Kala Azar or Black Fever has existed in India and China for centuries, but its cause was not discovered until 1900 when William Leishman, a Scottish army doctor, found *Leishmania donovani* in stained smears from the spleen of a soldier suffering from a fever contracted at Dum-Dum in India. His observations were published in 1903. At the same time Charles Donovan, a Professor of Physiology at Madras University, described a similar parasite in smears made from a splenic biopsy. Prior to Leishman and Donovan's discovery kala azar was considered to be a communicable malaria-like disease that showed relapses, emaciation, as well as enlargement of the liver and spleen, and spread slowly across the continents along the trade routes.
In 1924 the Kala-Azar Commission noted that the distribution of a sandfly (*Phlebotomus argentipes*) in India closely overlapped the distribution of the disease. But directly demonstrating transmission by sandflies was more difficult until 1939 when Smith, Haldar and Ahmed made the discovery that if sandflies after taking a bloodmeal were fed on raisins instead of being given additional blood meals, the flagellates grew so numerous that they blocked the pharynx as happens with plague bacilli in fleas. These workers then subjected hamsters to the bite of blocked sandflies and each became infected. Human transmission of leishmaniasis was demonstrated in 1941 when Adler and Ber successfully infected “volunteers” with *L. major* by the bite of *P. papatasi*, and in 1942 Swaminath, Shortt and Andersen allowed 6 human “volunteers” to be bitten by infected *P. argentipes* and all developed kala-azar.
The insect vector

Sandflies (*Phlebotomus* in Old World and *Luzotomia* species in New World) are “pool feeders”. The female sand flies insert their saw-like mouthparts into the skin and agitate them to produce a small wound into which blood flows from capillaries. This tissue damage releases skin macrophages and freed *Leishmania* amastigotes into the pool of blood, which are taken up into the fly posterior midgut. The *Leishmania* then develop into several distinct developmental stages as they migrate anteriorly to the stomodeal valve, which forms a junction with the foregut.

Takes 6-9 days to complete their development.
Life cycle of *Leishmania* in Sandfly – Coevolution of vector and host

1. The amastigotes transform into weakly motile “procyclic promastigotes”. These replicate in the “bloodmeal”.
2. Within 4 hours, the bloodmeal is enclosed by the peritrophic matrix (PM) – a chitin and protein mesh secreted by the midgut. The PM protects the promastigotes from digestive enzymes until the parasites transform to resistant nectomonads.
3. *L. major* secretes a neuropeptide that arrests hindgut peristasis and prevents the cells from being excreted.
4. Within 48-72 hrs the PM is degraded by chitinases (some from the parasite), releasing the elongated very motile non-replicating nectomonad promastigotes. These migrate towards the anterior midgut, some attaching to the microvilli, until they reach the stomodeal valve.
5. The nectomonads transform into leptomonad promastigotes that resume replication. These secrete the promastigote secretory gel (PSG). The PSG contains a high MW filamentous proteophosphoglycan which fills the anterior midgut into the foregut.
6. Some transform into non-replicating metacyclic promastigotes.
7. The fly regurgitates and expells the PSG and the metacyclic promastigotes.
Developmental Stages of *Leishmania* in Sand Fly

(a) amastigote

(b) promastigote

*abdominal midgut* the bloodmeal phase

*thoracic midgut & foregut* the suggarmal meal phase
Fig. 1. Developmental profiles of *Leishmania mexicana* and *Leishmania infantum* in vivo and in vitro. Flies were infected with tissue amastigotes of *L. mexicana* (A) or *L. infantum* (B) and the relative proportions of amastigotes (■ - ■), procyclic promastigotes (Δ-Δ), nectomonad promastigotes (▲ - ▲), leptomonad promastigotes (○ - ○), and metacyclic promastigotes (● - ●) determined. Similarly, in vitro cultures were initiated with tissue amastigotes of *L. mexicana* (C) or *L. infantum* (D). Each experiment was repeated and the results combined to yield the data shown.
Promastigote secretory gel (PSG)

The plug must be partially egested before blood feeding can occur, thereby injecting both metacyclic promastigotes and PSG into the skin of the mammalian host. (b) Detail of the anterior midgut and foregut. The PSG plug (shaded) forces the stomodeal valve open and extends into the pharynx region. Metacyclic promastigotes (stippling) are concentrated at the anterior pole of the plug but are found along the foregut in both the cibarium and proboscis.
Role of sandfly saliva and parasite PSG in exacerbation of disease

Saliva:
Required for blood feeding activity since it has potent vasodilatory and antihaemostatic properties. Modulates the immune response to favor parasite survival and replication

PSG:
Leads to increase in pathology and parasite numbers when co-inoculated with metacyclic promastigotes.
Leishmaniasis

- **Leishmaniasis** - Zoonotic infection affecting 12 million people worldwide in 88 countries.
  - Annual incidence is 1.5 million with the cutaneous form and 0.5 million with the visceral form.

