularly in collaboration with IL-12. A very important fact is that the sustained release of IL-12 allows the perpetuation of Th1 immunity (83).
*L. amazonensis* infection induces relatively low levels of DC maturation/activation (in terms of expression of surface markers as well as the production of IL-12p40), while *L. braziliensis* infection induces high levels of activation in both infected DCs and bystander DCs. Both infected and bystander DCs are capable of priming naïve CD4⁺ T cells *in vitro*; therefore, strong DC activation is a characteristic for *L. braziliensis* infections (84).

IL-10 is another cytokine involved in the immune response to *Leishmania*; this cytokine can suppress Th1 responses with the consequent effect on the activation of macrophages. IL-10 was not considered to be important in *L. major* infection, because treatment of BALB/c mice with an anti-IL-10 monoclonal antibody had almost no effect on disease progression (85). On the contrary, IL-10-deficient mice with a BALB/c background were markedly more resistant to *L. major* infection than wild-type BALB/c mice (86,87). IL-10-receptor blockade has been shown to confer partial resistance to *L. major* in wild-type BALB/c mice. Finally, in humans overproduction of IL-10 in lesional tissue from VL patients and in plasma from patients with PKDL has been found (86-89).

3. **Induction of adaptive immune responses**

When DCs present antigens to naïve CD4⁺ T cells, they stimulate them to proliferation and promote the polarization towards a different immune response, mainly Th1, Th2, Th17 and T regulatory (Treg) response. The interaction of co-stimulatory molecules with their respective ligands and the local cytokine environment directs the differentiation. It has been proposed that there are two distinct subsets of dendritic cells, known as DC1 and DC2, which in turn, direct a Th1 or Th2 differentiation pathways, respectively. In Th1 polarization, certain pathogens or PAMPs trigger APCs, through TLRs, to secrete IL-12, which promotes the
differentiation of naive T cells into Th1 cells (29). In this model Th2 polarization might be due to an inability of antigen to activate DCs to produce IL-12 (Fig. 10). Th1 immune responses are characterized by the production of IFN-γ and tumor necrosis factor (TNF), among others (28, 29).

Fig. 10. Polarization of Th1 and Th2 cells by DCs. The interaction of co-stimulatory molecules with their respective ligands and the local cytokine environment stimulates the differentiation Th1/Th2. It has been proposed that there are two distinct subsets of dendritic cells, known as DC1 and DC2, which in turn, direct to a Th1 or Th2 differentiation pathways (Nat Rev Immunol. 2002 Nov;2(11):845-58). Reprinted with permission from Nature Publishing Group.

IFN-γ is the signature cytokine of a Th1 immune response, mainly acting on macrophages, NK cells and B cells. Macrophages stimulated with IFN-γ induce direct antimicrobial activity and production of TNF as well as up-regulating antigen processing and presentation.
pathways. In addition IFN-\(\gamma\) enhances NK cell activity and regulates B-cell functions, such as immunoglobulin (Ig) production and Ig class switching (90). TNF is a pleiotropic cytokine linked to Th1 immune responses and to inflammation. TNF is a powerful pro-inflammatory agent that regulates many facets of macrophage function and induces the production of other cytokines, e.g. IL-1, IL-8, IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (91).

Th1 immune responses are critical for the control of *Leishmania* infection, mainly because of the production of IFN-\(\gamma\), which activates macrophages and DCs, leading to parasite death.

Most studies on leishmaniasis have been done in experimental models with *L. major* and the importance of the Th1/Th2 paradigm in resistance or susceptibility to infection has been demonstrated. According to this paradigm, some strains of mice such as C57BL/6 (resistant strain) develop a self-limiting infection when infected with *L. major*, mediated by Th1-immune response while BALB/c mice (susceptible strain) develop uncontrolled infection mediated by Th2 immune response (29,92,93). However, the Th1/Th2 paradigm usually applies in the case of *L. major* but it does not apply to mice infected by other *Leishmania* species such as *L. braziliensis* or *L. amazonensis* (94). In the view of new discoveries on CD4\(^+\) T cell populations among others, it seems that this paradigm may not be relevant anymore (Fig. 11) (95).
Fig. 11. The mechanisms that influence the expansion of different CD4+ T cell populations as part of the adaptive immune response following *Leishmania major* infection, and their role in determining the outcome of disease (Front Immunol. 2012;3:80).

