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2019

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Dedication

To our fathers & our mother s

Acknowledgement

I am grateful to the Medical staff of Gadarif teaching hospital, certainly blood bank staff for their cooperation and kindness and professionalism way of dealing with researchers.

Also I am very thankful to my professional and creative supervisor Dr. Samar Osman for her great advices, which benefit me in research work, professional work and the entire life.

Abstract

Visceral leishmaniasis is an endemic disease in Sudan; affect numerous populations certainly in Gadarif State. Hospital based cross sectional study was conducted at Gadarif teaching hospital, 100 healthy apparent blood donors were included in the study and their blood specimens were examined microscopically by buffy coat technique and serologically by rk39 and there was no positive reported in the study. Further study must be conducted with large sample size and molecular diagnostic method (PCR) must be used.

Background

Visceral Leishmaniasis (VL), also known as kala-azar in the Indian sub-continent, is caused by the protozoan parasites *Leishmania donovani* and *Leishmania infantum* (= *Leishmania chagasii*), and is a potentially fatal disease with a worldwide distribution, in Asia, East Africa, South America and the Mediterranean region. The parasites are transmitted through the bite of female phlebotomine sand flies and in the human host are obligate intracellular parasites of the reticuloendothelial system, surviving and multiplying in different macrophage populations. In patients who develop symptoms, presentation is insidious with development of splenomegaly, irregular fevers, anaemia, pancytopaenia, weight loss and weakness occurring progressively over a period of weeks or even months. Almost all clinically symptomatic (non-immune) patients die within months if untreated. Sub-clinical infection in partially immune human carriers may be an important reservoir of infection. Other mammals, often canids, either domesticated or wild, act as an additional zoonotic reservoir of infection especially of *L. infantum*.

Visceral leishmaniasis persists today in poor, remote, and in certain politically unstable areas, where there is limited health care and patients have little access to affordable medications. Almost half of clinical cases occur in children. It is estimated that about 90% of the 50,000 to 90,000 new cases arising each year occur in the rural areas of India, Sudan, South Sudan, Kenya, Somalia, Ethiopia and Brazil, and that only 30% of cases are reported. Epidemics with high mortality in both adults and children occur, and have, in some areas, been associated with mass movements of non-immune populations from or into endemic areas, as occurred in the Sudan in 1990's, with hundreds of thousands of lives being lost. Of increasing concern is that visceral leishmaniasis co-infection with HIV is causing the disease to spread into previously unaffected areas of southern Europe, Ethiopia, and India.

Chemotherapy remains the most important element in the control of anthroponotic visceral leishmaniasis. Definitive diagnosis is invasive and is still based on demonstration of the parasites in biopsy material. Recently introduced dipstick diagnostics (antibody based) offer considerable improvement in case finding and epidemiology but have a limited role in determining drug efficacy and cure. In all areas visceral leishmaniasis control is limited by patient access to treatment centres with trained staff (especially in rural Africa), as well as cost and availability of drugs. The recent introduction of an oral treatment for visceral leishmaniasis in India, miltefosine, has raised hopes for improved treatment, but at the same time also raised concerns about safety, patient compliance and possible sub-optimal use leading rapidly to the development of resistance.

Better diagnostics, particularly based on antigen rather than antibody, which are simple to use and non-invasive, together with a safe, efficacious and affordable short course treatment could lead to effective disease control. The political will of afflicted countries and the required infrastructure are other key factors needed for elimination of kala-azar as a public health problem [1]

History

Leishmaniasis has a long history dating back as far as the first century AD. As early as this period, pre-Incan pottery from Ecuador and Peru 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-18 th 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."

The disease became known as Leishmaniasis after William Leishman, a Glasgwegian doctor seving 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 kal-azar patients and published his discovery a few weeks after Leishman. 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 kal-azar, Leishman and Donovan discovered the genus, *Leishmanias* [(2)].

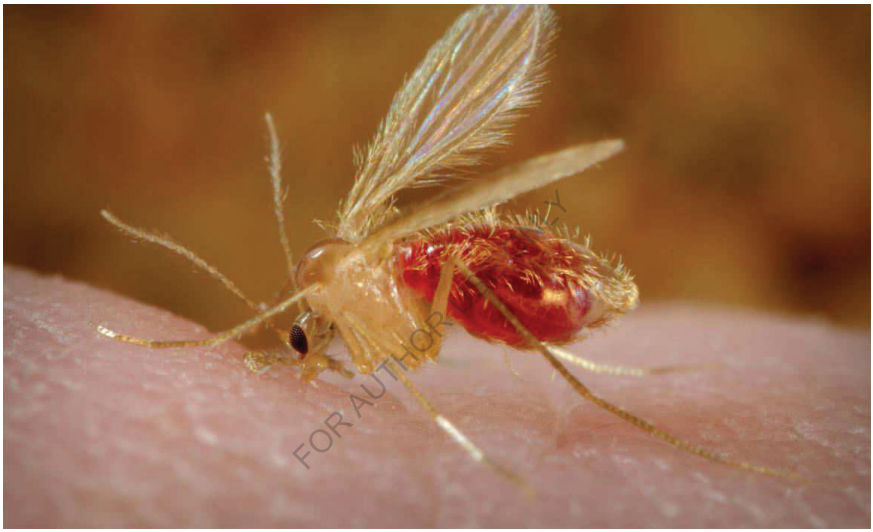
Vector

The vector of various leishmaniasis world over belongs to Order: Diptera; Class: Insecta; Family: Psychodidae; and Phylum: Arthopoda. The parasite is transmitted by the bite of infected female sand flies: Phlebotomus in the Old World and Lutzomyia in the New World (central and south America). Morphologically they resemble very closely with each other. The name 'sand fly' can be confusing as this name is sometimes used for other species as well. Sand flies in the genus Phlebotomus are vectors of a bacterium (Bartonella bacilliformis) that causes Carrion's disease (oroyo fever) in south America. In parts of Asia and north Africa, they spread a viral agent pappataci virus (an arbovirus) that causes sand fly fever (pappataci fever) as well as protozoan pathogens (Leishmania spp.) that causes leishmaniasis. Only some 10% of the approximately 600 known species of sand flies are vectors, and only 30 of these are important. Fauna of Indian sub-zone is represented by 46 species, of these 11, belong to Phlebotomine species and 35 to Sergentomyia species. Phlebotomus argentipes is the proven vector of kala-azar in India.

Habitat and behaviour of sand fly:

In general, the Old World sandfly species live in desert or semi-arid ecosystems and the New World species in forest dwelling. Some of the Old World species breed in peridomestic situations and enter human habitations, whereas disease transmission in the New World is associated with humans living or working near the forest. The insect vector of leishmaniasis, the phlebotomine sand fly, is found throughout the world's inter-tropical and temperate regions. The sand flies are small (approximately 2–3 mm in length), hairy and soundlessly flying insects. They are found around human habitations and breed in specific organic wastes such as feces, manure, rodent burrows, leaf litter and in dark corners in the crevices of the walls having high humidity and temperature, although they can be observed in dry regions with a favourable local microclimate (crevices, termite mounds, caves, hollows and holes in tree roots, etc.) where 15 to 80 tiny eggs can be laid. So far, knowledge on the breeding sites of P. argentipes is poor. The larval stages of sand fly present in alluvial or alkaline soil. The damp and dark corners of cattle sheds, where humus is present, and the cracks and crevices in the walls are favourable conditions for P. argentipes breeding. The larvae cannot survive drying out; they will feed on organic waste and then pupate. The female sand fly lays its eggs in the burrows of certain rodents, in the bark

of old trees, in ruined buildings, in cracks of house walls, in animal shelters and in household rubbish, or in such environments where the larvae can find the organic matter, heat and humidity which are necessary for their development. The body and the small wings are very hairy and when at rest the insects hold their wings upright in a V-shape above them. They are poor flyers and have a flight range of a few kilometers, usually fly quite low and remain in the vicinity of their breeding ground. They are unable to fly in the presence of any wind produced by fan or ventilator also. They are usually most active at dawn and dusk.



Female of phelebotomine sand fly

Physiology of sand fly:

The female sandfly needs blood in order to obtain the protein necessary to develop its eggs. In its search for blood they cover a radius of a few to several hundred metres around its habitat. They bite especially at night and dusk, there are exceptions to this such as Lutzomyia wellcomei, which bites mainly during daytime. They have short mouthparts and are pool feeders.

