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Visceral leishmaniasis in Ethiopia: Innate immune functions, biomarkers of cure and potential roles of cattle for transmission

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ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CD62L	Cluster of differentiation 62L
CD66b	Cluster of differentiation 66b
CGD	Chronic granulomatous disease
CO_2	Carbondioxide
CR3	Complement receptor -3
CRP	C-reactive protein
DAT	Direct agglutination test
DC	Dendritic cells
DCL	Diffuse cutaneous leishmaniasis
DHR 123	Dihydrorhodamine 123
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EHC	Endemic healthy control
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
*f*MLP	Formyl-methionine-leucine-phenylalanine
G-CSF	Granulocyte colony stimulating factor
GIPL	glycoinositolphospholipids
GM-CSF	Granulocyte macrophage colonystimulating factor
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HIV1/2	Human immuno deficiency viruses 1/2
IFN	Interferon
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL-1	Interleukin-1
IL-1β	Interleukin-1 beta
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-2	Interleukin- 2
IL-6	Interleukin- 6
IL-7	Interleukin- 7
IL-8	Interleukin- 8
iNOS	Inducible nitric oxide synthase
IP-10	IFN-inducible protein-10
KCl	Potassium chloride
L. aethiopica	*Leishmania aethiopica*
L. braziliensis	*Leishmania braziliensis*

L. chagasi	*Leishmania chagasi*
L. donovani	*Leishmania donovani*
L. enrietti	*Leishmania enrietti*
L. infantum	*Leishmania infantum*
L. major	*Leishmania major*
L. mexicana	*Leishmania mexicana*
L. tropica	*Leishmania tropica*
LCL	Localized cutaneous leishmaniasis
LN	Lymph nodes
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
M. tuberculosis	*Mycobacterium tuberculosis*
MALP-2	Macrophage-activating lipopeptide-2
MCF	Mononuclear cell factor
MCL	Mucocutaneous leishmaniasis
MCP-1	Monocyte chemotactic protein 1
MDM	Monocyte derived macrophages
MHC	Major histocompatability complex
MIP-1α	Macrophage inflammatory protein-1 alpha
MIP-1β	Macrophage inflammatory protein-1 beta
MMP- 9	Matrix metalloproteinase-9
MPO	Myeloperoxidase
MR	Mannose receptor
MyD88	Myeloid differentiation primary response 88
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium bicarbonate
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa B
NK	Natural killer
NNN	Novy-MacNeal-Nicolle
NO	Nitric oxide
O_2^-	Superoxide anion
P. orientalis	*Phlebotomus orientalis*
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction restriction fragment length polymorphism
PE	Phycoerythrin
PKDL	Post kala azar dermal leishmaniasis
PMN	Polymorphonuclear
Poly (I: C)	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptors
RANTES	Regulated on activation, normal T cell expressed and secreted
rK39	Recombinant fragment of kinesin gene 39
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute Medium 1640

S. aureus	*Staphylococcus aureus*
sCD40L	Soluble CD40 ligand
SD	Standard deviation
SEM	Standard error of the mean
TAE buffer	Tris-acetate-EDTA buffer
TB	Tuberculosis
TCR	T cell receptor
TEM	Trans-endothelial migration
TGF–β	Transforming growth factor beta
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TRIF	TIR Toll/interleukin-1 receptor domain containing adapter inducing interferon-beta
VEGF	Vascular endothelial growth factor
VL	Visceral leishmaniasis
WHO	World Health Organization

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1. INTRODUCTION

1.1. Visceral leishmaniasis

Leishmaniasis is a vector-borne neglected tropical disease caused by unicellular protozoan parasites of the genus *Leishmania.* The genus *Leishmania* comprises over 20 different species, which cause different clinical forms of leishmaniasis in humans. Clinically the disease ranges from different spectra of cutaneous forms such as: mutilating mucocutaneous leishmaniasis (MCL), disfiguring diffuse cutaneous leishmaniasis (DCL), and self-healing localized cutaneous leishmaniasis (LCL) to life threatening visceral leishmaniasis (VL or Kala-azar)[1,2].

Cutaneous leishmaniasis (CL) is mainly caused by *L. aethiopica*, *L. major, L. tropica, L. mexicana* and *L. amazonensis*. Diffuse cutaneous leishmaniasis, the anergic variant of CL, is mainly caused by *L. (mexicana) amazonensis* in the New world and *L. aethiopica* in the Old world, which is known to present disseminated skin lesions all over the body [3,4]. The severe form of CL known as MCL or espundia, is mainly caused by *L. (viannia) braziliensis* and *L. (viannia) guyanensis*. In MCL the skin lesions involve mucous membranes and may lead to disfiguring destruction of nose, mouth and throat cavities [2]. Induced by *L. aethiopica,* MCL is also relatively common in Ethiopia as a clinical form of CL [5].

Visceral leishmaniasis (VL) is mainly caused by species of *L. donovani* complex that consists of *L. donovani, L. infantum,* and *L. chagasi* [2]. Visceral leishmaniasis due to *L. donovani* complex has also other different names such as Kala azar, dumdum fever, febrile tropical splenomegaly, cachectic fever and non-malaria remittent fever. The disease may be endemic, sporadic or epidemic with different clinical features [2,6]. Post kala azar dermal leishmaniasis (PKDL), a clinical form associated with VL, is mainly reported from Sudan and India in 50% and 10% of treated VL cases respectively [7]. If left untreated VL is a fatal infectious disease.

1.2. Epidemiology of visceral leishmaniasis

Visceral leishmaniasis has diverse epidemiological distributions in tropical countries (Figure 1a) with different clinical manifestations. Approximately 90% of VL cases exist in six countries of the globe which include Ethiopia, Sudan, South Sudan, India , Bangladesh, and Brazil [8]. Globally, the distribution of VL causative species are limited to geographical boundaries as *L. chagasi/infantum* in south America , *L. infantum* in Europe and Middle East , *L. donovani* sensu lato in East Africa including Ethiopia, and *L. donovani* sensu stricto in the Indian subcontinent [9]. East Africa is the second largest focus for VL, contributing 15% of the estimated annual global burden of 0.2–0.4 million cases. In this region, Sudan, South Sudan, and Ethiopia are the major contributors to the kala azar burden (8). As estimated by the Federal Ministry of Health (FMoH) of Ethiopia, in Ethiopia there are about 4,500 to 5000 new VL cases annually and over 3.2 million people live at risk of VL infection [10,11]. The distribution of VL in Ethiopia is mainly concentrated in the lowland areas of the Northwest, Southwest and Southeast areas of the country (Figure 1b and 1c). The red color shaded areas in Figure 1b depict the VL endemic areas in Ethiopia.

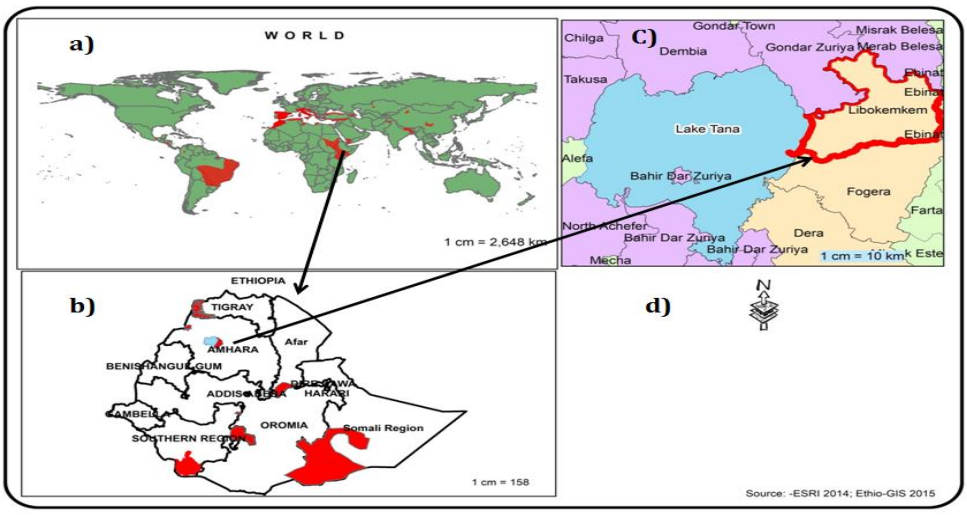

Figure 1. Epidemiology of VL and study area map.
a) The global distribution of VL, areas in red color show the endemic areas. b) Endemic foci of VL in Ethiopia: Ethiopia, with an area of 1,104,300 km^2, is a country in the horn of Africa bordered by Djibouti and Somalia in the east, Sudan in the west, Eritrea in the north, and Kenya in the south. It is the second

most populous country in sub-Saharan Africa with an estimated population of over 88 million in 2010. The Northern and Central highland plateaus are surrounded by lowland areas. The climate of the country ranges from cold and wet weather in the highlands with average annual rainfall of more than 1,000 mm to hot and dry in the lowlands with unreliable rainfall usually below 500 mm [12]. The VL endemic areas of the country are as shaded in red color. C) Map of Libo Kemkem and Fogera districts affected by the VL outbreak in 2005, where the current study is focusing. These maps were adapted from [13–16].

The Northwestern VL foci in Ethiopia cover the semi-arid low land areas such as Metema and Humera plains in Amhara and Tigray regional states bordering Sudan. A marked increase of VL cases occurred during the 1970s when migrants from the non-VL endemic highlands began to arrive in these areas to harvest crops according to the large-scale agricultural schemes introduced at that time. As an extension of the endemic area in North Sudan, post kala azar dermal leishmaniasis (PKDL) was also reported to be endemic to the Metema and Humera foci [12]. Settlers who were being relocated from highland areas to Kafta Humera, Tsegede and Armacho districts represented a vulnerable population in the area where relocation corresponds with a dramatic increase in VL cases. In 2005, an outbreak of VL in Libo Kemkem district, a highland area of Amhara regional state, was identified. The outbreak began in Bura village in 2003, with escalating cases in 2005 and occurring mainly in Libo Kemkem and Fogera districts [15,17]. These cases and some reported in the 1970s in Belessa, an area in the Gondar zone North of Libo Kemkem, are the only recorded VL cases from the highland areas in Ethiopia [18]. Cases of VL have also been recorded in the Awash Valley in Amhara and Afar regional states in the Northeast of the country [19].

The Southwest VL foci in Ethiopia include the Omo and Aba Roba plains and Weyto River Valley in the Southern Nations and Nationalities People's Regional (SNNPR) state [12]. The majority of people in these foci are nomadic or semi-nomadic pastoralists and have been exposed to the disease and acquired some immunity, as indicated by a positivity rate of up to 64% by the leishmanin skin test [20]. The cases of VL have also been reported from further East in the Moyale area and Genale river basin near the Kenyan border, Oromia Regional state [21]. In the Southeast Ethiopia, an outbreak of VL on the border between Kenya, Somalia and Southeast Ethiopia was identified, affecting Afder and Liban zones in Ethiopia's Somali Region [22].

In Ethiopia, various risk factors have been associated with VL transmission. The majority of VL cases throughout the country occur in males, due to increased exposure to the vector during agricultural or pastoral activities. In areas where the disease has been endemic for many years, more cases occur in younger age groups as they have yet to develop the acquired immunity. In areas where the disease has recently been introduced, all ages are susceptible, and most cases occur in groups that have regular contact with appropriate habitats [23]. It is speculated that agricultural workers returning from Humera and Metema introduced the disease and contributed to the outbreaks [17,24]. The transmission of VL seems to be associated with humidity as in the Southwest foci most infections occur in the rainy season. However, in the Northwest, transmission peak is just after the rainy season when the majority of migrant workers leave the highlands to work on lowland farms during the harvest. Similarly, it has been indicated that habitual outdoor sleeping, maintaining cattle indoors, lack of mosquito net ownership , un-plastered walls, living proximity to termite hills and poor nutritional status are associated with increased VL risk in Ethiopia [12]. In Ethiopia, VL is caused by infection with *L. donovani* where *P. orientalis* and *P. martini* have been confirmed as vectors of VL. Both sand flies species breed and rest in the termite mounds common to the areas[25].

1.3. Life cycle of *Leishmania donovani*

The life cycle for all *Leishmania* parasites is apparently similar. *Leishmania* parasites are naturally transmitted to their vertebrate hosts by the bite of blood sucking female sand flies [2,26]. The bite of sand fly creates a blood pool in dermis of the mammalian host where it deploys its salivary contents containing the promastigote form of *Leishmania* parasites. At the site of inoculation, *Leishmania* parasites are taken up by phagocytic cells, in which they transform into the amastigote form, and multiply in the phagolysosomes of recruited macrophages. *Leishmania* infection caused by *L. donovani*, can disseminate to visceral organs via the lymphatic system [27].

Figure 2 describes life cycle for *L. donovani.*

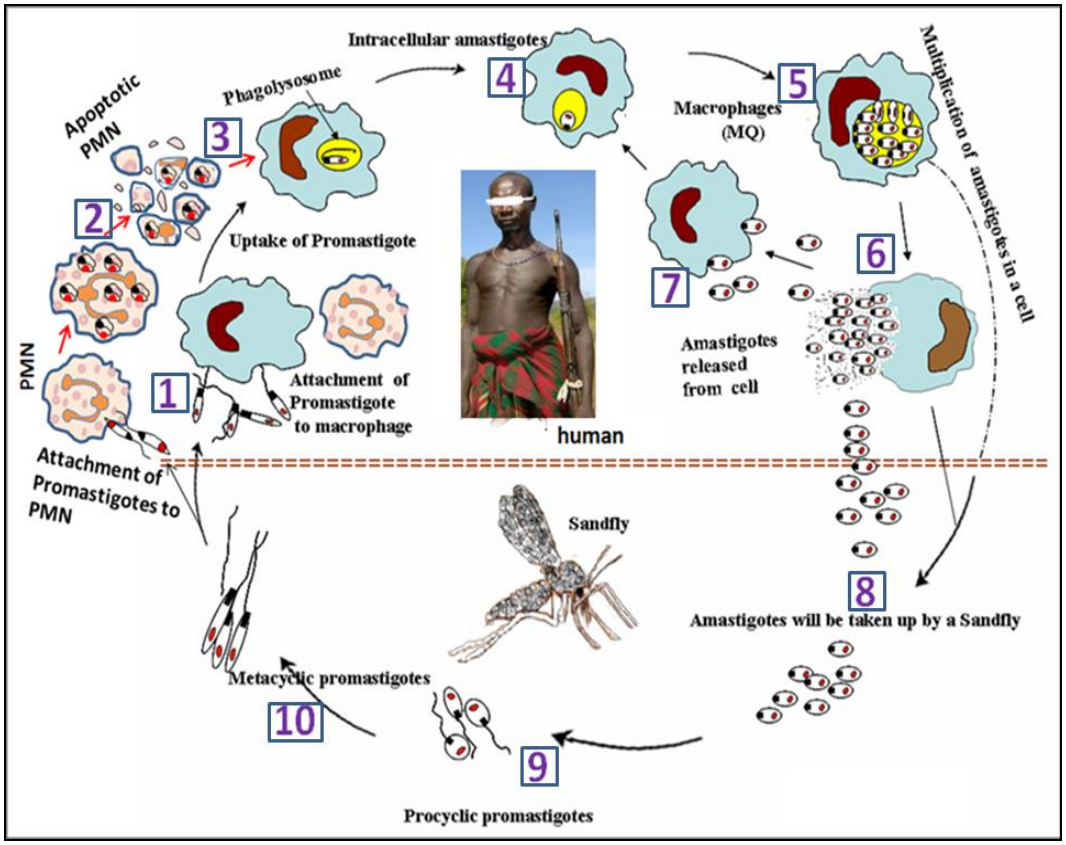

Figure 2. The lifecycle of *L. donovani* in brief.

1) During a blood meal, an infected phlebotomine sandfly releases metacyclic promastigotes into the dermis of a human host. 2) The parasites then interact with host PMN, and monocytes. The neutrophils swarm around the extracellular metacyclic promastigotes and engulf many of them, 3) Metacyclic promastigotes from the initial inoculums (or those have been released from infected neutrophils) are phagocytosed by macrophages, 4) Within the phagolysosome engulfed promastigotes transform to amastigotes within 12-24 hours, 5) In infected macrophages, amastigotes multiply and fill the phagolysosome. 6) Multiplication of amastigotes results in macrophage rupture and release of amastigotes, which infect another bystander macrophage. 7) The bystander macrophage engulfs the newly released amastigotes. 8) Amastigotes along with other host cells will be ingested by sandfly during its blood meal. 9) Amastigotes transform into procyclic promastigotes within 24-48 hours and multiply. 10) The procyclic promastigotes transform into metacyclic promastigotes within 5-7 days and inoculation of these promastigotes into to new host cells complete the lifecycle. Adapted from [13] with some modifications.

The amastigotes must overcome two environmental challenges: the battery of lysosomal enzymes and a low pH of 4.5-5.5. However low pH seems to facilitate amastigote multiplication since they are metabolically more active at low pH [28,29]. The transformation of amastigotes to promastigotes begins in the sandfly within 24-48 hours of ingestion of free or intracellular amastigotes and occurs exclusively in the gut. These transformed motile promastigotes continue to divide by binary division in the sand fly gut and latter the metacyclic promastigotes accumulated in the mid gut and foregut and are inoculated into the vertebrate host during a subsequent blood meal [3,30].

1.4. Modes of *L. donovani* transmission

Understanding the mode of transmission of *Leishmania* is essential for designing control and prevention strategies. The most common and natural mode of transmission of *Leishmania* is through the bite of sand fly; other modes of transmissions such as occupational exposures (needle stick) (19) or from mother to child during pregnancy [32] could also be possible, but have received little emphasis. Incidental transmission of *Leishmania* was previously reported as laboratory acquired infection caused by *L. tropica*, *L. braziliensis* and *L. donovani* [33–35]. Furthermore, VL transmissions from an infected person to other person can occur during blood transfusion and needle sharing among intravenous drug users [36–43].

A case of congenital leishmaniasis was reported in 1926 by Meinecke and colleagues [44], and sexual transmission of leishmaniasis was also recently reviewed [45]. Similarly, cultures of urine and prostatic fluid from VL patients have yielded *Leishmania* promastigotes, indicating the potential for sexual transmission of leishmaniasis [33,34,46–49]. In addition, transmission of leishmaniasis among homosexuals with AIDS who had rectal lesions and anal intercourse was reported

from endemic areas of Spain [33,34] which confirms the possibility of sexual transmission of leishmaniasis.

Generally, based on the source of infection, VL transmission is grouped into two as zoonotic and anthroponotic. Though the transmission of *L. donovani* infection has been considered as anthroponotic in East Africa[2], published reports have concern that there would be increased risks of VL in humans living in close proximity to domestic animals [2,50].

1.5. *Leishmania donovani* parasite

Leishmania is a flagellate protozoan parasite that belongs to the family of Trypanosomatidae [52]. It exists in two forms: the intracellular amastigote form in mammalian host cells and the extracellular motile promastigote form in the sand fly (Diptera, Psychodidae). Morphologically, the amastigote form is ovoid and non-flagellated measuring 3-5 μm in length [26,53]**.** It has round or oval nucleus adjacent to its smaller round or rod shaped kinetoplast. The non-functional flagellum in amastigotes does not extend beyond the cell body. The flagellum pocket functions as a site of endocytosis and exocytosis as well as anchoring the flagella [54]. The kinetoplast, which contains a dense mass of mitochondrial DNA, lies below the origin of the flagellum and is composed of several thousand circular DNA molecules [55].

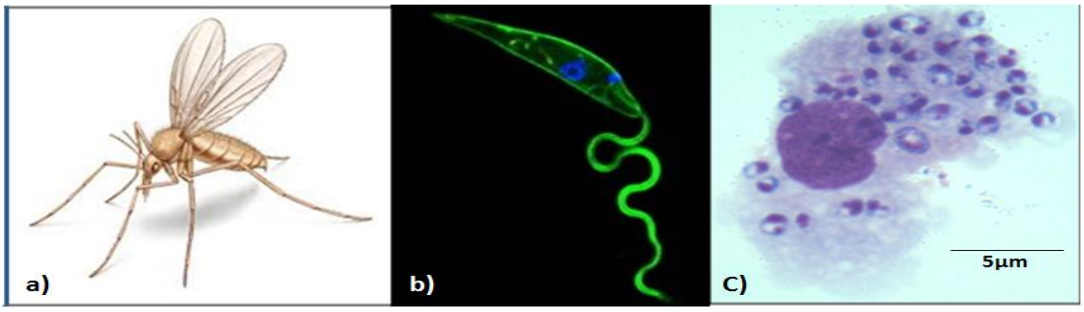

Figure 3. Morphological forms of *Leishmania* parasite, and the sand fly.
a) Sand fly image. Adapted from https://www.orkin.com/flies/sand-flies/, b) *Leishmania* promastigote (fluorescence stain). Adapted from http://www.emedmd.com/content/leishmaniasis, c) intracellular amastigotes in an infected macrophage.

Morphologically, the motile promastigote form is elongated with a cell body length of 8-15 µm (Figure 3b) with a single flagellum. Promastigotes can be classified as procyclic, nectomonad, haptomonad, paramastigotes and metacyclic promastigotes. The transformation of amastigotes to procyclic promastigotes is the first developmental event occurring in the sand fly. Procyclic promastigotes multiply and develop into metacyclic promastigotes in the posterior mid-gut of the sand fly [1,3,56–59].

1.6. Diagnosis of visceral leishmaniasis

Clinical diagnosis: The clinical onset of VL symptoms induced by *L. donovani* is gradual with common symptoms such as fever, malaise, shivering or chills, weight loss, anorexia, and discomfort in the left hypochondrium. The common clinical signs for VL are usually non-tender splenomegaly, with or without hepatomegaly, wasting, pallor of mucous membrane and lymphadenopathy. Moreover signs of malnutrition like oedema, skin and hair changes may also be apparent during VL. In addition, other infections such as pneumonia, dysentery and pulmonary tuberculosis may occur as complication to VL [2,60,61]. For non-indigenous people of any age who visit VL endemic areas, the onset of VL signs and symptoms begin with an abrupt onset of

fever from 3 weeks to 2 years after exposure. The disease may progress acutely with chills, high undulating fever, often with two peaks per day, drenching sweats, rapid weight loss and profound malaise [2].