The ability of DCs to induce Th1 or Th2 immunity may depend on the characteristics of the microenvironment present when DCs first interact with the antigen. However, in *Leishmania* cutaneous infections, DCs preferentially induce Th1/Tc1 immunity, priming both CD4+ as well as CD8+ T cells (96-100). Activated CD4+ T cells can interact with macrophages through CD40 and CD40L and induce IL-12 expression and production of NO from iNOS. CD40L deficient mice are susceptible to *Leishmania* infection and produce less IL-12, IFN-γ and NO compared with its wild type counterparts suggesting that CD40-CD40L interaction is required for the clearance of the infection (96).

In *Leishmania* infection IFN-γ is critical for macrophage activation as demonstrated by the fact that knock out resistant mouse strains (either for IFN-γ or IFN-γ receptor genes), were
unable to restrict growth of *L. major* in vivo and suffered fatal infection. It has also been shown that, recombinant IFN-γ was capable of activating infected macrophages from both resistant and susceptible mice to clear *L. major* in vitro (81,101).

The importance of IFN-γ in the induction of NO was demonstrated by the finding that IFN-γ, among a number of cytokines, was capable of independently enhance iNOS transcription and NO release from stimulated mouse peritoneal macrophages (102). These results are consistent with the impaired production of NO by macrophages from IFN-γ or IFN-γ –receptor gene knock-out mice and the crucial role of IFN-γ in macrophage activation (74).

The role of TNF as anti-leishmanial cytokine has been extensively studied. The participation of TNF in the development of a protective immune response to *L. major* infection has been analysed in experimental CL and the results were contradictory. Administration of recombinant murine or human TNF to resistant or susceptible strains of mice infected with *L. major* ameliorated the course of disease by reducing lesion size and parasitic burden. However, neutralizing with anti-TNF antibody transiently exacerbated disease in resistant mice, although the course of infection in BALB/c mice was unaltered or affected minimally (103-108).

Th17 cells have recently been described as a third subset of Th cells, and have provided new insights into the mechanisms important in immune responses essential for effective antimicrobial host defence (109). Th17 responses are critical for mucosal and epithelial host defence against extracellular bacteria and fungi. However, recent studies have reported that Th17 responses can also contribute to viral persistence and chronic inflammation associated
with parasitic infection. IL-17 has long been implicated in several inflammatory diseases (110).

The role of Th17 cells in leishmaniasis is still not well understood. Some studies have associated elevated levels of IL-17 together with IFN-γ production with healing in *L. donovani* model (111). In humans, sub-clinical infection due to *L. braziliensis* correlated with high levels of IL-17 (112), conversely the neutrophil infiltrate induced by IL-17 cause tissue destruction in patients with mucosal leishmaniasis (113-115).

The role of CD8+ T cells in cutaneous leishmaniasis is not clear, some studies associated these cells with control of the infection, such as in the case of VL, but studies of CL indicate that CD8+ T cells were not important for the control of a primary infection but for the resistance to reinfection (116). Other studies showed that recruitment of CD8+ T cells expressing the granule-associated serine protease granzyme B is correlated with lesion progression in patients infected with *L. braziliensis* (117).

### 3.1 Regulation of the inflammatory response.

Although the importance of a Th1 immune response in leishmaniasis has been demonstrated, in humans the resulting inflammatory reaction produced might be associated with disease exacerbation. The immunologic characteristics of disease vs. protection in VL and control vs. chronicity in CL and MCL are very different and not so well understood.

In VL, many studies have shown that IFN-γ and IL-12 are decreased during the acute phase of the disease, showing that immunosuppression occurs during VL (78,118-121). However, data from these studies must be analyzed carefully because the immune system is in fact highly
activated during acute phase of VL, producing IFN-γ and TNF as well as IL-10 and TGF-β. But these processes are expected to occur mainly in the spleen and liver and not in peripheral blood. In this way peripheral blood mononuclear cells (PBMCs) respond poorly to *Leishmania* antigens because reactive lymphocytes are trapped in the lymphoid organs (120). Recently, high levels of IL-10 are suggested to play a role in counterbalancing the exacerbated polarized response that may develop following a cure (122,123).