- **Old World cutaneous leishmaniasis** - *L. tropica* - in urban areas of the Mediterranean basin, the Middle East, Pakistan and parts of India.
- **Cutaneous leishmaniasis** - *L. major* - in rural areas, usually dry desert regions of Central Asia, Southern Russia, Middle East and Africa.
- **New World cutaneous leishmaniasis** - *L. braziliensis* and *L. mexicana* - in Mexico and central and South America.
- **Visceral leishmaniasis** - >80 countries in Africa, Asia, southern Europe (*L. infantum*) and S. America (*L. chagasi*). About 90% of the 500,000 new cases each year occur in 5 countries: Bangladesh, India, Sudan, Nepal, Brazil. India alone contributes to ~50% of all cases in the world.

- **Leishmania/HIV** co-infection is considered to be a real “emerging disease”, especially in southern Europe, where 25-70% of adult VL cases are related to HIV infection, and 1.5-9.5% of AIDS cases suffer from newly acquired or reactivated VL
CUTANEOUS LEISHMANIASIS:
Old World Cutaneous Leishmaniasis (CL)

*L. major*

Causes a moist, cutaneous, ulcerlike lesion at the site of the bite; it starts as a papule that runs an acute course (1-3 weeks) with early ulceration and a surrounding zone of inflammation, that usually heals in two months to a year leaving a depressed unpigmented scar, and lasting immunity.

It is transmitted by *Phlebotomus spp.* from gerbil, dogs or rodents to human or human-to-human. It is generally found in sparsely populated rural areas.
**L. tropica**

causes a dry cutaneous lesion that persists for months before ulcerating; it is also called Oriental sore, Delhi boil, Baghdad boil, Aleppo button, Jericho boil. The disease is characteristically found in more densely populated urban areas. Reservoir = dogs.

**L. aethiopica**

Causes a diffuse cutaneous form of disease (DCL).

Reservoir = Hyrax
New World Cutaneous Leishmaniasis

*L. mexicana*

The ulcer usually heals spontaneously in a few months. However, when the bite occurs on the ear it results in chronic lesions known as chiclero's ulcer. Because the cartilage of the ear pinna is poorly vascularized the immune response is weak, and in 40% of cases the result is mutilation of the pinna. Found principally in Central America and Mexico where it occurs in the forest dwelling people who harvest latex from the chicle trees to be used in the manufacture of chewing gum. In Belize it is also known as *bay sore*. 
Animal Reservoirs

202 Rodent reservoirs of Brazilian rain forest. Most cutaneous leishmaniasis in the New World is a zoonosis associated with rodents of the rain forest. Rodents such as Proechimys guyanensis are typical animal reservoirs for forest leishmaniasis in both Brazil and Central America. L. mexicana amazonensis has been isolated from this species. The Brazilian rain forest is the typical habitat of L. m. amazonensis.

203 Animal reservoirs in Panama. In Panama a wide variety of animals serve as reservoirs for L. b. panamensis. The hosts include species of monkeys, marmosets, and even such bizarre animals as the three-toed sloth shown here.
Muco-cutaneous leishmaniasis

*L. braziliensis*

Inoculation of promastigotes by the bite of a sandfly results in a small, red, skin papule that is itchy and ulcerates in 1-4 weeks as in the case of cutaneous leishmaniasis; this ulcer usually heals spontaneously within 6-15 months.

However, there is metastatic spread of the promastigotes from the site of the bite via the lymphatics. Disease is called espundia; the metastatic lesion involves the nasal and buccal mucosa causing destruction and malformations of the cartilage and soft tissues. The ulcerations can involve the nose, pharynx, palate and lips resulting in camel nose or parrot beak. Invasion of the larynx may result in a loss of speech. In Brazil 1/3 of the espundia cases are in children. May take many years for it to spread from the initial site of bite. Death may occur from secondary infections or respiratory complications.
Mucocutaneous leishmaniasis, Peru 1983
**L. guyanensis:**
In Uruguay and Venezuela, the disease is called *pian bois*, and the lesions are flattened ulcerated plaques that remain open and oozing all over body. Similar to wet lesions of *L. major*. 