The bite produces a rose-coloured papule surrounded by erythematous area about 10–20 mm in diameter. They can suck blood both from animals (cats, dogs, various rodents, cattle, birds and lizards, etc.) and human. Because of their small dimensions, they can get through standard

mosquito nets. Mosquito nets with a very fine mesh have the disadvantage that they make ventilation difficult, which is unpleasant in warm conditions.

As vector density is sensitive to climate variability, with vector densities varying seasonally. Parasite developmental time in vectors is also sensitive to environmental conditions, decreasing with high temperatures. We can also expect there to be contextual effects of climate on transmission, such as those mediated by natural disasters, which could increase the risk of acquiring an infectious disease.

Identification of sand fly species and Leishmania infection:

Epidemiological studies on leishmaniasis often begin with vector identification, though taxonomic identification of adult insects is difficult. Because of the wider breeding distribution and large species diversity of sand flies, it is important to combine multiple collection methods in a survey. Commonly used methods include castor oil sticky traps, light-traps, emergence traps, Shannon traps, human bait landing collection, human mouth aspirators on resting sites, household insecticide knockdown collection, and malaise traps. Conventional microscope is commonly used to identify the sand fly species. Closely related species can be morphologically differentiated in one sex only. In laboratory, live sand flies were frozen to death. Later, all sand flies were stained with 20% carbol fuchsin solution and then identified to species by microscope-based on some typical morphologic characteristics: mainly internal structures (such as hair on abdominal tergites, buccal capsule, pigment patch, pharyngeal basket and spermatheca, ciborium, pharynx for females and terminal genitalia for males). This method requires refined storage conditions for samples, a highly skilled technique, and taxonomic expertise. Morphologically identical species can sometimes be differentiated only with sophisticated techniques (e.g. analysis of the cuticle hydrocarbons, polymerase chain reaction (PCR), isoenzymes, etc.). Understanding the genetic variability of the vectors is still in its infancy. In recent years, molecular techniques have been used to differentiate the sibling species of sand flies that are similar in morphology7. The sand fly species can be identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the 18S rRNA gene using the individual specimen. The method requires minimum effort and thus may be a powerful tool for research on prevalent sand fly species and the relationships between Leishmania species and the vectors. Similarly, the infection of sand flies with Leishmania promastigotes has usually been examined by dissecting individual sand flies under a microscope. The sand flies should be fresh, and considerable skill and expertise are needed for the study of tiny individuals. Although the procedure takes a relatively long time, a large number of specimens have to be examined to obtain informative data for each area, because the rate of infection of sand flies with Leishmania is generally very low (0.01–1%). , even in endemic areas. In recent years, molecular techniques such as PCRRFLP of the 18S rRNA gene6, kDNA-PCR9, fluorescent quantitative PCR6 and mini exon PCR assay10 are used to identify Leishmania infections both in experimentally infected and field-captured phlebotomine sand flies, and could be a useful tool in

epidemiological studies and strategic planning for the control of human leishmaniasis. In addition, a real time PCR can also be used to detect the sand fly infection of Leishmania [3].

Immunity to visceral leishmaniasis

In murine VL, the terms resistance and susceptibility refer to the ability of the host to rapidly control parasite growth. Indeed, susceptible strains such as the Balb/c develop a life-long chronic infection, which unlike humans is not fatal to the host. Most studies in mice are based on intravenous or intraperitoneal injection of a high dose of parasites, hence bypassing the early events on the skin and parasite navigation to the viscera.

Some comparative studies suggest that the parasite dose and inoculation route influence the kinetics of parasite colonization of the viscera and the ensuing immune response. Acknowledging these limitations, researchers are starting to employ alternative animal models of the disease to perform in-depth immunologic studies, such as the extremely susceptible Syrian hamster model, through sand-fly-mediated parasite inoculation. Also, the recent use of rhesus macaques as models of VL takes advantage of the close phylogeny between humans and non-human primates and provides a window to the early events after infection which are silent in humans and hence not accessible.

In mice, VL provides a clear example of organ-restricted immunity. In the liver, infection is self-resolving, in a manner that is dependent on the development of T cell-mediated immunity and formation of granulomas. In contrast, in the spleen the immune system fails to clear parasites and instead, a lifelong chronic infection persists associated with immunopathology. The compartmentalized immune responses clearly observed in murine VL are not evident in human patients, where infection is progressive and varying degrees of parasite load are observed in the viscera. Also, in the Syrian hamster, parasites grow unimpaired in the spleen, liver and bone marrow (BM), until animal demise. Finally, in our recent study in *L. infantum*-infected rhesus macaques, we observed a progressive increase in the parasite load in visceral organs as the infection advanced toward the chronic phase (8 months). Nevertheless, the compartmentalized immune response observed in mouse VL has been instrumental in defining the immune networks that dictate parasite elimination vs. persistence during visceral Leishmania infection.

Mechanisms underlying the control of hepatic infection in mice:

Liver resident Kupffer macrophages harbor most parasites after intravenous injection of mice with L. donovani or L. Infantum. Kupffer cells have reduced innate capacity to kill intracellular Leishmania and hepatic parasite burden increases rapidly during the first weeks [25, 26]. Restriction of liver parasite numbers parallels the assembly of inflammatory structures, known as granulomas, constituted by a central core of fused and parasitized Kupffer cells and an outer cuff of motile lymphocytes and variable amounts of other immune cells. Granulomas allow the local concentration of inflammatory cytokines that in turn efficiently activate the leishmanicidal mechanisms of Kupffer cells. The kinetics of granuloma maturation during experimental infection of mice with L. donovani has been dissected in detail. Interestingly, Kupffer cells

exposed to the inflammatory environment during infection, but not directly infected by the parasite, appear activated a few hours after parasite inoculation and play a crucial role in initiating the protective response. , by secreting several chemokines and cytokines that recruit immune populations, including monocytes, neutrophils and invariant natural T killer (iNKT) cells. INKTcells, in particular, play a major role in coordinating initial granuloma formation. Via their invariant T-cell receptor (TCR), iNKT cells recognize CD1d-bound lipophosphoglycan (LPG), the most abundant surface glycolipid of Leishmania spp., triggering early production of IFN-γ. Additionally, iNKT cells rapidly secrete several cytokines upon activation, including C-X-C motive chemokine-10 (CXCL10), which attracts T cells and promotes maturation of granulomas. However, the role of iNKT cells during VL is unclear, as their activation with selective ligands was contradictorily associated to disease amelioration or aggravation in independent studies. It is possible that these cells are important in the orchestration of the initial response after infection, but their chronic activation is detrimental to the host.

By one week after infection, Tcells are recruited to the granuloma and eventually become the predominant immune cell type. Given the low level of innate parasite killing in the early stages of infection in the liver, it has long been assumed that the majority of parasitespecific T cells were primed in the spleen and subsequently migrated to the liver, guided by chemotactic gradients. Nevertheless, a recent study demonstrated that specific CD4 T cells can be primed in the liver and suffice to confer hepatic immunity. Both CD4 and CD8 Tcells appear indispensable for the development of mature granulomas. Two-photon imaging revealed that antigen presentation to CD8 T cells is restricted to Kupffer macrophages , whereas CD4 T cells may be activated by both Kupffer cells and some granuloma-associated dendritic cells (DCs).

A number of cytokines play critical roles in granuloma development and parasite killing. IL-12 is produced by activated Kupffer macrophages and induces IFN-γ by granuloma-associated lymphoid cells [43]. In turn, IFN-γ maximizes the leishmanicidal capacity of Kupffer cells. Arguably, the most important soluble factor for granuloma development and hepatic control of Leishmania infection is Tumor Necrosis Factor (TNF), which plays a crucial role in coordinating the assembly and maturation of granulomas.

In the absence of TNF, parasite growth in the liver proceeds unimpaired during the first weeks due to completely absent granuloma formation. However, later in infection (6–8 weeks) there is an abrupt assembly of granulomas causing rapid death due to fulminant hepatic necrosis. Additionally, lymphotoxin-α, a TNF-related cytokine, promotes the recruitment of leukocytes from the perivascular space to the sinusoidal areas, where infected Kupffer cells reside.

Granulomas attain full maturation by 2–4 weeks after infection and hepatic parasite burden rapidly declines up to 8 weeks post-infection. Importantly, sterile immunity in the liver is not achieved.

However, the presence of a residual parasite population is thought to incite a small but enduring immune response that provides long-term immunity to reinfection.