MCL is associated with a strong Th1-type immune response to *Leishmania* antigens with high levels of TNF and IFN-γ. Larger lesions correlated with a higher frequency of the IFN-γ- and TNF-producing lymphocytes, even if parasites were often absent or rarely found in lesions. Exacerbated immune responses are not so favorable to the patient since it leads to an uncontrolled inflammatory reaction causing tissue destruction (123-130).

The association of *Leishmania*-induced pathology and parasite persistence and production of TNF has lead attempts to block TNF. Indeed, TNF blockade with pentoxyfyllin has some remarkable clinical effects in drug resistant cases of CL (129-130). Although Th1-type immune responses protect against most forms of leishmaniasis, it is likely that some types of immune responses directed against *Leishmania* can lead to more severe clinical forms, while others lead to resolution of the infection with little pathology. It seems as if a balance between Th1 and Th2 type of immune responses against *Leishmania* is critical for the establishment of effective control of the disease (131-134).

In summary it can be concluded that uncontrolled inflammatory reaction, as part of the immune response, may lead to tissue damage, which means that inflammation must be strongly regulated to avoid its destructive effects.
4. Experimental models of leishmaniasis

Murine experimental models of leishmaniasis have been developed to contribute to the understanding of the pathogenesis of this disease. These studies have provided a great amount of information about the host-parasite relationship and the factors involved in the development of susceptibility or resistance and have defined mouse strains as being either resistant or susceptible to leishmaniasis (135).

Genetic studies in mice can give valuable information about the complex interactions between the parasite and the host, which indicate host genes that might control the outcome of the infection by the induction of distinct components of the immune response. The existing information about genetics of leishmaniasis is based mainly on mouse models with *L. major* and *L. donovani*. These studies have shown a multigenic basis of susceptibility which correlates well with the complexity of the immune response to infection (93,136).

Susceptibility and resistance data of various *Leishmania* species on some mouse strains are summarized in Table 1. In mice, different forms of the disease depend on the species of *Leishmania* and on the genetic background of the mouse (82,137). For example BALB/c mice are susceptible to *L. amazonensis* infection and develop non-healing skin lesions with high parasite loads and have a predominantly Th2-type of response (137). On the other, hand *L. braziliensis* causes only a transitory disease, in mice, which depends on the production of a Th1-type immune response. Studies have shown that the difference on the behavior is due to immunoregulatory virulence factors produced by *L. amazonensis*, but not by *L. braziliensis* (e.g. a parasite-derived serine protease activity) apart from the genetic background (138).
Table 1. Susceptibility of mice strains to *Leishmania* infection.

<table>
<thead>
<tr>
<th>Leishmania specie</th>
<th>Susceptible mouse strain</th>
<th>Resistant mouse strain</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. braziliensis</em></td>
<td>----</td>
<td>BALB/c, C57BL/6</td>
</tr>
<tr>
<td><em>L. amazonensis</em></td>
<td>BALB/c</td>
<td>C3H/HePas</td>
</tr>
<tr>
<td><em>L. major</em></td>
<td>BALB/c, P/J, SWR/J and CcS-16</td>
<td>AKR, B10.D2, CBA, C57BL/6, C3H, DBA/2J</td>
</tr>
<tr>
<td><em>L. donovani</em></td>
<td>BALB/c, C57BL/6, C57BL/10, CE/J, DBA/1J, SWV</td>
<td>A, AKR/J, 129/Sv, C3H/HeJ, CBA, DBA/2</td>
</tr>
<tr>
<td><em>L. panamensis</em></td>
<td>The BALB/c and DBA/2J</td>
<td>C57BL/6</td>
</tr>
</tbody>
</table>

Hamsters and guinea pigs are also used as experimental models to study leishmaniasis; hamsters provide an excellent susceptible host model which can be used for histopathological, drug efficiency and vaccine studies. One of the problems is the lack of high quality immunoreagents that limit immunological studies. Guinea pigs have been a traditional model for studies of delayed type hypersensitivity and also to test potential antigens for use in diagnostic tests.

