Review of Literature
Leishmaniasis.

Leishmaniasis is a disease caused by protozoan parasites that belong to the genus ‘*Leishmania*’ and is transmitted by the bite of certain species of sand fly (subfamily Phlebotominae). Two genera transmit *Leishmania* to humans: ‘*Lutzomyia*’ in the New World and ‘*Phlebotomus*’ in the Old World. Leishmaniasis is a multiform parasitic disease that results from an infection of one of the many species of *Leishmania*. Most forms of the disease are transmissible only from animals (zoonotic), but some can be spread between humans (anthroponotic). Human infection is caused by about 21 of 30 species that infect mammals. These include the *L. donovani* complex with three species (*L. donovani, L. infantum,* and *L. chagasi*); the *L. mexicana* complex with 3 main species (*L. mexicana, L. amazonensis,* and *L. venezuelensis*); *L. tropica, L. major,* *L. aethiopica,* and the subgenus *Viannia* with four main species (*L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) panamensis,* and *L. (V.) peruviana*). The different species are morphologically indistinguishable, but they can be differentiated by isoenzyme analysis, DNA sequence analysis, or monoclonal antibodies.

**Historical perspective**

**Parasite causing quinine resistant fever: Enemy identified**

Leishmaniasis has a long history dating back as far as the first century AD and has been the cause of great suffering and death for hundreds of years. As early as this period, pre-Incan pottery from Ecuador and Peru dating back to first century AD displayed depictions of skin lesions and facial deformities that are typical of cutaneous and mucocutaneous leishmaniasis. Incan text from the 15th and 16th century and accounts from Spanish conquistadors noted the presence of skin lesions on agricultural workers returning from the Andes. These ulcers resembled leprosy lesions and were labeled, “white leprosy,” “Andean sickness,” or “valley sickness.” In Africa and India, reports in the mid-18th century describe the disease now known as visceral leishmaniasis, as “kal-azar” or “black fever.” In 1756, Alexander Russell made an important advance in the discovery of Leishmaniasis after examining a Turkish patient. According to Russell, "After it is cicatrised, it leaves an ugly scar, which remains through life, and for many months has a livid colour. When they are not irritated, they seldom give much pain." Russell called this disease, "Aleppo boil."

Around 1858, when British ruled India, an epidemic of quinine resistant fever received serious attention of Government of Bengal. Then 1860 onwards, the Government in Calcutta received many reports of such endemic fever from Burwan, which turned some areas of Burwan and Hoogly into a “valley of death” surprisingly this fever continued to spread and in 1869 further reports of this endemic fever reported from Assam. This Assam endemic proceeded eastwards along the line of communication through the district of Goalpara, Kamrup, Nowgaun and Darrong where it developed into a full bloom epidemic between 1890 and 1900. In 1898 Sir Ronald Ross was appointed by Government to initiate an investigation about the nature of the disease. In 1903, William Boog Leishman, an English military
surgeon and Charles Donovan separately reported the presence of an organism, yet identified, in the smear preparation from spleen pulp of dead persons who died from this endemic fever. The disease became known as Leishmaniasis after William Leishman, a Glaswegian doctor serving with the British Army in India, developed one of the earliest stains of Leishmania in 1901. In Dum Dum, a town near Calcutta, Leishman discovered ovoid bodies in the spleen of a British soldier who was experiencing bouts of fever, anemia, muscular atrophy and swelling of the spleen. Leishman described this illness as “dum dum fever” and published his findings in 1903. Charles Donovan also recognized these symptoms in other kala-azar patients and published his discovery a few weeks after Leishman. Ross also did the same observation like them but, identified them as a new parasite, causing the disease that Donovan described as Kala-azar, disregarding with Leishman’s theory that the bodies found in the spleen pulp were degenerated Trypanosomes (Donovan, 1903; Leishman, 1903). In May issues of British Medical Journal, 1903 Ross proposed “As I suppose, they are found to belong to a new genus, it would be fair to give the name Leishmania to that genus. In that event the full name would be Leishmania donovani, instead of the name Piroplasma donovani given by Dr. Laveran to these organisms” (Ross, 1903). After examining the parasite using Leishman's stain, these amastigotes were known as Leishman-Donovan bodies and officially, this species became known as, *L. Donovani*. By linking this protozoan with kala-azar, Leishman and Donovan discovered the genus, *Leishmania*. (Fig 1, 2)

**Figure 1:** William Leishman and Charles Donovan. By linking the protozoan parasite with kala-azar, they discovered the genus, *Leishmania*.

**Figure 2:** Sir Ronald Ross. Named the protozoan parasite “*Leishmania donovani*” instead of “*Piroplasma donovani*”

Once the enemy identified as the causative agent for Kala-azar, news of its existence came in from other endemic areas like Sudan, Egypt etc. Leonard Rogers in 1904 discovered that like Trypanosomes, *Leishmania* also multiplied in a blood specimen kept at 27°C and nearly 40 years
after the discovery of parasite, Sandfly was identified as a vector of Kala-azar (Swaminath et al., 1942). So, finally debate on the nature of parasite is recorded (Gibson, 1983).

**Clinical Spectrum**

*Visceral Leishmaniasis*

Visceral leishmaniasis is typically caused by *L. donovani* in India and Africa and by two closely related species, *L. infantum* in the Mediterranean littoral and *L. chagasi* in Latin America (Lainson R and Shaw JJ, 1987). On occasion, *Leishmania* species that are predominantly associated with cutaneous disease, such as *L. mexicana* and *L. major*, are isolated from patients with classic visceral leishmaniasis. In addition, a small group of American troops who were infected with *L. tropica* in Saudi Arabia developed a disseminated viscerotropic syndrome that included some of the manifestations of classic visceral leishmaniasis (Magill AJ et al., 1993).

Visceral leishmaniasis due to *L. donovani* is a major problem in eastern India, particularly in Assam and Bihar states, and in Bangladesh (Addy M and Nandy A, 1992). *L. donovani* is endemic in Ethiopia and the Sudan. A large epidemic of visceral leishmaniasis occurred recently among refugees in the Sudan (Zijlstra EE et al., 1994). Visceral leishmaniasis due to *L. infantum* occurs sporadically in children and immunocompromised persons in southern Europe, the Middle East, and North Africa. An increasing number of cases of visceral leishmaniasis have been diagnosed in adults with concurrent HIV infection in Spain, southern France, and Italy.

Visceral leishmaniasis in Brazil, Venezuela, Colombia, and other areas of Latin America typically occurs in rural areas (Evans TG et al., 1992), but large urban outbreaks have recently been reported in northeastern Brazil (Jeronimo SMB et al., 1994). The majority of cases occur in children <10 years of age. American visceral leishmaniasis is usually caused by *L. chagasi*. In addition, *L. mexicana* has been isolated from a few patients with classic visceral leishmaniasis. Visceral leishmaniasis is also endemic in areas of eastern and northeastern China, but the number of cases appears to be small.

*Cutaneous Leishmaniasis*

Cutaneous leishmaniasis is typically a sporadic disease in the areas where it is endemic, but it occasionally follows an epidemic pattern, particularly when large groups of susceptible persons are exposed during military or construction operations or during settlement in the area. Cutaneous leishmaniasis is also diagnosed periodically in Americans or Europeans who have traveled or lived in areas where it is endemic.

Most cases of cutaneous leishmaniasis in the Mediterranean basin, the Middle East, southern Asia, India, and Africa are caused by three *Leishmania* species: *L. major*, *L. tropica*, and *L. aethiopica* (Lainson R and Shaw JJ, 1987). *L. major* is endemic in rural desert areas in central Asia, the Middle East, and North Africa. It causes cutaneous lesions that tend to be exudative or "wet" and large. Infection due to *L. major* has also been reported from sub-Saharan West Africa. The epidemiology there is less clear. *L. tropica* is endemic in urban areas of the Middle East, the Mediterranean littoral, India, Pakistan, and central Asia. The lesions
are usually "dry," with a central crust. *L. aethiopica* is endemic in the Ethiopian highlands and Kenya, where it causes simple cutaneous leishmaniasis as well as diffuse cutaneous leishmaniasis. In the New World, cutaneous leishmaniasis is caused by *L. mexicana*, *L. amazonensis*, *L. braziliensis*, *L. panamensis*, *L. guyanensis*, *L. peruviana*, and several other species including *L. chagasi* (a species more commonly associated with visceral leishmaniasis) (Lainson and Shaw, 1987; Grimaldi Jr et al., 1989). Humans become infected when they work or live in forested areas where the organisms are endemic or when they enter these areas for recreational or military activities. *L. mexicana* is found in scattered areas throughout Latin America, extending from Argentina to Texas, where a few cases of autochthonous transmission have been reported (Shaw et al., 1976). *L. mexicana* tends to produce small chronic ulcers on the face, ears, or other exposed areas. On rare occasions this species is isolated from patients with diffuse cutaneous leishmaniasis. *L. amazonensis* produces cutaneous lesions and, occasionally, diffuse cutaneous leishmaniasis. It is found in jungle areas in the Amazon basin. *L. braziliensis* is endemic in many areas of Central America and South America. It is an important cause of uncomplicated cutaneous leishmaniasis, and it produces mucosal disease in a subset of persons who become infected. *L. panamensis*, *L. guyanensis*, *L. peruviana*, and several other *Leishmania* species are found in focal geographic areas. *L. panamensis* has been an important problem in jungle areas of Panama.

**Mucocutaneous Leishmaniasis**

MCL generally refers to the South American disease called Espundia (Escomel E. 1911). It is primarily caused by *Leishmania braziliensis braziliensis*, and is a major health problem in Central America. 90% of MCL occur in Bolivia, the Brazilian state of Bahia and Peru. MCL evolves in two stages: a primary cutaneous lesion eventually followed by secondary mucosal involvement. The disease is characterized by a metastatic spread of infected macrophages to pharyngeal, buccal or nasal tissues by lymphatic or hematogenous dissemination. The evolution leads to spontaneous cure, the time of which varies according to the country and case. Once primary lesions are cured, leishmanial infections may remain dormant for a very long time. In the absence of effective early treatment, MCL can lead to a serious problem like permanent face mutilation for the patients.

**Post Kala-azar Dermal Leishmaniasis (PKDL)**

It is a type of non-ulcerative cutaneous lesion prevalent in endemic areas of Kala-azar in India chiefly in Bengal, less so in Madras and Assam. It develops in about 20% of Kala-azar patients generally one or two years after the treatment of the acute disease (Thakur and Kumar, 1992). The usual sites of distribution of these maculae are the face, upper trunk, thighs, forearms and legs. The nodules are soft, painless granulomatous growth and might persist for several years after parasitological cure; spontaneous cure does occur (Zilstra et al., 1995) but courses of treatment may be necessary. This reappearance of parasites after cure is a suggestion of the parasite survival in the host in a dormant stage. However, there
are reports of PKDL occurrence in patients with no previous history of leishmaniasis. The PKDL patients are of considerable epidemiological importance (Napier, 1946). (Fig 3)

**Figure 3: Clinical spectrum of Leishmaniasis.** Patients infected with different species of *Leishmania* show various morphological manifestations such as (A) Cutaneous, (B) Mucocutaneous, (C) Visceral and (D) Post Kala-azar Dermal Leishmaniasis (PKDL)

**General biology of Leishmania parasite:**

**Parasite's Identity:**

The genus Leishmania is divided into two subgenera- Leishmania and Viannia depending on the distribution of parasites in the gut of sandfly vector. In the subgenera Leishmania, the promastigotes develop in the midgut and foregut of the insect (suprapylaria section), whereas in the subgenera Viannia, parasites are restricted to the hindgut (Peripylaria Section) of sandfly. The parasite exists as two forms- one is flagellate extracellular promastigote in the sandfly vector and another is aflagellate intracellular amastigotes within mononuclear phagocytes of the vertebrate host. Species of the subgenus Leishmania
belong to the *Leishmania donovani* complex, *L. tropica* complex, *L. mexicana* complex. On the other hand *L. braziliensis* complex belongs to subgenera Viannia. These two subgenera belong to kingdom Protista. In brief systematic position of *Leishmania* is as follows (Levine *et al*., 1980):

Kingdom : Protista (Hakel, 1886)

Subkingdom: Protozoa (Goldfuss, 1817)

Phylum: Sarcomastigiphora (Honigberg and Balamua, 1963)

Subphylum: Mastigophora (Deising, 1866)

Class: Zoomastigophora (Calkins, 1909)

Order: Kintoplastida (Honigberg, 1963)

Suborder: Trypanosomatina (Kent, 1880)

Family: Trypanosomatidae (Dolfein, 1901)

Genus: *Leishmania* (Ross, 1903)

*Morphology:*

*Leishmania* exists in two stages:

**Amastigote stage:** The amastigote or aflagellar stage occurs in vertebrate hosts with the parasite residing in the cells of reticulo-endothelial system of man, dog and hamster. The parasite is oval or round shaped 2-4 µm long along the longitudinal axis with little or low motility. Although the cellular organelles are similar to the promastigote form, in place of flagellum, there is a delicate filament extending from the basal body to the margin of the body, which represents the root of the flagellum in the amastigote.

**Promastigote stage:** The promastigote is the extra cellular form of the parasite only detected in cultures or insect vectors. Fully developed promastigotes have long, slender spindle-shaped body measuring 15-20 µm in length.

**Figure 4:** The amastigote and promastigote form of *Leishmania* spp.

The nucleus is situated centrally and the kinetoplast lies transversely near the anterior end. The flagellum may be of the
same length as the body or even longer, projection from the front.

**The vector:**

The leishmaniases are caused by 20 species pathogenic for humans belonging to the genus *Leishmania*, a protozoa transmitted by the bite of a tiny 2 to 3 millimetre-long insect vector, the *phlebotomine sandfly*.

Of 500 known phlebotomine species, only some 30 of them have been positively identified as vectors of the disease. Only the female sandfly transmits the protozoa, infecting itself with the *Leishmania* parasites contained in the blood it sucks from its human or mammalian host in order to obtain the protein necessary to develop its eggs. During a period of 4 to 25 days, the parasite continues its development inside the sandfly where it undergoes a major transformation.

*Figure 5: Sandfly vector of Leishmania spp.*

When the now infectious female sandfly feeds on a fresh source of blood, its painful sting inoculates its new victim with the parasite, and the transmission cycle is completed. The insect vector of leishmaniasis, the phlebotomine sandfly, is found throughout the world's inter-tropical and temperate regions. The female sandfly lays its eggs in the burrows of certain rodents, in the bark of old trees, in ruined buildings, in cracks in house walls, in animal shelters and in household rubbish, as it is in such environments that the larvae will find the organic matter, heat and humidity which are necessary for their development.