Early events in the spleen during visceral Leishmania infection:

The spleen is the body's largest blood filter. Splenic macrophages are strategically placed to remove any exogenous particle or pathogen that enters the spleen through the blood stream. Following intravenous injection of L. donovani, about 95 % of the parasites are phagocytized by three distinct splenic populations; red pulp macrophages, marginal zone macrophages (MZM) and marginal metallophilic macrophages (MMM). Unlike liver Kupffer cells, macrophage populations of the spleen demonstrate a remarkable innate capacity to kill the parasite. Indeed, it is estimated that 50 % of the initial parasite inoculum is killed by macrophage populations of the marginal zone within the first 24 hours after infection. For both MZMs and MMMs this was shown to depend on the recruitment of the Interferon Regulatory Factor (IRF)-7 to parasitecontaining phagosomes and may involve leishmanicidal mechanisms independent of NO.

A few hours after mice infection, mature DCs appear in T cell areas at the periarteriolar lymphoid sheaths (PALS) and produce IL-12 to initiate protective Tcell responses [48, 50, 51]. Interestingly, priming DCs do not contain viable parasites, which led to the notion that protective T cell responses are induced by DCs activated in a bystander manner, hence resembling the early events in the liver mediated by bystander Kupffer cells.

It is not clear how bystander DCs acquire parasite antigens for T cell priming. DCs may phagocytize parasite debris present in the splenic marginal zone or ingest macrophages containing digested parasites. In mice infected with L. donovani, CD4 T cell activation can be detected in the first day after infection and the pool of parasite-specific splenic CD4 T cells increases several fold during the first weeks contributing to splenomegaly.

In CD4 T cells, IL-12 signaling leads to nuclear translocation of Signal Transduction and Activator of Transcription-4 (STAT-4) resulting in induction of the transcription factor T-box transcription factor-21 (T-bet) and upregulation of IL-12 receptor (IL-12R) to prime for Th1 differentiation. T-bet, in turn, induces the cardinal Th1 cytokine IFN-γ that through autocrine signaling activates STAT-1 and further stabilizes the Th1 lineage.

There is substantial evidence indicating that all the components involved in Th1 differentiation are necessary for an effective response against visceral Leishmania.

Shortly after infection, splenic DCs are also capable of producing other members of the IL-12 family, including IL-23p19, which may pair with IL12p40 to form biologically active IL-23.

Along with additional cytokines present in the VL spleen, such as the Transforming Growth Factor-β (TGF-β), IL-6 or IL-1β; IL-23 promotes the differentiation of Th17 cells from naïve CD4 T cells.

Recent evidence suggests an important protective role for Th17 cells during VL. Mice deficient for the IL17 receptor A (IL17RA) were more susceptible to L. infantum infection, exhibiting decreased numbers of splenic IFN-γ-producing CD4 T cells. Furthermore, IL17A acts synergistically with IFN-γ to potentiate NO production in infected macrophages. These studies in mice are supported by observations in human patients. For instance, analysis of cytokine responses in peripheral blood mononuclear cells (PBMCs) from symptomatic and asymptomatic VL patients revealed that the expression of Th17 cytokines was strongly associated with the asymptomatic state. Also, another study noticed negligible transcript levels of Th17-associated cytokines or transcription factors in splenic tissue from patients with active disease. Interestingly, IL-17A appears to play a host-detrimental role during infections by cutaneous Leishmania species.

During parasite development in the sand-fly vector, Leishmania produces extracellular vesicles (also known as exosomes) which accumulate in the midgut and become part of the inoculum injected by the fly during feeding. By exacerbating the inflammatory response, particularly through the induction of IL-17A, exosomes cause larger lesion size and longer footpad swelling.

It would be interesting to explore whether exosomes described in visceral Leishmania species, similarly induce an exacerbation of the immune response in the skin or instead down regulate inflammation to allow a silent navigation to the viscera.

CD8 T cell-mediated immunity has been comparatively less studied than the CD4 T cell response during VL. The expansion of splenic CD8 T cells after infection is impressive and may reach 10-fold within a 2 month period. CD8 T cells respond to IL-12 and type I IFNs by upregulating the T-bet and Eomesodermin (Eomes). These promote the expression of type I cytokines such as TNF or IFN-γ and cytotoxic molecules such as granzymes and perforin which allow CD8 T cells to perform effector function. There is sustained evidence pointing to an important role of CD8 T cellderived IFN-γ in the activation of infected macrophages to control parasite growth.

Less clear is whether CD8-mediated cytotoxicity plays any protective role. Some studies in mice demonstrate that parasite-specific cytotoxic T lymphocytes (CTLs) generated during infection are capable of killing infected cells ex vivo, via mechanisms relying on the perforin/granzyme and Fas/FasL pathways. However, it is not clear whether the parasite is killed concomitantly with the demise of the host cell. In vitro experiments indicate that parasites remain viable after CD8-mediated host cell lysis, but are eventually killed after infecting neighboring macrophages pre-activated with CD8derived IFN-γ. These observations suggest that the cytotoxic and cytokine-secreting functions of CTLs collaborate for efficient parasite killing, but such mechanism has so far not been demonstrated in vivo. The role of CD8 T cell effector function has been studied with more detail in infections with cutaneous Leishmania species. In mouse models of CL and human CL patients, the current paradigm indicates that CD8 T cells producing IFN-γ contribute to the protective response against the parasite while CD8-mediated cytotoxicity leads to tissue

pathology and promotes skin ulceration. Whether a similar dichotomy in the effector functions of CD8 T cells is operative in VL remains unknown and is a matter of great interest in future In mice, after the initial period of parasite elimination by splenic macrophages, the parasite population is maintained at a constant size throughout the following two weeks, suggesting that parasite replication is balanced by parasite killing studies.

While able to control infection, the immune response appears to be far from optimal, as it cannot eradicate the parasite. By the third week after infection, parasite load in the spleen starts to increase slowly, signaling the onset of the chronic phase. By this time, infection is starting to resolve in the liver. In the following sections, we will review the main immune networks responsible for parasite persistence in the spleen during chronic VL.

Chronic visceral leishmaniasis: regulatory cytokines:

Mice deficient in the immunosuppressive cytokine IL-10, or in which IL-10 signaling is blocked, are highly resistant to L. donovani infection. IL-10 is found in elevated levels in the serum, splenic aspirates, lymph nodes and bone marrow of VL patients and is produced after Leishmania antigen stimulation of whole blood cultures from patients with active disease, but significantly decreases after drug cure. Neutralization of IL-10 augments IFN-γ production in whole blood assays and promotes amastigote clearance in cultured splenic cells from VL patients. Together, all these pieces of evidence led to the conclusion that IL-10 is the major mediator of the immunological defects observed in the spleen during chronic VL .

IL-10 is a general suppressive cytokine with a range of anti-inflammatory effects in several immune lineages. During VL, IL-10 deactivates the leishmanicidal mechanisms of the macrophage and down regulates the expression of co-stimulatory molecules and MHC expression. It also decreases the production of IFN-γ in T cells and inhibits DC migration to T cell areas.

IL-10 can be produced by multiple immune lineages. During VL, several cell types have been identified as sources of IL-10, including CD4 and CD8 T cells, B cells, NK cells, macrophages and DCs. A major topic of interest in the past decade has been the identification of the relevant sources of IL-10 during VL. The best available evidence points to conventional IFN-γ-secreting Th1 cells as the most relevant source of pathological IL-10 during chronic experimental and human VL.

In mice, splenic CD4 T cells producing both IFN-γ+ IL-10+(sometimes denoted as type I regulatory T cells, Tr1) can be detected already at two weeks after parasite infection and attain a plateau by one month, representing 2 to 5 % of the total splenic CD4 T cell pool.

A matter of upmost interest is to decipher the mechanisms underlying the regulatory switch that results in the induction of the IFN-γ+ IL-10+double producer CD4 T cells. Recent studies are unveiling a cytokinic network that works to maintain the suppressive environment during chronic

VL. One study, employing splenocytes from human VL patients evidenced a role for T cell-derived IL-21 and myeloid cell-derived IL-27 in the induction of IL-10 in CD4 T cells. Moreover, recent data points to a crucial role for DCs in promoting the regulatory switch in CD4 T cells.