4.1 Experimental infection with *L. braziliensis*.

Several studies with murine models of *L. braziliensis* have demonstrated that all mouse strains tested are resistant to infection by this parasite (138,139). It has been shown that the weak infectivity of *L. braziliensis*, in mice, is mainly dependent on IFN-γ activity. Many authors have indicated that the resistance of mice to infection is due to the inability of the parasite to elicit strong and sustained IL-4 production or production of other cytokines that inhibit the
development of a Th1-type immune response, but data are controversial. For example, IL-10 inhibits Th1 development; however, BALB/c mice produce equivalent amounts of IL-10 following infection with either *L. braziliensis* or *L. major* (140-144).

A study carried out in Brazil showed different behavior of two strains of *L. braziliensis* isolated from different regions in the country. One of them (H3227) could induce detectable lesions in BALB/c mice whereas the other strain (BA788) could not do so. The cells recovered from the draining lymph nodes of BA788-parasitized mice produced higher levels of IFN-γ, showing different immunological characteristics of the parasite strain. The authors attributed the different behavior to their different genomic profiles (142).

As indicated before, infection with *L. braziliensis* in humans might lead to a self healing infection, but it may also progress, later on, to a destructive, disfiguring disease. Efforts to develop suitable experimental models that allow mimicking the biology of natural transmission of *L. braziliensis* led to the use of peptides found in the saliva of the mosquito. The saliva peptides are injected in conjunction with the parasite during blood feeding. *Lutzomyia longipalpis* saliva has been shown to affect the course of the infection caused by *L. braziliensis* which has been associated with an elevated IL-4 production and a reduction in the IFN-γ/IL-4 ratio (145,146). It has been shown that mice inoculated with *L. braziliensis* together with sand fly saliva developed lesions with heavily parasitized macrophages, neutrophil and eosinophil infiltration, demonstrating that sand fly saliva changes the inflammatory response. These effects are attributed at least in part to maxadilan, a polypeptide that induces vasodilation and presents a range of immunomodulatory activities (147). It has been found that maxadilan can inhibit T-cell proliferation and delayed-type hypersensitivity, decrease TNF release by macrophages and increase LPS-induced IL-6 and IL-10 production,
both \textit{in vitro} and \textit{in vivo} (147). In addition, maxadilan inhibits the intracellular killing of \textit{Leishmania} by macrophages suggesting that the ability of the sand fly saliva to reduce nitrogen oxidation in response to IFN-\(\gamma\) may be responsible for the inhibitory effect of the saliva on the intracellular killing (148). In summary, maxadilan allows the parasite to establish a more long lasting infection (149,150).

Although a great number of studies have been trying to elucidate the biology of \textit{Leishmania} infections and have helped to understand many aspects of the immune response to intracellular pathogens, the immunopathogenesis of human leishmaniasis is still not fully understood (95).
CHAPTER III

1. The present study

Detection of new active molecules for treatment of leishmaniasis has received considerable attention over the past few decades (4,5,151,152). Evanta, a tree found in Bolivia, has been associated with traditional medicine in the treatment of leishmaniasis and has been selected among the most promising medicinal plants of Bolivia (24). Alkaloids purified from the plant Evanta, have demonstrated leishmanicidal effect \textit{in vitro} on various species of \textit{Leishmania} parasite, e.g. \textit{L. amazonensis, L. infantum} and \textit{L. donovani} (21,22). In \textit{in vivo} studies some of the alkaloids showed activity against \textit{Leishmania} infected mice (23). In the present study we hypothesized that the total alkaloid extract of Evanta (AEE) might have immunomodulatory activity.

Specifically we examined the effect of AEE on:

- Viability and proliferation of \textit{L. braziliensis} promastigotes.

- Activation of T-cell, including two different activation pathways: a) polyclonal and b) \textit{Leishmania}-specific activation.

- Infection caused by \textit{L. braziliensis} in mice.

- Maturation and function of DCs.

2. Materials and Methods

The materials and methods for these studies are described in the separate papers.
3. Results and discussion

3.1 Paper I

In Latin America leishmaniasis is a major public health problem, with CL, mostly caused by *L. braziliensis* in Bolivia, being the most frequent form of the disease (153,154).