In its search for blood (usually in the evening and at night), the female sandfly covers a radius of a few to several hundred metres around its habitat.

**General Life Cycle**

*Leishmania* being digenetic parasites circulate between two hosts-human and sandfly. Infection begins when a sand fly carrying metacyclic promastigotes in their anterior midgut and thorax area bites and injects parasites into the host along with the dipteran’s salivary secretions while they are taking the blood meal. They are then engulfed by macrophages and either remains there, such as in those species that cause CL, or are carried by the macrophages to the mucocutaneous junctions or the reticuloendothelial tissues. Inside the macrophages promastigotes transform rapidly into the amastigote stage (non-flagellated) and proceeds to replicate. Amastigotes from infected macrophages reinfect new macrophages. Now, macrophages in blood that contains amastigotes are taken up by the vector, even from individuals
suffering from visceral forms of leishmaniasis. The parasite undergoes a complex series of developmental changes inside the gut tract of the sand fly, which are discussed as follows. (Fig 6)

**Development in the Sand fly Host:** Dipteran sandfly of the subfamily Phlebotominae are the only vectors of the various *Leishmania* species, with the genus *Phlebotomus* hosting the Old World species and the species of *Lutzomyia* for the New World (Shaw et al., 1987; Killick-Kendrick, 1990) Shortly, the parasite differentiates from a dividing procyclic promastigote stage that attached to the midgut wall, to a non-attach to the midgut and migrates to the mouth parts (Sacks and Perkins, 1985) reported, for example, that clones of *L. (L.) amazonensis* in *L. longipalpis* were not able to infect BALB/c mice when inoculation was carried out 3 days after the insect’s infection, but the inoculums became progressively infective from the 4th until the 7th day of development in the sand fly midgut. Coincidentally, the maximum infective capacity occurs at the time of a new blood meal. When the haematophagous insect bites a vertebrate host it introduces its mouthparts into the skin, lacerates blood vessels causing hemorrhages and then feeds in the haemorrhagic pool formed (Ribeiro, 1987). At this site of bite, a battle takes place between the host resistance and the insect’s strategies to obtain blood. At the site of inoculation, the natural defense mechanisms will be activated by host due to the presence, of agents like complement system, thrombin, kinins, platelets, natural antibodies, phagocytes, etc. and by the vector’s side, the pharmacological agents...
of the saliva are maxadilan, adenosine, interleukin 2 (IL-2) binding factor, apyrase, prostaglandins, etc. (Stiffel et al, 1987; Gillespie et al, 2000).

After sand fly bite a haemorrhagic pool is formed at the site of insect bite, where parasite growth is mainly allowed by a less abundant phagocytic cell – the monocyte, instead of most abundant and short lived neutrophils because they play a major role in parasite elimination instead of its hosting (Chang, 1981). However, neutrophils may also present antigens via class II MHC in the presence of GM-CSF (Gosselin et al., 1993) and secretes a Th1 cytokine IL-12 and a pleiotropic cytokine TGF-β. Thus, platelets might have important host-protective agents in Leishmaniasis. Immature DCs being more endocytic than mature DCs might uptake apoptotic infected neutrophils. However, platelets were described to interact directly with monocytes by inducing monocytes to secrete chemokine MCP-1 and IL-8 and thus facilitate macrophage infection.

Events of Transformation from Amastigote to Promastigote: Although it’s worth remembering that sandflies are relatively short lived, more so the phase of *Leishmania* life cycle in female sand flies is of shorter duration than in mammalian host. That’s why amastigotes can to some extent afford to “sit & wait” policy in their mammalian host and can counteract the immune response. This is not true of promastigotes in their sand fly hosts and therefore, much of the interesting developmental biology occurs in the vector phase of life cycle. There are about 5 developmental forms recognized during development in the subgenus *Leishmania* (*Leishmania*) namely procyclic promastigotes, nectomonad promastigotes, leptomonad promastigotes, haptomonad promastigotes and metacyclic promastigotes. The first major developmental event in sandfly is the transformation of amastigotes to procyclic promastigotes followed by their multiplication in the posterior midgut in the blood meal that is itself encased in a peritrophic matrix (PM). The PM is a lattice of chitin fibrils, proteins and glycoproteins secreted by the midgut epithelium in response to a bloodmeal in hematophagous insects (Shao et al., 2001; Pimenta et al., 1997). Thus PM offered some protection to *L. major* during this transformation by limiting trypsin exposure by midgut epithelium. Transforming amastigotes of *L. mexicana* experienced some mortality in *Lu. longipulpis* (Rogers, 2002). Procyclic promastigotes are short ellipsoid dividing cells, 6-8μm in length with weakly motile short flagellum. The second major developmental event 2-3 days after blood feeding is the elongation of procyclic forms to distinctive non dividing nectomonad promastigotes of 12-20μm body length. These forms are strongly motile and accumulated in the anterior part of the PM (Rogers, 2002). To become properly established the parasites escape from PM and attach them to the midgut epithelium and those that escape faster produce higher infection rates (Ciháková and Volf, 1997). Thus PM whilst initially providing some protection to the parasite is also a barrier that must be navigated (Pimenta et al., 1997). As PM breaks down since, flies continue to take sugar meals over the several days. Infection by nectomonad promastigotes is achieved by two means. One, some of the nectomonad forms are attached to the midgut epithelium by inserting their flagella between microvilli (Bates and Rogers, 2004) thus allows them to produce mature infections in a particular species of sandfly (Sacks, 2001). Note that the
nectomonad and haptomonad mechanisms and sites of attachments are different. It is thought that leptomonads are derived from a nonattached subpopulation of nectomonad promastigotes that reach the barrier of the stomodeal valve. The main function of leptomonads is in the production of promastigote secretary gel (PSG), which plays an important role in transmission of infection.

Global Distribution

Leishmaniasis is found in parts of about 88 countries. Approximately 350 million people live in these areas. Most of the affected countries are in the tropics and subtropics. The settings in which leishmaniasis is found range from rain forests in Central and South America to deserts in West Asia. More than 90% of the world's cases of visceral leishmaniasis are in India, Bangladesh, Nepal, Sudan, and Brazil. Leishmaniasis is found in some parts of the following areas:

- In Mexico, Central America, and South America—from northern Argentina to Texas (not in Uruguay, Chile, or Canada)
- Southern Europe (leishmaniasis is not common in travelers to southern Europe)
- Asia (not Southeast Asia)
- The Middle East
- Africa (particularly East and North Africa, with some cases elsewhere)

Leishmaniasis is not found in Australia or Oceania (that is, islands in the Pacific, including Melanesia, Micronesia, and Polynesia).

Figure 7: Geographical distribution of visceral leishmaniasis in the Old and New world

Drug resistance
Pentavalent antimonial drugs were used worldwide for the treatment of VL and CL for over six decades with little evidence of resistance. Although the selection of resistant *Leishmania* has long been a part of laboratory studies, it is only in the past 15 years that acquired resistance has become a clinical threat. In most parts of the world, over 95% of previously untreated patients with VL respond to pentavalent antimonials, the recommended first-line treatment. However, the region endemic for VL in North Bihar, India, has the unique distinction of being the only region in the world where widespread primary failure to Sb(V) has been reported. Even in this geographical region a variation in Sb(V) sensitivity occurs with significant drug resistance at the epicenter of the epidemic and a high level of sensitivity only 200 miles away (Sundar et al., 2000). This resistance is so far unique to *L. donovani*; all isolates from a large number of refractory as well as responding patients in India were identified as this species (Thakur et al., 2001). Until the late 1970s, a small daily dose (10 mg/kg; 600 mg maximum) for short duration (6 to 10 day) was considered adequate, when unconfirmed reports suggested a 30% treatment failure with this regimen from four districts most severely affected, Muzaffarpur, Samastipur, Vaishali, and Sitamarhi (Peters, 1981). Following this, an expert committee revised recommendations to use Sb(V) in two 10-day courses with an interval of 10 days and a significant improvement in cure rates (99%) was observed (Aikat et al., 1979). However, only a few years later, another study noted 86% cure rates with 20 days of continuous treatment with this regimen (Thakur et al., 1984). In 1984, a World Health Organization (WHO) Expert Committee recommended that Sb(V) should be used in doses of 20 mg/kg/day up to a maximum of 850 mg for 20 days, with a repeat of the same regimen for 20 days in cases of treatment failure. Four years later, Thakur et al. evaluated the WHO recommendations and reported that 20 days of treatment with 20 mg/kg/day (maximum 850 mg) cured only 81% of patients, although with an extension of the treatment for 40 days, 97% of patients could be cured (Thakur et al., 1988). Three years later, the same group noted a further decline in cure rate to 71% after 20 days of treatment, and recommended extended duration of treatment in non-responders (Thakur et al., 1991). Jha et al. (Jha et al., 1992) found that extending the therapy until 30 days could cure only 64% of patients in a hyperendemic district of Bihar. From these findings it became clear that Sb(V) refractoriness was increasing although the reports came from studies that were not strictly controlled. In two following studies carried out under strictly supervised treatment schedules, it was observed that only about one-third of all VL patients could be cured with the currently prevailing regimen (Sundar et al., 2000). Figure 8: Map of Bihar State, India, showing distribution of resistance to pentavalent antimonials in kala-azar-endemic areas. (Croft et al., 2006)
The incidence of primary unresponsiveness was 52%, whereas 8% of patients relapsed. During the same period only 2% of patients from the neighboring state of (Eastern) Uttar Pradesh failed treatment (Sundar et al., 2000). These studies confirmed that a high level of Sb(V) unresponsiveness exists in Bihar, though the drug continues to be effective in surrounding areas.

There are reports of antimony resistance spreading to the Terai regions of Nepal, especially from the district adjoining hyperendemic areas of Bihar, where up to 24% of patients seem to be unresponsive, though in eastern Nepal a 90% cure rate has been reported (Rijal et al., 2003). The reason for the emergence of resistance is widespread misuse of the drug. Sb(V) is freely available in India, and is easily accessible over the counter. Most patients (73%) first consult unqualified medical practitioners, who might not use the drug appropriately (Sundar et al., 1994). It has been a common practice to start with a small dose and gradually increase the dose over a week. Drug-free intervals are given with the belief that they will prevent renal toxicity. On many occasions the daily dose of drug is split into two injections, to be given twice daily. These practices presumably expose the parasites to drug pressure, leading to progressive tolerance of the parasite to Sb(V). It has been observed that only a minority of patients (26%) were treated according to prescribed guidelines: irregular use and incomplete treatments were a common occurrence. These facts point to the mishandling of antileishmanial drugs in Bihar as a significant contributor to the development of drug resistance. (Fig. 9)

Figure 9: Proposed mechanisms of antimony action and resistance in *Leishmania* spp. Levels of ornithine decarboxylase (ODC), γ-glutamylcysteine synthetase (GCS), and an intracellular P-glycoprotein (PgpA) are elevated in some laboratory-derived resistant lines (thick lines), whereas decreased Sb reductase is observed in others. Dotted lines indicate nonenzymatic steps.
implicated in resistance. The red arrow indicates inhibition of trypanothione reductase and other targets. Uptake of Sb(III) is mediated via an aquaglycoporin (AQP1). (Decuypere et al., 2012)

The factors involved in drug resistance:

Two important considerations in an analysis of the importance of a drug resistance problem are the ease with which resistant individual microbes can be selected by a particular drug, and the potential spread of resistance in a population and, hence, the importance to public health. First, the spread of drug-resistant genotypes through a population of microorganisms is primarily governed by certain measurable parameters: (i) the volume (dose and frequency) of drug used, (ii) the probability that a drug-sensitive infection becomes resistant upon infection, (iii) the duration of infection in individuals, (iv) the fitness costs (division rate and transmissibility) for the pathogen incurred by being resistant in the absence of drugs, and (v) the degree to which compensatory mechanisms develop that offset these fitness costs (Bjorkman et al., 2000). In zoonotic diseases, such as most cases of cutaneous leishmaniasis and most L. infantum/L. chagasi visceral leishmaniasis, the parasite is primarily an infection of a feral or domestic mammalian host and only occasionally infects humans. In zoonoses, the time that a parasite population is exposed to a drug is insignificant unless the mammalian reservoir host is also treated. This could be of great importance if control methods for canine leishmaniasis included extensive treatment of the domestic canine host. Treatment of canines has led to a reduction in parasite drug sensitivity as determined in assays on L. infantum isolates (Gramiccia et al., 1992). Current knowledge of the epidemiology and transmission of leishmaniasis suggests that the spread of acquired drug resistance is not a factor to be considered in cutaneous leishmaniasis except in anthroponotic foci of L. tropica. However, it is a factor that requires consideration in L. infantum leishmaniasis, where transmission is from human to human by needle (Cruz et al., 2002) and a factor of major importance in anthroponotic disease foci such as L. donovani in Bihar State, India (Sundar et al., 2001). This does not mean that there is no selection of resistant parasites in zoonotic infections in animals (Gramiccia et al., 1992) or in humans during long courses of treatment, especially in immunocompromised patients (Escobar et al., 2001). Rather these events must be considered in relation to chances of transmission of resistant parasites to the wider human population. These factors must also be separated from observations that indicate that in zoonotic leishmaniasis there are populations of parasites that are highly insensitive to a drug, as determined in drug sensitivity assays on isolates (Yardley et al., 2005). These populations probably have a highly stable “resistance” phenotype (and genotype) and are transmitted from host to host.