In some countries, the number of subclinical cases are considered to be more numerous than the number of clinical cases by about 5:1 ratio. Cases of VL occur in increasing numbers among people with immunosuppression due to acquired immunodeficiency syndrome (AIDS) or anti-tumor chemotherapy, and these cases are difficult to treat [34]. A portion of VL patients develop skin rash known as post kala azar dermal leishmaniasis (PKDL) after successful treatment, which is characterized by a macular, maculopapular, and nodular rash in VL patients. The rash usually starts around the mouth from where it spreads to other parts of the body depending on severity. PKDL is largely restricted to areas where *L. donovani* is the main causative parasite [7].

Figure 4. Different clinical forms of VL.
Left: VL patient presented with hepatosplenomegaly. Right: VL patient presented with post kala azar dermal leishmaniasis (PKDL). Adapted from http://www.who.int/ leishmaniasis /visceral _leishmaniasis/en.

Not only clinical signs and symptoms of VL but also its epidemiological distribution overlap with other disease conditions such as malaria, typhoid, tuberculosis, and schistosomiasis. Clinical signs and symptoms alone are not specific enough to differentiate VL from chronic malaria, schistosomiasis or other systemic infections. Since clinical diagnosis alone could not able to rule out VL, reliable *Leishmania*-specific diagnostic confirmation tests are required [2,11].

Parasitological diagnosis: Parasitological diagnosis of VL can be achieved by direct microscopical demonstration of *Leishmania* amastigotes in tissue samples or biopsies [62,63] or in the peripheral blood buffy coat [64]. Laboratory diagnosis for PKDL is usually performed using examination of stained smears from skin lesions, and cultures from dermal scrapings or stained sections obtained from a skin biopsy [62]. In smears or touch preparations of infected tissue stained with Giemsa's stain, preferably at pH 7.2, amastigotes are readily seen with the cytoplasm typically pale blue and the nucleus and kinetoplast of purple-pink color [30]. The size of amastigotes ranges from 3 x 5 μm to 4.5 x 5 μm and appear purple blue with central nucleus with a rod shaped structure at the right angle of nucleus [3]. Spleen aspirates from immunocompetent VL patient is regarded to be a better sample than other tissue aspirates [3,30,65]. The splenic aspirates can also be used for determining parasite load by counting the number of amastigotes in the smears in relation to the white blood cell counts [65]. The most specific diagnostic method for VL is isolation of the causative agent of VL from clinical specimens in culture medium which helps to characterize the organisms up to species or genotype level [3].

Serological diagnosis: The direct agglutination test (DAT) is an important serological tool for the diagnosis of VL, which is a highly specific and sensitive test. It is relatively cheap and simple to perform, and ideal for both field and laboratory use [64,66]. Other important laboratory method used for the diagnosis of VL includes enzyme linked immunosorbent assay (ELISA), and rapid diagnostic test (RDT) developed from recombinant antigen known as rK39 [62,66–68].

Molecular diagnosis: In recent years, polymerase chain reaction (PCR) based diagnostic methods are used with a wide range of sensitivities and specificities for detection of leishmanial infections. However PCR based techniques can not differentiate between asymptomatic and acute VL disease in endemic areas, since many carriers of the infection in these areas will be PCR positive without developing VL disease. This technique remains complex and expensive, and in most VL-endemic countries, restricted to a few teaching hospitals and research centers [69,70].

1.7. Pathogenesis of visceral leishmaniasis

The incubation period, the period between the time of the initial infection and the appearance of clinical symptoms, for most leishmaniasis is usually from 2 weeks to 18 months [71]. In VL symptoms may take more years to appear and the gross inflammatory reactions within the viscera often develop between 2–8 months after infection [72]. In infected macrophages**,** *L. donovani* amastigotes multiply and affect different tissues [2]. Upon destroying host cells, amastigotes are released and infect other phagocytic cells. These amastigotes then disseminate through the lymphatic and vascular systems, ultimately infiltrating the bone marrow, liver and spleen. Since *Leishmania* parasites multiply in macrophages, the rapid proliferation of these cells in the spleen and liver, consequently, may lead to organomegaly [71–73]. This contributes to the depressed production of blood cells (red blood cells, white cells and platelets) due to *Leishmania* infection of bone marrow and results in immunosuppression which in turn makes the host susceptible to co-infections [71,73]. When the disease is advancing, untreated symptomatic infection can lead to a mortality rate of 75%–95% [71,72]. As a sequel of VL, patients infected by *L. donovani* may develop post-kala-azar dermal leishmaniasis (PKDL) after successful treatment of VL mainly in India and Sudan [71,73,74]. Lesions may appear anywhere on the body, but they usually occur on the face [72]. These may remain asymptomatic for months to years and then develop to a progressive proliferation of parasites within the skin, giving rise to diffuse macular, maculopapular, or nodular lesions [73].

In most of *Leishmania* endemic countries, HIV co-infection has been emerged as a major complication of leishmaniasis. Among HIV-positive individuals, VL was previously considered as the fourth most common opportunistic parasitic disease reported [34]. In Southern Europe and Africa particularly in Ethiopia and the Sudan, HIV/*Leishmania* co-infection is regarded as an emerging disease [75]. In co-infected patients, it is believed that the clinical course of leishmaniasis is modified by HIV [76]. Another contributing factor to pathogenicity of VL is malnutrition. A study result from

an animal model shows malnutrition induced innate immune defense alteration resulted in increased visceralization after cutaneous infection by *L. donovani* [77].

1.8. Immunity during visceral leishmaniasis

The immune system is an integrated body system that is comprised of various organs, tissues, cells and cell products that mediate host defense against pathogenic microorganisms. The immune responses to *Leishmania* infection are highly complex and while they may accelerate cure, some responses exacerbate the disease depending on the particular circumstances [78]. Immunological response to *Leishmania* parasite involves the participation of both innate and adaptive immune system. In addition to humoral components, innate and adaptive immune cellular responses [79] are known to be crucial components to fight *Leishmania* parasites. The initial tissue damage caused by sand fly bite promotes the recruitment of neutrophils to the site of injury [80,81] as the first line of immune defense mechanism. Inoculated parasites are then taken up by recruited neutrophils, and eventually by macrophages or dendritic cells which also engulf parasites or parasitized neutrophils [82]. This enables *Leishmania* parasites to survive and replicate inside macrophages by modulating the antimicrobial machinery as well as by increasing the host cell membrane fluidity and disrupting lipid rafts, which in turn affects the antigen presentation capability of host antigen presenting cells (APCs) [83]. During these processes, the parasitized APCs interact with T cells to stimulate several cytokines and chemokines, which help *Leishmania* to hijack the whole immune system for its own survival [84].

It is believed that Th1 cell mediated immunity is an effective immune response to control intracellular *Leishmania* infection [85–88]. In the murine model, it has been demonstrated that in CL there is a clear dichotomy between Th1-mediated protection (mediated by IFNγ, IL-2, TNF-α) and Th2-mediated disease progression (mediated by IL-10, IL-4) [89,90]. However, this Th1/Th2 dichotomy is not so clear in VL infection

of both mice and humans [91]. The immune response and the pathology of VL are complex, involving a number of factors in the process of susceptibility or resistance to parasites [92]. Previously, adaptive immunity was considered as the crucial immune response for the resolution of infection. Currently, however, there is increasing evidence that innate immune mechanisms contribute to the defenses against parasitic infections [93].

The earliest interaction of *Leishmania* parasites begins with innate immune cells (neutrophils and monocytes) in the blood pool made during sand fly bites which create a hemorrhagic pool in the host skin for feeding [94]. Therefore, using a whole blood based assay is an appropriate approach to study the function of innate immune cells during VL infection.

1.8.1. Innate immunity and *Leishmania*

In general terms innate or inborn refers to the non-antigenic specific immune system, which plays a critical role in host recognition and response to bacterial, fungal, and parasitic pathogens. It comprises cellular components such as neutrophils, macrophages, dendritic and natural killer cells; and humoral components such as C-reactive protein (CRP), lysozyme, and complement factors to fight against foreign invading microorganisms. This type of host immune defense consists largely of professional phagocytes including polymorphonuclear (PMN) leukocytes, circulating monocytes, and tissue-based macrophages [95,96]**.** Upon pathogen challenge, the cellular component of innate immunity recognizes a wide range of pathogen associated molecular motifs through pattern recognition receptors (PRR). Toll-like receptors (TLRs) are considered as the most important PRRs in triggering the activation of innate immunity [96]**.** In perspective to leishmaniasis**,** evidence shows that *L. major* derived molecules can activates TLRs. The best studied *Leishmania* molecule is lipopolyglycan (LPG) , which is known to activate TLR-2 [97]. Initially, the

biting of sand fly causes damage of the microvasculature that creates a hemorrhagic pool, where metacyclic promastigotes are inoculated together with sand fly saliva. These inoculated metacyclic promastigotes are sufficient to establish disease progression. Sand fly saliva itself contains well-characterized molecules that have several bioactive compounds, which lead to vasodilation, inhibition of coagulation and immunomodulatory effects. It also contains uncharacterized molecules that could attract neutrophils and macrophages and recruit them to the site of infection [98,99]. These recruited neutrophils and macrophages rapidly phagocytose metacyclic promastigotes in the local inflammatory area. After establishing an intracellular niche, metacyclic promastigotes are transformed to non-motile amastigotes in macrophages. These amastigotes replicate within the host cells, and eventually rupture the cells to escape and infect other bystander phagocytes [100]. The amastigotes can be transported to visceral organs which results in organomegaly. Hence due to these interactions, the innate immune response develops after the initial sensing of invading microbes leading to production of effector molecules that contribute to the containment of the initial infection [101].

There is a growing perception that innate immune responses have immunoregulatory roles and shape the downstream acquired immune response. However, roles of innate immune cell functions during VL are not well explored. Innate immune cell functions during infection are demonstrated in the following figure.

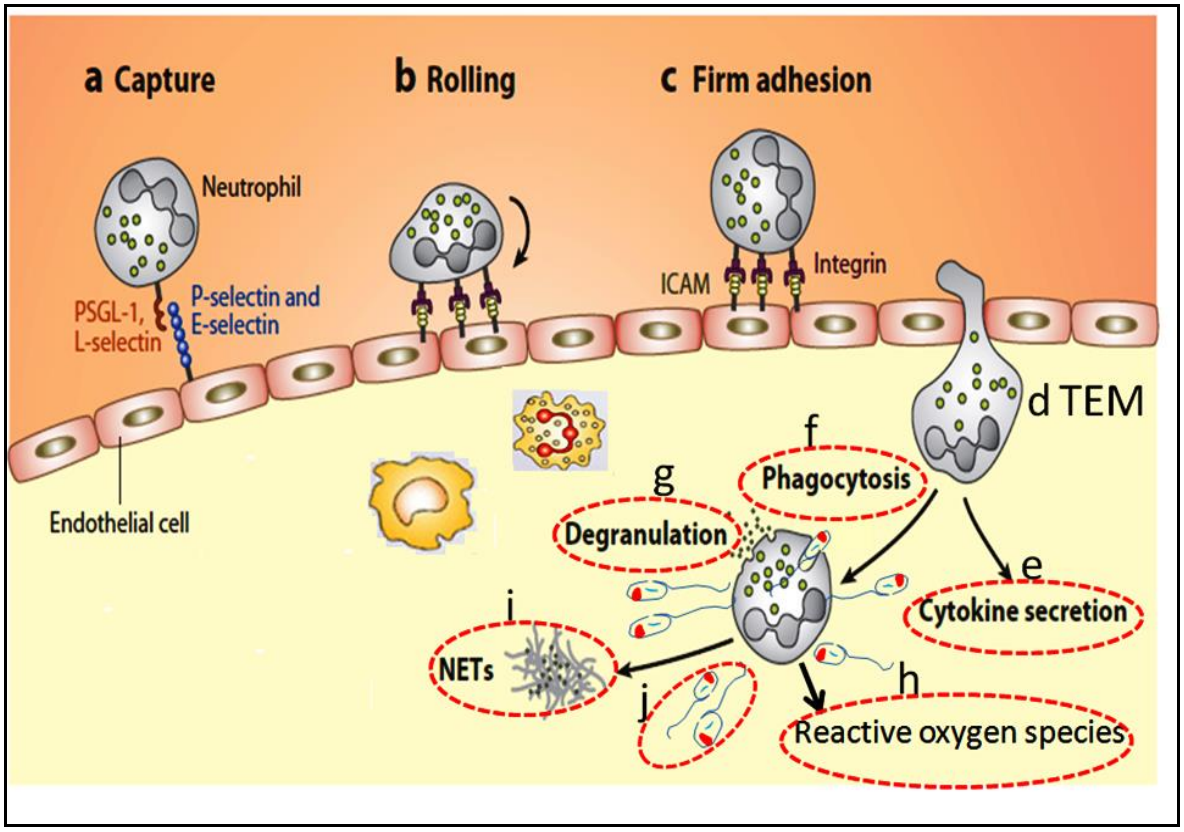

Figure 5. Leukocyte recruitment to sites of inflammation.
Circulating neutrophils recognize signs of inflammation and migrate to areas where the antimicrobial arsenal is required for the elimination of infection. (a) Close to the inflammatory sites, stimulated endothelial cells expose a class of molecules, the selectins, which serve to capture circulating neutrophils and tether them to the endothelium. (b) Selectin-mediated rolling along chemoattractant gradients then ensues, followed by (c) integrin-mediated firm adhesion and d) TEM (trans- endothelial migration). The neutrophils pass through the endothelium and arrive at the site of inflammation. e) The neutrophil releases cytokines that recruit other immune cells and it begins to implement its antimicrobial activities. f) Among the processes employed are engulfment of microbes via receptor-mediated phagocytosis, (g) release of granular antimicrobial molecules through degranulation, h) production of reactive oxygen species, and (i) formation of neutrophil extracellular traps (NETs) and j) Leishmania promastigotes initiating inflammation. In this dissertation innate immune cellular functions (as illustrated in the Figure 5 e, f, g, and h) was investigated. Adapted from [102].

1.8.1.1. Toll-Like Receptors (TLR)

After discovery of Drosphila Toll, a mammalian homologue family of structurally related proteins was subsequently identified and collectively referred to as the Toll-like receptors (TLRs)[103]. To date, there are 13 mammalian TLRs described so far that recognize different classes of pathogen derived molecular motifs (Table 1). While

TLRs 1–9 are functionally conserved between humans and mice, TLR10 seems to be functional only in humans, and TLR11 is functional in the mouse. The conserved TLRs can be divided into extracellular: TLR1-2, TLR4-6, and TLR11 or intracellular: TLR3,TLR7-9 and TLR13 [96,104,105]. The extracellular and intracellular receptors recognize ligands at the cell surface and in the endosomal compartment, respectively [106]. Each TLR detects distinct sets of molecules from viruses, bacteria, fungi and parasites, which upon binding recruit different adaptor proteins such as MyD88 (myeloid differentiation primary response 88) or TRIF (Toll/interleukin-1 receptor domain containing adapter-inducing interferon-β) [106]. They are primary sensor molecules of cellular receptors of the innate immune system that function by recognizing pathogen-associated molecular patterns(PAMPs) [103,107].

Toll like receptors are known to play an important role in the initiation of innate immune responses and T cell-mediated adaptive immune responses through up-regulation of MHC and co-stimulatory molecules, as well as through the induction of Th1-polarizing cytokines [108]. Toll like receptors expressed by innate immune cells are known to induce the production of proinflammatory cytokines and type I interferon (IFN) [106]. Neutrophils, a major innate immune cell population, express the majority of TLR family members, but they fail to express intracellular TLR3 and TLR7 [109]. In these cells, TLR activation often leads to the generation of reactive oxygen species (ROS), cytokine production, increased cellular survival, receptor expression, and phagocytosis [110]. The conserved microbial molecules such as bacterial lipopolysaccharide (LPS), peptidoglycan, bacterial DNA, and double-strand viral RNA are recognized by TLRs. Since TLR recognition is often associated with the production of proinflammatory cytokines and with the generation of antimicrobial effector molecules, it is decisive to determine the implications of TLR activation during *Leishmania* infections. In line with this, a few *Leishmania* derived molecules has been reported to activate TLRs and the majority of the studies to date reported the recognition of *Leishmania* parasites by TLR2,TLR4 and TLR9 [93]. The contribution of innate immune system in controlling protozoan parasites in mammals has been not investigated in comprehensive manner.

Table 1. TLRs and their microbial ligands

TLRs*	Ligands	Microbes
TLR1	Triacyl lipopeptides	Mycobacteria and Gram-negative bacteria
TLR2	Peptidoglycans	Gram-positive bacteria
	GPI-linked proteins	Trypanosomes
	Lipoproteins	Mycobacteria and other bacteria
	Zymosan	Yeasts and other fungi
	Phosphatidlyserine	Schistosom/es
TLR3	Double-stranded RNA	Viruses
TLR4	LPS	Gram- negatives bacteria
	F-protein	Respiratory syncytial virus (RSV)
	Mannans	Fungi
TLR5	Flagellin	Bacteria
TLR6	Diacyl lipopolypeptides	Mycobacteria and Gram-positive bacteria
	Zymosan	Yeasts and other fungi
TLR7	Single-stranded RNA	Viruses
TLR8	Single-stranded RNA	Viruses
TLR9	CpG unmethylated dinucleotides	Bacterial DNA
	Dinucleotides	
	Herpes virus components	Some herpesviruses
	Hemozoin	Malaria parasite heme by product
TLR10	Unknown	Unknown
TLR11	Unknown	Uropathogenic bacteria
	Profilin	Toxoplasma
TLR12	Unknown	Unknown
TLR13	Unknown	Vesicular stomatitis virus

*All function as homodimers except TLR1, 2, and 6, which form TLR2/1 and TLR2/6 heterodimmer. Ligands indicated for TLR2 bind to both; ligands indicated for TLR1 bind to TLR2/1 dimmer , and ligands indicated for TLR6 bind to TLR2/6 dimers. Source Kuby Immunology, 7th ed. New York, 2009 [205].

1.8.1.2. Phagocytosis

Phagocytosis is the ingestion and destruction of microbes by cells called phagocytes. The main types of phagocytes of the innate immune cells are the monocytes/ macrophages, neutrophils and dendritic cells (DCs). The way in which phagocytic cells phagocytose particles is essentially the same and can be divided into four stages: attachment, ingestion, killing and degradation. During attachment stage *Leishmania* binds to the macrophage surface and stimulate their own phagocytosis [111,112].

Although early studies described the polarization of *Leishmania* parasites during attachment and phagocytosis by macrophages as random process [112], subsequent

reports show a preferential orientation of *L. mexicana, L. tropica, L. braziliensis, L. major* or *L. aethiopica* promastigotes during macrophage entry. These differences may be to some extent species specific. For example, *L. donovani* promastigotes adhere either through their flagellar tip or aflagellar (posterior) pole [113,114] whereas *L. enrietti* promastigotes enter mouse macrophages primarily from the posterior pole [115].

Attachment of *Leishmania* to host macrophages is mediated by interactions between specialized receptors on the phagocyte surface and presumed complementary ligands on the parasite surface [113,116]. Receptors such as CR3, CR1, mannose receptor (MR), Fc gamma receptors (FcγRs) and fibronectin receptors (FnRs) facilitate *Leishmania* internalization [117–119]. In particular, CR3 has been implicated in the safe uptake of *Leishmania* promastigotes, guiding them toward a pathway that delays phagolysosome maturation [120]. Subsequently to undergo phagocytosis the parasites require host cell cytochalasin-inhibitable actin as a passive partner [111,121,122]. This is a critical step for promastigote survival, which hides it from humoral effector molecules. Studies have claimed that *Leishmania* parasites attain at cytosolic location after phagocytosis, despite the conventional belief that these protozoa remain exclusively in phagolysosomes [113,114,123].

According to a recent study, the type of macrophage receptors utilized during phagocytosis influence the intracellular fate of the parasite [117]. A definitive understanding of the roles of various receptors in parasite survival during natural infection remains unclear [120,124]. The surface membrane components of the extracellular promastigote differ from the intracellular amastigote [125]. The surface membrane development of virulent promastigotes often happened in the sand fly gut by a developmental process known as metacyclogenesis, which leads to further modifications in surface proteins and other glycoconjugates [126,127].

The professional phagocytic cells, neutrophils and monocytes, can recognize pathogens directly by sensing conserved PAMPs, and of course, recognition of pathogens can be facilitated by opsonins. Thus, phagocytosis is either through pattern

recognition receptors (PRRs) or via receptors to components of complement or to IgG (CR1, CR3 and FcR) [128–130]. It has recently been shown that TLRs are important PRRs in triggering the activation of innate immunity [96]. Thus TLRs are likely contribute to the defense against parasites as reviewed in Kaye and Aebischer [107]. Although the likelihood of being bitten by infected sand flies is high for all inhabitants living in the VL endemic areas, only a small ratio of the population is susceptible to *Leishmania* infection and develop disease [2]. According to my working hypothesis, one possible reason for the susceptibility is a dysfunction of professional phagocytes such as neutrophils and monocytes. The contribution of innate effector cell functions to the susceptibility to VL infection has not been investigated to date.

1.8.1.3. Reactive oxygen species (ROS)

Reactive oxygen species are reactive chemical species containing oxygen, used as antimicrobial substances by phagocytes and other cell types, such as lung epithelial cells, to eliminate pathogens. It is a collective term used for oxygen derived free radicals such as superoxide, hydroxyl radical and non-radical oxygen derivatives of high reactivity (singlet oxygen, hydrogen peroxide, peroxynitrite, hypochlorite)[131]. The conversion of oxygen to antimicrobial products is mediated by a plasma membrane-associated enzymes [132]. In response to activating signals, ROS is generated from molecular oxygen by phagocytes' nicotinamide adenine dinucleotide phosphate (NADPH) dependent phagocyte oxidase (also named as phagosome oxidase) [133,134]. NADPH oxidase converts oxygen to superoxide ion, which is converted to hydrogen peroxide by superoxide dismutase. The produced H_2O_2 partially converted to hypochlorous acid ($HClO^-$) by myeloperoxidase, as demonstrated in the schematic diagram, Figure 6.