Therapy of leishmaniasis is usually based on pentavalent antimonial drugs, but due to their toxicity and increased resistance new therapeutic alternatives are needed (35). Additionally, anti-leishmanial drugs are unaffordable in most of the affected countries and as there is no effective vaccine available yet, chemotherapy remains the only alternative. In this sense Evanta could have a great potential because of its leishmanicidal effects either used singly or in combination with conventional anti-leishmanial drugs.

Plant-derived drugs are mostly used by modern medicine in a way that is similar to their traditional use by native cultures, which is why we studied the effects of a total alkaloid extract of Evanta (AEE) rather than isolated molecules. To optimize our procedures we first determined the toxicity of AEE on eukaryotic cells *in vitro*. This first step allowed us to find the extract dose that would not affect the viability of the cells. We found that at 50 µg/ml AEE was already toxic, so lower doses were used in the following experiments with eukaryotic cells.

The second step was to analyze the effect of AEE on parasite growth. We found, as expected, that doses of 50 µg/ml and higher caused a total elimination of the parasite. However, at 25 µg/ml a strong effect (97 %) was still detected while at 10 µg/ml a 69 % inhibition of parasite
growth was observed. It is important to underline that at this dose (10 µg/ml) the viability of eukaryotic cells was practically not affected.

The most frequent alkaloid in AEE, 2Ph, could be an interesting candidate for the development of an alternative treatment used as a single compound. In order to find out if the isolated 2Ph was as efficient as AEE, we compared their effects on \textit{in vitro} culture of \textit{L. braziliensis}. We found that AEE had stronger inhibitory effect than 2Ph on the parasite growth, indicating possible additive anti-leishmanial effects among the alkaloids found in AEE.

Many studies have demonstrated the importance of the immune response on the control of \textit{Leishmania} infections. These studies have indicated a beneficial effect of a Th1 type of immune response to control the infection properly. Several mechanisms are triggered during T- and B-cell activation: proliferation/expansion, differentiation/maturation, production/secretion of active molecules (e.g. cytokines, chemokines, Igs) and memory formation. To examine this we investigated the effects of AEE on T and B cells. As a measure of T and B-cell activation we studied proliferation and production of cytokines and Igs. We also tested the direct effect of AEE on macrophages. Our results indicated that AEE was not directly stimulating B-cells, T-cells or macrophages.

Thereafter, we analyzed the effects of AEE on activated murine spleen cells and human PBMCs and observed that AEE affected proliferation of spleen cells at 25 µg/ml, while PBMCs were affected already at 10 µg/ml. 2Ph showed similar effect compared with AEE. The effect of AEE was shown to be specific and not due to an effect on the viability of the cells in contrast to what has been seen for curcumine (alkaloid molecule).
According to the well-known Th1/Th2 paradigm of *Leishmania* infection, IFN-γ is one of the most important cytokines for an effective immune response. We tested the effect of AEE on polyclonally induced IFN-γ production of murine spleen cells and human PBMCs. We used two different polyclonal T-cell activators, Concanavalin A (ConA) and monoclonal anti-CD3 antibody, to evaluate different activation pathways.

IFN-γ production by murine T cells was decreased by AEE in a dose dependent manner, regardless of the polyclonal activator used (ConA or anti-CD3) (P=0.0005 or 0.001, respectively). We also observed that the effect was more evident when the cells were pretreated with AEE and subsequently stimulated with ConA or anti-CD3 as compared with cells treated with AEE and simultaneously stimulated with polyclonal activators. IFN-γ production by ConA-activated human PBMCs was reduced at lower concentrations of AEE than was seen with murine spleen cells, which may due to the different cellular composition of murine spleen cells as compared with PBMCs.

Our results showed an unexpected effect of AEE on the immune response, parameters that, according to the literature, are decisive to control *Leishmania* infection and to eliminate the parasite: polarization to a Th1-type immune response with the production of IFN-γ, TNF and NO (102-104,106,118,121). Although the development of a Th1-type immune response is important in the control of leishmaniasis, many studies have shown that the maintenance of a chronic inflammatory response could potentially lead to pathological manifestations and tissue damage (126-132).
Our results suggest that AEE has direct leishmanicidal effect as well as inhibitory effects on T-cell activation, as measured by proliferation and IFN-γ production, and might contribute to the control of the chronic inflammatory reaction seen in *Leishmania* infection.