Host-related factors: The immune status of leishmaniasis patients has long been known to affect drug efficacy. This has proven to be of particular importance in relation to pentavalent antimonial treatment of DCL (Ercoli, 1966) and coinfections with HIV in the visceral form (Berhe et al., 1999), where there is an absence of a specific T-cell mediated immune response and mutual exacerbation of infection. The basis for this lack of activity of pentavalent antimonials has been explored in immunodeficient mouse models for which the effects are probably due to deficiencies of both Th1-cell-mediated and macrophage responses (Murray et al., 1989). Experimental models have shown that the antileishmanial activity of
pentamidine is also T-cell dependent whereas those of amphotericin B and miltefosine are T-cell independent (Murray et al., 1993). Irrespective of the findings of the experimental models, it is now known that intact immunity holds the key to the curative ability of antileishmanial drugs, including amphotericin B. Experiences with HIV/VL coinfection in the Mediterranean region, most frequently caused by *L. infantum*, suggest that CD4- deficient individuals tend to relapse frequently (Laguna et al., 2003). In randomized controlled trials in Spain, cure rates in both antimonial and amphotericin B-treated coinfected patients were as low as 66% and 62%, respectively, compared with 90% cure rates in non-HIV patients (Laguna et al., 1999). Similar figures have been reported in Ethiopia. In addition, 60% relapse of responders 12 months after completion of treatment was reported. In recent years there has been a decline in the incidence of VL in HIV-infected patients following the introduction of highly active antiretroviral therapy (Lira et al., 1999), again suggesting an important role for CD4 lymphocytes in preventing relapses and controlling the infection. Moreover, ATP binding cassette (ABC) transporters have been widely reported to export xenobiotics (Homolya et al., 2003) and cause drug resistance in various diseases such as cancer (Gottesman et al., 2002). Earlier studies have reported the expression of analogs of ABC transporters on the surfaces of antimony resistant strains of *Leishmania* promastigotes (Legare et al., 2001), believed to efflux antimonials. However, the demonstration of these transporters in promastigotes may not be very relevant to clinical situations. There are a few reports available on the expression of similar transporters in laboratory isolates of in vitro-developed Sb* strains of leishmanial amastigotes (El Fadili et al., 2005) or on amastigotes from field isolates of antimony resistant *L. donovani* (Vergnes et al., 2007). Although sodium antimony gluconate (SAG) kills leishmanial amastigotes directly at higher doses in vitro as reported previously (Wyllie et al., 2004), a much lower dose is required for killing the parasite within macrophages (Mφ) (Ibrahim et al., 1994). It has been recently reported that infection by “antimony resistant” Leishmania elevates ABC transporter expression in host cell surface. These transporters efflux out antimonials from the infected cells, resulting in prevention of elimination of intracellular amastigotes.

*Leishmania*-related factors: Given the known biochemical and molecular differences between species it is perhaps unsurprising that there is variation in intrinsic sensitivity between *Leishmania* species to several drugs. Although such variation has been reported in laboratory studies, careful interpretation is of prime importance as different assay conditions can lead to several fold differences in activity values (Croft and Brun, 2003). Despite this caveat, there is ample evidence of variation. The second element of variation in response comes from selection due to drug pressure. The selection of drug-resistant pathogens is a major and well-known threat to the treatment of bacterial, viral, and fungal infections as well as some parasitic infections, such as malaria. The primary effect in cell killing is the interaction of a drug with one or more targets. Thus, the alteration of the intracellular drug level or the ability of the drug to affect the target is commonly observed in a wide variety of organisms. Drug levels at the target site of action can be lowered by a variety of mechanisms, including decreased uptake, increased export, and inactivation by metabolism or sequestration. Likewise, alterations in levels of primary target can occur due to decreased target affinity.
for the drug or complete loss of target, usually associated with a bypass mechanism. Complex downstream events leading to cell damage and death are often triggered by inhibition of a primary target. For example, many antiparasitic drugs (e.g., nifurtimox, primaquine) undergo futile-redox cycling, producing reactive oxygen species that can peroxidatively damage membrane lipids, proteins or DNA (Docampo and Moreno, 1986). Thus, overexpression of various repair systems can also play a role in drug resistance. Multiple mechanisms are frequently involved.

Antimonials and their mechanism of resistance

Variation in the clinical response to the pentavalent antimonials sodium stibogluconate, and meglumine antimonate (Glucantime) in VL, CL, and MCL has been a persistent problem in the treatment of leishmaniasis over the past 50 years. One explanation for this phenomenon is the intrinsic difference in species sensitivity to these drugs. In general, studies using the amastigote-macrophage model, *L. donovani* and *L. braziliensis* were found to be three- to fivefold more sensitive to sodium stibogluconate than *L. major*, *L. tropica*, and *L. mexicana* (Berman, 1981). This was also shown in earlier studies by Berman et al., using another amastigote-macrophage model, which also demonstrated a wide variation in the sensitivity of isolates from cutaneous leishmaniasis cases to pentavalent antimonials (Berman et al., 1982). In one controlled clinical trial in Guatemala that compared the cure rate to antimonials of CL caused by different species (Navin et al., 1992), sodium stibogluconate produced a significantly higher cure rate in patients with *L. braziliensis* (96%) lesions than those with *L. mexicana* lesions (57%).

Pentavalent antimonial drugs were used worldwide for the treatment of VL and CL for over six decades with little evidence of resistance. Although the selection of resistant *Leishmania* has long been a part of laboratory studies, it is only in the past 15 years that acquired resistance has become a clinical threat. In most parts of the world, over 95% of previously untreated patients with VL respond to pentavalent antimonials, the recommended first-line treatment. However, the region endemic for VL in North Bihar, India, has the unique distinction of being the only region in the world where widespread primary failure to Sb(V) has been reported (Sundar, 2003). Even in this geographical region a variation in Sb(V) sensitivity occurs with significant drug resistance at the epicenter of the epidemic and a high level of sensitivity only 200 miles away (Sundar, 2003). This resistance is so far unique to *L. donovani*; all isolates from a large number of refractory as well as responding patients in India were identified as this species (Thakur et al., 2001). Until the late 1970s, a small daily dose (10 mg/kg; 600 mg maximum) for short duration (6 to 10 day) was considered adequate, when unconfirmed reports suggested a 30% treatment failure with this regimen from four districts most severely affected, Muzaffarpur, Samastipur, Vaishali, and Sitamarhi (Peters, 1981). Following this, an expert committee revised recommendations to use Sb(V) in two 10-day courses with an interval of 10 days and a significant improvement in cure rates (99%) was observed (Aikat et al., 1979). However, only a few years later, another study noted 86% cure rates with 20 days of continuous treatment with this regimen (Thakur et al., 1984). In 1984, a World Health Organization (WHO) Expert Committee recommended that Sb(V) should be used in doses of 20 mg/kg/day up to a maximum of 850 mg for 20 days, with a repeat of the same regimen for 20 days in
cases of treatment failure. Four years later, Thakur et al. evaluated the WHO recommendations and reported that 20 days of treatment with 20 mg/kg/day (maximum 850 mg) cured only 81% of patients, although with an extension of the treatment for 40 days, 97% of patients could be cured (Thakur et al., 1988). Three years later, the same group noted a further decline in cure rate to 71% after 20 days of treatment, and recommended extended duration of treatment in nonresponders (Thakur et al., 1991). Jha et al. (Jha et al., 1992) found that extending the therapy until 30 days could cure only 64% of patients in a hyperendemic district of Bihar. From these findings it became clear that Sb(V) refractoriness was increasing although the reports came from studies that were not strictly controlled. In two following studies carried out under strictly supervised treatment schedules, it was observed that only about one-third of all VL patients could be cured with the currently prevailing regimen (Sundar et al., 2000). The incidence of primary unresponsiveness was 52%, whereas 8% of patients relapsed. During the same period only 2% of patients from the neighboring state of (Eastern) Uttar Pradesh failed treatment (Sundar et al., 2000). These studies confirmed that a high level of Sb(V) unresponsiveness exists in Bihar, though the drug continues to be effective in surrounding areas. There are reports of antimony resistance spreading to the Terai regions of Nepal, especially from the district adjoining hyperendemic areas of Bihar, where up to 24% of patients seem to be unresponsive, though in eastern Nepal a 90% cure rate has been reported (124). The reason for the emergence of resistance is widespread misuse of the drug. Sb(V) is freely available in India, and is easily accessible over the counter. Most patients (73%) first consult unqualified medical practitioners, who might not use the drug appropriately (Sundar et al., 1994). It has been a common practice to start with a small dose and gradually increase the dose over a week. Drug-free intervals are given with the belief that they will prevent renal toxicity. On many occasions the daily dose of drug is split into two injections, to be given twice daily. These practices presumably expose the parasites to drug pressure, leading to progressive tolerance of the parasite to Sb(V). It has been observed that only a minority of patients (26%) were treated according to prescribed guidelines: irregular use and incomplete treatments were a common occurrence. These facts point to the mishandling of antileishmanial drugs in Bihar as a significant contributor to the development of drug resistance (Sundar et al., 1994).

In a study to determine whether acquired drug resistance was present in Bihar, *L. donovani* isolates were taken from responders and nonresponders (Lira et al., 1999). Using an in vitro amastigote-macrophage assay, isolates from patients who did respond to sodium stibogluconate treatment were threefold more sensitive, with 50% effective doses (ED$_{50}$) (around 2.5 g Sb/ml) compared to isolates from patients who did not respond (ED$_{50}$ around 7.5 g Sb/ml). There was no difference in the sensitivity of isolates when the promastigote assay was used (Lira et al., 1999). The significant difference in amastigote sensitivity supports the concept of acquired resistance in Bihar. However, more biological evidence is required to support the temporal and spatial parameters of the Bihar phenomenon. The sample size in this first study (Lira et al., 1999) was small (15 nonresponders and 9 responders), and a threefold difference in sensitivity can be seen between experiments in this model (Croft and Brun, 2003). Other
reports on VL isolates from Sudan have also shown that the clinical response to sodium stibogluconate was reflected in isolates in the amastigote-macrophage model (but not in promastigotes) (Abdo et al., 2003). Other observations support the notion that Sb resistance can be acquired. In *L. infantum* isolates taken from immunodeficient and immunocompetent VL patients in France both before and after meglumine antimoniate treatment, isolates from 13 of 14 patients posttreatment had decreased sensitivity in an amastigote-macrophage assay (Faraut-Gambarelli et al., 1997). A similar decreased sensitivity was observed in *L. infantum* isolates taken from dogs before and after meglumine antimoniate treatment (Gramiccia et al., 1992). In the laboratory *L. donovani* resistance to antimonials is easily generated in culture, most recently in axenic amastigote of *L. donovani* and *L. infantum*, and a rodent model (Ephros et al., 1997). Although the in vitro data suggest that increasing the dose of Sb(V) could overcome the unresponsiveness, even the current doses produce unacceptable toxicity and further increase in the quantity of drug could seriously jeopardize the safety of the patients (Sundar et al., 2001). What we still do not have is a marker of clinical antimony resistance in *L. donovani* isolates. Several laboratory generated markers of Sb resistance have now been identified (Sundar et al., 2001), but evidence of their existence in field isolates from refractory patients has yet to be found. Although an amplicon was observed in a few isolates from Sb-refractory patients, the significance of this observation has yet to be determined (Singh et al., 2001). The development of Sb resistance in the anthroponotic cycle in Bihar suggests that resistance could also develop to other antileishmanial drugs as they are introduced. A similar potential for resistance to develop exists in East Africa, especially in Sudan, another anthroponotic focus of VL with intense transmission, where poverty, illiteracy, and poor health care facilities portend misuse of the drug and consequent emergence of resistance. Resistance seems to be a feature of intensive transmission of anthropo- notic *L. donovani* as epidemic turns to endemic in foci where Sb(V) has been used as monotherapy for long periods, often with poor supervision and compliance (Sundar et al., 2001). In other parts of the world, Sb(V) continues to be effective (Collin et al., 2004). Another concern is that increasing numbers of HIV/VL-coinfected patients will be a potential source for emergence of drug resistance. These patients have high parasite burden and a weak immune response, respond slowly to treatment, have a high relapse rate, and could be a reservoir of drug-resistant parasites. Furthermore, the reports of transmission of infection via needle sharing in HIV/VL-coinfected patients in southern Europe, identify another route for spread of resistant parasites (Cruz et al., 2002).