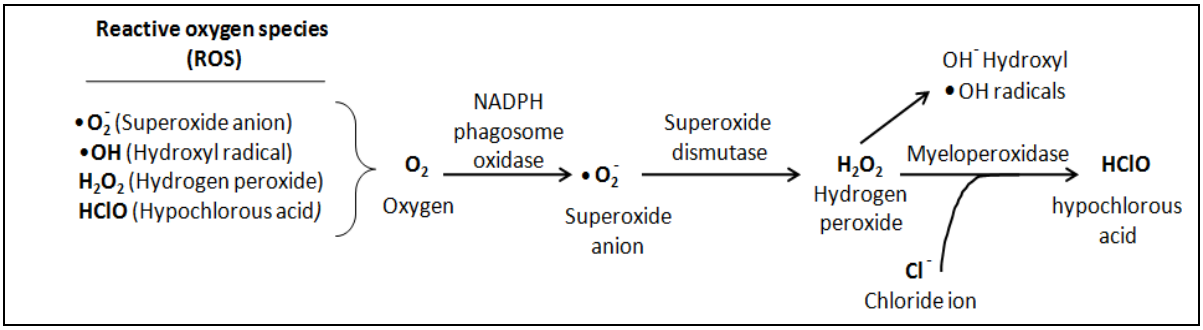

Figure 6. Generation of antimicrobial reactive oxygen species in phagocytic cells. In phagocytic cells, phagosome NADPH oxidase, transform molecular oxygen into highly reactive superoxide, which is converted to hydrogen peroxide and then to hypochlorous acid by superoxide dismutase and myeloperoxidase enzymes respectively (Adapted from Kuby Immunology, 7th ed. New York, 2009).

In response to phagocytosis and ligands of PRRs, as well as in response to IFN-γ and a second signal provided by a PAMP or TNF-α, neutrophils and macrophages produce ROS [135–138]. The patterns recognized by PRRs can be either of pathogenic origin, PAMPs, or induced by danger patterns, DAMPs, that signal tissue damage. DAMPs are intracellular molecules generally hidden from PRRs, such as adenosine triphosphate (ATP) [139–141]. Activation of endothelial cells can also induce production of ROS by neutrophils [135]. ROS can kill pathogens directly by causing oxidative damage or indirectly by stimulating pathogen elimination through various non-oxidative mechanisms, such as PRR signaling, autophagy, neutrophil extracellular traps (NETs) formation, and T-lymphocyte responses [142]. Polymorphonuclear leukocytes employ a system comprised of MPO, H_2O_2 and oxidisable halide co-factor to kill a variety of micro-organisms [143]. Myeloperoxidase is believed to be involved in augmenting the cytotoxic activity of H_2O_2 and O_{2^-}. Babior [143] has demonstrated that, during endocytosis, MPO is released into the phagosomes as a result of fusion of the phagosome membrane with the azurophil granules. *Leishmania* parasites encounter ROS during infection [144]. Despite the strong ROS responses initiated by activated macrophages, a subset of metacyclic promastigotes is still able to transform into amastigotes and eventually establish an infection. The exact mechanisms how *Leishmania* used to resist the toxic effects of ROS is not well known, however, several parasite surface molecules that include lipophosphoglycan (LPG) and glycoprotein-63

(GP63) have been found to exert protecting properties by scavenging toxic oxygen products [144]. A recent study based on experiments in mice reported that induction of ROS production in *L. amazonensis* infected peritoneal macrophages did not lead to parasite killing but led to smaller lesion sizes at early stages of infection [145]. In other case *L. major* was reported to use the produced ROS for modulation of M2 macrophage differentiation to ensure its intracellular survival [146].

Detection and measurement of free radicals in biological samples are generally accepted methods for analysis of ROS level. Among the different methods developed, the reduction of cytochrome c assessed by photometry and changes in the fluorescence properties of dihydrorhodamine (DHR)123, which can be assessed by flow cytometry is widely used due to its convenience and accuracy [147]. The general principle of ROS measurement using DHR 123 is shown in the figure below.

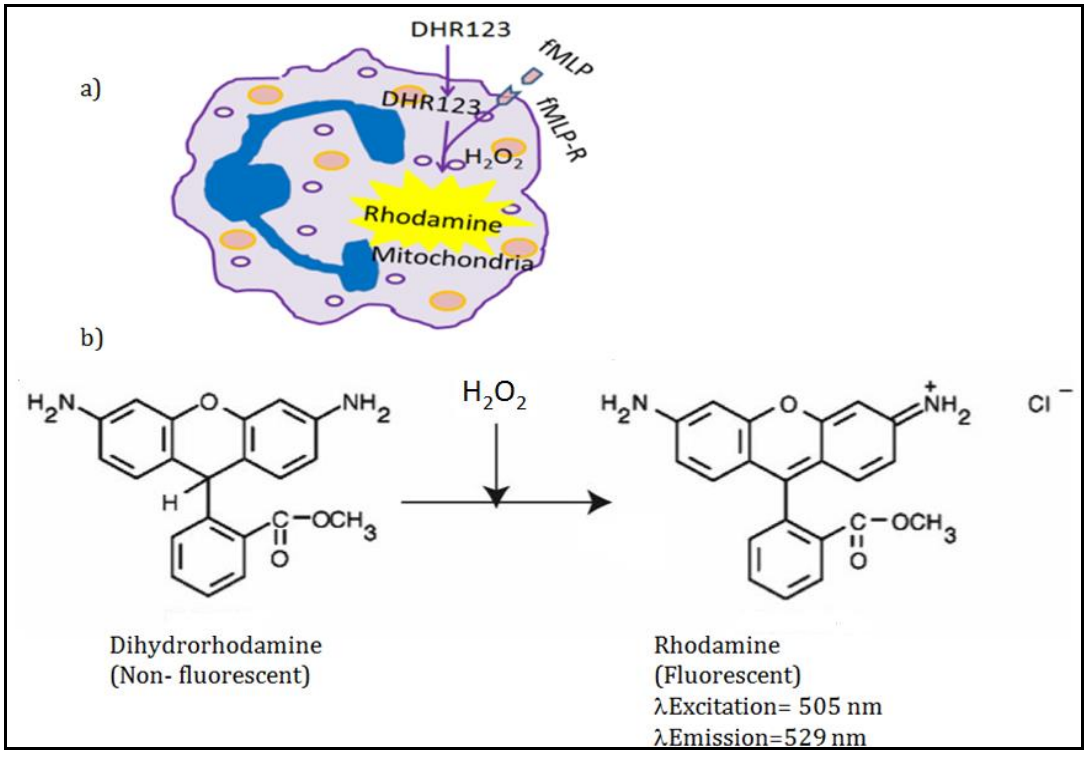

Figure 7.Schematic representation showing the principle of ROS measurement.
The principle of ROS measurement is the oxidation of dihydrorhodamine 123 (DHR 123) to rhodamine 123: (a) the freely permeable, non-fluorescent DHR 123 enters cells. Upon cell stimulation, DHR 123 is

oxidized by hydrogen peroxide (H_2O_2) resulting in the formation of fluorescent dye localized in mitochondria [148], (b) the chemical structures of DHR 123 (non-fluorescent) and the product rhodamine (fluorescent). Dihydrorhodamine 123 is a non-fluorescent molecule that, by oxidation, yields rhodamine 123, a fluorescent cationic and lipophilic probe (λ excitation=505 nm, λ emission=529 nm). Hydrogen peroxide oxidizes DHR 123 in the presence of peroxidases, adapted from [147,149].

1.8.1.4. Neutrophils

Neutrophils or PMN are the most abundant human blood leukocytes, constitute 40 to 60 % of leukocytes in the circulation[150,151]. They are the first cells to arrive at the site of *Leishmania* infection[152] and are essential part of the innate immune system. They are cells known to express TLRs that aid in the clearance of a wide range of microbial pathogens and their products. Neutrophils are phagocytic cells and important sources of cytokines, participating in host defenses through a variety of mechanisms [153].

The presence of granules in neutrophils was recognized by Metchnikov and Ehrlich as cited by Dale et al. [153] and their associated proteins were first defined through biochemical and histochemical studies at the beginning of the 20th century. Neutrophil granule proteins are produced in sequence, with the earliest proteins produced in myeloid progenitors and packaged in primary granules [154]. Subsequently, granule proteins are packaged in secondary and tertiary granules under the control of genes that in turn are regulated by distinct transcription factors [155].

Neutrophil granules serve as reservoirs for digestive and hydrolytic enzymes prior to delivery into the phagosome. The azurophilic granule contents, first released to the phagosome, possess microbicidal activity and may play an important role in the tissue destruction observed during inflammatory reactions [153]. Although neutrophils have other microbicidal mechanisms including antimicrobial peptides (example defensins and broadly acting proteases), phagocytosis with generation of ROS is still regarded as the critical killing mechanism for most invading pathogens [156,157] as described in the above section(1.8.1.3). The surfaces of the neutrophils are complex with diverse sites for interaction with its surroundings [158]. Both neutrophils and monocytes express receptors for interaction with opsonins to facilitate phagocytic movement and ingestion through pathways affecting cytoskeletal reorganization [159,160].

Neutrophils and monocytes express cell adhesion molecules such as selectins and integrins, which affect the trafficking of these cells by impeding their rolling and subsequent adhesion to the capillary vascular wall in the process of diapedesis and eventual migration into tissue [161–163]. Neutrophils bear a family of receptors that facilitate the migration of phagocytes after they leave the vascular compartment [153]. In addition to the complement receptors (such as receptors for C5a), neutrophils have several other chemotactic receptors. These include receptors for bacteria derived or synthesized *N*-formyl peptides, platelet activating factor (PAF), leukotriene B-4 (LTB-4), and a variety of chemokines [164–166]. Neutrophils also have surface receptors for the colony stimulating factors, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), from early in development to the mature circulating neutrophils [167].

Patients with neutrophil deficiencies suffer from severe infections that are often fatal underlining the importance of these cells in antimicrobial defense. Neutrophils from chronic granulomatous disease (CGD) patients fail to generate the products of the respiratory burst. A microbicidal defect in the neutrophils of affected children caused recurrent bacterial and fungal infections associated with early mortality [168]. The lack of function of NADPH oxidase components leads to a failure to generate hydrogen peroxide in response to bacterial or fungal infection [169,170]. In spite of neutrophils' relevance in immunity, research on these cells has been hampered by their experimentally intractable nature since they are not easily cultured *in vitro* [102].

1.8.1.5. Monocytes

Peripheral blood monocytes represent 3 to 7% of total white blood cells in healthy human adults[150]. Monocytes are produced by the bone marrow and soon after maturation enter the bloodstream as quiescent cells [171,172]. Bone marrow and blood monocytes retain a proliferative capacity and can differentiate into resident phagocytic cells, which broadly termed as macrophages and histiocytes in the spleen, liver, lungs and other tissues. This tissue based mononuclear phagocyte system is called the reticuloendothelial system reviewed in Bainton et al [154] and as cited by Dale et al [153]. Monocytes are known to serve as the precursors of certain populations of dendritic cells, which play an important role in host defense as potent antigen-presenting cells during T-lymphocyte activation [173–176]. Mononuclear phagocytes of the blood and tissues survive far longer than neutrophils. This feature of phagocytes is clinically very important since it protects patients from an overwhelming risk of fatal infections when neutrophil production is transiently interrupted [153].

A preserved capacity of monocytes to augment production of granule proteins through new protein synthesis is another feature that is lost in mature neutrophils. In addition, there are significant differences in their chemotactic responses and metabolic burst activity during phagocytosis [153,177]. Monocytes accumulate more slowly at a site of acute inflammation, but persist longer [153]. They have Fc receptors and express the IgG receptor FcγRI (CD64) constitutively in contrast to neutrophils, which express this receptor only in response to inflammatory stimuli [153,177].

Monocytes and macrophages play important regulatory and effector roles in both arms of the human immune system [178–180]. Circulating monocytes, which are derived from myelomonocytic stem cells in bone marrow, have two main functions in the immune system: (i) to replenish resident macrophages and dendritic cells in peripheral tissues under normal states and (ii) to patrol healthy tissues through long

range crawling on the resting endothelium [181]. In response to inflammatory signals, monocytes move to sites of infection in the tissues, engage in phagocytosis of foreign substances, and initiate an early immune response through the recruitment of PMN leukocytes [150]. Mononuclear phagocytes ingest invading pathogens to kill and ingest materials to degrade and eliminate as waste and debris. They remove defective and aged red cells, red cell inclusions in the spleen, and by doing so clean up debris at sites of infection or tissue damage [153,182,183]. Activated monocytes are known to release IL-1, IL-6, TNF-α, and IFN-α/β cytokines that are important for the regulation of hematopoiesis [153]. Monocytes can activate nitric oxide synthase, which leads to the synthesis of nitric oxide [184].

Activated monocytes eradicate invading pathogens after recognizing a wide range of microbial pathogens by their TLR [153]. Toll like receptors are expressed to a far higher degree by monocytes than neutrophils. Upon binding to specific ligands, TLRs signal via pathways dependent or independent of the adaptor protein MyD88, activate NF-κB and stimulate proinflammatory cytokine production [185,186]. Other cell based receptors such as CD14 cooperate with specific TLRs to enhance pathogen recognition. For instance, upon binding to LPS, CD14 interacts with TLR4 to facilitate recognition and enhance eradication of Gram-negative bacilli from the circulation and tissue sites [187].

1.8.1.6. Macrophages

Macrophages and their precursor (monocytes) are the big eaters' of the immune system, and they produce microbicidal agents such as NO and ROS to eliminate infecting microbes. Macrophages are the second wave of cells that enter the site of *Leishmania* infection following neutrophils [137]. They are also the main host cells for *Leishmania* parasite replication and source of cytokines that modulate T cell-mediated

immune responses. Upon activation by Th1 cells, macrophages can act also as effector cells for intracellular parasite killing. *Leishmania* promastigotes are rapidly phagocytosed by dermal macrophages through a CR-3 dependent mechanism [188,189] and eventually transform to amastigotes, which settle in acidic parasitophorous vacuoles [98].

Macrophages are the long-term final host cells for *Leishmania* parasites. In experimental leishmaniasis, it has been shown that macrophages are crucial for parasite survival but are also important for their elimination [190]. The function of these cells depends on the type of activation and the vulnerability of the parasite to the effector mechanisms. In the murine model, killing of parasites by macrophages is dependent on the activation of macrophages by IFNγ and a second signal that triggers production of TNF-α. The signals for activation may be from amastigotes, promastigotes or parasite-derived components. Upon activation macrophages stimulate inducible nitric oxide synthase(iNOS), which leads to the production of NO [191–193]. The clear role of NO in killing *Leishmania* was established by pharmacological inhibition of the production of NO *in vitro* and by the observation of a higher susceptibility of iNOS knockout mice to infections with *L. major* [193–196]. Using an *in vitro* mouse model experiment, macrophages were shown to destroy both *L. donovani* promastigotes and amastigote forms [197]. Nonetheless, *Leishmania* parasites can multiply and survive in the microbicidal environment of mononuclear phagocytes [198].

Though several activated forms of macrophages are believed to exist, there are two main groups designated as M1 and M2 subtypes. This subdivision was based on functional polarization of macrophage as M1 (classical) and M2 (alternative) activation. While M1 polarized cells display a strong pro-inflammatory microbicidal response, M2 polarization is linked to production of an anti-inflammatory condition leading to tissue regeneration and wound healing [199]. During stimulation with Th1 associated cytokines, particularly IFNγ, macrophages acquire an effector function against intracellular pathogens, referred to as a classically activated or M1 phenotype. On the contrary, macrophages generated in the presence of Th2 associated cytokines

such as IL-4, IL-13, IL-33, TGF-β and IL-10 [200] are referred to as alternative activated or M2 macrophages [201].

1.8.1.7. Cytokines

Cytokines are small glycoproteins produced by a number of cells, predominantly leukocytes, that regulate immunity, inflammation and hematopoiesis and have a fundamental role in communication within the immune system [202]**.** A series of proinflammatory cytokines are derived from stored intracellular granules or synthesized de novo following microbial infections [203,204]. These cytokines are capable of activating monocytes, macrophages, neutrophils and endothelial cells to react against, or bind to micro-organisms, and to recruit other immune cells to the site of infection [204]. Interaction of the cytokines with their receptors on a target cell can cause changes in the expression of adhesion molecules and chemokine receptors on the target cell membrane. Allowing cells to move from one location to another, cytokines regulate host responses to infection by signaling an immune cell to increase or decrease the activity of particular enzymes, thereby altering and enhancing its effector functions [205,206]. Cytokines signaling induces their effects in three main ways: autocrine, paracrine and endocrine. Cytokines such as IL-2 are considered to have an autocrine effect when their effects act on the cells that produce them. Some cytokines such as IL-17 have paracrine effects acting on other cells in their vicinity while cytokines having endocrine effects such as IL-1 and TNF-α are known to act systemically [207].

Scientists in the field of immunology recognized cytokines as key elements in the host immune response against *Leishmania*. During murine leishmaniasis, with respect to inflammatory responses, macrophages produce IL-1β, TNF-α and IL-12 where Th-1

cells produce IFN-γ, and Th2 cells produce IL-4. Other cells such as DCs produce IL-12 while natural killer (NK) cells produce IFN-γ [208,209]. Cytokines are involved in the induction of chemokines, which exerts a secondary effect on leukocyte recruitment [210]. Following *Leishmania* infections, proinflammatory cytokines such as TNF-α and IL-12 are abrogated, whereas anti-inflammatory cytokines, such as IL-10 and transforming growth factor beta (TGF–β) are induced [211,212]. Table 2 summarizes sources and activity of selected cytokines included in this dissertation.

Table 2. Sources and activity of selected cytokines investigated in this work.

Cytokine: Synonyms	Sources	Activities
IL-1α, IL-1β : Lymphocyte activating factor, mononuclear cell factor (MCF), endogenous pyrogen	Many cell types, including monocytes, macrophages, dendritic cells, NK cells, and non-immune system cells such as epithelial and endothelial, fibroblasts, smooth muscle cells and so on.	Displays a wide variety of biological activities on many different cell types. The *in vivo* effects include induction of local inflammation and systemic effects such as fever, the acute phase response, and stimulation of neutrophil production.
IL-12: NK cell stimulatory factor; cytotoxic lymphocyte maturation factor	Macrophages, B cells , and dendritic cells.	Important factor in inducing differentiation of Th-1 subset of helper T cells; induces IFN-γ production by T cells and NK cells and enhances NK and cytotoxic T cell activity.
IL-6: B-cell stimulatory factor 2; hybridoma /plasmacytoma growth factor; hepatocyte-stimulating factor	Some T cells and B cells, several nonlymphoid cells, including macrophages, bone marrow stromal cells, fibroblasts, endothelial and muscle cells, adipocytes, and astrocytes.	Regulates B and T-cell functions; *in vivo* effects on hematopoiesis. Induces inflammation and the acute phase response.
TNF-α: Cachectin, TNF ligand superfamily member 2	Monocytes, macrophages, and other cell types, including activated T cells, NK cells, neutrophils, and fibroblasts.	Mediator of inflammatory and immune functions. Regulates growth and differentiation of a wide variety of cell types. Cytotoxic for many types of transformed and some normal cells. Promotes angiogenesis, bone resorption, and thrombotic processes. Suppresses lipogenic metabolism.
IL-17:cytotoxic T lymphocyte associated antigen 8	$CD4^+$T cells (particularly those of the TH17 subset), $CD8^+$, γδT cells, NK cells, intraepithelial lymphocytes, and some other cells	Promotes inflammation by increasing production by epithelial, endothelial, and fibroblast cells of proinflammatory cytokines such as IL-1, IL-6, TNF-α, G-CSF, GM-CSF, and chemokines that attract monocytes and neutrophils.
IL-10: cytokine synthesis inhibitory factor	Activated subsets of $CD4^+$ and $CD8^+$ T cells, macrophages, and dendritic cells.	Enhances proliferation of B cells, thymocytes, and mast cells; in cooperation with TGF-β, stimulates IgA synthesis and secretion by human B cells. Anti-inflammatory; antagonizes generation of the TH1 subset of helper T cells.

source: adapted from [205]

1.8.1.8. Chemokines

Chemokines are small protein molecules that attract cells to move towards higher concentrations of the stimuli and so recruit cells into, within, and out of tissues for subsequent activation of immune cells at local tissue sites [204]. They are members of a superfamily of low molecular weight (6–17 kDa) cytokines that recruit and activates leukocytes through increased adhesion, degranulation and respiratory burst [213,214]. Most chemokines are secreted proteins and their production is induced by a variety of stimuli, mitogens including LPS, proinflammatory cytokines and several pathogens [214,215]. Chemokines were initially recognized for their effects on cell activation, differentiation, and trafficking [216]. Based on position of their conserved cysteine residues, chemokines are divided into two major (CXC and CC) and two minor (C and CX3C) subfamilies [217].

The release of TNF-α and IL-1β from activated neutrophils and macrophages have been related to chemokine synthesis in several cell types, including PMNs, fibroblasts, endothelial and epithelial cells [212]. In murine CL, cytokines seem to synergize with leishmanial elements to regulate chemokines production. In line with this opinion, TNF-α and IL-1β together with macrophage inflammatory protein (MIP)1α (also known as CCL3) were reported to regulate Langerhans cell mediated transport of *Leishmania* parasites from the infected skin to regional lymph nodes (LNs) [218]. Interleukin-12 is required for the induction of Th1 related chemokines such as lymphotactin (also known as XCL1), IFN-inducible protein 10 (also known as CXCL10) and monocyte chemotactic protein 1 (MCP-1) also known as CCL2 in lymph nodes of resistant *L. major* infected mice [219]. Table 3 summarizes sources and activity of selected chemokines included in this dissertation and keeps on their old nomenclatures**.**

Table 3.Sources and activity of selected chemokines investigated in this work.

Chemokine	Receptor Expression on Leukocytes	Cell affected	Parasite	Biological Effect	Reference
IP-10 (CXCL10)	Some memory B cells, eosinophils, NK cells, plasmacytoid DC, mast cells, plasma cells, activated T cells, Th-1cells	T- cells	*L. donovani*	Attracts $CD4^+$ and $CD8^+$ cells and prolonged expression contributes to granuloma formation and parasite elimination in liver	[220]
			L. major	Attracts Th1 cells and activates them to release IFN-γ	[219]
IL-8 (CXCL8)	Basophils, eosinophils, mast cells, monocytes, neutrophils, NK cells , some T-cells	Neutrophils	*L. major*	Attracts neutrophils to lesion	[221,222]
MCP-1 (CCL2)	B cells, basophils , immature dendritic cells, monocytes, macrophages, NK, Plasmacytoid DC, activated T cells, memory T cells	T- cells, Monocytes	*L. infantum*	Induction of Th2 cells and parasite persistence in the spleen	[223]
MIP-1β (CCL4)	B cells, basophils, iDC, Mast cells, Monocytes, macrophages, NK cells, plasmacytoid DC, activated T cells, Th-1 cells, regulatory T cells	T-cells, Monocytes , DCs	*L. major*	Attracts macrophages to lesion site	[80]

Source: Adapted from [205]

Chemokines are known to play roles in the recruitment of innate immune cells to the site of *Leishmania* parasite entry and in adaptive immunity during macrophage activation and parasite killing. The immune response is initiated at the site of inflammation by cells equipped with TLRs [224] and phagocytic receptors [225] to enable the detection of PAMPs [226] and uptake of opsonized pathogens. Thus at the cutaneous site, sentinel cells express various receptors for cytokines and together with tissue cells produce numerous chemokines initiating a cascade of innate immune responses [227]. The sand fly injects the mammalian host with *Leishmania* within its saliva containing molecules that have several activities including vasodilation, inhibition of coagulation and immunomodulatory effects [228] in addition to other molecules that attract PMNs as well as macrophages [99,229]. The parasite itself also produces a chemoattractant protein called *Leishmania* chemotactic factor (LCF), which can attract PMNs [230]. Polymorphonuclear cells are the first cells to arrive at the site of *Leishmania* infection [152]. In humans, PMNs containing *Leishmania* secret chemokines such as IL-8 [231] that are essential in attracting more PMNs to the site of infection.