*Figure 4–12. Cutaneous leishmaniasis. A. Lesions of outer nose and corner of eye due to *L. braziliensis guyanensis*. B. “Wet” lesion of arm caused by *L. major* acquired in Senegal.*
In some patients, especially in Venezuela, a diffuse cutaneous form develops which is characterized by a macrophage granuloma and thickening of the skin. This condition looks like lepromatous leprosy and sometimes like fungal disease, and is often misdiagnosed. Probably due to a failure of immune response. May be due to *L. mexicana*.
*L. amazonenesis* (diffuse cutaneous leishmaniasis or DCL)

Skin smear

*L. peruviana* = uta.

*L. panamensis*

Reservoir: dogs, sloths, monkeys

Vector: *Lutzomyia* species.
VISCERAL LEISHMANIASIS

*L. donovani* causes the classic type found in India. This is a metastatic disease. Parasites are only occasionally seen in blood, but are present in the spleen and lymph nodes. The incubation period is 1-4 months. Disease is characterized by fever, anemia, splenomegaly, wasting, imbalance of serum proteins (A/G ratio is reversed) and hyperpigmentation of the skin. The death rate is very high if left untreated. *L. infantum* causes the Mediterranean form of kala azar and has dogs, jackals and foxes as reservoirs. Humans are accidental hosts.
Role of host immune response

Similar to leprosy, leishmaniasis is a disease in which the clinical diversity reflects a complex interplay between the virulence of the infecting species and the host's immune response.

• **Localized cutaneous disease**
  A vigorous immune response, with most cases resolving without intervention. This form of disease exhibits a helper T-cell subtype 1 (TH1) immune response, with interleukin-2, interferon-gamma, and interleukin-12 as the prominent cytokines that induce disease resolution.

• **Visceral or diffuse cutaneous disease**
  Patients exhibit relative anergy to the *Leishmania* organism and have a prominent helper T-cell subtype 2 (TH2) cytokine profile.
Summary of the clinical aspects of Leishmaniasis

- **Localized cutaneous leishmaniasis (LCL):** Crusted papules or ulcers occur several weeks to months (and in rare cases) after sandfly bite inoculation on exposed skin. Lesions usually heal spontaneously.
- **Diffuse cutaneous leishmaniasis (DCL):** Analogous to lepromatous leprosy, individuals with DCL cannot mount a cell-mediated immune response to the *Leishmania* parasite. Consequently, patients develop multiple, widespread cutaneous papules and nodules, and they are anergic to leishmanin skin testing (LST).
- **Recidivans cutaneous leishmaniasis (RCL):** A relatively uncommon clinical variant of leishmaniasis, RCL appears as a recurrence of lesions at the site of apparently healed disease years after the original infection. RCL lesions typically occur on the face, and RCL presents as an enlarging papule, plaque, or coalescence of papules that heals with central scarring. Relentless expansion at the periphery may cause significant facial destruction similar to the lupus vulgaris variant of cutaneous tuberculosis.
- **Post-kala azar dermal leishmaniasis (PKADL):** Endemic to India and the Sudan, this form of leishmaniasis develops months to years after the patient’s recovery from VL. Cutaneous lesions demonstrate great variability, ranging from hypopigmented macules to erythematous papules and from nodules to plaques. As in leprosy, the wide clinical spectrum of PKADL reflects the immune response of the individual to the *Leishmania* organism. Lesions may be numerous and persist for decades. Isolated parasites from the lesions are identical to those causing the original visceral disease.
- **Mucocutaneous leishmaniasis (MCL):** Predominantly a New World disease, this form of leishmaniasis may not manifest clinically until years after localized cutaneous disease apparently has healed. In a poorly understood manner, certain species of *Leishmania* migrate to the upper respiratory tract where relentless destruction of the oropharynx and nose ensues. Gradually, the migration results in extensive mid facial destruction and, occasionally, in death.
- **Visceral leishmaniasis (VL) (kala azar):** *Leishmania* parasites localize to the reticuloendothelial system, rather than to the skin, and produce a potentially lethal widespread systemic disease.

A “Cocktail Moment”

Has Leishmaniasis Become Endemic in the U.S.?

Believed to be all but absent from the U.S., the Leishmania parasite has infected more than 1000 hunting dogs.

Unwanted visitor. Leishmania parasites can kill.

Fly bites dog? Normally, Leishmania is transmitted by sandflies, but it's not clear how U.S. foxhounds become infected.