**Mechanisms of action and resistance.** After 60 years of use, the antileishmanial mechanism of action of pentavalent antimonials is only now nearly understood. Interpretation of some of the earlier reports on mode of action and drug sensitivity to antimonials is complicated by the fact that liquid formulations of sodium stibogluconate contain the preservative 3-chlorocresol, itself a potent antileishmanial agent (Roberts and Rainey, 1993). Unfortunately, much of this literature does not specify whether the liquid form or additive-free powder form was used. Nonetheless, it is now generally accepted that all pentavalent antimonials are prodrugs that require biological reduction to the trivalent form [Sb(III)] for antileishmanial activity. The site (amastigote or macrophage) and mechanism of reduction (enzymatic or
nonenzymatic) remain controversial. However, several studies have reported that axenic amastigotes (i.e., cultured in the absence of macrophages) are susceptible to Sb(V), whereas promastigotes are not, suggesting that some stage-specific reduction occurs in this life cycle stage (Ephros et al., 1997). However, there are reports to the contrary (Sereno et al., 1998). Certainly, a proportion of Sb(V) may be converted to Sb(III) in humans (Goodwin and Page, 1943) and in animal models (Lugo de Yarbuh et al., 1994), so both mechanisms maybe operative. Further studies are required to resolve this issue. Although stage-specific reduction has been demonstrated recently (Shaked-Mishan et al., 2001), the mechanism by which amastigotes reduce Sb(V) is not clear. Both glutathione and trypanothione can nonenzymatically reduce Sb(V) to Sb(III), particularly under acidic conditions (Frezard et al., 2001). However, the physiological relevance of these observations is open to question since the rates of reduction are rather slow. Moreover, promastigotes contain higher intracellular concentrations of trypanothione and glutathione than amastigotes (Ariyanayagam and Fairlamb, 2001) and both stages maintain intracellular pH values close to neutral, independent of external pH (Glaser et al., 1988). Thus, it is difficult to account for the selective action of Sb(V) against the amastigote stage by a nonenzymatic mechanism. As both stages can take up Sb(III) and Sb(V) the insensitivity of promastigotes to Sb(V) cannot be attributed to drug exclusion (Brochu et al., 2003). Two possible candidates for the enzymatic reduction of Sb(V) to Sb(III) in amastigotes have recently been identified. The first is a thiol-dependent reductase related to glutathione S-transferases that is more highly expressed in amastigotes (Denton et al., 2004). The second is a homologue of a glutaredoxin-dependent yeast arsenate reductase (Zhou et al., 2004). The levels of expression of this protein in promastigotes and amastigotes were not reported and the low specific activity of the recombinant enzyme with glutaredoxin raises questions as to the physiological nature of the electron donor in Leishmania spp. The importance of these candidate proteins in conferring sensitivity to Sb(V) in amastigotes needs to be addressed. There have been comparatively few studies on the mode of action of these drugs. Initial studies suggested that sodium stibogluconate [Sb(V)] inhibits macromolecular biosynthesis in amastigotes (Berman et al., 1985), possibly via perturbation of energy metabolism due to inhibition of glycolysis and fatty acid β-oxidation (Berman et al., 1987). However, the specific targets in these pathways have not been identified. More recent studies have reported apoptosis in Sb(III)-treated amastigotes involving DNA fragmentation and externalization of phosphatidylserine on the outer surface of the plasma membrane (Sereno et al., 2001). However, these effects do not involve the classical caspase mediated pathway (Sereno et al., 2001) and do not meet the more recent stringent definition of apoptosis (Jiang and Wang, 2004). The mode of action of antimony in drug-sensitive L. donovani involves several effects on glutathione and trypanothione metabolism (Wyllie et al., 2004). Exposure to Sb(III) causes a rapid disappearance of trypanothione and glutathione from isolated amastigotes and promastigotes in vitro. A significant portion of these thiols are effluxed from cells in approximately equimolar amounts with the remainder being converted intracellularly to their respective disulfides (trypanothione and glutathione). The formation of the latter was ascribed to continuing oxidative metabolism in the face of inhibition of trypanothione reductase Sb(III), but not Sb(V), has
previously been shown to be a time-dependent reversible inhibitor of trypanothione reductase in vitro (Cunningham and Fairlamb, 1995). Since Sb(III) also inhibits recovery of intracellular thiols following oxidation with diamide, this is consistent with inhibition of trypanothione reductase in intact cells (Wyllie et al., 2004). The profound loss of these thiols (<90% in 4 h) coupled with the accumulation of disulfide (up to 50% of the residual within 4 h) causes a marked decrease in cellular thiol redox potential. Similar effects on thiol levels and thiol redox potential were observed when amastigotes were exposed to Sb(V), intrinsically linking the effects of the biologically active Sb(III) with the clinically prescribed Sb(V). The mechanism by which *Leishmania* spp. acquire resistance to antimonials has been the subject of intensive research for several decades, often yielding apparently contradictory results. It should be borne in mind when evaluating the literature that (i) *L. tarentolae* is quite different to species that infect mammals, and (ii) some laboratory-derived promastigote resistant lines were initially generated by selection for resistance to arsenite (Ouellette et al., 1991) and subsequently found to be cross-resistant to Sb(III), whereas others have been directly selected for resistance by exposure to Sb(III). While Sb and As are both metalloids, the selection method may affect the resulting resistance mechanism. As promastigotes are not sensitive to Sb(V), lines that were reportedly selected for resistance with Sb(V) preparations may have been selected for resistance to the *m*-chlorocresol preservative instead (Roberts et al., 1993). Alternatively, Sb(V) preparations could be partially reduced due to prolonged storage at acidic pH or in culture media containing thiols such as cysteine or glutathione (Frezard et al., 2001). It is also not inconceivable that some *Leishmania* spp. constitutively express higher amounts of “antimony reductase” activity in the promastigote stage than others. Diminished biological reduction of Sb(V) to Sb(III) has been demonstrated in *L. donovani* amastigotes resistant to sodium stibogluconate (Shaked-Mishan et al., 2001). This line also shows cross-resistance to other Sb(V) drugs, but the same susceptibility to Sb(III) as the wild type (Ephros et al., 1999), distinguishing it from the trypanothione pathway mutants described below. It is not known whether this mechanism occurs in clinical isolates at present. The accumulation of Sb(V) and Sb(III) in promastigotes and amastigotes has been shown to be by different transport systems (Brochu et al., 2003), and although Sb accumulation was lower in resistant forms than in sensitive forms, levels of accumulation could not be correlated to sensitivity in wild-type cells. Aquaglycoporins have recently been demonstrated to mediate uptake of Sb(III) in *Leishmania* spp. and overexpression of aquaglycoporin 1 renders them hypersensitive to Sb(III) (Gourbal et al., 2004). Transfection of aquaglycoporin 1 in an Sb(V)-resistant field isolate also sensitized it to sodium stibogluconate when in the amastigote form in a macrophage. Increased levels of trypanothione have been observed in some lines selected for resistance to Sb(III) or arsenite (Mukhopadhyay et al., 1996). This is due to increased levels of the rate-limiting enzymes involved in the synthesis of glutathione (γ-glutamylcysteine synthetase) (Grondin et al., 1997) and polyamines (ornithine decarboxylase) (Haimeur et al., 1999), the two precursor metabolites to trypanothione. Increased synthesis of glutathione and trypanothione from cysteine could help to replace thiols lost due to efflux as well as to restore thiol redox potential perturbed by accumulation of disulfides (Wyllie et al., 2004). Spontaneous formation of Sb(III) complexed with either
glutathione, trypanothione or both has been demonstrated by proton nuclear magnetic resonance spectroscopy (Yan et al., 2003) and by mass spectrometry (Mukhopadhyay et al., 1996). Since glutathione S-transferase (GST) is elevated in mammalian cells selected for resistance to arsenite (Lo et al., 1992), it has been proposed that formation of the metalloid-thiol pump substrates in Leishmania spp. could be rate-limiting and that GST could mediate this activity (Mukhopadhyay et al., 1996). However, GST is not detectable in Leishmania spp., although there is an unusual trypanothione S-transferase activity associated with the eukaryotic elongation factor 1B complex (Vickers and Fairlamb, 2004). The precise nature of the Sb-thiol complex remains uncertain, but two routes of elimination of the complex can be envisaged. The first involves sequestration in an intracellular compartment or direct efflux across the plasma membrane. Early studies noted that PgpA, a member of the ATP-binding cassette (ABC) transporters, is amplified in some resistant lines (Callahan and Beverley, 1991). However, it soon became apparent that this transporter is not responsible for drug efflux across the plasma membrane. First, overexpression of PgpA was reported to decrease influx of Sb rather than increase efflux, possibly due to a dominant-negative effect through interactions with other membrane proteins (Callahan et al., 1994). Second, overexpression of PgpA did not mediate increased efflux of radioactive arsenite from cells (Dey et al., 1994) or transport of arsenite across plasma membrane preparations (Mukhopadhyay et al., 1996). Finally, PgpA plays a relatively minor role in resistance (Papadopoulou et al., 1996) and is localized in membranes that are close to the flagellar pocket, the site of endocytosis and exocytosis in this parasite (Legare et al., 2001). Thus, the identity of the efflux pump in the plasma membrane and its role in resistance to antimonials remain to be determined. However, the studies described above have identified PgpA as functioning to sequester Sb(III) in an intracellular vacuolar compartment in Leishmania. It is worth noting that resistance due to intracellular sequestration of Sb(III) as a thiol conjugate would show higher rather than lower intracellular levels of Sb(III). Thus, either sequestration plays a minor role in resistance or the conjugates must be rapidly exocytosed from the cell. The next important step is to relate mechanisms observed in laboratory studies to clinical resistance. In one study on field isolates, no amplification of the genes found in laboratory studies was observed; rather amplification of a gene on chromosome 9 possibly involved in protein phosphorylation was identified (Singh et al., 2003).

**Major host immunological responses in Leishmaniasis**

**Primary host response against parasite invasion**

In order to develop a successful parasitic relationship with it’s host, the *Leishmania* must evade both the innate and adaptive immune responses. When *Leishmania* first enters the human body, it is in the promastigote form. Promastigotes are engulfed by macrophages but are resistant to proteolysis and degradation in the phagosome. Within mammalian host *Leishmania* resides as amastigotes in phagocytic cells such as macrophages, dendritic cells (DCs) and neutrophils. The complement protein C3b is one of the most potent immune opsins. C3b binds to foreign material and promotes its uptake via C3b receptors on phagocytic cells. C3b binds to *Leishmania* parasite which results in uptake by the macrophage. *Leishmania* has a special surface protein called gp63, which converts C3b into iC3b (Hermoso et al., 1991).
From the parasitic standpoint this conversion would favour phagocytic clearance rather than lytic clearance as *Leishmania* is very resistant to degradation once phagocytosed. Therefore, this conversion is crucial for *Leishmania*'s survival. After being engulfed, the *Leishmania* must endure harsh conditions inside the phagosome like the oxidative burst used by the macrophage to destroy the foreign material inside the phagosome. Macrophage plays a primary role in the host defense and regulation of immune responses upon activation (Unanue and Allen, 1987). This process consists of an attack by superoxide and hydroxyl radicals on the parasite. *Leishmania* produces acid phosphatases on its surface which inhibits this burst, macrophages often attempt to degrade parasites with acidic enzymes. This occurs when lysosomes fuse with the phagosome. The *Leishmania* resists this attack through a proton pump present on the surface and allows its intracellular pH to remain neutral. Also the protozoan molecule lipophosphoglycan (LPG) plays an active role by inhibiting lysosomal enzymes. The parasites interact with its host in a complex way inside the severe environment of phagolysosomes and eventually evade this immune defense mechanism (Alexander and Russell, 1992)

*Humoral responses in leishmaniasis*

Infection of Leishmania in human is characterized by the appearance of anti-leishmanial antibodies in the sera of the patients. In CL, usually they are present at low levels during the active phase of the disease (Behin and Jacques, 1989). In contrast, strong anti-leishmanial antibody titres are well documented in VL (Neogy et al., 1987). The role of elevated anti-leishmanial antibodies in kala-azar patients towards protection or pathogenesis is still unclear. Critical analysis of Leishmania antigen-specific immunoglobulin (Ig) isotypes revealed elevated levels of IgG, IgM, IgE and IgG subclasses during disease (Anam et al., 1999). IgG not only fails to provide protection against this intracellular pathogen, but it actually contributes to disease progression. Passive administration of anti-leishmanial IgG resulted in larger lesions in BALB/c mice with greater amount of IL-10 protection (Miles et al., 2005). This result can be correlated with the highly elevated titres of anti-leishmanial antibodies during the active phase of the disease and a consecutive fall in the antibody titre after a successful cure. The elevated antibody titres against promastigote or amastigote antigens have been extensively exploited for specific serodiagnosis in last two decades.

*Cell mediated immune response*

Host cells for *Leishmania* spp. also have a pivotal role in bridging the gap between innate and adaptive immunity. However, although many of the survival strategies noted above may indirectly affect the three signals that are required for inducing T cell activation and differentiation (antigen processing and presentation, the expression of co-stimulatory molecules and the production of host cell cytokines), there have been few studies over the past few years that directly address these pathways in an *in vivo* context and/or in relation to defined virulence factors. Notably, few examples have been found of bona fide virulence factors that selectively target antigen presentation, although these are abundant in other
intracellular pathogens. The tools of in situ analysis hold great promise for addressing some of these questions in coming years. By contrast, there has been, and continues to be, much interest in defining the cellular mediators of acquired resistance. The following section describes some of the more recent nuances of the acquired immune response to *Leishmania* spp. (Fig 10)

Figure 10: Fig 19. Major chemokine mediated events occurring in the spleen and liver during visceral leishmaniasis. Production of CCL2 and CXCL10 followed by accumulation Mf and T\textsubscript{H}1 cells in the infected liver results in formation of granuloma and eventual resolution of infection. In contrast impaired chemokine driven cellular encounters between DCs and T-cells due to downregulation of CCL19, CCL21 and CCR7 coupled with a lack of persistant production of CXCL10 results in parasite persistence in the spleen (Kumar and Nylén, 2012).

Host resistance and adaptive immunity.

CD\textsuperscript{4+} T\textsubscript{H}1 cells are critical for the control of *Leishmania* infections, owing to their ability to make IFN\textgamma, which activates macrophages and DCs, leading to parasite death. The role of CD\textsuperscript{8+} T cells in cutaneous leishmaniasis has been less well appreciated. Although early studies indicated that CD\textsuperscript{8+} T cells were important to control visceral leishmaniasis (Stern et al., 1988), the initial studies with *L. major* indicated that CD\textsuperscript{8+} T cells were not important for control of a primary infection but they participated in the resistance to reinfection (Muller et al., 1993). However, when mice were infected with low doses of parasites, CD\textsuperscript{8+} T cells appeared to be essential for resolution of primary infection (Belkaid et al., 2002), owing in part to the ability of IFN\textgamma to promote a T\textsubscript{H}1-type response (Uzonna et al., 2004). Nevertheless, CD\textsuperscript{8+} T cells are not always associated with disease resolution. For example, the recruitment of CD\textsuperscript{8+} T
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cells that express the granule-associated serine protease granzyme B is correlated with lesion progression in patients infected with *L. braziliensis* (Faria et al., 2009). Moreover, it appears that CD8+ T cell exhaustion following infection with *L. donovani* may contribute to the chronicity of infection (Joshi et al., 2009). Importantly, CD8+ T cells that are activated during *Leishmania* infections may not all be specific, as uninfected DCs that have matured during inflammation can stimulate CD8+ T cells to proliferate without the expression of their cognate ligands (Maroof et al., 2009). The role of these non-leishmanial-specific CD8+ T cells in the infection is not clear, but when previously activated CD8+ T cells are expanded during infection, they can provide increased resistance to previously encountered pathogens (Polley et al., 2005).

**Parasite persistence.** In spite of the development of a robust immune response in resistant mice, as well as in many patients, a small number of parasites persists following disease resolution. The production of IL-10 has a large role in dampening the immune response and thus allowing some parasites to escape destruction (Belkaid et al., 2001). Recent studies have shown that IL-10 can come from a variety of sources following leishmanial infections, including regulatory T (T<sub>Reg</sub>) cells (Belkaid et al., 2002), Th1 cells (Anderson et al., 2007), CD8+ T cells (Belkaid et al., 2001), B cells (Ronet et al., 2010), natural killer cells (Maroof et al., 2008), regulatory DCs (Svensson et al., 2004), macrophages (Miles et al., 2005) and neutrophils (Charmoy et al., 2007). Which of these is most important as a source of IL-10 is less clear, but may depend upon differences in the parasites and the stages of the infection. In *L. donovani* infection, CD8+CD40+ T cells may act as contra-T<sub>Reg</sub> cells by limiting the production of IL-10 during the early phase of infection, but themselves become susceptible to IL-10-induced apoptosis as the disease progresses (Martin et al., 2010). Thus, the question of how immunoregulatory mechanisms limit immune responses, and as a consequence lead to the There have been many mechanisms described by which parasites attempt to modulate the ability of their host cells to respond to the signals that lead to induction of leishmanicidal activity. From these observations one might conclude that completely eliminating parasites may not be possible regardless of the magnitude of the immune response. However, earlier studies in which IL-10 was eliminated have suggested that sterile cure could be achieved in certain situations (Belkaid et al., 2001). More recently, in mice lacking BCL-2-interacting mediator of cell death (BIM), a pro-apoptotic BCL-2 family member, not only did *L. major* infections resolve, but also this resolution was associated with the clearance of all detectable parasites (Reckling et al., 2008). Hence, given a sufficient immune response, sterile cure can be achieved in leishmaniasis.