Immunotherapy using administration of chemokines has shown promising results in the treatment of VL [232]. In mouse experimental VL model, treatment with chemokines leads to a strong Th-1 immune response [233]. Interferon-inducible protein-10 is known as antitumor, antiviral, and antifungal activities [234–236]. During healing process, in *Leishmania* infected B6 mice an early but strong induction of IP-10 was observed [152]. This chemokine promotes the recruitment and activation of Th1-polarized cells and, therefore, considered as a potential candidate for immunotherapy to cure VL [237,238]. *In vivo* mouse model, IP-10 treatment was shown to mediate effective parasitic killing and induces the shift from Th2 to Th1 immune response along with generation of NO from restimulated splenocytes [239]. Other studies revealed that treatment with chemokines have demonstrated restoration of the impaired effector response, thereby rendering protection against *Leishmania* infection [233,240]. *Leishmania* promastigotes believed to repress IP-10 but induce IL-8 production [241]. The probable reason is that some *Leishmania* species owe their virulence partly due to their ability to repress the induction of pro-

inflammatory cytokines and chemokines genes and so making their entry less detectable to the host [242,243].

The induction of chemokines is a critical step in VL infection as successful immune response since it dictates the migration of appropriate cell populations to the infected sites. Thus pathogenesis in VL is often associated with altered chemokine expression profiles and defective migration of immune cells [244]. Elevated concentrations of monokines induced by gamma interferon (MIG) and IP-10 were detected in sera of VL patients during active infection and has been suggested that these chemokines along with IFN-γ play an important immunopathogenic role in VL [245]. In addition, chemokine mediated antileishmanial activity against *L. donovani* has been demonstrated both *in vitro* (human) and *in vivo* (murine) infections with *L. donovani.* In macrophages primed with MIP-1α and MCP-1, multiplication of *L. donovani* amastigotes is inhibited through the induction of the respiratory burst and nitric oxide [232,240,246]. Furthermore, treatment of *L. donovani* infected BALB/c mice with MIP-1α or MCP-1 significantly suppressed parasite burden in the liver and spleen [240]. However, VL associated with impaired proinflammatory cytokine responses and impaired generation of free radicals helps parasites establish a niche within the hostile environment of host macrophages [247].

1.9. Can *L. donovani* have bovine reservoir host cells?

Depending on the source of infection, VL transmission is grouped as zoonotic or anthroponotic transmission type. The transmission of *L. donovani* infection has been considered as anthroponotic in endemic countries in East Africa [2]. However, *L. donovani* infected wild and domestic animals were reported in several foci [2,50,51]. Epidemiological reports also indicated the increased risk of VL in humans living in close proximity to domestic animals [2,50]. Additional studies have emerged which

strengthen the notion that domestic animals may serve as hosts for the parasites, or possibly, as reservoirs for human *L. donovani*. A study from Nepal [50] detected VL parasite DNA in animals such as cows, buffaloes, and goats. Furthermore, a recent study in Northwest Ethiopia detected *L. donovani* DNA from cattle and reported a positive correlation between anti-*P. orientalis* saliva and anti-*L. donovani* IgG levels in cows, goats and sheep [51]. In East Africa, *P. orientalis* is considered as the major vector for VL transmission [2]. Though parasitic DNA and antibodies against *L. donovani* were detected in domestic animals such as cattle, no study has yet demonstrated the presence of *L. donovani* parasites *in vivo* or *in vitro* in bovine cells in which these obligate intracellular parasites would be predicted to reside [248]. Therefore, studies are required to prove whether bovine neutrophils and macrophages can be infected with *L. donovani* parasites. Such an observation would provide additional evidence supporting the hypothesis that domestic animals may serve as reservoir host for *L. donovani* parasites.

1.10. Test of cure for visceral leishmaniasis

According to report from the WHO 2015 expert group, the key challenges in VL control are the inconsistent performance of rapid diagnostic tests (RDTs), lack of drug resistance monitoring, insufficient access to treatment and lack of test of cure [249]. Parasite detection is the most recommended test in the Ethiopian guidelines for diagnosis, treatment and prevention of leishmaniasis [11]. However, parasite detection involves invasive procedures of spleen, bone marrow or lymph nodes aspirates. In addition these procedures need highly skilled personnel with well-equipped facilities, which are not found in most of the health care facilities in VL endemic regions in Ethiopia. Thus availing simple and reliable tools for assessing therapeutic success is among the high priorities in the field of leishmaniasis. A recent advance in immunology has led to the identification of potential biomarkers for different infectious diseases. Both innate and adaptive arms of the immune system are known to play roles in VL control. Neutrophils that express matrix metalloproteinases (MMP)-9 and inflammatory monocytes were shown to be important in the splenic

tissue remodeling that occurs during clinical VL [250]. Moreover, it has been documented that the serum profile of MMP-9 is inversely correlated with spleen size and parasite load in human VL [251]. In experimental *Leishmania* infection, healing correlated with CD40 Ligand (CD40L)-dependent T-cell responses primed by DCs at the site of infection [252]. Serum soluble CD40L (sCD40L) level was reported to negatively correlate with spleen size and parasite load in human VL [251]. Moreover, sCD40L from VL exposed individuals was shown to help control *Leishmania* infection in macrophages *in vitro* [253]. Among the cytokines demonstrated to correlate with VL progression and recovery is IL-10. IL-10 levels were shown to directly correlate with parasite load [254,255] under different geographic and transmission settings.

1.11. Aims of the study

1. It is a known fact that only few individuals living in VL endemic areas develop VL although most/all people are likely to have been exposed to bites of infected sand flies. Since the innate immune response is regarded to control pathogens early after infection and to drive the adaptive immune response I hypothesized that a dysfunction of innate immune responses is the reason for diseases development after infection with *L. donovani*, and may contribute to disease dissemination. Therefore, I aim to investigate selected cellular innate immune functions in VL patients and endemic healthy controls (EHC) to assess:

- neutrophil activation status, degranulation, ROS production and phagocytic capacity in response to *L. donovani* and defined TLR ligands.
- monocyte ROS production and phagocytic capacity in response to *L. donovani* and defined TLR ligands.
- secretion of cytokines/chemokines in response to *L. donovani* and defined TLR ligands in whole blood cultures.

2. The transmission of VL induced by *L. donovani* infection in Ethiopia is usually considered to be anthroponotic [2]. However recently in Ethiopia, within a VL endemic area *L. donovani* DNA and antibodies to *L. donovani* and to *P. orientalis* saliva were detected in cattle [51]. However, no study has yet demonstrated the presence of viable *L. donovani* parasites *in vivo* or *in vitro* in bovine cells [248]. Therefore the aim of this study is to:

- investigate the infection of whole blood bovine PMN, bovine monocytes derived macrophages (MDM) cells by *L. donovani* parasites.

- isolate and culture *L. donovani* parasites from infected bovine MDM.

3. In clinical VL both innate and adaptive arms of the immune system were shown to play a role in the outcome of the disease. The present findings from other countries show that activation or regulation of both immune system results in production of biomolecules which could have potential biomarkers for VL clinical cure. Therefore, I hypothesized that the biomolecules sCD40L, MMP-9 and IL-10 can also be used as biomarker candidates for VL clinical cure in Ethiopia. Thus I aim to investigate the serum level of sCD40L, MMP-9 and IL-10 in serum of the VL patients before initiating any treatment and after completing treatment (clinical cure) to identify biomarker candidates to be used as test of VL clinical cure in Ethiopia.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. *Leishmania* parasites

Species	Source	Reference number
L. donovani eGFP	Paul-Ehrlich-Institute, Lagen, Germany	MHOM /ET/ 67/ HU3
L. donovani	Paul-Ehrlich-Institute, Langen, Germany	MHOM /ET/ 67/ HU3
L. donovani	WHO Center for Leishmaniasis, Institute de Salud Carlos III, Madrid, Spain	MHOM/IN/80/DD8
L. chagasi	WHO Center for Leishmaniasis, Institute de Salud Carlos III, Madrid, Spain	MHOM/BR/00/1669
L. infantum	WHO Center for Leishmaniasis, Institute de Salud Carlos III, Madrid, Spain	MHOM/FR/LEM-75
L. aethiopca	WHO Center for Leishmaniasis, Institute de Salud Carlos III, Madrid, Spain	MHOM/ET/72/L100)
L. major	WHO Center for Leishmaniasis, Institute de Salud Carlos III, Madrid, Spain	MHOM/ SU/73/5-ASKH)
L. tropica	WHO Center for Leishmaniasis, Institute de Salud Carlos III, Madrid, Spain	MHOM/SU/74/K27,

2.1.2. Chemicals, antibiotics, media and buffers

Name of items	Company, city/town, Country	Cat. number
Absolute methanol	Sigma Aldrich GmbH, Munich, Germany	322415
AIM-V	GIBCO, Thermo scientific, Waltham, USA	31035025
Bovine serum albumin	Carl Roth GmbH, Karlsruhe, Germany	8076.2
Buffer pH -4	Fisher scientific, Loughborough , UK	SB101-500
Buffer pH -7	Fisher scientific, Loughborough, UK	SB108-500

$CaCl_2$	MERCK, Darmstadt, Germany	102378
D (+)- Glucose	Merck, Darmstadt, Germany	Art 8346
DNA gel loading dye	Thermo scientific, Waltham , USA	R0611
FACS lysing solution	BD Bioscience, CA, USA	349202
Fetal bovine serum (FBS)	GIBCO, Thermo scientific, Waltham ,USA	16000-044
Ficoll	H.C Bioscience, Uppsala, Sweden	17-1440-03
Giemsa stain	Sigma Aldrich , Munich, Germany	G4507-25G
HCl	Sigma Aldrich, Munich, Germany	295426
Human serum	Biowhittaker, Lancaster, USA	14.490e
Hygromycin B	Sigma Aldrich, Munich , Germany	H3274
KCl	Sigma Aldrich , Munich , Germany	7447-40-7
L-glutamine	GIBCO, Thermo fisher, USA	21051-04
NaCl	Sigma Aldrich, Munich , Germany	73575
$NaHCO_3$	Sigma Aldrich , Munich, Germany	S-8875
NaN_3	Sigma Aldrich, Munich, Germany	S2002-25G
Nutrient agar	HiMedia, Mumbai, India	M001
PCR Buffer (10x)	Thermo Fisher scientific, Waltham, USA	BY5
Penicillin/Streptomycin	Biochrom AG, Germany	A2213
Phosphate buffer saline (PBS)	Thermo Fisher scientific, Waltham, USA	18912-014
RPMI 1640	Sigma Aldrich, Munich, Germany	R8758
TAE buffer	Sigma Aldrich , Munich, Germany	LSKMTAE50
Trypan blue (0.4%)	GIBCO, Thermo scientific, Waltham , USA	15250061
Tween -20	Sigma Aldrich , Munich, Germany	P9416
2-Mercapto ethanol	Sigma Aldrich , Munich, Germany	M-7522

2.1.3. Antibodies, stimulants, enzymes and fluorescent agents

Name of item	Company, city/town, country	Cat. number
Lipopolysaccharide (LPS)	Sigma Aldrich , Munich, Germany	SIGMA L4391
Agarose powder	Sigma Aldrich, St. Louis, USA	A9539
Anti- CD11C-PE	BD Biosciences, Thermo fisher, Waltham, USA	333149
Anti- CD62L-PE	BD Biosciences, Thermo fisher, Waltham, USA	BD-555544
Anti- CD66b-FITC	BD Biosciences, Thermo fisher, Waltham, USA	BD-555724
DHR 123	Invitrogen, Thermo fisher, Waltham, USA	D-23806
Ethidium bromide	Sigma Aldrich, St. Louis, USA	E1510
Fluospheres carboxylate modified microspheres 1.0 µm	Invitrogen, Thermo fisher, Waltham, USA	F 8823

HaeIII	Sigma Aldrich, St. Louis, USA	R5628
HhaI	Promega, Fitchburg, USA	R644A
Macrophage activating lipopeptide-2 (MALP-2)	Enzo, Alexis, Lausen, Switzerland	ALX-162-027-C050
M-CSF human, recombinant	Sigma Aldrich, St. Louis, USA	SRP3110-10UG
Microcentrifuge	Beckman coulter, Brea,USA	BKA46472
Molecular size markers (100 bp)	Promega, Fitchburg, USA	G2101
N-formyl-L-methionyl-L-leucyl-phenylalanine (*f*MLP)	Sigma Aldrich, St. Louis, USA	Prod. No. F3506
Polyinosinic-polycytidylic acid (poly I:C)	Invivogen, California, USA	invivogen tlrl-picw
QIAamp DNA Mini Kit	Qiagen, Chatsworth, CA, USA	69506
S. aureus Bioparticles, Alexa Fluor 488conjugate	Invitrogen, Thermo fisher, Waltham, USA	S-23371

2.1.4. ELISAs and ready to use kits

Name of item	**Company, city/town, country**	**Cat. number**
Rapid diagnostic test		
rK39 rapid VL test	InBios International, Seattle, USA	DiaMed-IT Leish
Direct agglutination test (DAT) for VL	Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium	0410.057.701
HIV1/2 rapid test	KHB, Shanghai Kehua, Shanghai, China	KH-R-02
DNA Isolation and PCR kits		
DNeasy	QIAGEN GmbH, Hilden, Germany	69582
HotStarTaq Master Mix Kit	Qiagen GmbH, Hilden, Germany	203446
ELISA kits		
Human CCL2/MCP-1	R&D systems , Minneapolis, USA	DY279
Human CCL4/MIP-1β	R&D systems , Minneapolis, USA	DY271
Human CXCL10/IP-10	R&D systems , Minneapolis, USA	DY256
Human CXCL8/IL-8	R&D systems , Minneapolis, USA	DY208
Human IL-10	R&D systems , Minneapolis, USA	DY217B
Human IL-12p70	R&D systems , Minneapolis, USA	DY1270
Human IL-17	R&D systems , Minneapolis, USA	DY317
Human IL-1β/IL-1F2	R&D systems , Minneapolis, USA	DY201
Human IL-6	R&D systems , Minneapolis, USA	DY206-05
Human MMP-9	R&D systems , Minneapolis, USA	DMP900
Human SCD40L	R&D systems , Minneapolis, USA	SCDL40
Human TNF-α	R&D systems , Minneapolis, USA	DY210-05
Reagent diluents (10X)	R&D systems , Minneapolis, USA	DY995
Streptavidin -HRP	R&D systems , Minneapolis, USA	DY998
Substrate reagent pack	R&D systems , Minneapolis, USA	DY999

2.1.5. Equipments

Name of items	Company, city/town, country	Cat. number
Bench top centrifuge	Hettich, Rotina 48, Balingen, Germany	D-78532
CO_2 incubator	Binder,Tuttlingen, Germany	12-06841
Digital light microscope	Leica -S2, Wetzlar, Germany	Type: 11020518016
Electrophoresis apparatus	BioRAD, Hercules ,USA	Ser.No. 041BR 48464
ELISA washer	Dialab, Wiener Neudorf, Austria	Diawasher ELX50
FACS Calibur	Becton, Dickinson, Franklin Lakes, USA	342975
FACS Canto -II	Becton, Dickinson, Franklin Lakes, USA	338962
Inverted microscope	Leica DMIL, Wetzlar, Germany	ENARP 162-00052
Light microscope	Leica, CME, Mumbai ,India	Model : 1349522x
Micropippette(0.5-10 µL)	Biohit, Helsinki , Finland	No.9093935
Microcentrifuge	Accuspin, Thermo fisher, Waltham, USA	75003241
Micropipette 0.5-10 µL	Biohit Mechanical, Helsinki, Finland	Ref.725050
Micropipette 0.5-10 µL	Dragon MED, Beijing, China	71111104
Micropipette 10 µL	Pipetman , Gilson, Villiers-le-Bel, France	F144802
Micropipette 1000 µL	Pipetman , Gilson, Villiers-le-Bel, France	F123602
Micropipette 10-100 µL	Biohit Mechanical, Helsinki, Finland	Ref.725050
Micropipette 20 µL	Pipetman , Gilson, Villiers-le-Bel, France	F123600
Micropipette 200 µL	Pipetman , Gilson, Villiers-le-Bel, France	F123601
Micropipette 20-200 µL	GmbH, Wertheim, Germany	Ref.704778
Microplate Reader	SpectraMax 190, CA, USA	Molecular Devices
Multichannel pipette 10-100 µL	Brand GmbH, Wertheim, Germany	703708
Multichannel pipette 50-300 µL	Camlab, Cambridge, UK	Part No: 1171811
Multichannel pipette 30-300 µL	Brand,Wertheim, Germany	08J30345
Neubauer-improved counting chamber (0.02 mm depth)	Marienfeld, Lauda-Königshofen, Germany	0642110
Neubauer-improved counting chamber (0.1 mm depth)	Marienfeld , Lauda-Königshofen, Germany	0640031
Micropipette eppendorf 10-100 µL	Sigma Aldrich , Munich, Germany	S.No.110117
Micropipette (100-1000 µL)	Sigma Aldrich , Munich, Germany	S.No.086417
Micropipette (20 µL)	Gilson , Villiers-le-Bel, France	K23716H
Micropipette (200 µL)	Gilson, Villiers-le-Bel, France	CE52724
pH Meter	HANNA, Michigan, USA	HI902
Pipette boy (pipette AID)	Drummond, Alabama, USA	Serial No:40980 s
Pipette boy (pipette AID)	Falcon, Thermo fisher, Waltham, USA	Cat.No. 1367542
Sonicator	Ultrasonic cleaner Elmasonic, Pontiac, USA	S025EL
Suction machine	Sue 30, Heto, Ikast, Denmark	478684-4
T3000-Thermocycler-	Biometra, Gottingen, Germany	Ser.No.2307189
UV trans-illuminator	BioRAD, Hercules , USA	Ser.No.721BR01632
Water bath	Memmert, Schwabach, Germany	DE 66812464

2.1.6. Plastics and consumables

Types of items	Company, city/town, country	Cat. number
16 well chamber slides	Nunc Lab-Tek , Thermo fisher, Waltham, USA	154534
24-well culture plates	Nunc, Thermo fisher, Waltham, USA	142485
96-micro plates	R&D systems , Minneapolis, USA	ELISA DY990
Bottle top Filter 0.22µm	Thomas Scientific ,Swedesboro, USA	1226S75
Cell Scrapers	Corning™ Falcon, fisher scientific, USA	08-771-A
Centrifuge tubes (15ML)	Globe Scientific, NJ, USA	6285
Centrifuge tubes (50 ML)	Globe Scientific, NJ, USA	6288
Cryotubes (Nunc tubes)	Sigma Aldrich , Munich, Germany	Z760951
Culture Flasks (25 cm^2)	Nunc, Thermo fisher, Waltham, USA	156340
FACS tubes (5ML)	VWR, Radnor, PA, USA	352054
Pipette Graduated 3ml Sterile Pastette	Alpa laboratories, Welland, Canada	LW4112
LeucoSep	Bioscience, Greiner, Germany	227290
Serological pipette (1, 2, 5, 10, 25 mL)	Laboratory, NJ, USA	LS-1331100-4
Syringe Filter 0.22 µm	Thomas Scientific , Swedesboro, USA	1211K48

2. 1.7. Primers and PCR reaction

Primers

Region	Primer pair	Product size	Primer sequence	
ITS1	LITSR / L5.8S	300-350	fwd	5'- CTGGATCATTTTCCGATG -3´
			rev	5' -TGATACCACTTATCGCACTT- 3´

PCR reaction

HotStar Taq Master Mix	
[dNTPs, 10 x Amplification buffer, Taq polymerase 5U/µL and 15mM $MgCl_2$]	12.5 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL
Extracted DNA	2 µL
Distilled water	9.5 µL

2. 1.9. Buffers and media prepared in the laboratory

FACS buffer

Bovine serum albumin	5 g
PBS pH 7.2	495 mL
N_3Na (10%)	500 µL
Heat inactivated human serum	5 mL

Complete medium (RPMI 1640)

RPMI 1640	440 mL
L-glutamine 200mM	5 mL
Penicillin/streptomycin10,000 U/ 10,000 µg/mL	5 mL
Fetal bovine serum	50 mL

Novy-MacNeal-Nicolle (NNN) medium

Nutrient agar	9.2 g
Glucose	0.6 g
NaCl	2.4 g
Blood	100 mL
dH_2O	300 mL

Locke's Overlay Solution

NaCl	9.0 g
KCl	0.6 g
$CaCl_2$	0.2 g
$NaHCO_3$	0.2 g
Glucose	2.5 g
Penicillin /Streptomycin	100U/100 µg / mL
dH_2O	900 mL

Wash buffer

1 x PBS
5 % complete-Medium

Agarose gel (1.5%) preparation

Agarose powder	1.5 g
1x TAE buffer	100 mL
Heat in microwave	Higher to mediun
Dissolve the agarose	Until transparent
Cooling the agarose gel	Keep it in water at 55^{o}C

Agarose gel (2%) preparation

Agarose powder	2 g
1x TAE buffer	100 mL
Heat in microwave	Higher to mediun
Dissolve the agarose	Until transparent
Cooling the agarose gel	Keep it in water at 55^{o}C

2.1.10. Software

FlowJo LLCv10.0.8, Ashland, USA

Prism GraphPad 6 Software (Inc., Oberlin Drive, San Diego, USA)

BD FACSDiva software © Becton, Dickinson and Company, CA. USA

BD CellQuest Pro software © Becton, Dickinson and Company, CA. USA

SoftMax® Pro Microplate Data Acquisition and Analysis Software, Version 6, 5014178 D, 1311 Orleans Drive, Sunnyvale, California, USA.