### 3.2 Paper II

In this paper we studied how AEE affected *Leishmania* specific reactivation of lymphocytes. More importantly, we analysed the capacity of AEE to control *Leishmania* infection in mice.

Our previous studies showed that AEE, in addition to its leishmanicidal activity on *L. braziliensis*, could reduce polyclonal induced cellular proliferation and IFN-γ production. In order to determine if AEE could similarly interfere with a *Leishmania*-specific activation of lymphocytes we examined whether AEE could affect the antigen specific reactivation of lymphocytes *in vitro* using different mechanisms of activation.

Therefore, mice were immunized with total *Leishmania* lysate (TLL), TLL-primed spleen cells were restimulated *in vitro* either with TLL or bone marrow derived macrophages infected with *L. braziliensis* (L-BMM).

We first tested the effect of AEE *in vitro* on spleen cells from TLL-immunized mice. We found that *Leishmania*-specific restimulation was affected by *in vitro* pre-treatment with AEE, similarly to that observed for polyclonally activated cells.

To test the effect of AEE *in vivo*, mice immunized with TLL were treated with AEE two weeks post infection and then the spleen cells were restimulated *in vitro*. We found that restimulation of spleen cells with TLL and L-BMM had different effect. In AEE-treated mice, restimulation with L-BMM showed a clear reduction in IFN-γ production, whereas IFN-γ
production after restimulation with TLL did not differ between treated and untreated mice. The reasons for the differences observed using these two ways of stimulation are not known but could be due to different antigenic epitopes being presented by the BMMs that has actively phagocytized and processed a microorganism (a more natural process) as compared with the splenic APCs (155).

We further showed that AEE considerably inhibited the production of IL-12 and TNF while no effects were seen on other cytokines, e.g. IL-10.

Pentavalent antimonial has remained the first choice to treat leishmaniasis for decades, so it was of interest to investigate the effect of meglumine antimoniate (SbV) in our models. We found that SbV had no effect on antigen-specific reactivation of cells, and that the combined treatment AEE/SbV did not alter the effect seen using AEE alone. The results indicated that the combination of AEE plus SbV did not synergize or antagonize the effects on the Leishmania-specific reactivation of lymphocytes in vitro.

An important question to address was whether the administration of AEE might be able to control an in vivo infection. We tested the effect of AEE on BALB/c mice infected with L. braziliensis. Similarly to humans, BALB/c mice are relatively resistant to infection with Leishmania. It has been reported that in L. braziliensis-infected BALB/c mice there is an intense inflammatory reaction due to T-cell sensitization and enhanced production of IFN-γ and TNF (the major cytokines involved in the pathogenesis) (86,134). The development L. braziliensis skin lesions is also accompanied by the expression of a broad spectrum of chemokines that are known to attract neutrophils, monocytes/macrophages, NK cells, and CD4+ and CD8+ T-cells (156).
When BALB/c mice were injected with *L. braziliensis*, a transient inflammation that resolved in few days was obtained. Therefore, we co-injected maxadilan with the parasite to resemble a natural infection by means of a sand fly bite. It has been reported that the salivary gland polypeptide maxadilan enhances *Leishmania* infections.

Inoculation of *L. braziliensis* in combination with maxadilan modified the inflammatory response induced by the infection in BALB/c mice, increased the infectivity and exacerbated the infection (both lesion size and parasite survival) as shown previously (145,146). Other studies have reported that the observed effects of maxadilan are modulation of the host immune response, e.g. inhibited TNF release and increased IL-6 production, which leads to extensive accumulations of heavily parasitized epithelioid macrophages, with persistent neutrophilia and eosinophilia in the lesions (147-151).

We showed that i.p. treatment with AEE, SbV and AEE/SbV differently affected the kinetics of infection. AEE treated mice controlled efficiently the inflammation during the first weeks post infection (p.i.) compared with PBS-treated mice. In contrast, SbV-treated mice did not seem to control inflammation as efficiently as AEE treated mice (p<0.1).