Indeed, DC-derived IL-27 and IL-12 appear to be involved in the induction of IL-10 in CD4 T cells. Interestingly, the suppressive-promoting capacity is restricted to infected DCs [53] and DC depletion between the third and fourth week after infection reduces pathology and enhances resistance to infection.

Finally, IL-10 signaling may contribute to additional IL-10 and IL-27 secretion by the infected.

macrophage, to continuously fuel this suppressive loop. Expression of IL-10 by Th1 cells is a widespread phenomenon that ensures a tight control over excessive activation that may cause pathology. During infections with the apicomplexans Plasmodium or Toxoplasma, the emergence of IL-10 + IFN-γ+ CD4 T cells is required to limit excessive pathology. Even during VL some evidence suggests that IL-10 may be host protective; particularly, in regulating a detrimental inflammatory response in the liver. Indeed, the extensive hepatic necrosis accompanying L. donovani infection in TNF-deficient mice may result from a concomitant defect in IL-10 induction.

Likewise, the severe hepatic pathology that follows L. donovani infection in IL27R−/− mice involves CD4 T cells and may result from curtailed IL-10 induction.

The suppressive role played by DCs during chronic VL is not limited to the induction of Tr1 cells. Indeed, early work evidenced the expansion of a CD11low CD45RBhi DC population during L. donovani infection in mice that dampened T cell responses and induced antigen-specific tolerance in vivo. The transfer of these $CD11c^{low}$ DCs to DC-depleted and infected mice was able to restore splenomegaly and parasite burden to levels present in non-depleted mice, via a mechanism that did not involve the induction of Tr1 cells. Finally, a recent study demonstrated that the early inflammatory milieu during VL promotes the activation of IRF-5 in DCs, which leads to upregulation and stabilization of the transcription factor Hypoxia Inducible Factor-1α (HIF-1α). HIF-1α, in turn, promotes the secretion of IL-10 by DCs, while limiting IL-12, which results in delayed expansion of specific CD8 T cells and their limited effector function, thus further supporting the suppressive role of DCs and IL-10 during VL.

Whilst much less studied than IL-10, TGF-β is another suppressive cytokine that has been linked with parasite persistence in VL [90, 110, 111]. Additionally, mice resistant to L. infantum infection become significantly more susceptible when injected with a viral vector expressing TGF-β.

Chronic visceral leishmaniasis: T cell exhaustion:

Chronic infections are characterized by a prominent impairment of T cell function, known as T cell exhaustion, which precludes an effective response in the long term. Exhaustion proceeds progressively, paralleling the increase in pathogen burden. Some functions, such as cytotoxicity, IL-2 production or proliferation are lost initially. Severe exhaustion is characterized by an inability to produce TNF, IFN-γ or to degranulate. Apoptotic deletion is usually the final fate of an exhausted T cell. Nevertheless, exhausted T cells are capable of long-term survival, if their specific antigen remains present. Evidence has convincingly linked the occurrence of T cell exhaustion with progressive and sustained expression of inhibitory receptors on effector T cells.

These include programmed death-1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA-4) or lymphocyte-activation gene-3 (LAG-3). These receptors act by inhibiting T cell activation, thus precluding optimal effector function. T cell exhaustion was initially noticed over a decade ago in models of chronic viral infections, but recent work unveiled a similar paradigm during chronic protozoan infections.

In mice infected with L. donovani, splenic CD8 Tcells exhibit signs of functional exhaustion by the third week after infection, and severe functional impairment is evident after the fourth week, with abrogated production of IFN-γ, TNF, IL-2 and granzyme B. Exhaustion is paralleled by increased expression of PD-1 in CD8 Tcells and its ligand PD-L1 in splenic DCs. Treatment with an antibody blocking the PD-1/PD-L1 interaction rescued the functionality of parasite-specific effector/memory CD8 T cells, resulting in lower splenic parasite burden. Interestingly, the recovery of CD8 T cell effector function after αPD-1 treatment was only partial.], suggesting that additional inhibitory receptors may contribute to the functional attrition of CD8 T cells during VL. In agreement, mice treated with a CTLA-4 blocking mAb 1 day after infection demonstrate significantly lower parasite burden by 1 month post-infection, consistent with the timing of CD8 Tcell exhaustion. The relevance of these findings in mice has been confirmed in human VL patients, whose splenic CD8 T cells similarly exhibited functional impairment and augmented expression of PD-1 and CTLA-4. Contrasting with the wealth of evidence demonstrating CD8 Tcell exhaustion, in CD4 Tcells the phenomenon has been far less studied and is less understood. In chronic canineVL, splenic CD4 Tcell exhaustion is less severe than CD8 exhaustion and appears only in aggravated clinical stages of the disease.

Suppressive cytokines, such as IL-10 and TGF-β, have been consistently linked with T cell exhaustion in viral infections and cancer. For instance, TGF-β directly enhances PD-1 expression in CD8 T cells. Due to the elevated levels of these cytokines in the VL spleen it would be interesting to explore how these cytokines influence T cell exhaustion during VL and whether their blockade leads to an amelioration of the functionality of effector Tcells.

Chronic visceral leishmaniasis: loss of splenic lymphoid architecture:

The most striking clinical feature of both human and experimental VL is the impressive splenomegaly. Concomitant with increased organ mass and size, a number of changes in the splenic microarchitecture occur. These include disorganization of the white pulp, hypertrophy of the red pulp and disruption of the marginal zone. In the white pulp, germinal centers (GCs) disappear, and the PALS collapses. Neovascularization is also prominent in both red and white pulp.

n mice, the structural changes in the spleen start as the infection enters the chronic phase (around 3 to 4 weeks post-inoculation). Disorganization of the PALS is mediated by TNF and results mainly from the loss of gp38+ stromal cells. These are crucial for establishment and maintenance of the PALS by producing the chemokines Chemokine (C-C motif) Ligand-19 (CCL19) and CCL21, which attract naïve and memory T cells [95, 130, 131]. Concomitant with the disassembly of the PALS, an extensive remodeling of the splenic marginal zone also occurs, characterized by depletion of the MZM population.

Again, TNF appears to mediate the loss of MZMs, through a mechanism that is not clearly elucidated but may involve a direct apoptotic effect. It is intriguing to note that TNF, the crucial cytokine responsible for the maturation of protective granulomas in the liver, is similarly the major factor responsible for the histopathological sequelae of chronic infection in the spleen.

A final significant alteration in the lymphoid architecture of the spleen during chronic VL is the loss of follicular dendritic cells (FDCs), leading to loss of GCs and B cell follicles, which become occupied by parasitized macrophages and plasma cells.

The structural changes that lead to loss of splenic lymphoid architecture during chronic VL disrupt cell-cell interactions that are crucial for effective immune responses, thus contributing to the suboptimal responses during chronic VL. For instance, the deletion of FDCs and concomitant disorganization of GCs impedes the long-term interactions between B cells and T follicular helper cells (Tfh cells) that are necessary for the production of specific antibodies capable of neutralizing the parasite.

Antibodies, B cells and T follicular helper cells in visceral leishmaniasis:

Experimental work performed over the past decades led to a prevailing view that considers B cells and antibodies of minimal importance for the protective immunity during VL. Indeed, hypergammaglobulinemia has long been recognized as one of the cardinal signs of VL, correlates positively with disease severity and decreases upon drug cure.

Early studies also demonstrated that most of the circulating IgGs are not parasite-specific, but instead result from polyclonal B cell activation. Indeed, autoantibodies are a recurrent finding in VL patients, frequently associated with proliferative glomerulonephritis.

In our recent study, employing a non-human primate model of VL, hypergammaglobulinemia was established early after infection and persisted during the chronic phase. Yet, the production of Leishmania-specific IgG was short lived and decreased at chronic infection, implying that most antibodies produced are not specific for the parasite.

Analyzing the splenic B cell population we observed the expansion of memory B cells expressing CD27 after infection that contracted at the chronic phase, hence closely following the production of specific antibodies. We further observed the persistent expansion of a splenic B cell population with the atypical CD21−CD27− phenotype that appeared responsible for the non-specific hypergammaglobulinemia.

These observations incited us to explore the dynamics of T follicular helper cells (Tfh cells) in the spleen of rhesus macaques infected with L. infantum. Tfh cells are a CD4 T cell helper subset specialized in coordinating GC reactions and providing crucial help to B cells in the production of high affinity antibodies.