**Th1/Th2 paradigm in Leishmaniasis**

The immunology surrounding *Leishmania* infection is complicated both from the standpoint of the host response to a given *Leishmania* species and the fact that different species can elicit very different responses. Particularly difficult from a vaccine development standpoint is the fact that it is not entirely understood what constitutes a protective response in humans. During the past decade, several investigators have used the Th1/Th2 paradigm to design strategies to design strategies for antigen
discovery/selection in vaccine development against leishmaniasis. Thus, leishmanial antigens that predominantly stimulate Th1 responses in patient cells or spleen or lymphnode cells from mice infected with \textit{L. major} have commonly been accepted as ‘potential protective antigens’ and therefore promising vaccine candidates. Conversely, antigens that predominantly stimulate a Th2 response from these cells have been regarded as of lesser interest because they are likely to be associated with pathology (Campos-Neto et al., 1995). Paradoxically, leishmanial antigens against which a Th1 response is developed during infection may not necessarily be protective antigens. For example, lymph node cells of BALB/c mice chronically infected with \textit{L. major}, upon stimulation with Ldp23 antigen, produce high levels of IFN-\(\gamma\) and undetectable amounts of IL-4, a typical Th1 response (Campos-Neto, 2005).

\begin{center}
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\textbf{Figure 11: }\textit{T}_\text{H}1\textit{ and }\textit{T}_\text{H}2\textit{ dichotomy in Leishmaniasis} (Sharma and Singh, 2009)

\textbf{Organ specific immunity in VL.}

Experimental infection with \textit{L. donovani} is characterized by distinct organ-specific immune responses (Engwerda and Kaye, 2000). The liver is the site of an acute resolving infection, minimal tissue damage and resistance to reinfection, whereas the spleen becomes a site of parasite persistence. Infection results in an impaired cell-mediated immune response that is associated with decreased Th1 cytokine production (Liew FY and O'Donnell, 1993). Both CD4\(^+\) and CD8\(^+\) T cells are activated following \textit{L.}
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*donovani* infection (Engwerda and Kaye, 2000), and both Th1 and Th2 responses are generated (Kaye et al., 1991). Broadly speaking, CD4+ T cells play a key role in controlling hepatic parasite growth in a primary infection (Alexander et al., 2001), whereas CD8+ T cells are critical for rapid resolution of infection in the liver following rechallenge. As CD4+ and CD8+ T cells are both required for optimal resistance, nude (McElrath et al., 1988), severe combined immunodeficiency disease (SCID) (Kaye and Bancroft, 1992) and recombinase activating gene (Rag)−/− mice are unable to control *L. donovani* growth in the liver, and reconstitution experiments demonstrate a critical role for CD4+ and CD8+ T cells in the resolution of infection (Alexander et al., 2001). Control of hepatic infection in mice requires a coordinated host response involving the development of cellular infiltrates known as inflammatory granulomas around infected macrophages (Murray et al., 1992). In contrast, the spleen and bone marrow become chronically infected by mechanisms that are less well understood. In the mouse, the spleen becomes grossly enlarged and can account for up to 15% of body weight within 6–8 weeks post-infection. Parasite persistence is accompanied by a failure of granuloma formation, splenomegaly, and disruption of lymphoid tissue microarchitecture including disruption of B-cell follicles and the marginal zone (MZ) (Smelt et al., 1997), and enhanced haematopoietic activity (Zijlstra and el-Hassan, 2001). Immunodeficiency commonly occurs during the chronic stages of the disease in humans and leads to increased susceptibility to secondary infections (Ho et al., 1983). Pathology is intimately linked to parasite persistence, and it is hoped that understanding the development of immunity in the liver may lead to potential means to improve parasite clearance in the spleen.

**The evolution of hepatic granulomas**

In most cases, efficient immune responses to *L. donovani* in the liver depend on the formation of granulomas, a process dependent on chemokine production, subsequent recruitment of monocytes, neutrophils, CD4+ T cells and CD8+ T cells, production of inflammatory cytokines and activation of infected cells. Two exceptions occur in mice deficient in TNF and reactive nitrogen intermediates (RNI) (Murray and Nathan, 1999), where granuloma formation occurs, albeit less efficiently in TNF-deficient mice (Murray et al., 2000), but parasite growth is not controlled. Kupffer cells (KC) are the major tissue macrophage found in the liver lining the sinusoids, and are a major target for *Leishmania* infection (Crocker et al., 1984). Following infection, chemokines including CCL3 (MIP-1α), CCL2 (MCP-1) and CXCL10 (γIP-10) are rapidly secreted, possibly by the infected KC (Cotterell et al., 1999), resulting in the initial recruitment of monocytes and neutrophils, both of which are critical for the effective control of parasite growth (Cervia et al., 1993). It is likely that other cells of the innate immune system such as NK cells and natural killer T (NKT) cells are also important in the early stages of granuloma formation due to their ability to rapidly produce large quantities of inflammatory cytokines such as IFN-γ. The fusion of some infected T cells are required for the resolution of *L. donovani* infection in the liver, and are recruited to the developing granuloma in response to various chemokine and cytokine stimuli following monocyte and neutrophil recruitment. The number of hepatic CD4+ and CD8+ T cells is seen to increase by 1 week
post-infection, probably reflecting both local expansion and recruitment from the spleen (Kaye et al., 2004). Studies have shown that the development of hepatic immunity can be readily manipulated (Murphy et al., 1997) suggesting that T-cell responses are continually generated during infection. This appears to be different from infection with L. major, where the pattern of T-cell cytokine secretion is fixed within 3 days following infection (Scott, 1991). High rates of apoptosis are also seen in T cells in mice infected with L. donovani (Alexander et al., 2001). Conversely, B cells appear to have a regulatory role in granuloma formation and show enhanced resistance to L. donovani with more rapid parasite clearance from the liver, accelerated granuloma formation and markedly reduced parasite burden in the spleen (Smelt et al., 2000). There are a number of key proinflammatory (Th1) cytokines required for efficient granuloma formation, and these include IL-12 (Murray, 1997), IFN-γ (Squires et al., 1989), TNF, lymphotxin (LT) (Engwerda et al., 2004), granulocyte/macrophage colony-stimulating factor (GM-CSF) (Murray et al., 1995) and IL-2 (Murray et al., 1993). These Th1 cytokines are required for the stimulation of chemokine production and the generation of leishmanicidal molecules by the infected KC (Kaye et al., 2004). The Th2 cytokine IL-4 is also required for the resolution of hepatic infection and for the priming of CD8+ T cells that are critical for long-term protection (Stager et al., 2003). As a result of T-cell dependent macrophage activation, reactive oxygen intermediates (ROI) and RNI are generated. These are primarily responsible for parasite killing within the infected macrophage (Murray and Nathan, 1999). However, gp91phox−/− mice ultimately resolve hepatic infection whereas NOS2−/− mice do not, indicating an important role for ROI and RNI in the early control of L. donovani, but a critical role for RNI alone in the eventual resolution of infection (Murray).

![Figure 12: Changes in splenic function and architecture during L. donovani infection. (a) In the initial acute stages of infection (<28 days) splenic structure is maintained and the spleen is an important site for the generation of immune responses. Blood](image-url)
containing Leishmania parasite flows through the marginal sinus into the MZ. Parasites are taken up by two unique populations of macrophages in the MZ, the MMM and the MZM, and by red pulp macrophages. DC acquire Leishmania antigen in the MZ, and subsequently migrate into the T-cell areas of the PALS. Mature DC and naïve T cells express the chemokine receptor CCR7 and migrate into the PALS in response to the chemokines CCL19 and CCL21 expressed by gp38+ stromal cells and the central arteriole. Once in the PALS, DC secretes IL-12 and present Leishmania antigen to T cells, resulting in the generation of antigen-specific T-cell responses. (b) During the chronic stages of infection (428 days) the splenic architecture breaks down, contributing to an immunocompromised status in the host. TNF is responsible for a number of these changes, and appears to be produced by heavily parasitized macrophages that migrate into the PALS and produce TNF locally. TNF leads to a loss of gp38+ stromal cells and their reticular matrix in the PALS, and a subsequent loss of CCL19 and CCL21 expression on both the stroma and the central arteriole, leading to reduced cellular recruitment to the PALS. TNF also leads to the selective loss of MZM but not MMM, and positively regulates IL-10 production. IL-10 acts directly on DC, leading to the downregulation of CCR7 on the DC surface, further inhibiting their ability to migrate into the PALS. Finally, the function of the stromal cell network is altered in that it supports the development of regulatory DC from haematopoietic progenitor cells. These DC are immature and produce IL-10, thus further contributing to chronicity of infection in the spleen. (Stanley and Engwerda, 2007)

and Nathan, 1999). Granulomas become fully evolved by 2–4 weeks post-infection, and many functionally and structurally mature granulomas in which leishmanicidal mechanisms have been generated to kill parasites (known as sterile granulomas) are present at 8 weeks post-infection (Murray et al., 1987). After this time sterile granulomas gradually dissemble in an involution process (Murray, 2001). In the early stages of infection granulomas at various stages of maturation are apparent, and relatively mature granulomas can be readily seen alongside infected KC that have no associated cellular infiltrate. Thus granuloma formation occurs in an asynchronous manner for reasons that are not well understood. Although sterile cure is never achieved in the liver, it is resistant to reinfection.

Protection against immunopathology in the liver

A number of immunoregulatory cytokines are also produced in the developing granuloma. These cytokines are required to balance the successful development of protective immunity in the liver and the prevention of associated immunopathology. Cytokines including IL-10 (Murphy et al., 2001), TGF-β (Wilson et al., 1998) and IL-27 (Rosas et al., 2006) can suppress the control of L. donovani growth, yet they appear to have little impact on granuloma formation. Endogenous IL-10 regulates parasite killing by suppressing the production of, and responses to, IL-12 and IFN-γ in particular (Murray et al., 2003). High levels of IL-10 are observed both in infected humans (Ghalib et al., 1993) and in experimental models (Ato et al., 2002), and IL-10 is coexpressed with TNF in L. donovani-infected mice (Engwerda et al.,
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1996). The potent immunosuppressive activity of IL-10 during VL includes the suppression of macrophage activation (Bogdan et al., 1991), DC migration (Ato et al., 2002) and protective Th1 responses (Murphy et al., 2001). CD4+ T cells can contribute to IL-10 production during infection, and the majority of these lack CD25 and Foxp3 expression, indicating that they are not natural regulatory T cells (Stager et al., 2006).

*Development of immunity in the spleen in the acute phase of infection*

The murine spleen is a highly organized lymphoid organ consisting of red pulp regions that are separated by a specialized MZ from white pulp regions. The white pulp is composed of B-cell follicles and T-cell areas that surround the central arteriole termed per-arteriolar lymphoid sheaths (PALS). Owing to the contact the spleen has with the blood system it is a very important site for the generation of immunity against systemic infections, and the spleen appears to be an important site in the acute phase of experimental *L. donovani* infection for the generation of immune responses that are ultimately responsible for disease resolution in the liver. This is perhaps not surprising given the artificial route of parasite infection. However, it is possible that protective immune responses are also generated in other tissue sites in the body, such as hepatic lymph nodes, and the bone marrow.

*The establishment of a chronic infection in the spleen*

Although MZM and MMM are infected within hours of parasite injection, the production of TNF is not observed until 3 days later by both infected and uninfected macrophages (Gorak et al., 1998). The suppression of early TNF production may represent one way in which *L. donovani* parasites survive proinflammatory host responses. At later stages of infection, there is a high frequency of TNF-producing cells in the spleen, and very high levels of TNF are readily observed in a widespread distribution throughout red and white pulp regions (Engwerda et al., 1998). It is likely that many different cell types contribute to the production of TNF in the spleen at these later stages of infection. Following which, structural changes in the spleen begin from day 14 to 21 post-infection. This chronic phase of infection is associated with immune dysfunction and the development of CD4+ T cells producing regulatory cytokines (Gomes et al., 2000). The white pulp becomes disorganized and reduced in size, there is hypertrophy of the red pulp, and heavily infected macrophages are observed in multiple and unusual locations, such as in the PALS. The murine spleen shows many pathological changes associated with human VL (Zijlstra and el-Hassan, 2001), including GC involution and loss of follicular dendritic cell (FDC) networks. FDC are an important cell type in the developing GC for the proliferation and regulation of B-lymphocytes. B cells proliferate within GC and undergo isotype switching and subsequent somatic hypermutation following recognition of unprocessed antigen on FDC. Long-term persistence of unprocessed antigen on FDC also serves to maintain serum immunoglobulin levels and immunologic memory by enabling periodic restimulation of memory B-cell clones. From weeks 4–8 post-infection with *L. donovani* FDC become almost undetectable by both immunohistochemistry and by functional
immune complex trapping, and this loss is associated with the infiltration of heavily parasitized macrophages into the white pulp. This FDC destruction is likely to affect the regulation of B-cell function and contribute to the hypergammaglobulinaemia associated with VL. The mechanism of destruction of FDC during VL remains unknown but it is postulated to be CD8+ T-cell mediated, or due to other host factors or parasite-derived molecules from the heavily parasitized macrophages that infiltrate the region (Smelt et al., 1997).

Subversion of macrophage function and immune mechanisms

As Leishmania parasites inhabit an intracellular niche, it is perhaps not surprising that they have evolved various means to attenuate and/or subvert how their host cell integrates signals from the external 'immune' environment. Indeed the literature has a rich history of reports of Leishmania spp. affecting a range of diverse macrophage functions, including chemotaxis, cytokine production and immune synapse formation. However, until recently many of these studies have been overshadowed at a mechanistic level by the vexed issue of spatial segregation—that is, how do intra-phagosomes parasites affect the cytosolic signalling pathways of their host cell? These mechanisms include activation of the suppressor of cytokine signalling (SOCS)-3 (Bertholet et al., 2003); inhibition of p38 mitogen-activated protein kinases (p38 MAPK) (Junghae and Raynes, 2002); local activation of latent transforming growth factor β (TGF β) (Gantt et al., 2003) and/or ceramide-induced modulation of extracellular signal-related kinase (ERK), activated protein-1 (AP-1) and NF-κB transactivation (Ghosh et al., 2002). The delay in TNF production observed in vivo is likely to represent a requirement for early interferon γ (IFN-γ) for subsequent macrophage priming and TNF production (Gorak et al., 1998). Interestingly, limited cytokine release by dendritic cells (DC) can also occur following L. donovani infection (McDowell et al., 2002). L. donovani infected macrophages have also been reported to be less efficient at upregulating MHCII expression in response to exogenous IFN-γ, thereby reducing antigen presentation to and subsequent activation of T cells (Reiner et al., 1988), although this has not been confirmed by others (Lang et al., 1994). Infected macrophages also have an impaired ability to upregulate the costimulatory molecule CD80 and heat-stable antigen (HSA) (Kaye et al., 1994). Modulation of costimulatory molecules by Leishmania parasites might also have indirect effects on developing immune responses.