The parasite load, was also affected differently, with. AEE-treated mice being able to control the parasite load more efficiently compared with PBS treated mice. When we compared the effect of AEE with SbV we found that SbV could control the parasite load more efficiently.

Apparently, AEE could control the inflammatory reaction more efficiently than the parasite load but SbV reduced more efficiently the parasite load, but it could not control the inflammation in the same effective way as AEE during the first weeks p.i.
When we combined the treatment with AEE and SbV, the overall inflammatory process seemed to be controlled more efficiently; both the footpad thickness and the parasite load compared with when AEE and SbV were used separately. Thus, it looks like the inflammatory process is caused both by the parasite itself and also by the immune response developed in response to the infection. SbV controlled the parasite burden, while AEE controlled the parasite burden as well as the inflammatory reaction. This is in agreement with other studies that have shown the beneficial effects of combining the use of SbV with other drugs (129,130).

In summary, the data presented here suggested that AEE, in addition to its leishmanicidal effect, also could be considered as an immunomodulatory agent. We can conclude that the effect on the inflammatory reaction seen in mice treated with AEE most likely is due to the capacity of AEE to suppress the production of proinflammatory cytokines. Moreover, the combined use of AEE and SbV may be a more efficient alternative for the treatment of leishmaniasis.

3.3 Paper III

DCs are considered the bridge between innate and adaptive immunity, they can stimulate the adaptive immune response, both humoral and cellular. DCs play an important role in T-cell stimulation and polarization of naïve T-cells towards a Th1, Th2, Th17 or regulatory phenotype. The ability of DCs to induce Th1 or Th2 immunity may depend on the characteristics of the microenvironment present when DCs first interact with the antigen (157,158). In leishmaniasis, the interactions between the parasites and DCs are complex and involve contradictory functions that can stimulate or suppress T-cell responses, leading to the
control of infection or progression of disease (159,160). Thus, since DCs are pivotal cells in eliciting an immune response, it was of importance to find out whether AEE might induce or suppress the maturation/activation of DCs and/or affect their functions.

We first examined the effects of AEE and 2Ph on DCs maturation by examining the expression of maturation markers and production of cytokines. We found that neither AEE nor 2Ph altered the expression surface activation markers on the DCs, but both AEE and 2Ph were able to reduce the production of IL-12p40, an important Th1 signature cytokine (78), and IL-23, a Th17 promoting cytokine (110,161). On the other hand IL-10 and IL-6 were not significantly reduced by AEE or 2Ph treatments. These results are consistent with our previous results and indicate that AEE and 2Ph may have an anti-inflammatory effect.

Therefore, we analyzed the allostimulatory capacity of AEE- or 2Ph-treated DCs and we found that AEE or 2Ph-treated DCs did not affect the expression of various T-cell activation markers, such as CD54, CD62L, CD69 and CD40L on co-cultured allogeneic CD4⁺ T cells. This shows that CD4⁺ T-cells were fully activated in the presence of DCs, regardless of the treatment DCs received during their stimulation. In contrast, the allogeneic CD4⁺ T cells secreted lower levels of IFN-γ following co-culture with the AEE-DCs and 2Ph-DCs. As IL-12 is the main inducer of Th1 polarization, the reduction in IFN-γ secretion by the CD4⁺ T-cells observed in this study is most likely resulting from a reduced ability of the DCs stimulated in the presence of AEE or 2Ph to secrete IL-12.

DCs treated with AEE or 2Ph secreted reduced levels of IL-6 and IL-23, both cytokines that affect Th17 polarization and hence IL-17 secretion of Th cells (162). However, the levels of IL-17 secretion by the allogeneic CD4⁺ T-cells were not significantly affected by co-culture
with the AEE-DCs or 2Ph-DCs. Th17 differentiation is affected by other cytokines which may be counteracting the effect of IL-6 and IL-23, resulting in unchanged levels of IL-17.

The fact that IFN-γ, IL-12p40, IL-6 and IL-23 were significantly decreased following treatment with AEE or 2Ph suggests an effect of the drugs in the control of chronic inflammatory reaction.