Indeed, we observed the expansion of a splenic Tfh population in the first few weeks following parasite inoculation. Tissue imaging further evidence that Tfh cells were able to infiltrate B cell follicles and GC during the acute phase. However, Tfh cells were mostly absent from the spleen at the chronic phase, hence paralleling the decline in CD27+ memory B cells and specific IgG.

Thus, our study in non-human primates suggests that the inability to maintain a sustained Tfh response during the chronic phase of infection may underlie the defects in the humoral response during VL.

Thus, it will be important to decipher the immune mechanisms behind this failure to maintain Tfh cells. One possibility may relate to the existence of a strong Th1-polarizing environment in the spleen during VL, with induction of expression of T-bet in CD4 T cells that directly represses the expression of the Tfh master transcription factor B cell lymphoma-6 (Bcl-6). Furthermore, the destruction of FDC networks and loss of GCs that occurs during VL, may also preclude a sustained Tfh differentiation and preclude their effector function.

During chronic VL, amastigotes are exposed to antibodies when they egress from heavily infected macrophages to infect new ones. The view that antibodies are detrimental to the host is supported by the observation that, when ingested by macrophages, IgG-opsonized amastigotes promote IL-10 secretion and inhibit IL-12 production.

However, ligation of FcγRs on the surface of macrophages and DCs may lead to pro- or anti-inflammatory outcomes, depending on the identity of the Fc receptors activated, IgG subclass or cell type. For instance, one study demonstrated that parasite-specific IgG is required for efficient L. major uptake and IL-12 production by DCs, suggesting that FcγR-mediated uptake has contrasting outcomes in DCs and macrophages. Also, in a model of coinfection of L. amazonensis and L. major in mice, it was shown that resolution of the lesion required specific

antibody that was able to enhance the microbicidal mechanisms in the macrophage by promoting ROS production. As such, the role of antibodies and Fc receptors during Leishmania infections is more complex than previously appreciated, with the outcome of FcγR ligation being clearly context-dependent.

Furthermore, the role of antibodies as regulators of the inflammatory response is not necessarily detrimental to the host. While B cell-deficient mice resolve L. donovani infection more rapidly than WT mice, such increased resistance comes at the cost of hepatic pathology. However, administration of immune serum to infected B cell-deficient mice alleviates pathology without decreasing the efficiency of hepatic parasite clearance, suggesting a tissue-protective role for antibodies.

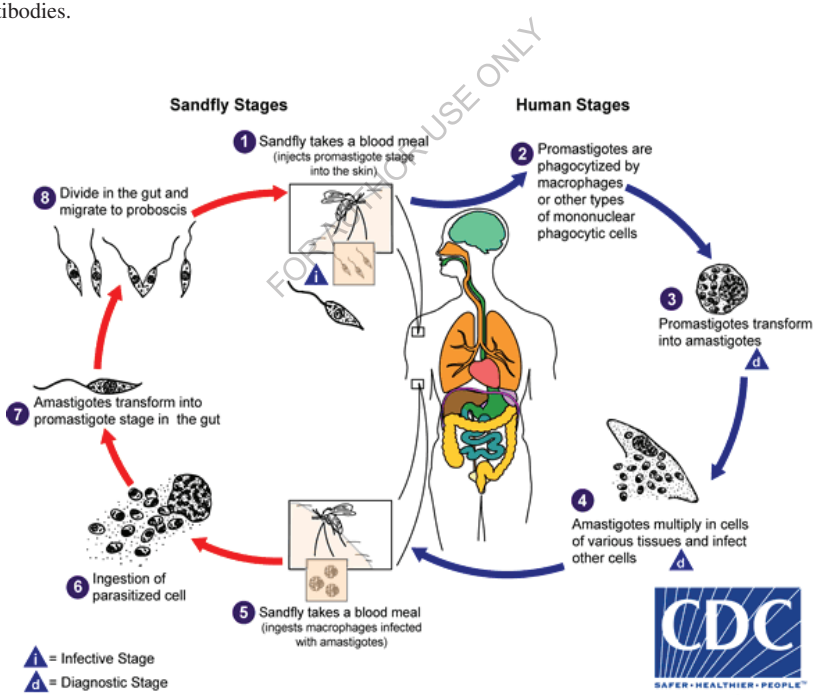
Even conceding that parasite opsonisation by specific IgG is deleterious for the host, there are alternative mechanisms through which antibodies may contribute to the protective response against Leishmania; for instance by neutralizing parasite virulence factors. An illustrative example comes from the intracellular bacteria Listeria monocytogenes. A monoclonal antibody against listeriolysin, the pore-forming toxin of L. monocytogenes, was capable of blocking bacterial replication inside macrophages and provided resistance to infection in mice. Antibodies against Leishmania virulence factors, such as the metalloprotease gp63, have been detected in the sera of VL patients, but it is not clear whether they are capable of neutralization or play any protective function. As such, it is imperative to identify antibodies with neutralizing capacity and to evaluate whether their administration is capable of modifying the course of the disease to the benefit of the host.

Rather than considering the role of antibodies solely as pathological or irrelevant, it is perhaps wiser to acknowledge that these molecules may play both protective and non-protective roles during VL [4] .

Biology of Leishmania parasite

Causal Agent:

Leishmaniasis is a vectorborne disease that is transmitted by sand flies and caused by obligate intracellular protozoa of the genus *Leishmania*. Human infection is caused by more than 20 species. These include the *L. donovani* complex with 2 species (*L. donovani*, *L. infantum* [also known as *L. chagasi* in the New World]); 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 4 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, molecular methods, or monoclonal antibodies.



Life cycle of Leishmania parasite

Leishmaniasis is transmitted by the bite of infected female phlebotomine sand flies. The sand flies inject the infective stage (i.e., promastigotes) from their proboscis during blood meals ❶. Promastigotes that reach the puncture wound are phagocytized by macrophages ❷and other types of mononuclear phagocytic cells. Promastigotes transform in these cells into the tissue stage of the parasite (i.e., amastigotes) ❸, which multiply by simple division and proceed to infect other mononuclear phagocytic cells ❹. Parasite, host, and other factors affect whether the infection becomes symptomatic and whether cutaneous or visceral leishmaniasis results. Sand flies become infected by ingesting infected cells during blood meals (❺, ❻). In sand flies, amastigotes transform into promastigotes, develop in the gut ❼(in the hindgut for leishmanial organisms in the *Viannia* subgenus; in the midgut for organisms in the *Leishmania* subgenus), and migrate to the proboscis ❽ [5] .

Leishmaniasis overview

Visceral leishmaniasis (VL) also known as kala-azar, is considered as one of the most significant neglected vector-borne zoonotic parasitic sickness with high mortality rate if left untreated. Sand fly is the vector, man and carnivores are the typical reservoir. In the human host, parasites are surrounded by macrophages, which are then carried by the circulatory system to other organs like liver, spleen and bone marrow where they cause hyperplasia and or macrophage cells [6].

World health organization (WHO) estimates that the disease is endemic at least in 60 countries of four continents with 0.5 million new cases and 40,000 cases death annually [7]. The vast majority (more than 90%) of cases are in Sudan, India, Bangladesh, Nepal and Brazil.

Visceral leishmaniasis remains occult in individuals with normal immune systems and appears to be very common in most of the VL endemic foci [3]. Visceral leishmaniasis is caused by *Leishmania donovani* complex including *Leishmania infantum* and *Leishmania donovani*. In general *Leishmania infantum* infections are asymptomatic, raising concern that the parasite could be available in blood donors from else healthy inhabitants in regions to which it's endemic [8]. The transmission of parasites through blood transfusion is relatively rare. Currently six parasitic infections have yet considered as transfusion- transmitted infections (TTI) including *Plasmodium spp*, *Trypanosoma cruzi*, *Leishmania spp,* Babesia *microti*, *Toxoplasma gondii* and Filaria [9].

The frequency of asymptomatic carriers among blood donors have been carried out in southern Europe, with a proportion ranging from 0% to 36.4% depending on the test used and number of individuals studied [10-22]. Several studies suggest that the presence of Leishmania in the peripheral blood of asymptomatic carriers is probably episodic, suggesting that such individuals are asymptomatic carriers for a variable period [23-47].