2.2. Methods

2.2.1. Part I. Establishment of a functional laboratory and optimization of methods

2.2.1.1. Establishment of a functional laboratory to carry out the planned studies in the VL endemic area in Ethiopia

At the University of Lübeck under the supervision of Prof. Dr. Tamás Laskay, I received training for four months on immunological techniques such as FACS based whole blood assay for neutrophils surface marker staining, phagocytosis, reactive oxygen species (ROS) and whole blood culture for cytokine/chemokine measurement in supernatants using ELISA. These techniques were chosen to carry out research on innate immune functions without the need of cell separation in field conditions in Ethiopia where isolation of cells is not feasible. For another one month I went to the Paul- Ehrlich Institute in Langen to learn techniques regarding macrophage infection with *Leishmania* parasites under the supervision of Prof. Dr. Ger van Zandbergen. After my training in Germany, I returned to Ethiopia, Addis Ababa, to transfer and establish the technology I learnt in Germany to the Armauer Hansen Research Institute (AHRI) and the Ethiopian Public Health Institute (EPHI).

My main research aim was to investigate selected innate immune cell functions in VL patients visiting Addis Zemen Health center for treatment. After collecting blood samples at this health center, the laboratory work was planned to be done at Bahir dar Regional Health Research Laboratory Center (BRHRLC) which is found in the capital city of Amhara regional state. Bahir dar is situated at the shore of Lake Tana and is 578 km far from Addis Ababa.

Amhara Regional state Health Bureau was communicated for permission to work at BRHRLC. Thus a letter of cooperation was written to BRHRLC which requested me to prepare a memorandum of understanding (MOU) how its laboratory team and I work together in harmony. An MOU was prepared and signed between BRHRL and AHRI, my institute in Addis Ababa. Afterwards, I was allowed to start to discuss with the

laboratory team, and I was provided a laboratory space and store. Except a flow cytometry, water bath, freezer and fridge, the laboratory space had no laboratory equipments, materials and consumables that would have been necessary to initiate my research work.

Addis zemen town (approximately 19,755 population), the capital of Libo Kemkem district, with average altitude of 2,000 m above sea level is 80 km from Bahir dar and located between Bahir Dar and Gondar city on the major road connecting Addis Ababa[15]. Addis zemen health center is one of the health institutes in the country where leishmaniasis cases are referred to for treatment and case management. Having a letter of cooperation from the Regional Health Bureau I went to Libo kemkem District Health Bureau to obtain a permission letter that allows working with the health center staff. Having the permission, I contacted the Addis Zemen health center for the commencement of the project. I visited Kala azar treatment ward where most young male patients were on treatment. At the initial step, we had several meetings with clinical staff, nurses, and laboratory staff to come to common understanding on how I can start my research project. This step took several months. Finally we agreed to start the project after having discussed over the benefit of the research to the community, for the patients, and for the staff of the health center.

I have made a list of all required laboratory materials that would be required to perform my experiments in BRHRLC. Thus all the laboratory materials were ordered but purchasing materials from abroad was a lengthy process which took more than 6 months. Meanwhile, in order to start establishing the laboratory at BRHRLC, I carried out a simple need assessment survey to obtain information whether establishing a cell culture and leishmaniasis diagnostic laboratory at the center was required and if it would have sustainable function for the region. I collected data from clinicians working at Addis Zemen Health center, Felege-Hiwot hospital, Bahir dar university and a private hospital (GAMBI hospital) regarding their need for a cell culture and leishmaniasis diagnostic laboratory in their region to fulfill their need for laboratory diagnosis and/or use it for research, too. Several departments of the health institutions like internal medicine, dermatology, and Addis zemen Health centre agreed to make use of the lab as a referral and diagnostic service. Having this consensus with health institutions, I organized a meeting with the acting director of

BRHRLC and the capacity building team leader about the issue on how to establish the cell culture and leishmaniasis diagnostic laboratory. We came to a common understanding and conclusion that establishing a cell culture and leishmaniasis diagnostic laboratory at BRHRLC was necessary. Then I went back to AHRI and Ethiopian public Health institute in Addis Ababa, where I organized materials required for the establishment of the lab. Discussion was made with the directors of the respective institutes on the need of establishing cell culture lab and leishmaniasis diagnostic service at BRHRLC. Both institutes cooperated and provided me all necessary support during the establishment of the laboratory. Armauer Hansen Research Institute provided CO_2 incubator, vacuum suction machine, CO_2 gas with its cylinder, micropipettes, necessary reagents, chemicals, consumables, car with driver and research assistant while the EPHI provided a class II biological safety cabinet, and a pH meter. This way I have established a cell culture and leishmaniasis diagnostic laboratory and connected with Felege-Hiwot referral and teaching hospital. I have given training on cell culture and FACS based whole blood assay for innate immune cell functions investigation to the staff at parasitological laboratory and so received letter of recognition from the regional health bureau (see Annex).

Figure 8.Parts of the established laboratory at Bahir dar Regional Health Research Laboratory center.
a) While practical training was given to BRHRLC lab staff on cell culture technique, b) While I was giving a training on laboratory diagnosis of leishmaniasis for district hospital lab staffs from Amhara regions using multi headed microscope, c) Parasitologist examining *Leishmania* culture under inverted microscope, and d) Taking microphotographs of leukocytes infected with *Leishmania* amastigotes from slit skin smear of a CL patient.

The laboratory was visited by his Excellency Mr. Degu Andargachew the president of Amhara regional state, by Dr. Amha Kebede, the former Director General, Ethiopian public health institutes and different organization delegates.

2.2.1.2. Optimization of methods

Before starting the actual experiment in the field, I optimized all laboratory protocols in Germany, at the Department of Infectious Diseases and Microbiology, University of Lübeck, and after returning to Ethiopia, at the Armauer Hansen Research Institute

(AHRI), Addis Ababa and BRHRLC, Bahir dar. Some of representative results of the optimization work are shown below.

1) Surface marker staining: CD62L as activation marker and CD66b as granulation marker on neutrophils

Optimization for neutrophils surface staining was done to select the appropriate blood volume, concentration of stimulant and technical applicability of the whole blood assay for the assessment of CD62L and CD66b expression. In Ethiopia the concentration of stimulants and the volume of each antibody were again optimized using blood samples from healthy individuals. As an example a representative experiment for CD62L antibody volume optimization is presented below, Figure 9.

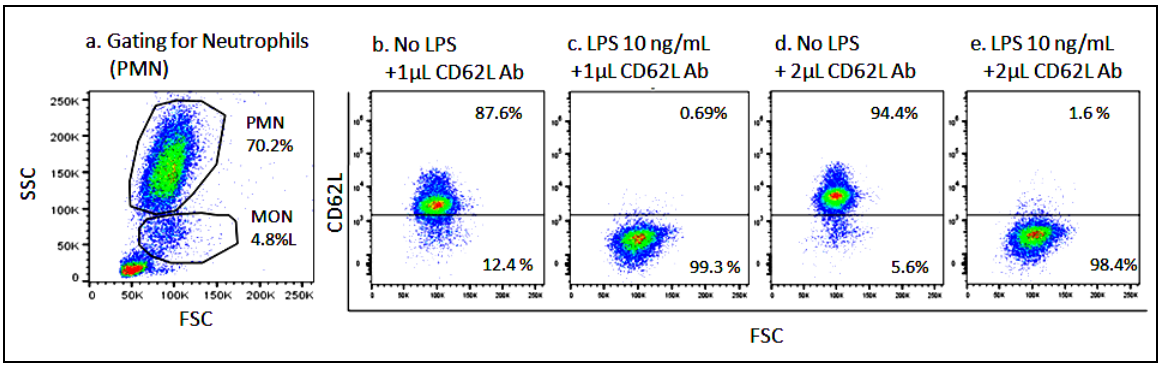

Figure 9.Whole blood surface staining for CD62L.
Flow cytometry analysis was carried out to assess the surface expression of CD62L on neutrophils without stimulation as well as after exposure to LPS. Whole blood was collected from a healthy individual. Fifty microliters of blood was incubated with LPS at 10 ng /mL or without any stimuli at 37^{o}C for 30 minutes. Subsequently, probes were stained with various amounts (1 µL or 2 µL) of anti-human PE-conjugated monoclonal antibody to CD62L for another 30 minutes at 4^{o}C. Erythrocytes were lysed by adding 500 µL of 1x FACS lysing solution and incubated for 15 minutes at room temperature (RT). Neutrophils were gated and the expression levels of CD62L were quantified by using flow cytometry. a) Gating strategy for neutrophils based on their SSC and FSC characteristics, b) no stimulus, 1µL CD62L Ab c) LPS 10 ng/mL, 1 µL CD62L Ab, d) no stimulus, 2 µL CD62L Ab, e) LPS 10 ng/mL, stained with 2 µL CD62L Ab. The % shows the proportions of cells expressing CD62L.

Since 1 µL of CD62L was not sufficient enough to stain CD62L, from this experiment I selected 2 µL of CD62L Ab which sufficiently stain CD62L and can give an optimal signal.

2) LPS concentration optimization for phagocytosis assay

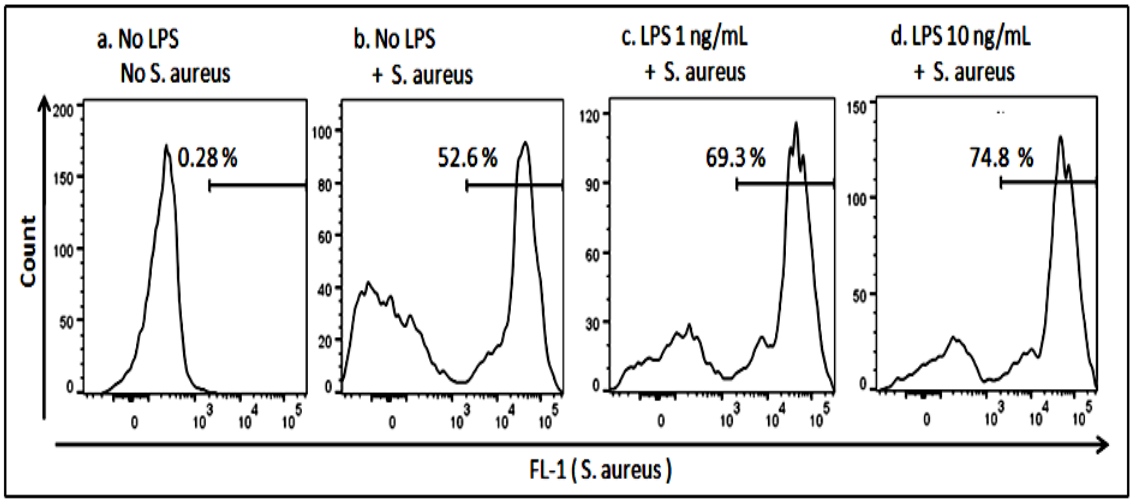

Figure 10.Optimization of LPS concentration for phagocytosis at laboratory conditions in Ethiopia.

Blood from healthy individual was collected. Fifty microliters of whole blood was distributed into eppendorf tube to which LPS was added in 50 µL volume resulting in a final LPS concentration of 1 ng/mL or 10 ng/mL. The tubes were incubated in water bath at 37°C for 30 minutes. Then 5 µL of 2 µg/mL of fluorescent-labeled *Staphylococcus (S.) aureus* Bio-particles (Alexa Fluor 488 conjugate) were added and incubated at 37°C in water bath for another 30 minutes. Erythrocytes were lysed by adding 500 µL of 1x FACS lysing solution and incubated for 15 minutes at room temperature (RT). The tubes were washed and 32 µL of 0.4% trypan blue (quenching solution) was added in order to quench the fluorescence of bacteria attached to the leukocyte surface. Cells were washed, the supernatant was removed and the pellet was washed once again with FACS buffer. The pellets were re-suspended in 400µL FACS buffer and the phagocytosis of *S. aureus* particles by neutrophils was assessed by flow cytometry. a) no S. aureus, no LPS, b) *S. aureus* 2 µg/mL, no LPS c) *S. aureus* 2 µg/mL, LPS 1ng/mL d) *S. aureus* 2 µg/m, LPS 10 ng/mL plus

Based on such experiments, LPS at concentration of 10ng/mL was selected for the subsequent studies.

3) Selection of optimal LPS concentration and *Leishmania* parasite form for ROS production assay

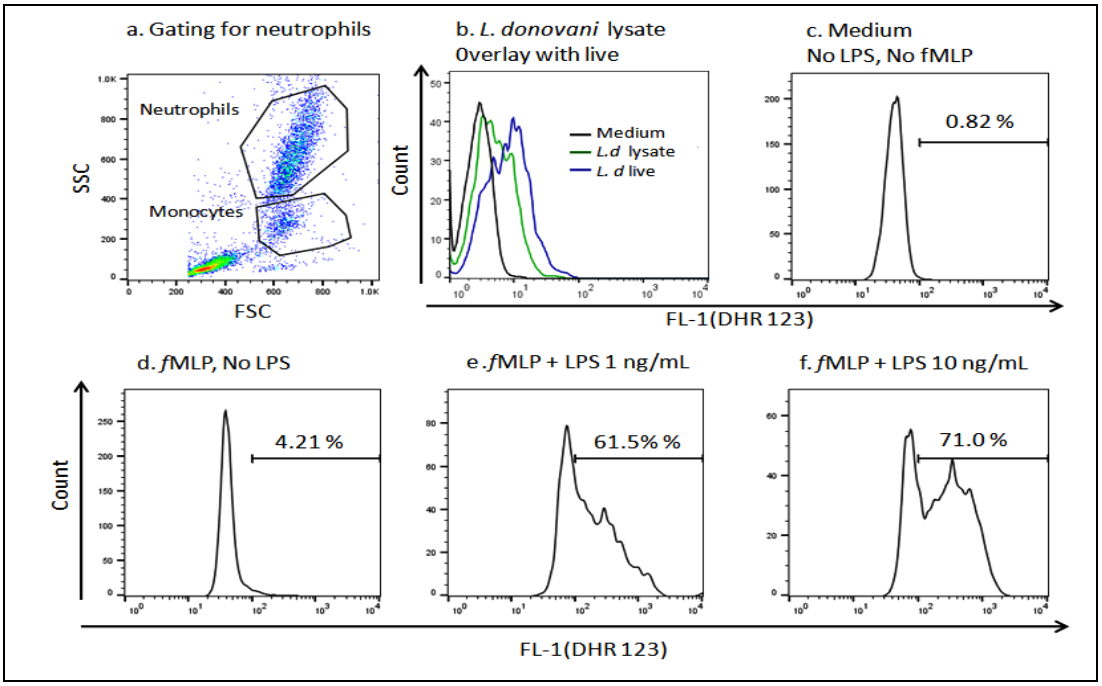

Figure 11. ROS production by blood neutrophils from healthy individuals' optimization.
a) Gating for neutrophils, b) Autofluorescence of neutrophils (fluorescence intensities in the absence of DHR) in medium alone (black line), after exposure to *L. donovani* lysate 1x10^7/mL (green line) and after exposure to viable *L. donovani* promastigotes 1x10^7/mL (blue line). c-f) fluorescence intensities of neutrophils loaded with DHR; c) without stimulation (Medium), d) after exposure to 8.7x10-^{4}mM *f*MLP, e) after exposure to 8.7x10-^{4}mM *f*MLP and 1 ng/ml LPS, f) after exposure to 8.7x10-^{4}mM *f*MLP and 10 ng/mL LPS. The % values show the proportion of DHR 123 positive cells.

From this experiment I selected *L. donovani* lysate 1x10^7/mL for the assay, because of the autofluorescence observed in neutrophils exposed to viable *L. donovani.* The autofluorescence in the FL1 channel would considerably affect/falsify the results obtained with DHR123. In addition, it is not feasible to obtain standardized batches of viable *Leishmania* every day when patients are available. A parasite lysate can be prepared in bulk, stored frozen and could be used from the same stock for all experiments. The concentration of LPS 10 ng/mL was selected for subsequent studies for ROS assay.

4) Optimization for cytokine/ chemokine assay

Optimization for *Leishmania* parasite concentration for the IL-8 release assay was carried out. Representative results are shown in Figure 12.

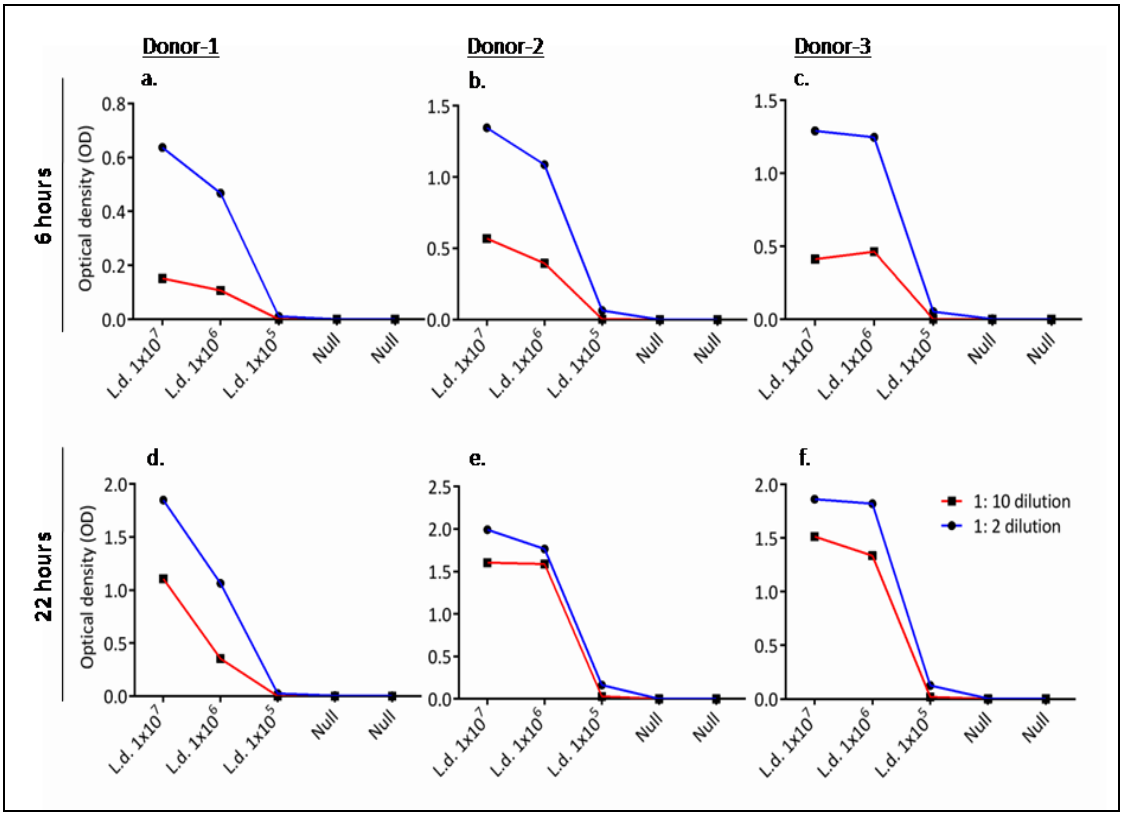

Figure 12. Effect of the amount of *Leishmania* parasite lysate for the release of IL-8 in whole blood culture.

Five hundred micro liters of diluted blood (1:5 with medium) was co-incubated with different concentrations of *L. donovani* lysate at 37°C. Supernatants were collected after 6 hours and 20 hours and diluted at 1:10 and 1:2 with reagent diluent. IL-8 ELISA was performed according to manufacturer's instruction. The upper panel (a-c) shows IL-8 release after 6 hours of incubation while the lower panel (d-f) after 20 hours of incubation for three blood donors.

Based on this experiment, *L. donovani* lysate at concentration at $1x10^7$ /mL was selected as an inducer of IL-8 in subsequent experiments.

Based on optimization done in Germany, similar optimization experiments were done in Ethiopia before commencing the actual experimental work. After starting the actual experiments, dilutions of all supernatants were first optimized before running the ELISA assay; a representative example is shown in the figure below.

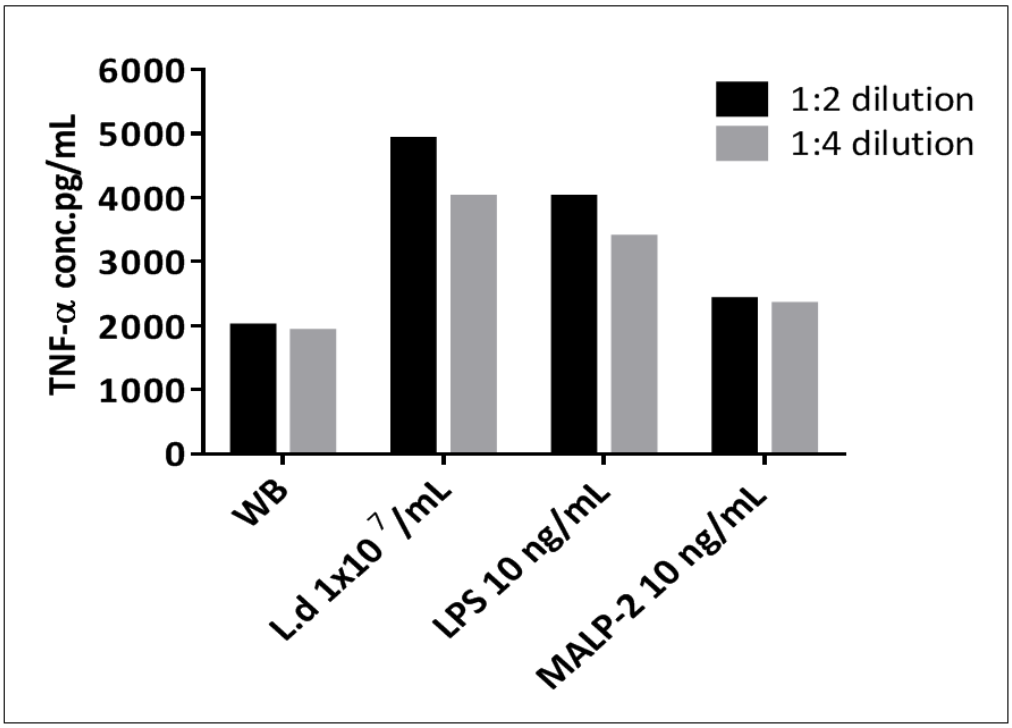

Figure 13.Optimization of the dilution of supernatants for TNF-α ELISA.
Blood was collected from an adult male EHC individual. Blood was diluted 1:5 with RPMI medium supplemented with L-glutamine and P/S antibiotics without FBS. Then 500 µL of diluted blood was distributed in 1.5 mL eppendorf tubes, the respective stimuli were added and incubated in water bath at 37°C for 22 hours. TNF- α content of the supernatants diluted 1:2 and 1:4 was determined by ELISA.