Cellular phosphotyrosine phosphatases. The role of a host protein phosphatase, SRC homology 2 domain phosphotyrosine phosphatase 1 (SHP1; also known as PTPN6), in regulating anti-leishmanial immunity has long been recognized (Gregory and Olivier, 2005). SHP1 has a central role in the regulation of inducible nitric oxide synthase (iNOS) and hence nitric oxide production, and it was recently shown that SHP1 binds to a conserved KTMM motif that is found in multiple kinases that act downstream of SHP1 and are associated with innate immunity (these kinases include extracellular signal-related kinase 1 (ERK1), ERK2 and IL-1 receptor-associated kinase 1 (IRAK1)). L. donovani increases SHP1 activity and expression in host macrophages, a process thought to contribute to parasite survival. But how is this
achieved? New evidence points to a well-known leishmanial virulence factor, major surface protease (MSP; also known as GP63 and leishmanolysin) (Joshi et al., 2002). MSP enters the host cell cytosol in a process that is dependent on the presence of cholesterol-rich lipid microdomains or 'lipid rafts' in the host cell membrane (Gomez et al., 2009), and appears to have an ability to selectively cleave intracytosolic protein tyrosine phosphatases such as SHP1, protein tyrosine phosphatase 1B (PTP1B) and T cell protein tyrosine phosphatase (TCPTP; also known as PTPN2), leading to their activation (Gomez et al., 2009). *Leishmania* spp. contains multiple MSP genes, and species differences in expression of these genes are well known (Hsiao et al., 2008). It will be important to determine whether all MSPs cleave intracytosolic protein tyrosine phosphatases. In *L. donovani*, MSP can be found on the promastigote surface as well as in the parasite cytoplasm, and it has been proposed that these pools of MSP are functionally distinct, with the surface MSP being involved in parasite development within the sandfly and the cytoplasmic MSP being a pre-formed store that is ready for rapid use in the mammalian host (Yao et al., 2007). MSP is somewhat promiscuous in its action; in fibroblasts, transforming growth factor-β-activated kinase 1 (an upstream activator of p38 mitogen-activated protein kinase (p38 MAPK)) is a target of enzymatic cleavage by MSP, providing a mechanism for inactivating p38 MAPK (Halle et al., 2009). Host cellular protein tyrosine phosphatases may also have broader roles in anti-leishmanial immunity. Of note, SHP2 (also known as PTPN11), which shares many downstream targets with SHP1, is coupled to signal-regulatory protein α1 (SIRPα1; also known as PTPNS1), which is a myeloid-restricted inhibitory receptor (Takizawa and Manz, 2007) that binds the membrane glycoprotein CD47 when macrophages encounter invariant natural killer T cells during early *L. donovani* infection (Beattie et al., 2010). Intriguingly, sodium stibogluconate also targets SHP1 at concentrations that are used for chemotherapy in humans (Pathak and Yi, 2001). (Fig 13, 14)

**Figure 13:**
GP63-mediated degradation directly
impacting host cells signalling and functions. *Leishmania* GP63 cleaves several targets of the infected macrophages such as: the myristoylated alanine-rich C kinase substrate (MARKS) a critical substrate for PKC-dependent signalling; the adaptor molecule p130CAS and PEST involved in the actin cytoskeleton remodelling; the mTOR kinase affecting translation initiation and consequently type 1 IFN production; and transcription factors such as NF-κB and AP-1 therefore greatly abrogating their capacity to do their transcriptional works. Interestingly cleavage of NF-κB p65 subunit generates a p35 subunit that can form heterodimers translocating to the nucleus concurring to chemokines production. Arrows indicate GP63 targets involved in signalling pathways; Red crosses indicate signalling cascade alteration; abrogated lines indicate functional inhibition. (Olivier et al., 2012)

![Figure 14: GP63-mediated PTP activation. *Leishmania* GP63 cleaves and activates host PTPs (SHP-1, PTP1B and TCPTP) leading to inactivation of various kinases bringing about the inhibition of several macrophage functions usually inducible by various agonist (LPS, IFNγ). SHP-1 was found to be involved in the downregulation of IRAK-1, MAPK and JAK/STAT signalling pathways. PTP1B on the other side has been found to inactivate JAK2, therefore also concurring, as SHP-1, to greatly affect the capacity of IFNγ to trigger this signalling pathway. In regard to TCPTP found to be mainly being present and activated in nucleus, its actual impact is still unravelled in *Leishmania* infection context. Arrows indicate activation; abrogated lines indicate downregulation of specific kinases; Red crosses indicate signalling alteration and functional inhibition. (Olivier et al., 2012)
Protein kinase C and nuclear factor-κB. Interference with host cell signalling at the level of macrophage protein kinase C (PKC) has also long been known (Olivier et al., 1995). An enzyme with PKC-like activity that is found in the infective promastigotes of *L. mexicana* has been recently reported to be upregulated during early contact with macrophages and to be involved in macrophage invasion (Alvarez-Rueda et al., 2009). Similarly, a role has been proposed for phosphatidylinositol kinases in a variety of leishmanial signalling responses that are linked to invasion. Hyporesponsiveness of host MAPK and nuclear factor-κB (NF-κB) leads to cross-tolerance to activation by bacterial lipopolysaccharide (LPS) and *L. major* (Ben-Othman et al., 2009) but does not appear to be due to the induction of cellular protein tyrosine phosphatases, suggesting alternative means for parasites to inhibit host responsiveness. *L. amazonensis* promotes the activity of the NF-κB p50–p50 transcriptional-repressor complex, which negatively regulates iNOS gene expression. Furthermore, in *L. amazonensis*-infected macrophages that had been previously stimulated with LPS, the NF-κB p50–p50 dimer replaced the NF-κB p65–p50 dimer (which is normally produced in LPS-stimulated macrophages). In addition, MSP cleaves the NF-κB p65 subunit (Gregory and Olivier, 2005). These results suggest an active process of immune deviation (Calegari-Silva et al., 2009).

Parasite-Mediated Modulation of Cytokine Secretion

Cytokine activation of macrophages is necessary for the induction of their killing responses. As described previously, IFN-γ and TNF-α are two major macrophage activating cytokines required for the elimination of intracellular parasites. During early stages of infection, macrophages exposed to protozoan parasites produce IL-12 and TNF-α that are responsible for initiating IFN-γ synthesis by NK cells. The production of IL-12 is vital to protection vs. Leishmania infections. As an evasion mechanism, parasites such as Leishmania spp. actively inhibit the production of proinflammatory cytokines. Alternatively, these pathogens can also up-regulated antiinflammatory cytokines that have potent macrophage deactivating activities resulting in exacerbation of disease. Enhanced intracellular survival by down-regulating the proinflammatory cytokine IL-12 and the up-regulation of the antiinflammatory cytokines (i.e., TGF-β and IL-10) are reviewed in this section.

Inhibition of IL-12 Production

The down-modulation of IL-12 production may be used by pathogens as a way to escape cell-mediated immunity (CMI). Three intracellular pathogens have been shown to suppress macrophage IL-12 production. Leishmania (Carrera et al., 1996) measles virus, (Karp et al., 1996) and HIV (Chehimi et al., 1994, Chougnet et al., 1996) inhibit the production of IL-12, and in all cases it was postulated that the down-regulation of this cytokine may interfere with or delay the development of CMI to these pathogens. The importance of IL-12 in host defense against Leishmania was demonstrated by treating susceptible BALB/c mice with recombinant IL-12 that rendered them resistant to *L. major* due to an up-regulation of IFN-γ and the initiation of a protective Th1- type response. Mice lacking the IL-12 gene or animals
treated with IL-12 mAbs are more susceptible to L. major (Scharton-Kersten et al., 1995) and macrophages taken from Leishmania lesions produced very little IL-12 if they harbored parasites (Belkaid et al., 1998). Amastigote forms of both L. Mexicana and L. major are also capable of suppressing macrophage IL-12 production induced by either LPS or CD40 ligation (Weinheber et al., 1998). Furthermore, the phosphoglycan moiety of L. major LPG was shown to regulate IL-12 synthesis in J774 cells at the transitional level, which appears to be mediated through NF-κβ. Parasite modulation of macrophage intracellular signaling pathways by altering PTK activities also appears to modulate secretion of IL-12. In macrophages, LPS stimulation activates all three classes of MAPK (i.e., Erk1/2, c-JNK, and p38). p38 MAPK activation promotes the induction of IL-12 mRNA, but activation of the extracellular signal-related kinase (Erk) suppresses LPS-mediated IL-12 transcription. Leishmania phosphoglycans, which promote parasite survival, have been shown to act by stimulating Erk MAPKs to actively inhibit the transcription and secretion of IL-12 (Feng et al., 1999). Finally, the STAT4-dependent IL-12 signaling pathway recently was shown to be essential for the development of protective immunity against L. major (Stamm et al., 1999). Parasite modulation of STAT4 activity may also be a potential mechanism for inhibiting IL-12 production.

Induction of the Inhibitory Cytokines (TGF-Beta and IL-10)

TGF-β is a pleiotropic cytokine involved in many functions of resident tissue cells, and it has been shown to inhibit some activity of immune cells. TGF-β can inhibit macrophage activation (Scharton-Kersten et al., 1995) and mediates immunosuppression by inhibiting IL-2-dependent T- and B-cell proliferation and IL-2-dependent Ig production by B cells (Kehrl et al., 1986). This inhibitory cytokine exerts potent regulatory effects on macrophage function, including the suppression of IL-12 production and the inhibition of IFN-γ-induced iNOS protein and NO production, (Lopez et al., 1993) events critical for the resolution of Leishmania infections. Immunosuppression observed in visceral leishmaniasis is believed to be partly due to the abundant production of TGF-β during the course of infection (Rodrigues et al., 1998). Antigen-presenting cells from L. donovani infected hamster produce high levels of TGF-β that has been implicated as a cytokine that promotes the in vitro replication and survival of Leishmania within macrophages. (Barral et al., 1993) TGF-β-producing cells are more prominent in lesions of susceptible BALB/c compared with resistant C57BL/6 mice and TGF-β levels appear to correlate inversely with iNOS levels within parasitized lesions. The inhibition of iNOS and NO production mediated by increased levels of TGF-β results primarily from alteration of iNOS mRNA stability and translation. (Ding et al., 1990, Tsunawaki et al., 1988). The severity of infections observed by two other intracellular protozoan pathogens, T. cruzi and T. gondii, are increased in the presence of TGF-β. Treatment with anti-TGF-β Abs, given through the first weeks of infection, promotes resistance to infection and induces a Th1-type response. Clearly, increased production of this inhibitory cytokine by Leishmania-infected cells significantly impairs macrophage responses and promotes parasite survival. Interleukin-10 is a pleiotropic immunomodulatory cytokine produced by a wide range of cells, including monocytes and macrophages, B
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cells, thymocytes, and keratinocytes. This cytokine plays a pivotal role in the augmenting Th2-dependent immune responses and suppressing Th1-dependent CMI. IL-10 reduces the antigen-presenting activities of monocytes (Fiorentino et al., 1991), down-regulates MHC I and II molecules and co-stimulatory molecules (Chang et al., 1994) and potently suppresses many effector functions of macrophages, including the release of cytokines such as TNF-α and IL-12, and the generation of NO. (Aloisi et al., 1997). It has been reported that IL-10 also inhibits the inflammatory response in alveolar macrophages by modulating PKC activity. Similar to TGF-β, increased secretion of IL-10 induced by infection significantly enhances pathogen survival and may be an evasion strategy used by the parasite to suppress the immune response. In fact, defective activation of PKC mediated signal transduction events have been linked to the immunomodulatory role of IL-10 in visceral leishmaniasis (Bhattacharyya et al., 2001).

Normal BALB/c mice infected with Leishmania developed progressive nonhealing lesions with numerous parasites in them, but IL-10 -/- BALB/c mice controlled the infection and had relatively small lesions compared with controls. Pretreatment of macrophages with recombinant IL-10, or supernatants from amastigote-infected macrophages, resulted in a dramatic enhancement in the intracellular survival of the parasite. Results indicate that IL-10 inhibits the intracellular killing of Leishmania, and it appears that this parasite causes the increased production of this inhibitory cytokine. IL-10 mRNA expression was shown to be increased 16.5-fold in L. donovani-infected macrophages (Melby et al., 1998) and IL-10 produced by the infected macrophages prevented macrophage activation and diminished their production of TNF-α and IL-12. In humans, the severity of Leishmania infections has been most closely associated with increased levels of IL-10, and a similar relationship has been identified recently using IL-10 transgenic mice (Groux et al., 1999). Infection with Mycobacterium avium and Klebsiella pneumonia also induces the production of IL-10 and the treatment of mice with anti-IL-10 mAbs promoted resistance to infection. Recently, the up-regulation of IL-10 production induced by Leishmania amastigotes has been shown to involve ligation of host Fc receptors. Leishmania amastigotes contain host-derived IgG on their surface that allows them to ligate FcγRc on inflammatory macrophages. The ligation of these phagocytic receptors on macrophages alters their cytokine profile when these cells are exposed to inflammatory stimuli. Ligation of the FcγRc by immune complexes inhibits the production of IL-12, and can also induce the synthesis of IL-10 (Sutterwala et al., 1999). Axenic amastigotes (i.e., no IgG on their surface) failed to induce IL-10 unless they were first opsonized with immune IgG. In addition, macrophages lacking the common -γ chain of the FcγRc produced less IL-10 following infection than did normal macrophages.