The evidence of human cases of transfusion transmitted leishmaniasis (TTL) is of interest,

especially VL, with clinical features and outcomes similar to those of the natural infection, not only in endemic but also in nonendemic areas [48]. Transmission of Leishmania by transfusion is most likely to occur in an endemic area because that is where infected donors most often reside. In those areas a case of transfusion transmission is usually impossible to differentiate from local transmission by sandflies. That, and the fact that visceral infection with Leishmania is asymptomatic in healthy individuals, is expected to result in underestimation of the occurrence of transfusion transmitted infection. Leishmania does produce clinical disease in infants and the Immunocompromised as demonstrated in documented cases of transmission by transfusion in Chung and colleagues [49]. An additional reason for the low number of reported cases is that clinical suspicion is low in nonendemic areas where symptomatic infection is often labeled as

fever of unknown origin, because diagnosis is difficult without a high index of clinical suspicion and a specific search for Leishmania. An additional level of complication for the donor pool is presented by the fact that transplacental and sexual transmission is possible [50-51]

The American Association of Blood Banks has recommended ban on blood donations from a selected group (soldiers returning to the United States from VL endemic parts, especially countries of Middle East) to prevent transfusion transmitted Leishmaniasis [52].

Visceral leishmaniasis in Sudan

Leishmaniasis has tremendous historical relevance, with recorded disease thousands of years before Common Era (BCE) [53]. An examination of ancient Egyptian and Christian Nubian mummies dating back to 3500 to 2800 BCE yielded successful amplification of *Leishmaniasis donovani* DNA. During a time period referred to as the Middle Kingdom, Egyptian trade and military excursions involving Nubia (modern Sudan) are thought to be responsible for the introduction of leishmaniasis into Egypt, as DNA-positive samples were not seen prior to this time period. Furthermore, some sources suspect Sudan as the original foci of visceral leishmaniasis (VL) [54].

Prof Ahmed Mohammed Elhassan achieved numerous studies about leishmaniasis in Sudan certainly in Elgadreif area and he established institute of endemic diseases to study tropical endemic diseases in Sudan including leishmaniasis.

Laboratory diagnosis of visceral leishmaniasis

The diagnosis of VL is complex because its clinical features are shared by a host of other commonly occurring diseases, such as malaria, typhoid, and tuberculosis; many of these diseases can be present along with VL (in cases of coinfection); sequestration of the parasite in the spleen, bone marrow, or lymph nodes further complicates this issue.

Laboratory diagnosis of leishmaniasis can be made by the following: (i) demonstration of parasite in tissues of relevance by light microscopic examination of the stained specimen, in vitro culture, or animal inoculation; (ii) detection of parasite DNA in tissue samples; or (iii) immunodiagnosis by detection of parasite antigen in tissue, blood, or urine samples, by detection of nonspecific or specific antileishmanial antibodies (immunoglobulin), or by assay for leishmania-specific cell-mediated immunity.

Demonstration and isolation of parasite:

The commonly used method for diagnosing VL has been the demonstration of parasites in splenic or bone marrow aspirate. The presence of the parasite in lymph nodes, liver biopsy, or aspirate specimens or the buffy coat of peripheral blood can also be demonstrated. Amastigotes appear as round or oval bodies measuring 2 to 3 μm in length and are found intracellularly in monocytes and macrophages. In preparations stained with Giemsa or Leishman stain, the cytoplasm appears pale blue, with a relatively large nucleus that stains red. In the same plane as the nucleus, but at a right angle to it, is a deep red or violet rod-like body called a kinetoplast.

After identification, parasite density can be scored microscopically by means of a logarithmic scale ranging from 0 (no parasite per 1,000 oil immersion fields) to +6 (>100 parasites per field).

The sensitivity of the bone marrow smear is about 60 to 85%. Splenic aspirate, though associated with risk of fatal hemorrhage in inexperienced hands, is one of the most valuable methods for diagnosis of kala-azar, with a sensitivity exceeding 95%. It requires no special equipment, from the patient's standpoint is generally preferable to the more painful bone marrow aspirate, and has proven to be safe and relatively easy to perform in experienced hands. For patients suspected to have VL, splenic aspirate can be performed even when spleen is not palpable, after demarcating the area of splenic dullness by percussion. The only risk of splenic puncture is bleeding from a soft and enlarged spleen. At our treatment center, fatal bleeding has occurred only twice in 9,612 splenic aspirate procedures performed over the last 10 years. To avoid the risk of excessive blood loss, splenic puncture should be avoided in patients with a platelet count of less than 40,000 platelets/μl and a prothrombin time of more than 5 s over the control.

A tissue specimen, e.g., a spleen, liver, or lymph node tissue specimen, may be subjected to imprint cytology by the repeated pressing of its cut flat surface on microscopic slides. The smear is fixed with absolute alcohol and stained with Giemsa stain. In imprint cytology, a monolayer of cells is formed and amastigotes are easily identifiable. The results are expressed as the number of leishmania per 100 host cell nuclei. Tissue specimens can also be subjected to histology, and the presence of parasites can be demonstrated by standard hematoxylin and eosin stain. Tissue specimens are usually uneven in thickness; consequently the amastigotes are unevenly distributed. Long searches may be required to demonstrate the parasite. The sensitivity of the test can be increased by staining the specimen with fluorescent dye-tagged antibodies to the surface receptors of the parasite. Fluorescein isothiocyanate isomer- or rhodamide B isothiocyanate-conjugated antiserum is usually used for this purpose. Fluorescent dye-conjugated monoclonal antibodies are also used for speciation of the parasite.

Culture of parasite can improve the sensitivity of detection of parasite, but leishmania culture is rarely needed in routine clinical practice. However, cultures are required for (i) obtaining a sufficient number of organisms to use an antigen for immunologic diagnosis and speciation, (ii) obtaining parasites to be used in inoculating susceptible experimental animals, (iii) in vitro screening of drugs, and (iv) accurate diagnosis of the infection with the organism (as a supplement to other methods or to provide a diagnosis when routine methods have failed). Leishmania strains can be maintained as promastigotes in artificial culture medium. The culture media used may be monophasic (Schneider's insect medium, M199, or Grace's medium) or diphasic (Novy-McNeal Nicolle medium and Tobies medium). We prefer diphasic medium containing modified diphasic rabbit blood agar overlaid with RPMI 1640.

for primary isolation, and we prefer M199 medium containing 20% fetal calf serum to amplify parasite numbers. Hockmeyer's medium, which is Schneider's commercially prepared culture medium supplemented with 30% heat-inactivated fetal calf serum with 100 IU of penicillin and 100 µg of streptomycin, is simple to use and satisfactory for diagnosis of VL, but it is expensive.

Culture tubes are inoculated with 1 to 2 drops of bone marrow or splenic aspirate and incubated at a temperature between 22 and 28°C. The tubes are examined weekly for the presence of promastigotes by phase-contrast microscopy or by wet mount of culture fluid for 4 weeks before being discarded as negative. If promastigotes are present, they are maintained by weekly passage to fresh medium. Blood can also be used to isolate the parasite, but the method is slow and takes longer. Aseptically collected blood (1 to 2 ml) is diluted with 10 ml of citrated saline, and the cellular deposit obtained after centrifugation is inoculated in culture media. Contamination of the culture media by bacteria or yeast species or other fungi usually complicates the culture but can be avoided by use of good sterile techniques and by the addition of penicillin (200 IU/ml) and streptomycin (200 µg/ml) to the medium (for bacteria), as well as 5-flucytosine (500 µg/ml) (as an antimycotic agent).

In vitro culture of the amastigotes is done for chemotherapeutic studies and to study the interrelationship of the amastigotes and macrophages. The amastigotes are grown in tissue or macrophage culture. These cell lines are produced from (i) human peripheral blood monocytes, after these are set apart by density sedimentation with lymphocyte separation medium (LSM; Organon-Teknika, Durham, N.C.), in which case a new batch of macrophages must be produced a new; (ii) macrophage cell lines, e.g., P388D and J774G8 lines from mice; and (iii) dog sarcoma and hamster peritoneal exudates of cell lines, in which case continuous culture can be achieved.

The parasite can also be demonstrated after inoculation of laboratory animals (such as hamsters, mice or guinea pigs) with infected specimen. Animal inoculation is not usually employed as a diagnostic test, since several months may be required to obtain a positive result. Golden hamster is the animal of choice for maintaining *L. donovani* complex. It can be infected via many routes, including across mucous membranes, but intraperitoneal and intrasplenic routes are preferred. Both amastigotes and promastigotes can infect the animal. After inoculation, the animal is examined weekly for signs of infection, such as cutaneous lesions, hepatosplenomegaly, or metastatic lesions. Amastigotes can be harvested by biopsy from the spleen and the liver of an animal that is under anesthesia and that is allowed to survive following the procedure as a source of infective parasite. In the absence of signs of obvious infection, the animal is generally sacrificed after 4 months, at which point liver and spleen samples are examined for the presence of the parasite.