Based on such experiments, in order to save the supernatants for additional cytokine assays, the 1:4 dilution was selected as an optimal dilution for this cytokine.

2.2.2. Part -II. Innate immunity in VL

2.2.2.1. Study area

Libo Kemkem district (Figure 1C) is located in the Amhara region of Northwestern Ethiopia at an altitude of 2,000 m above sea level. The district is made up of 30 kebeles with an estimated population of 196,813 in 2004. A kebele is the smallest administrative unit of Ethiopia, a delimited group of people which consists of at least five hundred families. Addis Zemen (the district capital, population 19,755) is located between Bahir Dar and Gondar on the major road connecting Addis Ababa. The district has one health center and 10 health posts [15], Addis Zemen health center is one of these.

2.2.2.2. Study population

Visceral leishmaniasis (VL) patients visiting the Addis Zemen Health Center for treatment participated in the study. Addis Zemen Health Center is found in Libo kemkem district located at 80 km North of Bahir dar in the Amhara regional state. Visceral leishmaniasis suspected cases were diagnosed according to 2013 Ethiopian national guidelines [11] and were enrolled in the study at the time they contacted the health centre for diagnosis and treatment. For this study, VL volunteer patients (n=29) and endemic healthy control (EHC) (n=26) volunteers were participated.

2.2.2.3. Inclusion and exclusion criteria

Visceral leishmaniasis patients were considered for study inclusion when they fulfilled World Health organization (WHO) recommendation for VL clinical case definitions [2]

plus positive serological tests (for both rK39 and direct agglutination test; DAT), sero-negative for HIV-1/2 and in the absence of any observable additional infectious and non-infectious disease. Endemic healthy control (EHC) donors were considered as controls if they were living in the VL endemic area for more than 6 months, and either a family member or a neighbour living within a 300 meter radius had a history of VL. Endemic healthy control donors did not have any apparent infectious or non-infectious diseases. Endemic healthy controls were selected to have the same sex, ethnic background, and age as their VL study group. All study subjects were between 18 and 50 years of age. Written informed consent form was obtained from each study participants. Those EHC individual testing DAT positive or rk39 positive, and/or HIV positive, were not included in the study.

2.2.2.4. Body mass index (BMI)

The weight and height of the study subjects were measured to calculate body mass index (BMI)(weight/height^2). The BMI was used to determine nutritional status of study subjects as per classification recommended by WHO (http://apps.who.int/bmi).

2.2.2.5. Collection of human whole blood samples

Five milliliters (mL) heparinized blood was collected from VL patients and EHC donors at Addis Zemen health center. The collected blood samples were transported at room temperature (20-25 ^{o}C) to Bahir Dar regional health research laboratory all the subsequent studies were carried out. The experiments were started immediately after the arrival of the samples. For the whole blood experiments 50 µL of undiluted or 500 µL of 5 times diluted whole blood samples were used.

2.2.2.6. Complete blood cell count (CBC)

Total leukocyte count, red blood cell (RBC) count , platelet count, haemoglobin (Hgb) determination, RBC indexes and differential blood cell counts were done using Cell-DYN 1800 hematology analyzer machine (Abbott Laboratories, USA).

2.2.2.7. Serological tests

Prior to collection of whole blood, VL patients and EHC were tested for VL using the rK39 rapid test and DAT (direct agglutination test). All positive VL cases and all negative EHC by rK39 rapid test were confirmed by DAT. The DAT titers for all VL cases included in this study were > 1:1600, while the DAT titers for all EHC included in this study were <1:400. Screening HIV-1/2 serological test was done according to the current test algorithm at Addis Zemen Health center laboratory which was based on Ethiopian national guidelines 2007 [256].

2.2.2.8. *Leishmania* parasite preparation

Leishmania donovani (MHOM /ET/ 67/ HU3) and episomal green fluorescent protein (eGFP) expressing parasites were donated by Prof. Dr. Ger van Zandbergen, Paul-Ehrlich-Institute, Federal Institute for Vaccines and Biomedicines, Langen, Germany. These parasites were cultured in Novy-MacNeal-Nicolle (NNN) medium and sub-cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100U/100 µg/mL Penicillin / Streptomycin and 2 mM L-glutamine. In addition, the culture medium was supplemented with 30 µg/mL hygromycin-B for the culture of eGFP expressing parasites. At seventh day in culture, when *L. donovani* promastigotes reached stationary phase, the culture was collected and washed with sterile PBS (pH

7.2) at 1000 x g for 10 minutes(three times). The final pellet was re-suspended in PBS and promastigotes were counted using a haemocytometer with 0.02 mm depth. *Leishmania donovani* promastigote lysate stock with a concentration of $2x10^8$ promastigotes /mL was prepared by freezing in liquid nitrogen and thawing in water bath at 37^{o}C for five consecutive cycles and stored at -20 ^{o}C until use.

2.2.2.9. Assessment of CD62L and CD66b expression

Flow cytometry analysis was carried out to assess the surface expression of cell activation markers in VL and EHC whole blood neutrophils without stimulation as well as after exposure to different stimuli. Briefly, whole blood activation assays for VL patients and EHC donors were carried out by using CD62L and CD66b as neutrophils surface activation markers. Fifty microliters blood was incubated with *L. donovani* lysate ($1x10^7$/mL), LPS (10 ng/mL) or MALP-2 (10 ng/mL) for 30 minutes at 37^{o}C. Subsequently, probes were stained with anti-human CD62L-PE and CD66b-FITC monoclonal antibodies. Following incubation for another 30 minutes at 4^{o}C, erythrocytes were lysed by adding 500 µL of 1x FACS lysing solution and incubating for 15 minutes at room temperature (RT). Neutrophils were gated and the expression levels of the surface markers were quantified using flow cytometry.

2.2.2.10. Phagocytosis assays

Whole blood phagocytosis assay was carried out to assess the ability of phagocytic cells (neutrophils and monocytes) of VL and EHC to phagocytose microorganisms. Fifty microliters of whole blood was distributed into eppendorf tubes to which *L. donovani* lysate, LPS, MALP-2 or poly I:C was added in 50µL volume of complete medium resulting in a final concentration of lysates of $1x10^7$/mL *L. donovani* promastigotes, 10 ng/mL LPS, 10 ng/ mL MALP-2 or 10 µg/mL poly I:C . Then tubes

were incubated in water bath at 37 ^{o}C for 30 minutes. Afterwards, 5µL of 42 µg /mL of fluorescent-labeled *Staphylococcus (S.) aureus* Bio-particles (Alexa Fluor 488 conjugate) was added to come up with 2 µg /mL final concentration and incubated at 37 ^{o}C in water bath for another 30 minutes. Then lysis of the erythrocytes was achieved by adding 500 µL of 1x FACS lysing solution and incubating for 15 minutes at room temperature (RT). Then the tubes were centrifuged for 8 minutes at 1000 x g at RT and the supernatants were removed. Afterwards, 32 µL of 0.4% trypan blue (quenching solution) was added at 1:19 dilution with FACS buffer in order to quench the fluorescence of bacteria attached to the leukocyte surface and centrifuged for 8 minutes at 1000 x g at RT. The supernatant was removed and the pellet was washed once again with FACS buffer. The supernatant was then removed and the pellets were re-suspended in 400µL FACS buffer and the phagocytosis was assessed by flow cytometry.

2.2.2.11. Assessment of the production of reactive oxygen species (ROS)

The intracellular production of ROS in VL and EHC blood neutrophils and monocytes was assessed by using the substrate DHR 123. Whole blood was incubated with lysates of 1x10^{7} *L. donovani* promastigotes/mL, 10 ng/mL LPS or 10 ng/ mL MALP-2 for 40 minutes at 37^{o}C prior to the exposure of 10 µM N-formyl-L-methionyl-L-leucyl-phenylalanine (*f*MLP) and 500 µM DHR123 for 5 minutes at 37^{o}C. Following incubation on ice for 2 minutes, erythrocytes were lysed and DHR-123 fluorescence of gated neutrophils and monocytes was quantified by using flow cytometry as previously described [257].

2.2.2.12. Assessment of cytokine/chemokine secretion

The secretion of cytokines/chemokines by leukocytes in whole blood was assessed in response to LPS, MALP-2, poly I:C and *L. donovani* promastigote lysate. Whole blood from VL and EHC donors was diluted to 1:5 with RPMI 1640 without serum, supplemented with 100U/100µg/mL Penicillin/ Streptomycin and 2mM L-glutamine. Diluted whole blood (1:5) was distributed in 1.5 mL eppendorf tubes at a volume of 500 µL to which *L. donovani* promastigote lysate at $1x10^7$/mL, LPS at 10 ng/mL, MALP-2 at 10 ng/mL or poly (I: C) at 10 µg/mL final concentration was added to bring the total volume of the probe to 550µL. The tubes were then incubated in water bath at 37°C. Supernatants were collected after 22 hours of incubation and stored at -70°C until the ELISA assay was performed. Human cytokines/chemokines in this study were measured using sandwich ELISA kits purchased from R&D Systems and included : IL-1β, TNF-α, IL-6, IL-12 p70, IL-10, IL-17, IL-8, IP-10, MIP-β and MCP-1. The assays were done according to manufacturer's procedure, optical density (OD) at 450 nm was measured by using a SpectraMAX-190 ELISA reader, and the results were expressed in pg/mL based on a standard curve. The assays were optimized and detection limits and ranges of the assay were determined in preliminary studies. The detection limits and ranges of cytokine detection were assessed as follows: IL-1β (0.9-250 pg/mL), TNF-α (4-1000 pg/mL), IL-12p70 (4-2000 pg/mL), IL-6 (2.3-600 pg/mL), IL-17(1.9-1000 pg/mL), IL-10 (31.2-2000 pg/mL), MIP-1β (15.62-1000 pg/mL), MCP-1(15.6-1000 pg/mL), IL-8 (31.2-2000 pg/mL) and IP-10 (31.2-2000 pg/mL). Graph pad prism 6 software was used to analyse data, and statistical significance was calculated by t-tests at the $p < 0.05$ level.

2.2.3. Part III. Interaction of bovine cells with *L. donovani*

2.2.3.1. Bovine whole blood collection

Whole blood (30 mL) was collected in EDTA tubes from pure Zebu (*Bos indicus*) (n = 6) and their cross with Holstein Friesian cattle (n = 6). All animals were females, age of 6 – 8 years, and all were tested negative for *Leishmania* exposure using the immunochromatographic (rK39) rapid diagnostic test (IT Leish, Bio-Rad). Whole blood cell count (WBC) and differential count for neutrophils and monocytes were performed using an automated hematology analyzer (Sysmex XT-1800i, Kobe, Japan).

2.2.3.2. Co-incubation of bovine whole blood and *L. donovani* promastigotes

Whole blood (100 µL) was distributed in 1.5 mL eppendorf tubes and 1 x10^6 *L. donovani* stationary phase promastigotes were added in 10 µL. The tubes were incubated in water bath at 37 °C for 22 hours. Red blood cells were lysed by adding 500 µL 1x BD FACS lysing solution for 15 minutes at room temperature. The tubes were centrifuged at 1000 x g for 8 minutes at room temperature, and then washed twice with 500 µL PBS (pH 7.2). Supernatants were discarded and the cells were re-suspended in 400 µL PBS (pH 7.2). Cytospin smears from the cell suspensions were stained with 10 % Giemsa staining solution and examined microscopically for PMN infection. Infection rate was calculated from the number of infected PMN per 200 PMN cells.

2.2.3.3. Bovine peripheral blood mononuclear cells isolation

Bovine peripheral blood mononuclear cells (PBMC) were isolated as previously described [258]. Briefly, 25 mL of whole blood was dispensed in sterile 50 mL centrifuge tube and centrifuged for 5 minutes at 298 x g to collect plasma into another sterile tube. Meanwhile 15 mL of Ficoll was added to leucosep tubes and centrifuged for 2 minutes at 133 x g. Then plasma free blood was diluted with equal volume of medium and transferred into 50 mL leucosep tubes containing 15 mL Ficoll. It was then subjected to spinning for 20 minutes at 298 x g without break. The buffy coat was carefully collected in a new sterile 50 mL tube and filled with wash medium up to the 45 mL level. This was again centrifuged for 10 minutes at 298 x g with low break, and this step was repeated for a second wash for 10 minutes at 298 x g with low break. The supernatant was removed and the PBMC were re-suspended in 2.5 mL of RPMI 1640 medium supplemented with 100U/100µg/mL of penicillin/streptomycin, 10 % fetal bovine serum (FBS) and 2 mM L-glutamine (complete RPMI 1640 medium).

2.2.3.4. Bovine monocyte differentiation to macrophages

Isolated PBMCs were washed and re-suspended in polystyrene culture flask containing complete RPMI 1640 medium with 1 % autologous plasma (obtained from whole blood), and incubated at 37 °C, 5 % CO_2 for 1.5–2 hours. The non-adherent cells were discarded and tubes were washed twice with pre-warmed sterile PBS (pH 7.2) containing 5 % complete medium (wash medium). Cells were then suspended in 5 mL of complete RPMI 1640 medium and incubated at 37°C, 5 % CO_2. Rinsing adherent cells with warm washing medium twice and replacing with complete medium were repeated for every two days up to 6–8 days. After 6–8 days of culture, cells were washed twice with pre-warmed sterile PBS and kept on ice for 30 minutes with gentle agitation to detach the majority of adherent cells. Additional adherent cells were

removed by a cell scraper. 91–97 % of the detached cells were viable when assessed by trypan blue exclusion. The detached cells were washed twice with wash medium (200 x g for 10 minutes) and re-suspended in 5 mL of complete RPMI 1640 medium on ice. The cells obtained by this procedure were considered monocyte derived macrophages (MDM).

2.2.3.5. Bovine MDM infection with *L. donovani* stationary stage promastigotes

Into wells of 16 well chamber slides containing 250 µL complete medium, 40 µL containing 1x 10^5 MDM was added. The culture was incubated for 30 minutes at 37 °C, 5 % CO_2 to allow cell adherence. *Leishmania donovani* stationary promastigotes (wild type) was added to the culture at a MOI of 10 promastigotes to 1 MDM in 10 µL volume. Into 1.5 mL eppendorf tubes containing 900 µL complete medium, 1 x 10^6 bovine MDMs in 80 µL complete medium volume were added. *Leishmania donovani* stationary promastigotes (eGFP expressing) were added to the cultures at a MOI of 10 promastigotes to 1 MDM in 20 µL volume. Eppendorf tubes were centrifuged at 200 x g for 5 minutes to promote contact. All cultures were incubated for an additional 90 minutes at 37 °C, 5 % CO_2 and then washed carefully with warm sterile wash medium until extracellular promastigotes were removed. For cultures in chamber slides, 200 µL complete medium was added and incubated for 3, 6, 24 and 48 hours, and 5 days at 37 °C, 5 % CO_2. After each time points of infections, cultures were washed with pre-warmed PBS for 2–3 times and air dried for subsequent staining. The chamber slides were then fixed with absolute methanol for 5 minutes and stained with 10 % Giemsa staining solution for 10–15 minutes and examined under a binocular light microscope. For MDM infected with eGFP expressing *L. donovani* in the eppendorf tubes, the infection rate was assessed by using flow cytometry. At each time points, tubes were washed carefully with warm sterile wash medium until extracellular promastigotes were removed and a drop of the suspension was visualized under the microscope to confirm that extracellular parasites were removed from each tube.

2.2.3.6. Co-incubation of infected bovine whole blood cells with MDM

Bovine whole blood (100 µL) was distributed in 1.5 mL eppendorf tubes and $1x10^6$ stationary phase promastigotes of *L. donovani* in 10 µL volume were added and incubated for 22 hours in a 37 °C water bath. Erythrocytes were then lysed using a sterile 155 mM NH_4Cl (ammonium chloride) solution for 15 minutes at room temperature, centrifuged at 1000 x g for 8 minutes and washed twice. Finally, pellets were re-suspended in 400 µL of RPMI 1640 complete medium. One hundred µL of the suspension was added to 24 well micro-plates and to chamber slide wells containing bovine MDM. These were kept in a humidified atmosphere at 37 °C, 5 % CO_2 for 16 days. Half of the plates were examined microscopically after staining with a 10 % Giemsa staining solution, while the other half were kept in culture at 37 °C, 5 % CO_2.

2.2.3.7. Culturing *L. donovani* promastigotes from infected bovine MDM

On the 16th day of culture, *L. donovani* infected bovine MDM (as described above) were washed with warm wash medium and examined under an inverted microscope for any motile extracellular parasites. Then supernatants were replaced by 300 µL of liquefied Novy-MacNeal-Nicolle (NNN) medium or complete RPMI 1640 medium and the cultures were incubated at 26 °C. The cultures were examined under an inverted microscope every day for the presence of motile promastigotes.

2.2.3.8. Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) for species determination

Leishmania donovani promastigotes cultured from *in vitro* infected bovine MDM and reference strains of *L. aethiopica* (MHOM / ET/ 72/ L100), *L. donovani* (MHOM / IN80/ DD8), *L. infantum* (MHOM / FR / LEM-75), *L. tropica* (MHOM / SU / 74 / K27) and *L. major* (MHOM / SU / 73 / 5-A SKH) were cultured in complete medium and their

stationary phase promastigotes were harvested by centrifugation at 1000 × g for 10 minutes at 4 °C. The genomic DNA was extracted by using Qiagen DNeasy (Qiagen, Hilden, Germany) for blood and tissue kits according to manufacturer's protocol. The extracted DNA was quantified using a spectrophotometer at OD of 260 nm. The PCR was performed using a T3000-Thermocycler (Biometra, Gottingen, Germany) based on the volume of each PCR reaction at 25µL where LISTR/L5.8S primer pairs were used at 0.5 µL of each primer (15 pmol) with 12.5 µL HotStar Taq Master Mix (Qiagen, Hilden, Germany) and 2µL (20 ng/µL of DNA template). The amplification conditions were set at 30 cycles at 94 °C for 30 seconds, at 50 °C for 30 seconds, and at 72 °C for 30 seconds. The PCR products were visualized in 1.5% agarose (Sigma Aldrich, St. Louis, USA) gel with 0.5µg/mL Ethidium bromide (Sigma Aldrich, St. Louis, USA). Four micro liters of each PCR product was loaded along with 2µL of 5x loading buffer (Thermo scientific, Waltham , USA) on the gel while 3 µL of 0.1 µg/ µL molecular size marker (Promega, Fitchburg, USA) was loaded as a molecular marker. Electrophoretic separation was performed at 100 V and 50 mA for 90 minutes and pictures were taken using a UV trans-illuminator (BioRAD, Hercules, USA) for further analyses. *Leishmania* species identification was achieved by PCR product amplification of the ribosomal internal transcribed spacer-1 (ITS-1) sequences and followed by restriction digestion with enzymes *Hha I* (Promega, Fitchburg, USA) as described before [259]. The reaction mix contained 17 µL PCR product, 2 µL of 10× PCR enzyme buffer (Thermo Fisher Scientific, Waltham, USA) and 1µL of restriction enzyme *Hha I*, and was incubated for 4 hours at 37 °C [260]. The restriction fragments were visualized in 2 % agarose (Sigma Aldrich, St. Louis, USA) gel in the presence of 0.5µg/mL Ethidium bromide and finally the PCR products and restriction fragments were visualized using a trans-illuminator.

2.2.4. Part-IV. Biomarkers investigation for test of VL

2.2.4.1. Assessment of serum concentration of MMP-9, sCD40L and IL-10 in VL patients before and after treatment

A case control study involved 49 VL cases from Addis Zemen treatment center and 30 EHCs from Bura kebele (the smallest administrative unit in Ethiopia), Libo Kemkem district. Clinical cure was defined as absence of any clinical sign and symptoms of VL after completion of the recommended dose (20mg/kg/day) and duration of treatment (30 days) with sodium stibogluconate (Generic SSG, Albert David Ltd, Calcutta). Endemic healthy controls (EHC) were of the same sex and age ± 5 years as the VL cases, had no treatment history for VL, and tested DAT negative. Both study subjects (VL cases) and EHC, were included in the study after qualifying the inclusion and exclusion criteria mentioned above. Five milliliters of venous blood samples from EHC cases and from VL cases, before and after treatment, were collected for serum separation, which was stored at -20^{o}C until use. Serum levels of MMP-9, sCD40L and IL-10 were assessed by sandwich ELISA as per the manufacturer's protocol (DuoSet ELISA development systems, R&D systems). Data analysis was done using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego CA, USA) and STATA version 11 (StataCorp LP, College, Station, Texas, USA).

2.2.5. Ethical consideration

Concerning human subject studies, National Ethical Clearance Committee at Federal Democratic Republic of Ethiopia Ministry of Science and Technology ethically reviewed and approved with No. 310/227/07 on date 30/05/2011 prior to implementation of the innate immune study project. All blood sample donors signed on informed consent form before data collection. All VL positive patients were treated in accordance with Addis Zemen Health center protocol based on national treatment guidelines.

Concerning the animal based study; ethical approval was obtained from the institutional review board of Armauer Hansen Research Institute with No. PO-05/12 on date 07/02/2012, and a veterinarian obtained consent from the owners of the domestic animals (Sebeta Agro Industry Farm) for the collection of blood samples. International animal experimentation guidelines were followed appropriately.

2.2.6. Statistical analysis

Data was analyzed using flowJo software (FlowJo LLCv10.0.8, Ashland, USA) and Prism Graph Pad Software for Windows (Inc. Oberlin Drive, San Diego, USA). Statistical significance was calculated using t-tests, paired t-test or Mann-Whitney non-parametric tests where appropriate and $p < 0.05$ was considered statistically significant. Sociodemographic, anthropometric and treatment outcome data were documented using a pretested questionnaire (attached to annex).

3. RESULTS

3.1. Part -I. Innate immune cell functions in VL

3.1.1. General description of study subjects

3.1.1.1. Demography of VL patients and EHC included in the study

Twenty nine male VL patients (mean age of 27 years) and 26 male EHCs (mean age of 29 years) were included in this study. All VL patients and EHC donors were from the same ethnicity background. All study participants were screened negative for HIV, using the rapid HIV1/2 test according to the Ethiopian national HIV diagnostic algorithm [261]. All VL participants were clinically VL suspected and tested positive for VL antibody by serology (rk39 and DAT), whereas all EHC (control) subjects were tested negative for VL antibody by serology. All VL patients 29/29 (100%) had a travel history for seasonal agricultural activities, where 93.1% of them travelled to an area previously reported as VL endemic area: Humera area 24 (82.8%) and Metema area three (10.3%). The remaining two (6.9%) of the cases had travel history to Benshangul-Gumuz area, an area previously not known as endemic for VL. The travel history of EHC groups showed that 10/26 (38.5 %) of them travelled to an area previously reported as VL endemic area in the north part of the country; of these 9 (90%) traveled to Humera, and one (10%) to Metema. The clinical onsets of symptoms of VL cases were manifested in all VL cases after they returned from endemic area. The majority of VL patients (16, 55.2%) who stayed less than two months in an endemic area contracted the disease; most VL patients (14, 48.3%) had fever for duration of 1.1 to 2 months (table.4).