KP Chang
Foxhounds infected with visceral leishmaniasis (*L. infantum*)

Foxhounds infected with *T. cruzi*
Treatment

• Kala azar is treated today essentially as it was in 1940. The major drug is Sodium stibogluconate or Pentastam, a derivative of antimony, which was developed in 1930! Severe reactions including death occur in 10% of those treated. It is very expensive, and the recommended one month treatment costs around $150. Drug resistance has also developed. Up to 70% of infected patients in India are resistant to this drug.
• Amphotericin is used with or after an antimony compound for visceral leishmaniasis unresponsive to the antimonial alone.
• Pentamidine isotionate has been used in antimony-resistant visceral leishmaniasis, but although the initial response is often good, the relapse rate is high and it is associated with serious side-effects.
• Recently a new drug was developed, miltefosine. This is a membrane signaling pathway inhibitor. This can be taken orally and is very effective against visceral leishmaniasis. In clinical trials has a 95% cure rate!
• Pentamidine is also used for New World cutaneous leishmaniasis, but it usually heals spontaneously.
• There is no treatment for muco-cutaneous leishmaniasis.
Diagnosis of Cutaneous Leishmaniasis

Microscopic detection of amastigotes (LD bodies) in stained smears made from the edges of the ulcer. Culturing parasites from the lesion can also be used in diagnosis.
The Parasite Surface

The surface of *Leishmania* promastigotes is covered by glycoproteins.
1. Galectins are beta-galactose binding family of lectins. Kamhawi et al. selected a galectin by bioinformatics from a cDNA library of sand fly midguts = PpGalec.

2. *Leishmania* promastigotes bound to recombinant PpGalec in vitro

3. Preincubation of promastigotes with antibody against PpGalec inhibited binding to fly midguts in vitro.

4. Incubation of amastigotes with anti-PpGalec decreased parasite survival in midgut in vivo after 6 days (when the fly excretes the bloodmeal remnants).

5. Metacyclic promastigotes did not bind to PpGalec - due to covering up of galactose residues by arabinose residues.
Lectins (Ricin, peanut agglutinin, PpGalectin): Bind to D-Galactose

Hence, the metacyclic promastigote LPG does not bind the PpGalectin in the midgut and out it goes.
Nectomonads bind to sand fly galactin receptor - PpGalec

Figure 2. A cluster of midgut epithelial cells of Phlebotomus papatasii showing a strong expression of PpGalec (red). Actin and nuclei are stained green and blue, respectively.
Nectomonads attached to microvilli in fly midgut
Trojan Horse Hypothesis

Metacyclic promastigotes taken up first by Neutrophils (PMN’s)
A method for silent delivery of parasites to macrophages

Promastigotes are targeted to two different compartments:

1. Highly lytic degradative compartment
2. Tight phagosomes with intact parasites

LPG not required for targeting, but is required for maintenance of parasites in non-lytic-ER derived compartments.

Apoptotic neutrophils can be engulfed by primary macrophages.
Promastigotes are taken up by phagocytosis of apoptotic neutrophils and the parasites differentiate into amastigotes inside the phagosome. This is a very hostile environment where cells taken up by the macrophage are normally destroyed by several mechanisms:

- Oxidative burst - Phagocytosis of a foreign body activates an NAD(P)H oxidase in the plasma membrane. This enzyme transfers protons to molecular oxygen, forming the highly reactive superoxide and hydroxyl radicals at the site of engulfment. These radicals react with the pathogen's phospholipid membrane and also with its macromolecules.

- Acidification - After fusion of the phagosome with the endosome, the vesicle is acidified by a proton ATPase. The low pH (4.5-5.0) causes denaturation of proteins, which become susceptible to acid hydrolases.

- Digestion - The endosome fuses with primary lysosomes, and acid hydrolases are released which degrade DNA, RNA, proteins and carbohydrates.
Phagosome-lysosome fusion in *L. donovani* infection of macrophage
Virulence factors

Virulence factors that promote colonization of the host:

1. Contact host cells
2. Adhere to host cells
3. Invade host cells
4. Compete for nutrients
5. Resist innate immune defenses such as phagocytosis and complement

Virulence factors that damage the host:

1. Membrane components that bind to host cells causing them to synthesize and secrete inflammatory cytokines
2. Parasite components that induce autoimmune responses.
3. Harmful toxins (?)
Transformation of Leishmania

1. Plasmid can replicate as episome
   or
1. Plasmid can recombine with genomic DNA

For expression of integrated resistance gene
Only need an upstream Splice Acceptor Sequence (SAS)
and a downstream Polyadenylation signal

Selection of transformants is performed by plating on agar
and selecting for drug resistance.
Double gene knockout by homologous recombination

Drug resistance markers available for *Leishmania*:

- Neomycin (Neo) R
- Hygromycin (Hygro) R
- Phleomycin (Ble) R
A digression
(or how I learned to love parasites)

Brasilian National Institute of Amazon Research in Manaus (INPA)

Spiny rat