In areas of endemicity, recognition of species of Leishmania is rarely required. However, identification of an organism to the species level is helpful epidemiologically and is also important for the treatment of and prognosis determination for global travelers who are not immune to the parasite and tend to develop unusual manifestations of the disease.

Identification of species of the *L. donovani* complex is particularly difficult, because morphologically the species are almost indistinguishable from each other. For species-level identification, a large amount of promastigotes is obtained by culture of the organism and the species-specific isoenzyme pattern is analyzed by cellulose acetate electrophoresis.

DNA detection method:
Due to the limitations inherent in techniques used for detection of parasites, new approaches to the detection of parasites, such as DNA hybridization, have been attempted since the early 1980s. Although these methods had considerable sensitivity (detecting as few as 50 to 100 parasites).

Their potential use in routine diagnosis is hampered by the complex procedure of hybridization. The development of PCR has provided a powerful approach to the application of molecular biology techniques to the diagnosis of leishmaniasis. Primers designed to amplify conserved sequences found in minicircles of KDNA of leishmanias of different species were tested in various tissues of relevance. Such a target was eminently suitable because the kinetoplast is

known to possess thousands of copies of minicircle DNA. In recent years, PCR-based diagnostic methods with a wide range of sensitivities and specificities have been described [55] .

Justification

Visceral leishmaniasis is a major health problem Sudan with the highest disease burden in Gadarif State. In addition to the basic route of transmission via the sand fly bites, leishmaniasis may also be transmitted through blood transfusion. No information is available about the prevalence of Leishmania infection between blood donors in Sudan; therefore we aimed to investigate this question.

Objectives

General objectives

To evaluate the prevalence of asymptomatic Leishmania infection among blood donors in Gadarif State, Sudan.

.Specific objectives

- To compare between the sensitivity of buffy coat and techniques rk39
- To compare between the specificity of rk39 and buffy coat technique.

Materials and methods

Study area

Gadarif State is one of the most important States in Sudan and plays a significant role in economic and agricultural activities of the country. It has a population of more than 1727404 residents, cover 75000 $Km^{2,}$ and lies between latitude 14 and 16 north and longitude 33 and 36 east. The average rainfall is 612 /millimeter. Four rivers pass through the State (Atbara, Elrahad, Saitit and Basalam) and there are many forests, which provide an excellent environment for the sand fly vector. There are 10 centers for the diagnosis and treatment of VL in the State.

Study design

Hospital based- descriptive cross-sectional study

Sample size

100 healthy appearing blood donors

Study period

January to April, 2018

Study population

Blood donors attended to the blood bank at Gadarif teaching hospital during the period of the study (January - April, 2018).

Inclusion criteria

Only Persons with normal blood pressure, normal hemoglobin level and free from HIV, hepatitis B and C viruses was included in the study.

Exclusion criteria

Each person with abnormal blood pressure, abnormal hemoglobin level, HIV positive, hepatitis B or C positive and those whom matched the WHO clinical features of visceral leishmaniasis (prolonged irregular fever, splenomegaly and weight loss) , will be excluded from the study.

Ethics

The study received ethical clearance the research ethical committee, Alneelain University.

Method

Collection of blood:

Under all aseptic precautions (cleaning the area with povidone iodine followed by adequate rubbing by alcohol pad containing 60% isopropyl) 5 ml of venous blood was collected from median ante-cubital or appropriate veins by gentle suction. The collected blood sample was equally divided immediately into plain vacutainer and a 3.2% Tri-sodium citrate coated vacutainer. Immediately after collection with proper labeling, blood was carried to the laboratory for buffy coat preparation and serum.

Serum preparation

2.5 ml of Whole blood was added in an untreated tube for serum. blood was allowed to clot for 20 to 30 min a t room temperature followed by centrifugation at2,500rpmfor5min.The serum was removed from the clot and collected in cryogenic vials and labeled consequently.

Buffy coat ((density gradient centrifugation) :

In a sterile round bottom disposable test tube (10 ml capacity), 2 ml of citrated blood from the vacutainer was poured slowly by the side of the test tube so that the blood completely

Overlay the separation fluid without any mixing. The test tube was centrifuged for

15 minutes. 3000 rpm referable in a swinging centrifuge machine. After centrifugation, a thick buffy coat was seen in the interface of plasma. RBCs were settled at the bottom part of the test tube. Now the plasma was gently sucked by a micropipette of 1ml capacity and was preserved in an eppendrof tube. Then by another of 100-200 l capacity/micropipette, the buffy coat material was sucked out very gently .

Staining of buffy coat smears

Buffy coat films were stained by leishman stain.

Procedure of leishman staining

An air dried thin film was made and placed on a staining rack and flooded with Leishman stain. The slide was left for 2 minutes to fix. Double amount distilled water was added over the flooded stain from a plastic wash bottle for better mixing of the solution. The stain was left for 10 minutes. The stain was washed with running tap water and air dried again the smear to examine under oil immersion objective.

Microscopic examination of the buffy coat film

At least 1000 fields per slide were examined under oil immersion lens to detect amastigote form of Leishmania donovani. Amastigotes appear as round or oval bodies measuring 2 to 3 m in length and are found in inside or outside the monocytes and macrophages. In preparations stained with Leishman stain, the cytoplasm appears pale blue, with a relatively large nucleus that stains red. In the same plane as the nucleus, but at a right angle to it, is a deep red or violet rod-like body called a kinetoplast. If any amastigote in 1000 fields was found, the slide was reported as positive. Grading of positive smear was done according to standard chart.

Score 0 = No LD bodies in 1000 fields

Score 1 = 1-10 LD bodies in 1000 fields

Score 2 = 1-10 LD bodies in 100 fields

Score 3 = 1-10 LD bodies in 10 fields

Score 4 = 1-10 LD bodies per fields

Score 5 = 10-100 LD bodies per fields

Score 6 = 100 LD bodies per fields.

rK39 rapid test

We used a ready-to-use Immunochromatographic strip manufactured by InBios Inc. (Seattle, WA). This strip has rK39 antigen immobilized as the lower band of the nitrocellulose pad of the strips, which contain protein A/colloidal gold as a detection reagent A band 1 cm above the rK-39 band contained antibody to protein A/colloidal gold and was used as a positive control to detect normal immunoglobulin G (IgG).

The rK39 immunochromatographic RDT, Kala- azar Detect (InBios International, USA) was performed at RMRIMS according to manufacturer's instructions. At room temperature, 20 µl of serum prepared from venous blood or one drop of finger stick blood was added to the dipstick. A single drop of blood was used in this study because this is what is routinely performed in the field. Three drops of the chase buffer solution was added to a test tube followed by addition of the dipstick into the test tube containing the chase buffer. The results were read after 10 minutes. The test was considered positive when both the control line and the test line appeared red in color.

Result

There is no positive cases among the study population (100% males) detected either by Buffy coat technique or rk39 rapid test.

Statistical analysis

Table statistics:

Frequency table				
Label	Value	Freq	%	Sum%
r1(sample size)	100	0	0	0
1 categories		0 cases	100%	

Mean: 0
Median: above purple row
Sum: 0
Cases-N: 0
Variance: 0
sd: 0
se: 0
95% CI: 0 >0> 0

Discussion

Our result is compatible to the result of many studies conducted in southern Europe of many investigators done in of and incompatible with the result of the study done by B. Sarkari et al showed that; Twenty-eight blood donors (1.4 %) were positive for Leishmania infection by DAT. Only one of these seropositive donors was positive for Leishmania infection by polymerase chain reaction also our result was differ from that obtained by Kiyoshi F Fukutani et al showed that, anti-*Leishmania* serology by ELISA was positive in 5.4% (74.5% of the study population was males) due to differences of species (*Leishmania donovani/ Leishmania infantum*), techniques, sample size, gender of the study population and personnel experiences.

Conclusion and recommendation

We conclude further studies must be conducted with more sample size, include both genders(males and females) and additional diagnostic techniques must be used chiefly polymerase chain reaction(PCR).