Table 4. Demography characteristics of VL patients and EHC groups.

Pre-tested structured questionnaire was used to collect data (see Annex).
SD = standard deviation

Characteristics	Category	VL (n=29)	Control(n =26)
Age (years)	No (mean ± SD)	27 ± 7.85	29 ± 6.98
Ethnicity (%)	Amhara	29 (100)	26 (100)
Duration of stay in VL endemic area (%)	0.5- 2 months	16 (55.2)	4 (15.4)
	2.1-3.0 months	5 (17.2)	2 (7.7)
	≥3.1 months	8 (27.6)	4 (15.4)
Onset of symptoms (%)	After return	29 (100)	NA
	Before return	0	NA
Duration of fever (in months) (%)	0.5-1	10 (34.5)	NA
	1.1-2	14 (48.3)	NA
	2.1 and above	5 (17.2)	NA
Travel history (%)	Yes	29 (100)	10 (38.5)
	No	0	16 (61.5)
Travel to specific areas (%)	Metema	3 (10.3)	1(10)
	Humera	24 (82.8)	9 (90)
	Benshangul-Gumuz	2 (6.9)	0

3.1.1.2. Clinical signs and symptoms of VL patients included in the study

The predominant clinical signs and symptoms among VL patients in this study were splenomegaly followed by skin pallor and history of bleeding. Other clinical signs and symptoms associated previously with VL such as jaundice, ascites and hepatomegaly were not observed in this study group (Table 5).

Table 5. Clinical sign and symptoms among VL study participants.

Characteristics	VL (n=29)	
	Yes (%)	No (%)
Splenomegaly	28 (96.5)	1 (3.5)
Skin pallor	18 (62.0)	11 (38.0)
History of bleeding	13 (44.8)	16 (55.2)
Past history of VL	1 (3.5)	28 (96.6)
Oedema	1 (3.5)	28 (96.5)
Lymph node enlarged	1 (3.5)	28 (96.5)
Hepatomegaly	0 (0.0)	29 (100)
Ascites	0 (0.0)	29 (100)
Jaundice	0 (0.0)	29 (100)

3.1.1.3. Malnutrition is more prevalent in VL cases than EHC

The classification of BMI (weight/height^2) was used to determine nutritional status according to ranges set as per WHO recommendation (http: // apps. who. int /bmi) [262]. According to body mass index (BMI), the majority (17, 58.6%) of VL cases were malnourished, comprising 24.1 % from severe malnutrition, 10.2 % from moderate malnutrition, and 24.1% from mild malnutrition. Similarly, one moderate and eight mildly malnourished subjects were observed in the EHC group (Table 6).

Table 6.Body Mass index (BMI) for VL and EHC included in the study

Extent of malnutrition	Range (BMI-kg/m2)	VL (n=29)	EHC (n=26)
Severe	<16.00	7(24.1%)	0(0.0)
Moderate	16.00 - 16.99	3(10.2%)	1(3.8%)
Mild	17.00 - 18.49	7(24.1%)	8 (30.8%)
Normal	18.50 - 24.99	12(41.3%)	17(65.4%)
Total		29(100%)	26 (100%)

3.1.1. 4. Peripheral blood leukocyte count is reduced in VL

A total leukocyte count of < 4500/μL and platelet count of <150 000/μL were used to define leukopenia and thrombocytopenia, respectively [279, 280]. Significantly lower mean leukocyte counts were observed in the VL study group than in EHC, p< 0.005 (Table 7). From the perspective of differential leukocyte count, the mean percentage of neutrophils for VL groups was significantly lower than EHC, p< 0.05. In contrast, the mean percentage of lymphocytes in VL was significantly higher than in EHC. There was no significant difference observed between the two groups for monocyte counts (Table 7).

Measurements of red blood indices such as haemoglobin (Hgb), haematocrit (Hct), and mean corpuscular/cell volume (MCV) showed significantly lower values in the VL group than in the control group (p < 0.005). There was no significant difference in MCH and MCHC values between the two groups (Table 7). There was significantly lower platelet counts observed in VL group than in EHC, p <0.005.

Table 7. Hematological parameters in VL versus EHC study group.

Abbreviations: fL= femtolitres, pg=picogram, g/dl= gram per deciliter, RBC=Red blood cells, Hgb= hemoglobin, Hct= hematocrit, MCV= Mean corpuscular volume, MCH= mean corpuscular hemoglobin, MCHC= mean corpuscular hemoglobin concentration, ns=no significant difference, SD=standard deviation. Data show mean ± SD and p- value. *P<0.05, **P<0.005, ***P<0.0005.

Hematological parameters	VL (n=29)	EHC(n=26)	p-value
WBC-counts/μL	2659 (2340)	6450 (2239)	***
Neutrophils %	34.9(14.9)	44.5 (9.7)	*
Monocytes %	14.8 (7.6)	16.5 (5.9)	ns
Lymphocytes %	48.3 (12.74)	38 (9.7)	**
RBC-counts million/μL	4.1 (1.7)	6.7 (7.2)	ns
Platelets counts $x10^3$/μL	80.9 (60.0)	137.2 (63.1)	***
Hgb- g/dL	9.2 (2.06)	15.5 (1.2)	***
Hct %	28.8 (5.9)	48.5 (3.7)	***
MCV- fL	80.5 (6.9)	91.7 (4.5)	***
MCH-pg	33.2 (40.4)	29.4(2.0)	ns
MCHC-g/dL	32.1 (2.8)	32.0 (2.0)	ns

3.1.2. Blood neutrophil and monocyte functions

3.1.2.1. Blood neutrophils of VL patients show lower levels of CD62L shedding and less degranulation after exposure to activating stimuli than neutrophils of control individuals (EHC)

A whole blood based *in vitro* assay was used to simulate conditions of cells in the circulation meaning that the cellular functions were determined in the presence of all cellular elements and soluble factors of the blood. Shedding of CD62L and enhanced CD66b expression were assessed as markers of neutrophil activation and degranulation, respectively. The response of blood neutrophils of VL patients to *L. donovani* promastigote lysates and to the TLR agonists LPS and MALP-2 was analyzed, and compared to the response of EHC neutrophils. The expression of the activation markers CD62L and CD66b was assessed on neutrophils by using flow cytometry. The gating strategy for neutrophils is shown in Figure 14a. Representative dot plots (Figure 14b) show that exposure to an activating stimulus such as LPS leads to down regulation (shedding) of CD62L-expression and up-regulation of CD66b expression on neutrophils from healthy individuals. However, significant differences were seen between the extent of response of neutrophils of VL patients and endemic healthy controls. After exposure to *L. donovani* lysate and the TLR-2/6 agonist MALP-2 the extent of CD62L shedding was significantly lower in VL neutrophils than in EHC neutrophils indicating a lower level of activation of VL neutrophils after exposure to these stimuli (Figure 14c). The exposure of VL neutrophils to *L. donovani* lysate resulted in significantly lower enhancement of CD66b expression than EHC neutrophils indicating a lower level of *L. donovani*-induced degranulation of VL neutrophils (Figure 14d). However, in response to LPS and MALP-2, neutrophils from both study groups show comparable degree of degranulation.

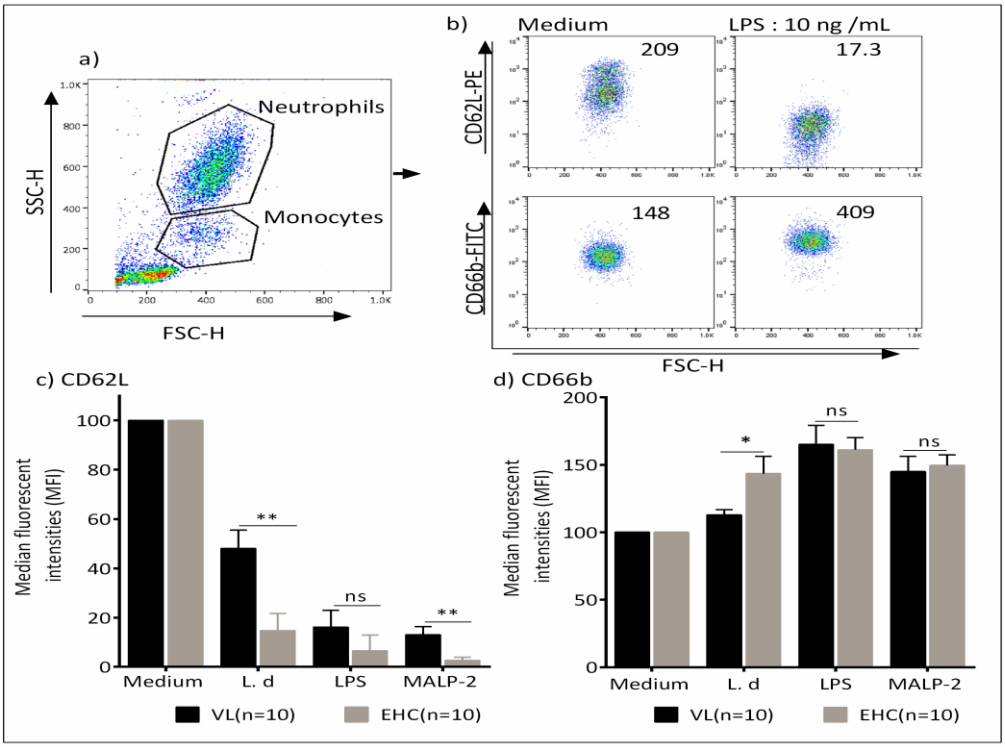

Figure 14.The effect of activating stimuli on the expression of CD62L and CD66b on neutrophils from VL patients and control individuals.

Whole blood samples from VL patients and EHC were incubated with *L. donovani* (*L. d*), LPS, or MALP-2 or without stimulation (Medium) for 30 minutes at 37°C. Subsequently, probes were stained with anti-CD62L-PE and anti-CD66b-FITC antibodies. After lysing the erythrocytes, the expression of the surface markers CD62L and CD66b was quantified by using flow cytometry. a) Gating strategy for neutrophils based on their SSC and FSC characteristics. b) Representative dot plots of CD62L and CD66b expression of EHC gated neutrophils without exposure to stimuli (Medium) and after exposure to LPS. The numbers in the boxes show the median fluorescent intensities (MFI) of the cells expressing the respective marker. c) Expression of CD62L (MFI) normalized to the expression levels on non-stimulated (Medium) neutrophils of VL and EHC neutrophils after exposure to *L. donovani* (L. d), LPS, or MALP-2. d) Expression of CD66b (MFI) normalized to the expression levels on non-stimulated (Medium) neutrophils of VL and EHC neutrophils after exposure to *L. donovani* (L. d), LPS, or MALP-2. Error bars show standard error of the mean (SEM). * $p<0.05$, ** $p<0.005$. ns=no significant difference.

3.1.2.2. Phagocytic capacity of neutrophils and monocytes from healthy individuals

Phagocytosis is the mechanism by which relatively large (over 0.5 µm) particles, such as bacteria and dead cells are internalized by phagocytic cells. In order to investigate if

the observed low-level activation of VL neutrophils in response to *L. donovani* (Figure 14) applies also to the phagocytic capacity of these cells, phagocytosis assays were performed by using fluorescent-labeled *S. aureus* particles. The gating strategy for neutrophils is shown in Figure 14a. It was observed that both neutrophils and monocytes from healthy individuals phagocytose *S. aureus* particles (Figure 15 b, e). The phagocytic capacity of both neutrophils and monocytes were enhanced after exposure to LPS (Figure 15 c, f).

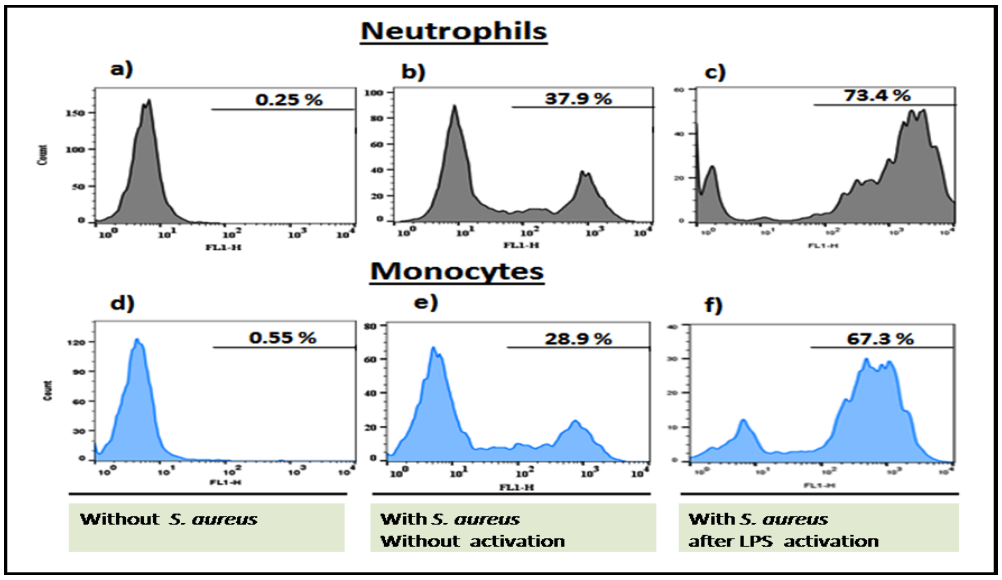

Figure 15.Ingestion of S. aureus particles by neutrophils and monocytes.
Whole blood samples from an endemic healthy control (EHC) individual were incubated in medium alone or in the presence of LPS (10 ng /mL) for 30 minutes at 37°C. *S. aureus* bioparticles (Alexa Fluor 488-labeled) were then added and the samples were incubated for an additional 30 minutes at 37°C. Erythrocytes were lysed and the ingestion of *S. aureus* bioparticles by neutrophils and monocytes as defined by light scatter differences was assessed by using flow cytometry. Representative histograms show: neutrophils (a) and monocytes (d) without co-incubation with *S. aureus* bioparticles; non-stimulated neutrophils (b) and non-stimulated monocytes (e) after co-incubation with *S. aureus* bioparticles; LPS stimulated neutrophils (c) and monocytes (f) after co-incubation with *S. aureus* bioparticles. The % values indicate the ratios of neutrophils and monocytes that engulfed bacteria.

3.1.2.3. Phagocytic capacity of neutrophils from VL patients is impaired

Activating stimuli; *L. donovani* (L. d) promastigote lysate, LPS, MALP-2 and poly I: C were used to test the phagocytic response of VL monocytes and neutrophils. By assessing the response of the phagocytic cells to these stimuli, I aimed to obtain information regarding the ability of the cells to respond to *Leishmania* parasites as well as to other stimuli such as TLR-ligands. There was no significant difference observed between EHC and VL patients regarding the ratio of neutrophils that phagocytosed *S. aureus* (Figure 16a). However, the median fluorescence intensity (MFI) of the cells that ingested *S. aureus* was significantly higher in the EHC group than in VL patients (Figure 16b). These results show that although the ratio of phagocytosing neutrophils is not different, VL neutrophils do not respond to the tested stimuli with an enhanced phagocytic capacity.

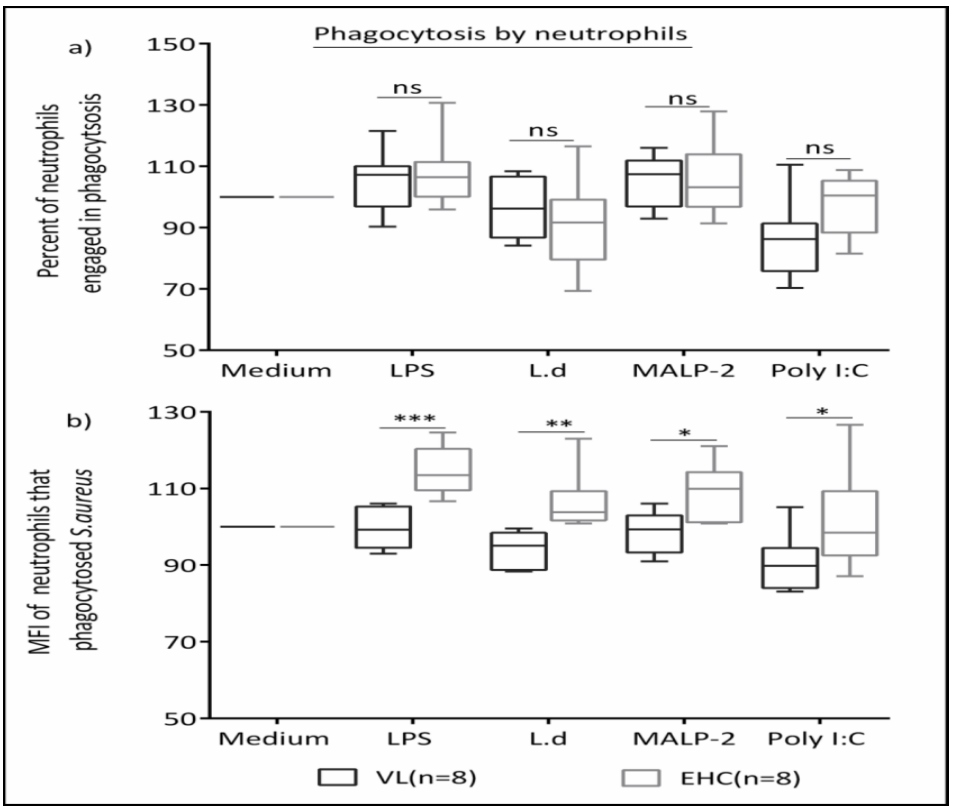

Figure 16.Phagocytosis of S. aureus by whole blood neutrophils.
Whole blood samples from VL patients and endemic healthy control (EHC) individuals were incubated with *L. donovani* (L. d) lysate: $1x10^7$/mL, LPS: 10 ng /mL, MALP-2: 10 ng /mL, poly I: C: 10 µg /mL or

without stimulation (medium) for 30 minutes at 37°C. *S. aureus* bioparticles (Alexa Fluor 488-labeled) were added following 30 minutes incubation at 37°C. Erythrocytes were lysed and *S. aureus* bioparticle fluorescence of gated neutrophils was quantified using flow cytometry. This figure depicts a) the percentage of neutrophils which ingested *S. aureus* bioparticles after exposure to various stimuli, and b) the MIF of neutrophils that phagocytosed *S. aureus* particles, as a measure of the number of phagocytosed *S. aureus* particles. Data in 'a' and 'b' were normalized to the phagocytosis level of non-stimulated neutrophils, cultured in medium alone. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$, and 'ns' stands for ' not significant'.

The number of bacteria ingested by phagocytosis positive VL neutrophils (MFI) was significantly lower after exposure to *L. donovani*, LPS, MALP-2 and poly I: C, as compared to EHC (Figure 16b). However, with regard to ratio of neutrophils engaged in phagocytosis, there was no significant difference between VL cases and EHC (Figure 16a).

3.1.2.4. Phagocytic capacity of monocytes from VL patients is impaired

As shown in Table 7, the differential count of peripheral blood monocytes in VL was similar to that of EHC**.** To investigate whether there was a difference in phagocytic capacity of monocytes between VL and EHC, whole blood from VL and EHC was exposed to *L. donovani* promastigote lysate, LPS, MALP-2 or poly I: C, and monocytes were identified by light scatter differences. The number of bacteria ingested by phagocytosis positive VL monocytes (MFI) was significantly lower after exposure to *L. donovani*, LPS, MALP-2 and poly I: C as compared to EHC (Figure17b).

The ratios of monocytes engaged in phagocytosis were not significantly different between VL patients and EHC (Figure 17a). Data in Figure 17a also show that the applied stimuli do not enhance the ratio of monocytes engaged in phagocytosis. The stimuli, however, resulted in an enhanced capacity of the phagocytosing monocytes from EHC to ingest *S. aureus*. The MFI values indicate a measure of the number of ingested bacteria.