**IL-10 and immunosuppression in VL**

The association of IL-10 and VL is now firmly established. Patients with active disease have elevated levels of IL-10 in serum as well as enhanced IL-10 mRNA expression in lesional tissue. IL-10 is a regulatory cytokine that can be produced by T cells, B cells, macrophages, dendritic cells (DCs) and epithelial cells. It has pleiotropic, primarily down-modulating, effects on innate as well as acquired
the induction of reactive nitrogen and oxygen metabolites (RNI and ROI). (b) In antigen-presenting cells (DCs and macrophages), IL-10 downregulates the expression of MHC class II, co-stimulatory molecules and IL-12, which will inhibit the effective generation and/or maintenance of antigen-specific Th1 cells. Moreover, IL-10 inhibits DC maturation and migration and can induce tolerogenic DCs, which produce IL-10 and promote the generation of IL-10-producing Tr1 cells. (c) IL-10 might also contribute to enhanced activation-induced T-cell death and (d) promote B-cell survival and plasma-cell differentiation. As disease progresses, B cells, which might also be a source of IL-10, and antibodies could be important contributors to VL pathology because self-reactive antibodies and immune-complex deposition might cause tissue damage. (e) Moreover, immune complexes can stimulate the macrophages and monocytes to produce IL-10 as well as proinflammatory cytokines (e.g. IL-6, TNF-α), a loop that will promote the generation of more immune complexes and more IL-10. Black arrows indicate sources of IL-10; the red lines indicate blocking/downmodulating activities of IL-10; the green arrows indicate differentiation/apoptosis promoted by IL-10. Abbreviations: ROI, reactive oxygen intermediates; RNI, reactive nitrogen intermediates. (Adapted from TRENDS in Immunology Vol.28 No.9, 2007)

in serum from VL patients enhances parasite replication in human macrophages and that blocking IL-10 reduced parasite growth. IL-10 also suppresses multiple antigen-presentation functions of DCs and macrophages. It inhibits the maturation of DCs from monocyte precursors, downregulates MHC II and co-stimulatory molecules and, perhaps most importantly, inhibits IL-12 production (Moore et al., 2001). The antigenspecific unresponsiveness in PBMCs from VL patients with respect to T-cell proliferation and IFNγ production has, in South American and Sudanese studies, been observed to be reversed by treatment with anti-IL-10 antibodies (Carvalho et al., 1994). It is not clear from these studies, however, which cells were proliferating or the source of IFNγ. Because the numbers of patients evaluated were few, the concentration of antibody used was extremely high or the study lacked adequate controls, the effect on recovery of antigen-specific T-cell responses is not so clear. In our own studies of a large series of VL patients from India, people were unable to detect antigen- driven IL-10 production by PBMCs or to recover an antigen-specific response with anti-IL-10 treatment. Further studies are clearly needed to elucidate whether antigen-specific T cells are present but suppressed in active VL, if they are lost and/or never generated appropriately or if they are recruited to the sites of infection and are therefore not detectable in the peripheral blood. Based on analyses of splenic aspirates, T cells are the main leukocyte subsets in the spleen of VL patients during the early stages of active disease, in contrast to healthy donors in whom B cells dominate.

Leishmania Modulates the Receptor Responsiveness in Macrophages

TLR Responsiveness

Of the growing number of receptors involved in the recognition of pathogen-associated molecular patterns (PAMPs) (Medzhitov et al., 2000), TLRs are considered key players of the innate immune
response (Netea et al., 2004). This family of receptors is comprised of thirteen members that recognize most of the molecular patterns on pathogens. The recognition of the ligands results in the secretion of inflammatory mediators such as TNF-\(\alpha\) and IL-12 as well as the induction of iNOS2 expression (Netea et al., 2004), leading to host protection. Following the recognition of a PAMP, the adaptor myeloid differentiation factor 88 (MyD88) is recruited to the TIR (toll-interleukin 1 receptor) domain of the TLR (Medzhitov et al., 1998). Next, IL-1 receptor-associated-kinase-1 (IRAK-1) is recruited to the complex and is phosphorylated by IRAK-4 and by autophosphorylation. IRAK-1 dissociates from MyD88 to interact with TRAF6 and activates various cascades, ultimately leading to the activation of MAP kinase pathways, the translocation of NF-\(\kappa\)B to the nucleus as well as the secretion of proinflammatory cytokines (Wesche et al., 1997, Suzuki et al., 2002). Another pathway, termed “MyD88-independent”, is implicated in signaling following engagement of TLR3 and TLR4. This cascade uses TRIF as an adaptor protein and allows the translocation of NF-\(\kappa\)B to the nucleus and the activation of MAP kinase pathways with a slower kinetics as well as the activation of IP-10 and IFN-\(\alpha/\beta\) via the activation of IRF3.

Substantial studies demonstrated that different receptors mediate the uptake and phagocytosis of *Leishmania* spp. by macrophages, although the initial signaling events are unknown. As LPG of *Leishmania* promastigotes interacts with NK cell-expressed TLR2, it is possible that the *Leishmania* LPG may interact with the macrophage expressed TLR2 and modulates cellular functions to ensure its survival within the host cell. For example, *L. major* induced IL-1\(\alpha\) expression was substantially decreased in MyD88-deficient mice. Similarly, the genetically resistant C57BL/6 mice became susceptible to *Leishmania* parasite in absence of MyD88 due to increased level of IL-4 and decreased level of IFN-\(\gamma\) and IL-12p40 (Muraille et al., 2003). Furthermore, silencing of TLR2, TLR3, IRAK-1, and MyD88 expression by RNA interference also revealed the involvement of both TLR2 and TLR3 in the production of NO and TNF-\(\alpha\) by macrophages in response to *L. donovani* promastigotes (Flandin et al., 2006). TLR2-mediated responses are dependent on Gal\(\beta\)1, 4Man\(\alpha\)-PO\(_4\) containing phosphoglycans, whereas TLR3-mediated responses are independent of these glycoconjugates. TLR3 also plays a role in the leishmanicidal activity of the IFN-\(\gamma\)-primed macrophages. It is quite possible that *Leishmania* may modulate MyD88 expression and recruitment to TLRs resulting in altered TLR responsiveness of the infected macrophages. An impaired resistance to *L. major* was also reported in TLR4-deficient mice. Compared to wild type controls, the growth of parasites in the cutaneous lesions was drastically increased in mice from a resistant background carrying a homozygous mutation of the tlr 4 gene (TLR4 e/e) as early as one day after inoculation of *L. major*. Later in the infection, an enhanced arginase activity leads to the production of compounds essential for parasite proliferation in macrophages and its increase in mutant mice indicating that TLR4 signaling could enhance the microbicidal activity of macrophages harboring parasites (Kropf et al., 2004). Results from studies comparing TLR4 deficient mice with TLR4 and IL-12\(\beta\)2 double deficient mice suggested an IL-12 independent role of TLR4 in anti-Leishmania immunity. The IL-12 dependent NK cell IFN-\(\gamma\) response was severely compromised in TLR9-deficient mice as well. In studies with *L. infantum* infection, in mature dendritic cells- (mDCs-) depleted mice, the IFN-\(\gamma\) response was abolished due to low IL-12
production that could be rescued by CpG and IL-12. *L. major* is also shown to modulate TLR9 signaling for activating NK cells. Likewise, *L. donovani* infection caused suppression of TLR2- and TLR4-stimulated IL-12p40, with an increase in IL-10 production in cells of monocyte/macrophage lineage by suppressing p38MAPK phosphorylation and activating ERK-1/2 phosphorylation through a contact-dependent mechanism (Chandra et al., 2008). These studies imply how *Leishmania* modulates the TLR responsiveness that might help their survival in macrophages.

### Role of miRNA in regulating TLR signaling

Toll-like receptor (TLR) signalling must be tightly regulated to avoid excessive inflammation and to allow for tissue repair and the return to homeostasis after infection and tissue injury. MicroRNAs (miRNAs) have emerged as important controllers of TLR signaling. Several miRNAs are induced by TLR activation in innate immune cells and these and other miRNAs target the 3′ untranslated regions of mRNAs encoding components of the TLR signaling system. miRNAs are also proving to be an important link between the innate and adaptive immune systems, and their dysregulation might have a role in the pathogenesis of inflammatory diseases. The signalling molecules that comprise each TLR signaling pathway are regulated by numerous mechanisms, including physical interactions, conformational changes, phosphorylation, ubiquitylation and proteasome mediated degradation. A more energy-efficient way to regulate the activity of TLR signaling molecules could be to destabilize the mRNA molecules that encode them. miRNA-mediated control of the expression of these signaling molecules — through either mRNA decay or translational inhibition — might not be as rapid as control through proteasomal degradation; however, this might be an advantage during infection, as miRNA mediated control of mRNA levels allows for a strong initial immune response that is gradually dampened down. However, it must be noted that for miRNA mediated targeting of the mRNAs encoding TLR signaling molecules to be effective, the signaling protein itself must also be targeted by a complementary method: either, it must be sufficiently unstable such that by the time it has passed one half-life no newly synthesized protein is available to take its place, or it must also be targeted for degradation by separate or complementary signaling mechanisms. The end result of the TLR signaling pathways is the activation of pro-inflammatory transcription factors that enhance the transcription of RNA polymerase II sensitive genes such as those encoding cytokines, chemokines and antimicrobial enzymes. Because miRNAs are also transcribed by RNA polymerase II (Cai et al., 2004, Lee et al., 2004), it stands to reason that miRNAs themselves are targets of TLR signalling pathways. miRNAs might provide a link between innate and adaptive immune signaling pathways and they might also have a role in controlling the switch from a strong early pro-inflammatory response to the resolution phase of the inflammatory process.

**Induction of miRNAs by TLR signalling**

Many studies have addressed the hypothesis that TLR signaling can modulate miRNA expression using various profiling techniques. Although a subset of miRNAs has emerged as strong targets of TLR
signaling, subtle differences in miRNA expression profiles have been observed depending on the TLR stimulus used, treatment time, technology used and, importantly, the cell type. TABLE 1 summarizes the results of these profiling experiments and lists those miRNAs that have been confirmed to be regulated by TLR signaling in independent studies. Multiple miRNAs are induced in innate immune cells, with a consensus emerging that miR-155, miR-146 and miR-21 are particularly ubiquitous. There is also evidence that the expression of certain miRNAs can decrease following TLR activation. Similar to other TLR-responsive genes, miRNAs can be classified as early or late response genes: some miRNAs (for example, miR-155) are highly induced 2 hours after treatment, whereas other miRNAs (for example, miR-21) are induced at later times. The expression of most TLR-responsive miRNAs described so far depends on nuclear factor-κB (NF-κB) activity. In all cases of miRNA induction by TLR activation that have been described so far, the transcription of miRNA primary transcripts is upregulated, although it remains possible that the processing of miRNA precursors could also be upregulated by TLR signaling (Ruggiero et al., 2009). Similar to other TLR responsive genes, it is also important that the induction of TLR-responsive miRNAs is regulated and mechanisms are now being discovered that negatively regulate miRNA induction by TLR signaling; for example, miR-155 in particular is subject to negative regulation by IL-10. Less is known about how TLR signaling can decrease miRNA expression, this could be through transcriptional repression or through post-transcriptional mechanisms that destabilize miRNA transcripts, and these areas are being actively investigated.

**Table 1: miRNAs Regulated by TLRs**

<table>
<thead>
<tr>
<th>miRNA</th>
<th>TLRs</th>
<th>Signalling molecules</th>
<th>Cell type</th>
<th>Other miRNA inducers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upregulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-155</td>
<td>TLR2, TLR3, TLR4, TLR9</td>
<td>MYD88, TRIF, JNK, AP1, NF-κB, KSRP</td>
<td>BMDMs, THP1 cells, monocytes, macrophages, DCs, B cells, TReg cells</td>
<td>Helicobacter pylori, KSHV, EBV, oxidized LDL, TNF, IFNβ</td>
</tr>
<tr>
<td>miR-146</td>
<td>TLR2, TLR3, TLR4, TLR5</td>
<td>MYD88, NF-κB</td>
<td>THP1 cells, macrophages, BMDMs, T cells</td>
<td>EBV, VSV, RIG-I, TNF, IL-1</td>
</tr>
<tr>
<td>miR-132</td>
<td>TLR4, TLR9</td>
<td>ND</td>
<td>THP1 cells, human monocytes and macrophages, BMDMs, splenocytes</td>
<td>KSHV</td>
</tr>
<tr>
<td>miR</td>
<td>TLR(s)</td>
<td>Regulation Factors</td>
<td>Cell Type</td>
<td>Pathogen</td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
<td>--------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>miR-21</td>
<td>TLR4</td>
<td>MYD88, TRIF, NF-κB</td>
<td>Inflamed lung tissue, RAW264.7 cells, BMDMs, B cells, H69 cholangiocytes</td>
<td>Cryptosporidium parvum, EBV (LMP1)</td>
</tr>
<tr>
<td>miR-223</td>
<td>TLR4</td>
<td>ND</td>
<td>Inflamed lung tissue, DCs</td>
<td>ND</td>
</tr>
<tr>
<td>miR-147</td>
<td>TLR2, TLR3, TLR4</td>
<td>MYD88, TRIF, NF-κB, IRF3</td>
<td>BMDMs, RAW264.7 cells, THP1 cells, alveolar macrophages</td>
<td>ND</td>
</tr>
<tr>
<td>miR-9</td>
<td>TLR2, TLR4, TLR7–TLR8</td>
<td>MYD88, NF-κB</td>
<td>Human monocytes and granulocytes</td>
<td>IL-1</td>
</tr>
<tr>
<td>miR-125b</td>
<td>TLR4</td>
<td>NF-κB</td>
<td>H69 cholangiocytes, rheumatoid arthritis synovial fibroblasts, LPS-tolerized THP1 cells</td>
<td>Cryptosporidium parvum</td>
</tr>
<tr>
<td>let-7e</td>
<td>TLR4</td>
<td>AKT1</td>
<td>Peritoneal macrophages</td>
<td>ND</td>
</tr>
<tr>
<td>miR-27b</td>
<td>TLR4</td>
<td>NF-κB</td>
<td>Human macrophages</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Downregulated**

<table>
<thead>
<tr>
<th>miR</th>
<th>TLR(s)</th>
<th>Regulation Factors</th>
<th>Cell Type</th>
<th>Pathogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-125b</td>
<td>TLR4</td>
<td>NF-κB, AKT1</td>
<td>Splenocytes, BMDMs, DCs</td>
<td>ND</td>
</tr>
<tr>
<td>let-7i</td>
<td>TLR4</td>
<td>NF-κB, C/EBPβ</td>
<td>H69 cholangiocytes</td>
<td>Cryptosporidium parvum</td>
</tr>
<tr>
<td>miR-98</td>
<td>TLR4</td>
<td>ND</td>
<td>H69 cholangiocytes</td>
<td>Cryptosporidium parvum</td>
</tr>
</tbody>
</table>
Targeting of TLR signalling pathways by miRNAs

Targeting TLR expression

The most obvious point at which to manipulate the TLR signaling pathway is at the level of receptor expression. Because TLR signaling induces a strong pro-inflammatory response, the expression of these receptors is restricted to certain cell lineages, including macrophages, dendritic cells (Dcs) and B and T cells (Rock et al., 1998). Furthermore, the expression of particular TLRs is restricted to specific cell types to adapt these cells for particular functions (Jarrossay et al., 2001). In addition, the expression of particular miRNAs in mammals has been shown to be limited to particular cell types, which indicates that miRNAs might have a role in controlling cell differentiation and cell-specific functions (Chen et al., 2004). An attractive hypothesis is that the differences in TLR distribution between different immune cell types could be the result of differential miRNA expression. However, so far, there is little evidence that TLRs themselves are directly targeted by miRNAs. Bioinformatic analysis of the 3’ untranslated regions (3’ UTRs) of human TLR mRNAs using the prediction program Targetscan shows that TLR-encoding genes have very few highly conserved target sites for miRNAs (review). It is possible that TLR genes can be targeted by miRNAs through weaker, non-conserved sites. A recent study20 that used a refined bioinformatic algorithm to predict active miRNA target sites in the 3’ UTRs of TLR and related genes showed that the myeloid-specific miRNA miR-223 (Chen et al., 2004), which has an important role in granulopoiesis (Chen et al., 2004) a strong candidate for regulating both TLR4 and TLR3 expression; TLR3 has been shown to be expressed at low mRNA levels in granulocytes (Muzio et al., 2000) possibly owing to increased levels of miR-223 in these cells. This implies that in resting cells, miRNA activity might regulate the potential of innate immune cells to respond to TLR activators. The mRNA encoding TLR4 is regulated by members of the let-7 miRNA family. In mouse peritoneal macrophages, the induction of let-7e expression decreases cell surface expression of TLR4, the mRNA of which contains a let-7 target site (Androulidaki et al., 2009). Furthermore, transfection of macrophages with antisense miRNA to let-7e leads to an increased lipopolysaccharide (LPS)-induced cytokine response (Androulidaki et al., 2009). Downregulation of let-7i expression was shown to increase TLR4 expression by human cholangiocytes (biliary epithelial cells) after Cryptosporidium parvum infection or LPS treatment.