Control of leishmaniasis

The aim of a vector control programme is to reduce or interrupt transmission of disease. An effective strategy for reducing human leishmaniasis is to control sand fly vectors, especially in domestic and peridomestic transmission habitats. A number of control methods are available, including chemicals, environmental management and personal protection. Although some methods can have a strong independent effect on sand fly populations, it is highly recommended that sand fly control involve more than one method, in an integrated vector management approach. Such a package depends on proper understanding of the local epidemiology of leishmaniasis (including whether transmission is anthroponotic or zoonotic) and detailed knowledge of the vector species involved, its habitats (peridomestic or sylvatic), flight range, host feeding preferences, resting sites, circadian rhythms and seasonality.

The planning of integrated vector management requires an initial assessment of the ecology of the area, formulation of operational targets, choice of proper methods and a monitoring and evaluation scheme. Moreover, rational decision-making for the optimal use of resources and adequate institutional arrangements, including a regulatory framework, are needed. Implementation of integrated vector management requires decision-making and quality assurance procedures that can be applied at the lowest administrative level in the health system. The cost–effectiveness and community acceptability of the control measures are central to integrated vector management. All such programmes should include a strong component of social mobilization, with sufficient time and resources dedicated to informing communities and enhancing their participation.

Leishmaniasis control has often been integrated with that of other vector borne diseases. For example, after intensive attempts to eradicate malaria in the 1950s and 1960s by indoor spraying with DDT, the prevalence of leishmaniasis fell dramatically in many countries. In this approach, integrated vector management programmes combine interventions and resources and target several vector-borne diseases (e.g. malaria, dengue, filariasis) in one area. Research and development in vector control is essential, and control policy should keep pace with technological advances.

Methods

The approach to sand fly control depends on the behaviour of the target vector, which may be strongly endophilic, peridomestic or sylvatic. Endophilic species can be attacked by spraying insecticide on indoor walls. For peridomestic species, outer walls and animal accommodations should also be sprayed. Sylvatic species have been attacked by spraying trees that are the resting sites of some Neotropical species, although this may not be cost– effective. The risk for infection

in settlements in the forests can be reduced by clearing trees and bushes over a radius of at least 1 km around houses.

Chemical control:

The main methods for controlling sand flies with insecticides are indoor residual spraying, spraying of resting sites of sylvatic species, use of insecticide-impregnated materials such as bed nets and curtains, and pyrethroidimpregnated dog collars. In selecting the insecticide for indoor residual spraying or impregnation of nets, consideration should be given to their safety for humans and the environment, their efficacy (including duration of effectiveness), the cost–effectiveness, the acceptability and availability of good quality products and the capacity and resources for safe, effective application and disposal of waste. The WHO Pesticide Evaluation Scheme (WHOPES) provides a list of WHO-recommended insecticides and dosages for indoor residual spraying.

Indoor residual spraying is one of the main means for controlling endophilic sand fly vectors and should be targeted to localities with active transmission (focal spray). Therefore, good knowledge of the epidemiology of leishmaniasis and local vector behaviour and ecology is needed. Its level of effectiveness depends on the class of insecticide used, the susceptibility of sand flies to the insecticide, the type of surface treated, the dosage and method of application and overall coverage. When exophilic or peridomestic sandfly species are involved, outer surfaces of domestic animal shelters and structures close to such dwellings (potential sandfly resting sites) must be sprayed. Sustaining a high coverage rate is essential for long-term control, and this requires a well-organized programme, including technical guidelines with standard operating procedures, management, efficient logistics, supervision, monitoring and evaluation of efficacy. Noncompliance with operational guidelines is a waste of money and may moreover lead to environmental damage. Quality assurance systems should be established as part of every indoor residual spraying programme. In the visceral leishmaniasis elimination initiative on the Indian subcontinent, a vector control monitoring toolkit has been prepared for this purpose.

Many classes of insecticide can be used in indoor residual spraying, including organochlorines (e.g. DDT), organophosphates (e.g. malathion), carbamates (e.g. propoxur) and synthetic pyrethroids (e.g. deltamethrin and λ-cyhalothrin). Malathion will potentially be included in a paint formulation (polyvinyl acetate-based suspension of malathion) for use against Lu.longipalpis.

The spectrum of susceptibility of sand flies to the range of insecticides used in vector control programmes is not completely known. Resistance to organochlorine insecticides has been reported (e.g. resistance of P. papatasi and P. argentipes to DDT in India), and sand flies may have also developed resistance to malathion and pyrethroids in areas where these insecticides have been used for a long time, such as concurrently for malaria control. The choice of

insecticide should be strictly regulated at national level, as the environmental legislation of some countries does not allow use of certain classes of insecticide. Recommendations for alternative insecticides should therefore be included in policies.

Insecticides should be rotated at appropriate intervals to prevent the emergence of resistance. All control programmes should include studies of susceptibility before the selection of insecticides, and resistance should be monitored at sentinel sites during the programme. Standard protocols for resistance monitoring and further information are available.

Insecticide-treated nets are an effective, relatively cheap, sustainable method for sandfly control. The term covers both nets that are impregnated at regular intervals and long-lasting nets, in which insecticide is incorporated or coated on the fibre and which remain effective for 2–3 years. (See WHOPES for further information.) Under ideal conditions, insecticide-treated nets with people sleeping under them act as baited traps that kill sandflies.

Acceptability and human sleeping behavior are critical to the effectiveness of insecticide-treated nets. It is often argued the nets are uncomfortable in the hot season due to lack of ventilation, but this idea stems from the false conception that nets must be of small mesh in order to protect against sand flies. Field studies show that insecticide-treated nets of normal mesh size are effective, and several studies have shown good acceptability in endemic areas, as the nets confer privacy and protect against nuisance from other insects. Screening of windows with insecticide-treated materials or impregnated curtains significantly reduced the numbers of sand flies entering houses in studies in Burkina Faso, Italy, the Sudan and the Latin American region.

Another method for control of cutaneous and zoonotic visceral leishmaniasis is the use of dog collars impregnated with pyrethroid insecticides. This method reduced the incidence of visceral leishmaniasis in children in a community trial in the Islamic Republic of Iran. In Brazil, their use under programme conditions revealed many operational problems, such as loss of collars. Pyrethroid insecticides can also be applied on dogs as shampoos, spotons and lotions, and these are being evaluated.

Environmental management:

Environmental management results in a reduction in sand fly–human contact or sand fly populations through interventions in ecological niches. Examples are relocation of human settlements away from sand fly habitats and physical modification of the habitats. Environmental management measures must be preceded by careful studies of local ecology and the environmental impact. Physical modification of P. papatasi sand fly breeding and resting sites by destruction of the burrows of the great gerbil (R. opimus) was used successfully in the Central Asian republics of the former Soviet Union. Similarly, in Colombia, French Guiana and Panama, clearing of forests around villages and settlements effectively reduced or eliminated vector–human contact and Leishmania transmission. Actual or potential sand fly breeding sites, such as rubble and rubbish tips, can be eliminated in sanitation programmes involving the local

community, especially in urban areas (e.g. Lu. whitmani in Brazil). It is important that any modification of vector habitats take into account environmental conservation and not create local ecological conflicts.

It is recommended that people entering or living in highly endemic foci use personal protection measures to avoid bites by sand fly vectors of leishmaniasis. These measures include avoiding times and places of sand fly activity and application of insect repellents on exposed skin.

Entomological monitoring and evaluation of vector control operations:

A well-designed scheme for monitoring and evaluating an integrated vector management programme should be prepared before any sand fly control operation is launched. The scheme should include clear definitions of the process, output and outcome indicators of the programme. The evaluation scheme should include methods to assess both the short- and long-term effects of the control measures on the vector population. A set of standard indicators is included in the tool kit prepared by WHO/TDR.

Routine indicators of quality include:

the performance of people conducting indoor residual spraying (or dipping insecticide-treated nets), by observation; the accuracy of spraying, i.e. the percentage of chemical on the wall in comparison with the target concentration (filter paper technique for indoor residual spraying or analysis of insecticide-treated nets); bioassay of the effectiveness of indoor residual spraying or insecticide treated nets (see WHOPES site); vector density, monitored by knock-down catches of sand flies resting indoors, light trapping, quantitative sticky-paper traps or standardized active catches of day-resting sand flies, depending on the sand fly species and its habits; and acceptability, as outlined in the TDR toolkit. Evaluation of the effectiveness of interventions on transmission should include studies with epidemiological end-points in humans (infection, disease) or studies of the effect on infection rates in sand flies [56] .

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