In addition, several studies have reported that extracts from plants are able to affect DC stimulation (163-167). Our results suggest that AEE and 2Ph interfere with LPS-induced stimulation of DCs, possible affecting any of a number of signaling pathways that are involved in LPS-induced DC activation and maturation, including MAP kinase, PI3 kinase, P38 SAP kinase, and NF-κB pathways (168,169). Whether the NF-κB signaling pathway or other pathways are affected in this case remains to be determined and will be the next part of this project.

In conclusion, our results showed that AEE and 2Ph affect the LPS-induced maturation of DCs and their ability to stimulate allogeneic CD4⁺ T-cells, indicating an immunomodulatory, predominantly anti-inflammatory, capacity, in addition to their leishmanicidal activity.
4. Overall conclusions

Treatment of leishmaniasis remains controversial and the need to develop new alternatives to the conventional used drugs is clear. Generally, the treatment of leishmaniasis has been based on the use of pentavalent antimonials but problems of toxicity and developing resistance have been reported. In this scenario many investigations started to look into the plant kingdom that could offer an unlimited source of new drugs, which in the past have demonstrated to be effective.

In contrast to the idea of using drugs to treat infectious diseases and only considering the effects on the microorganism, without taking into account the host, new interesting approaches are being developed in which the host also provides an environment that is dynamic and plays an important role in the outcome of the disease.

We studied the effects of a total alkaloid extract obtained from the plant known as Evanta (AEE) and postulated that due to its capacity to reduce cellular proliferation and production of the proinflammatory cytokines IFN-γ, IL-12 and, TNF (important mediators of inflammation) it might control the inflammatory response. This observation correlated with the effects seen on an in vivo infection with L. braziliensis, where AEE controlled inflammation at the site of the infection more efficiently than SbV. We could also show that AEE reduced the production of cytokines in mature DCs as well as their capacity to activate T lymphocytes. The most relevant results showed the reduction of proinflammatory cytokines IL-12p40 and IL-23 of AEE-treated and 2Ph-treated DCs. We also found that activation of DCs in the presence of AEE or 2Ph affected the capacity of DCs to stimulate CD4+ T cells observed by a reduction in
the production of IFN-γ. All these results, in line with our previous findings, suggest that AEE may be useful in controlling inflammatory responses at many levels (Fig. 12).

Fig. 12. Suggested model of the effects of AEE on Leishmania infection. AEE inhibits the parasite growth (1). At the same time AEE affects the production of IFN-γ by T-cells (2). AEE also affects the stimulation of DCs (3), causing a decrease in various cytokines (IL-12p40, IL-23) (4), which in turn affects the secretion of IFN-γ, IL-6 and also IL-10. The decrease in the production of proinflammatory cytokines produces indirectly the decrease in the secretion of acute phase proteins and chemotaxis of neutrophils (5), controlling inflammation. In this way AEE might control the parasite burden and the inflammatory response, in summary the overall infection process.

The analysis of the activity of the purified molecule 2Ph showed that due to the weaker effect on the parasite (albeit its similar effects on the immune response), it would not be beneficial to use it as compared with AEE. This is in line with our main suggestion that natural products should be used as close to its traditional use as possible.
Although we could not find any effect of SbV on the immune responses studied, we found that *in vivo* combined AEE/SbV treatment was extremely effective in the control of both inflammation and parasite load. This suggests that combined treatment might be an interesting approach that must be investigated further in the future. Using SbV at lower concentrations when used in combination with AEE might also be the solution to problems of toxicity as well as resistance.

5. **Future investigation**

We consider that two important questions need to be addressed in the future, first is to find out which signaling activation pathways are affected by AEE. Similar alkaloids have demonstrated to have immunomodulatory effects in the control of inflammatory reactions and have shown to affect different signaling activation pathways. Some of these studies pointed out that the activity on some signalling molecules and transcription factors such as MAP kinase phosphorylation and activation of NF-κB, Stat and others, all involved in the production of proinflammatory cytokines, were blocked (158-160). Whether AEE can also affect these signaling pathways remains to be determined.

Secondly, it is very important to find out if the complementary *in vivo* effects seen for the combined treatment could lead to a reduction in the dose of SbV which would mean less toxicity/costs for the patients. This would initially be investigated by combined treatment in mice infected with a resistant strain of the parasite. Answering these questions will demonstrate the real potential of the AEE in the treatment of leishmaniasis.
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