TLR2 mRNA has been shown to be regulated by miR-105, the expression of which is higher in oral keratinocytes derived from patients who respond weakly to TLR2 activation with low levels of cytokine induction, presumably owing to decreased TLR2 expression; this indicates that there might be a reciprocal relationship between TLR2 signaling and miR-105 expression. Although these data point to the regulation of certain TLRs by miRNAs, the absence of data in support of other TLRs being targeted by miRNAs underscores the importance of constitutive TLR expression.

Rather than shutting down the TLR signaling pathway completely by eliminating receptor expression, the trend for miRNA activity seems to be to tone down TLR activity through targeting key intracellular signaling proteins

Targeting TLR signaling proteins
The molecular targets of miR-146 are IL-1R-associated kinase 1 (IRAK1) and TNFR-associated factor 6 (TRAF6) (Taganov et al., 2006). These proteins are important components of the myeloid differentiation primary-response protein 88 (MYD88)-dependent pathway for NF-κB activation downstream of TLR2, TLR4, TLR5, TLR7–TLR9, which are the same TLRs that induce expression of miR-146 in the THP1 cell line. It was postulated that miR-146 can negatively regulate the MYD88–NF-κB signaling pathway after bacterial infection (Taganov et al., 2006). Recently, IRAK2, a kinase that is required for the persistence of NF-κB activation, has also been shown to be targeted by miR-146 (Hou et al., 2009), although the relevance of this observation for TLR signaling remains unclear. There is mounting evidence that miR-155 can negatively regulate TLR signaling pathways by targeting key signaling proteins. Inhibition of miR-155 in DC’s resulted in upregulated expression of components of the p38 mitogen-activated protein kinase (MAPK) pathway (Ceppi et al., 2009). TAK1-binding protein 2 (TAB2), a signaling molecule downstream of TRAF6 that activates mAPK kinases, was confirmed as a direct target of miR-155 (Ceppi et al., 2009). MYD88 has also been identified as a target of miR-155 in studies of miR-155 induction by Helicobacter pylori. Furthermore, MYD88 is a target of miR-155 in foam cells, which induce the expression of miR-155 when loaded with oxidized low-density lipoprotein. Another Toll/IL-1R domain-containing adaptor protein, MYD88 adaptor-like protein (MAL; also known as TIRAP), which functions as a bridging adaptor for TLR2- and TLR4-mediated MYD88-dependent signaling, has emerged as a target of miR-145. It remains to be determined whether the expression of miR-145 is also regulated during TLR2 or TLR4 signalling. However, it is known that MAL undergoes proteasomal degradation following TLR2 and TLR4 stimulation (Mansell et al., 2006). Therefore, perhaps an additional level of control of MAL expression exists through miR-145.

Molecules that are targeted by miRNAs is that relatively few proteins have been confirmed as direct targets of miRNAs (specifically, MYD88, MAL, IRAK1, IRAK2, TRAF6, BTK and TAB2; TABLE 2). However, these proteins are components of several TLR signaling pathways, which indicates that once one TLR is triggered, miRNA mediated targeting of common signaling proteins could silence signaling through multiple TLRs. Because most pathogens can engage several TLRs, miRNAs could help to avoid excessive pro-inflammatory responses after pathogen exposure by shutting down several TLR pathways. It is probable that miRNAs work together with multiple other mechanisms to control the expression of TLR signaling components. The combination of these mechanisms could result in timely and appropriate toning down and termination of the pro-inflammatory response (FIG. 16).
Figure 16: miRNAs function with other mechanisms to control the expression of TLR signaling components.

Targeting transcription factors

The targeting of transcription factors by miRNAs would have a global impact on TLR-induced gene expression. Many studies have highlighted the fact that miRNA-mediated targeting of transcription factors is an important aspect of miRNA function (Chen et al., 2007, Martinez et al., 2009). Higher basal levels of miRNAs in certain cell types might function as important epigenetic switches required for the functional maintenance of the cell type. For example, forkhead box P3 (FoXP3), a transcription factor that is required for the maintenance of regulatory T cells, was shown to drive the high level of miR-155 expression found in these cells; miR-155 then feeds back and targets FoXP3 to decrease its expression. More generally, transcription factors are usually expressed at low levels in cells, which might be the result of strict control by miRNAs. Evidence is emerging that the pro-inflammatory transcription factors activated by TLR signaling are targeted directly by miRNAs. NF-κB activity is mainly controlled by inhibitor of NF-κB kinases (IKKs). IKKα was recently shown to be targeted by a subset of miRNAs including miR-223 (Li et al., 2010) and IKKβ is targeted by miR-199 in human ovarian cancer cells38. However, an effect of these miRNAs on TLR signaling was not directly examined in these studies. Analysis of both miR-155 and its Kaposi's sarcoma-associated herpesvirus (KsHv) homologue has identified IKKα as a potential target, which supports the notion that miR-155 can negatively regulate innate immune signalling (Gottwien et al., 2007, Xiao et al., 2009). Recently, the TLR responsive miRNA miR-9 was shown to directly target NFKB1 mRNA (Bazzoni et al., 2009). NF-κB1 is cleaved to form the NF-κB p50 subunit, which has an important role in transactivation of the NF-κB p65 subunit. Therefore, the finding that miR-9 can target the NFKB1 gene identifies a key control point for TLR signaling. Other transcription factors downstream of TLR signaling have been identified as miRNA targets.
The transcriptional co-repressor CCAAT/enhancer binding protein-β (c/EBPβ) has been identified as a target of miR-155 by the analysis of B cells that constitutively express miR-155 or mice that had received antisense miR-155. The targeting of c/EBPβ by miR-155 was shown in these studies to decrease expression levels of granulocyte colony-stimulating factor (G-CSF), and possibly IL-6, in splenocytes. Peroxisome proliferator-activated receptor-γ (PPARγ) has anti-inflammatory effects and its expression is decreased after LPS treatment. This was shown to be the result of NF-κB-dependent induction of miR-27b, which directly targets Pparg mRNA; the inhibition of miR-27b resulted in increased PPARγ expression and blunted LPS-induced secretion of tumor necrosis factor TNF. Finally, the transcriptional co-activator p300, which is often associated with cAMP-responsive element-binding protein (cREB) and is required for the induction of antiviral genes, was shown to be a direct target of miR-132 in KSHV-infected lymphatic endothelial cells (Lagos et al., 2010). Multiple transcription factors can therefore be controlled by miRNAs, providing a direct mechanism to control the transcription of TLR-responsive genes.

Table 2: Verified Targets in TLR Signalling.

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>miRnA(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptors</td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>miR-223, let-7i, let-7e</td>
</tr>
<tr>
<td>TLR3</td>
<td>miR-223</td>
</tr>
<tr>
<td>TLR2</td>
<td>miR-105</td>
</tr>
<tr>
<td>Signalling molecules</td>
<td></td>
</tr>
<tr>
<td>MYD88</td>
<td>miR-155</td>
</tr>
<tr>
<td>MAL</td>
<td>miR-145</td>
</tr>
<tr>
<td>IRAK1</td>
<td>miR-146</td>
</tr>
<tr>
<td>IRAK2</td>
<td>miR-146</td>
</tr>
<tr>
<td>TRAF6</td>
<td>miR-146</td>
</tr>
<tr>
<td>BTK</td>
<td>miR-348</td>
</tr>
<tr>
<td>TAB2</td>
<td>miR-155</td>
</tr>
<tr>
<td>IKKα</td>
<td>miR-223</td>
</tr>
<tr>
<td>IKKβ</td>
<td>miR-199</td>
</tr>
<tr>
<td>IKKε</td>
<td>miR-155</td>
</tr>
<tr>
<td>Transcription factors</td>
<td></td>
</tr>
<tr>
<td>NF-κB1</td>
<td>miR-9</td>
</tr>
<tr>
<td>FOXP3</td>
<td>miR-155</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>miR-155</td>
</tr>
<tr>
<td>PPARγ</td>
<td>miR-27b</td>
</tr>
<tr>
<td>p300</td>
<td>miR-132</td>
</tr>
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</table>
Bioinformatic analysis has identified several miRNA-binding sites in cytokine- and chemokine-encoding mRNAs. Of note, IL6 mRNA contains a binding site for let-7; given the fact that let-7 family members can be negatively regulated by TLR signalling and NF-κB activation, this could potentially contribute to the increased IL-6 expression observed following TLR stimulation, although this has not been examined directly. Similarly, the 3’ UTR of TNF mRNA contains a binding site for the LPs-downregulated miRNA miR-125b, which indicates a mechanism by which TLR signaling might stabilize TNF expression (Tili et al., 2007). IL10 mRNA contains binding sites for 8 miRNAs in its 3’ UTR and overexpression of two of these — miR-106a and miR-106b— resulted in decreased IL-10 protein expression in a human Burkitt’s lymphoma Raji cell line (Sharma et al., 2009). However, a role for TLR signaling in the induction of miR-106a and miR-106b expression was not explored. mRNA encoding the IL-12p35 subunit contains a target site for miR-21 (Lu et al., 2009), as confirmed by reporter assays in which the 3’ UTR of the gene encoding p35 was linked to the luciferase gene, although the extent to which this might contribute to TLR responses remains undetermined. Although evidence for the direct targeting of cytokine mRNAs by miRNAs is limited, it is increasingly apparent that miRNAs can function together with RNA-binding proteins to regulate mRNA expression through the AU-rich elements (AREs) (Fig 17) that are found in numerous cytokine-encoding mRNAs. For example, TNF and IL10 mRNAs both contain long AREs that are targeted by the RNA-binding protein tristetraprolin (TTP), which has a key role in mRNA destabilization downstream of TLR signaling. miR-16 cooperates with TTP to mediate TNF destabilization, although this miRNA has not yet been shown to be TLR responsive (Jung et al., 2005). More relevant was a recent study (Gazar et al., 2010) that showed that miR-221, miR-579 and miR-125b are expressed following the induction of a state of LPS tolerance, during which TNF mRNA is degraded.

**Cytokines**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>MiRNA Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>let-7</td>
</tr>
<tr>
<td>TNF</td>
<td>miR-16, miR-125b, miR-155, miR-221, miR-579, miR-369-3</td>
</tr>
<tr>
<td>IL-10</td>
<td>miR-106, miR-466l</td>
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<tr>
<td>IL-12p35</td>
<td>miR-21</td>
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**Regulators**

<table>
<thead>
<tr>
<th>Regulator</th>
<th>MiRNA Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACHE</td>
<td>miR-132</td>
</tr>
<tr>
<td>PDCD4</td>
<td>miR-21</td>
</tr>
<tr>
<td>SHIP1</td>
<td>miR-155</td>
</tr>
<tr>
<td>SOCS1</td>
<td>miR-155</td>
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</tbody>
</table>
found to associate with TTP and to accelerate TNF mRNA decay, and miR-579 and miR-125b seemed to block TNF translation, possibly through recruitment of the translational inhibitor TIAR. However, it should also be noted that some of these effects could be mediated directly by the upregulation of miR-125b expression, which might destabilize TNF by direct binding (Tili et al., 2007). Conversely, miRNAs can also compete with RNA binding proteins to protect mRNA from destabilization. For example, miR-466l contains a seed region that is complementary to the canonical ARE ‘AUUUA’ sequence. Transfection of LPS-stimulated RAW264.7 macrophages with miR-466l resulted in the upregulation of IL-10 mRNA and protein expression by competing with TTP for binding to the ARE sequence in Il10 mRNA, which protected the mRNA from TTP-mediated degradation (Ma et al., 2010). In addition to the recruitment of specific RNA-binding proteins, mRNA stability can also depend on environmental factors. For example, miR-369-3, which associates directly with the ARE in TNF mRNA by base-pairing, could mediate translational activation of TNF only under conditions of serum starvation, and this activation depended on recruitment of the RNA-binding proteins fragile-X mental retardation-related protein 1 (FXR1) and argonaute 2 (AGO2). By contrast, miR-369-3 could repress TNF when the cells were actively proliferating. It has not been yet been investigated whether the activation of TLRs could affect the ability of a miRNA to degrade or stabilize mRNA sequences, but this possibility warrants further investigation. Although a direct binding site for miR-155 in the TNF mRNA has not been identified, miR-155 might be required for its stabilization, as miR-155-deficient B cells fail to produce TNF. Furthermore, a role for miR-155 in TNF mRNA stabilization has been shown in HEK293 cells and miR-155-transgenic mice have increased levels of circulating TNF after LPs injection. Although an exact mechanism has yet to be elucidated, it is possible that RNA-binding proteins might be involved. It will be interesting to determine whether other TLR-responsive cytokines are regulated by miRNAs.
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