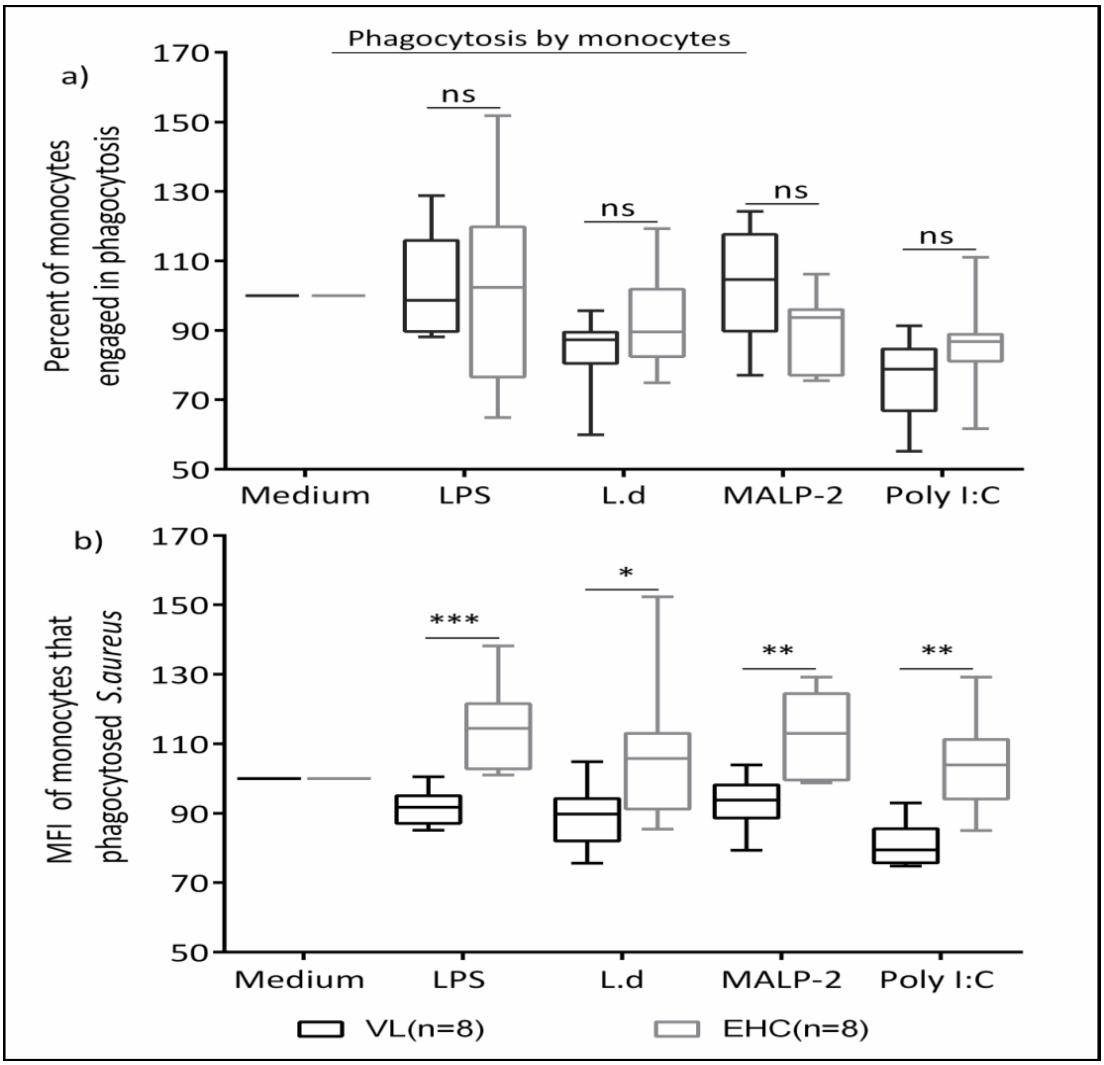

Figure 17. Phagocytosis of S. aureus by whole blood monocytes.
Whole blood samples from VL patients and endemic healthy control individuals (EHC) were incubated with *L. donovani* (L.d) lysate, LPS, MALP-2, poly I:C or without stimulation (medium) for 30 minutes at 37°C. *S. aureus* bioparticles (Alexa Fluor 488-labeled) were added following an incubation of 30 minutes at 37°C. Erythrocytes were lysed and fluorescence of gated monocytes was quantified by using flow cytometry. In this figure, a) shows ratio of monocytes having ingested *S. aureus* bioparticles, b) shows the MIF of monocytes that phagocytosed S. aureus particles indicating the number of phagocytosed *S. aureus* particles by the phagocytosis positive monocytes. Data shown are normalized to the phagocytosis level of non-stimulated (Medium) monocytes, where * $p<0.05$, ** $p<0.005$, *** $p<0.0005$, and 'ns' stands for ' not significant'.

Importantly, exposure to *L. donovani* or to the TLR ligands LPS, MALP-2 or poly I: C did not enhance the capacity of monocytes from VL patients to ingest *S. aureus.* These observations indicate that VL monocytes do not respond with an enhanced phagocytic capacity to the applied stimuli.

3.1.3. ROS production by neutrophils and monocytes is impaired in VL

As shown above, significantly lower numbers of bacteria were ingested by neutrophils and monocytes of VL patients compared to EHC (Figure 16b and Figure 17b). Next, I investigated if the reduced phagocytic capacity of VL neutrophils and monocytes after exposure to *L. donovani* and TLR agonists is accompanied by a reduced capacity of these cells to produce reactive oxygen species (ROS). Reactive oxygen species production is an essential antimicrobial effector mechanism which contributes to the killing of phagocytosed pathogens.

In view of the fact that *f*MLP is a priming stimulant for neutrophils and monocytes for enhanced ROS production[265,266], *f*MLP alone or in combination with *L. donovani*, LPS or MALP-2 was used to evaluate ROS production in VL neutrophils and monocytes. Priming of neutrophils and monocytes with *f*MLP alone led to significantly lower level of ROS release in VL than in control group (Figure 18 a, b).

In the presence of *f*MLP, when stimulated with *L. donovani*, LPS or MALP-2, VL neutrophils produced significantly lower levels of ROS than EHC neutrophils (Figure 18a). ROS production by VL patient's monocytes stimulated with *L. donovani* lysate, LPS or MALP-2 was also significantly lower compared to EHC monocytes (Figure 18b). Despite its priming effect, the costimulation of *f*MLP with *L. donovani*, LPS or MALP did not make VL neutrophils and monocytes to respond with higher ROS production except in EHC. Up on stimulation with *f*MLP alone or in combination with *L. donovani*, LPS or MALP-2, VL patient's neutrophils and monocytes did not show significant difference in ROS production. However, neutrophils from EHC showed significantly higher ROS production during stimulation with LPS plus *f*MLP than stimulation with *f*MLP alone (Figure 18 a). Stimulation of VL patients' neutrophils with *f*MLP plus *L. donovani* or MALP-2 resulted in higher ROS production than stimulating with *f*MLP alone. Similarly in response to *f*MLP plus LPS or *L. donovani,* VL patients' monocytes have shown slightly higher tendency of ROS production than monocytes stimulated

with *f*MLP alone, Figure 18b.

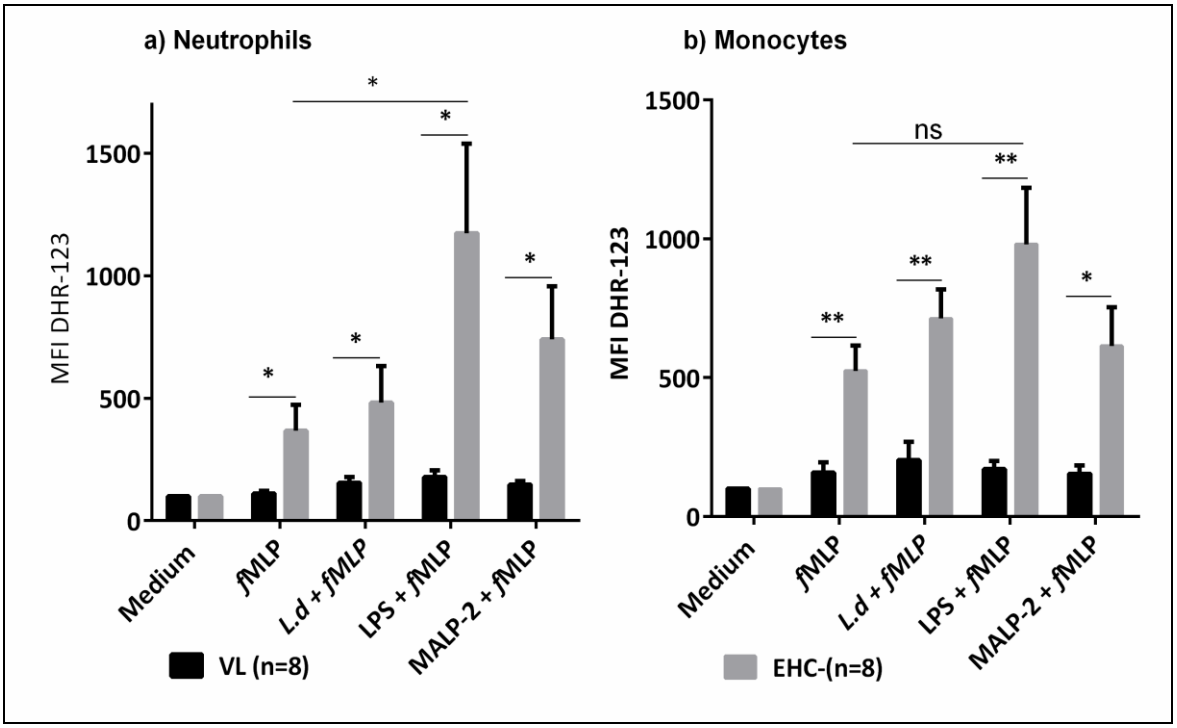

Figure 18.Generation of ROS by neutrophils (a) and monocytes (b) of VL patients and EHC.

Intracellular ROS production of neutrophils and monocytes was assessed using the substrate DHR-123. Blood was incubated with lysate of *L. donovani* (L. d) $1x10^7$ promastigotes /mL, LPS 10 ng /mL or MALP-2 10 ng /mL for 40 minutes at 37°C prior to exposure to *f*MLP 10 µM and DHR-123 500 µM incubated for 5 minutes at 37°C. Following incubation on ice for 2 minutes, erythrocytes were lysed and DHR-123 fluorescence of gated neutrophils and monocytes were quantified using flow cytometry. Shown are median fluorescent intensities (MFI) and bar graphs after normalization of ROS levels to fluorescent intensities of unstimulated (Medium) cells to 100 MFI. Error bars show standard error of the mean (SEM). * $p<0.05$, ** $p<0.005$, and 'ns' stands for ' not significant'.

The results demonstrated that neutrophils and monocytes of VL patients, in contrast to EHC, did not respond to *f*MLP, *L. donovani,* and TLR4 and TLR2/6 agonists in terms of ROS production. The presented data regarding the compromised response to stimulating stimuli and decreased phagocytic capacity, and decreased ROS production by VL neutrophils and monocytes suggest a decreased ability of VL leukocytes to kill *Leishmania* parasites in clinical VL.

3.1.4. Cytokines /chemokines production in VL whole blood culture

3.1.4.1. Baseline levels of IL-1β, TNFα, IL-6 and IL-10 production in whole blood culture are significantly lower in VL than in EHC

As described above I observed a lower level of cellular activation, lower phagocytic capacity and impaired ROS production by neutrophils and monocytes in VL patients as compared to EHC. To further analyse the alteration of innate immune cell functions in VL, secretion of cytokines/chemokines in whole blood culture was investigated, both without and with specific stimulants. To assess the baseline production of cytokines in the absence of stimulation, whole blood from VL and controls individuals was diluted 1:5 with medium and incubated for 22 hours without addition of any stimulant. In the supernatants collected, the baseline (unstimulated, US) level of the production of IL-1β, TNFα, IL-6, IL-17 and IL-10 was assessed. The baseline production of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6, was significantly lower in VL supernatants than in supernatants of EHC whole blood cultures (Figure 19). The baseline level of the anti-inflammatory cytokine IL-10 was also lower in VL supernatants than in EHC supernatants (Figure 19). The secretion of IL-17 and IL-12p70 did not differ significantly between VL and EHC (Figure 19).

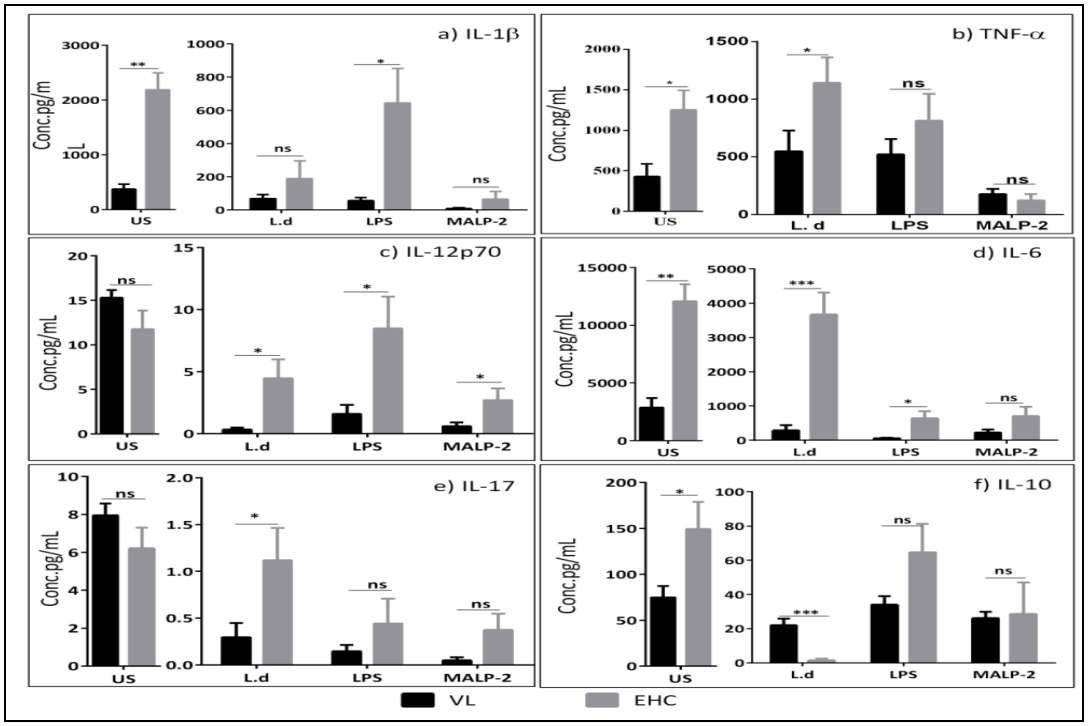

Figure 19. Secretion of cytokines by whole blood cells of VL and EHC after 22 hours without stimulation and after exposure to various stimuli.

Whole blood from VL (a-e : n=14; f:n=12) and EHC (a-e : n=14; f :n=12) was diluted 1:5 with medium and incubated without any additional stimuli (US) or with *L. donovani* promastigotes lysate $1x10^7$/mL , LPS 10 ng/mL or MALP-2 10 ng/mL at 37°C for 22 hours. Supernatants were collected and cytokines were measured using ELISA. The concentrations of the cytokines in un-stimulated culture (baseline value) were subtracted from the stimulated values. Statistical significance was calculated by using multiple t-test and differences were considered statistically significant when $p \leq 0.05$ where * $p<0.05$, ** $p<0.005$, *** $p<0.005$, and 'ns' stands for 'not significant'.

3.1.4.2. *Leishmania donovani* induced production of proinflammatory cytokines is lower in VL than EHC

To investigate the cytokine release of blood leukocytes upon exposure to various microbial stimuli, whole blood from VL patients and EHC individuals was diluted 1:5 with medium and stimulated with *L. donovani* promastigote lysate, LPS or MALP-2. As compared to the baseline cytokine levels in unstimulated cultures, the enhancement of the release of the proinflammatory cytokines TNF-α, IL-12p70 and IL-17 was significantly lower in VL after exposure to *L. donovani* as compared to the EHC group (Figure 19). A tendency of lower production of IL-1β was also observed in VL than EHC; however the difference was statistically not significant.

3.1.4.3. LPS stimulation induces lower secretion of proinflammatory cytokines in VL whole blood

Incubation with LPS for 22 hours resulted in significantly lower secretion of IL-1β, IL-12p70 and IL-6 in VL than in EHC group (Figure 19). There was a tendency of lower secretions of TNF-α and IL-17 in VL than EHC; however the difference was no statistically significant (Figure 19).

3.1.4.4. MALP-2 stimulation induces significantly lower level of IL-12p70 in VL than EHC

In response to MALP-2, VL blood cells produced significantly lower level of IL-12p70 than EHC (Figure 19). Although after stimulation with MALP-2 a tendency of lower secretion of IL-1β and IL-17 and higher secretion of TNF-α was observed in VL than EHC, the difference was not statistically significant.

3.1.4.5. IL-10 secretion was only significantly higher in VL than EHC in response to *L. donovani*

After stimulation with *L. donovani,* the level of the anti-inflammatory cytokine, IL-10, was significantly higher in the supernatants of VL blood cultures than in that of EHC blood cultures. However, stimulation with LPS and MALP-2 did not show significant difference in levels of IL-10 secretion between blood cultures of VL and EHC (Figure 19).

3.1.4.6. Baseline chemokine concentration secreted by whole blood without stimulation was lower in VL than in EHC

As it has been shown above, there were significantly lower baseline levels of proinflammatory cytokine production in whole blood cultures of VL patients than of EHC individuals. Next, I aimed to measure the release of chemokines such as MIP-1β, MCP-1, IL-8 and IP-10 in VL whole blood culture and compared to EHC individuals. The baseline level of chemokines release in VL and EHC whole blood culture supernatants was assessed after incubation for 22 hours without addition of stimulants. The result obtained from this experiment revealed that the concentrations of MIP-1β, IL-8 and IP-10 in VL supernatants were significantly lower than in EHC

(Figure 20). Similarly there was a tendency of lower MCP-1 production by VL than EHC, though the difference was not statistically significant.

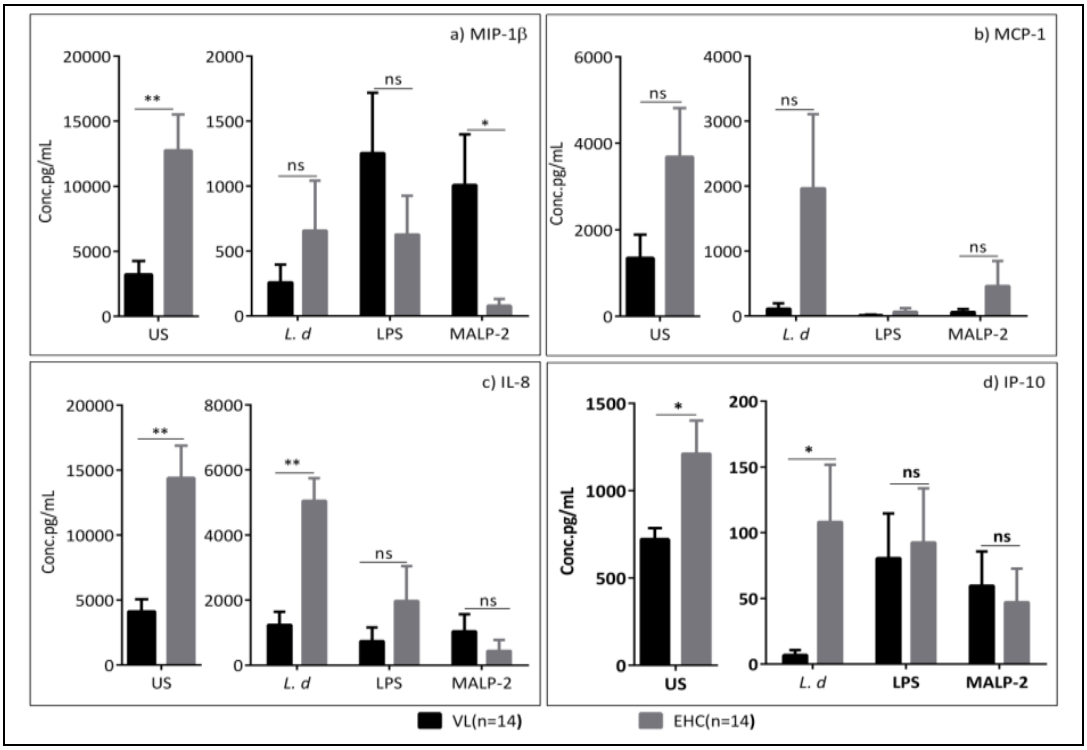

Figure 20. Chemokine secretion by whole blood of VL compared to EHC.
The release of chemokines by VL and EHC whole blood leukocytes without addition of stimuli (unstimulated, US) and after exposure to different stimuli were compared. Whole blood diluted 1:5 with serum/plasma free medium was co-incubated with *L. donovani* promastigotes lysate $1x10^7$/mL, LPS 10 ng/mL, and MALP-2 10 ng/mL or without any stimuli (US) at 37°C for 22 hours. Supernatants were collected and the chemokines were measured using ELISA. The concentrations of the chemokines in unstimulated cultures (baseline values) were subtracted from stimulated cultures to define stimulation specific chemokine production. Statistical significance was calculated by using multiple t-tests and differences were considered statistically significant when $p \leq 0.05$. * $p<0.05$, ** $p<0.005$, 'ns' indicates 'not significant'.

3.1.4.7. Stimulation with *L. donovani* induces lower secretion of IL-8 and IP-10 in VL than in EHC

In response to *L. donovani* VL whole blood leukocytes secreted significantly lower levels of IL-8 and IP-10 than the EHC group (Figure 20). In similar manner, upon stimulation with *L. donovani*, VL whole blood cells had a tendency to produce lower levels of MIP-β and MCP-1 than EHC, but the difference did not reach statistical significance (Figure 20).

3.1.4.8. In response to LPS, there was no significant difference in chemokines secretion between VL and EHC blood cells

Upon stimulation with LPS a tendency of higher level of MIP-1β release by VL blood leukocytes was observed. Similarly a tendency of lower level of IL-8 release was observed in VL blood cultures as compared to with those of EHC, but the difference was not statistically significant. In response to LPS, the secretion levels of MCP-1, and IP-10 in VL blood culture were similar to EHC blood (Figure 20).

3.1.4.9. MALP-2 stimulation induced higher level of MIP-1β in VL than in EHC

In response to MALP-2 VL blood leukocytes secreted significantly higher level of MIP-1β in whole blood culture than EHC blood leukocytes; in contrast, statistically significant differences were not observed in the secretion of MCP-1, IL-8 and IP-10 (Figure 20).

3.2. Part -II. Bovine innate immune cell infection by *L. donovani*

In Ethiopia, the transmission of VL is considered to be an anthroponotic disease, whereby the parasite can be transmitted from humans to animals. Recent evidence for this in Ethiopia was obtained from the detection of *L. donovani* DNA in domestic animals and from the identification of specific antibodies to *L. donovani* in cattle in VL endemic areas[51]. The common types of cattle breeds in Ethiopia are the Zebu (*Bos indicus*) and the Zebu cross with Holstein Friesian cattle. Zebu is the local Ethiopian cattle bred in large number while crossed cattle are fewer in number. Naturally *Leishmania* is transmitted through the bite of a sand fly into the dermis of mammalian host where neutrophils and macrophages become infected [81]. On one hand the existence of antibody to *L. donovani* indicates that cattle were exposed to *L. donovani*. On the other hand, it remains an unanswered question whether or not such cattle or other animals can serve as a reservoir host for *L. donovani* and can cause infection. I hypothesized that *L. donovani* can infect cattle. With this view, I collected blood from Zebu and their cross with Holstein Friesian cattle, and investigated *in vitro* whether phagocytes of these cattle breeds are permissive for an infection with *L. donovani.*

3.2.1. Bovine PMN can be infected by *L. donovani*

Since neutrophils are the first phagocytes that are infected after *Leishmania* infection [81], in the first series of experiments *L. donovani* promastigotes were added to blood of cattle and assessed for neutrophil internalization. The results showed that *L. donovani* can readily infect bovine PMN upon co-incubation with whole blood for 3, 6 and 22 hours at 37^{o}C. After 22 hours of incubation amastigote like forms were observed in bovine PMN; fewer amastigotes like forms were observed at earlier time points (data not shown). Not all PMN were equally infected, some PMN harbored more than five parasites per cell, and others had two to four parasites per cell, while no

parasites were seen in some PMN. The various morphologies of the infected PMN is shown in Figure 21.

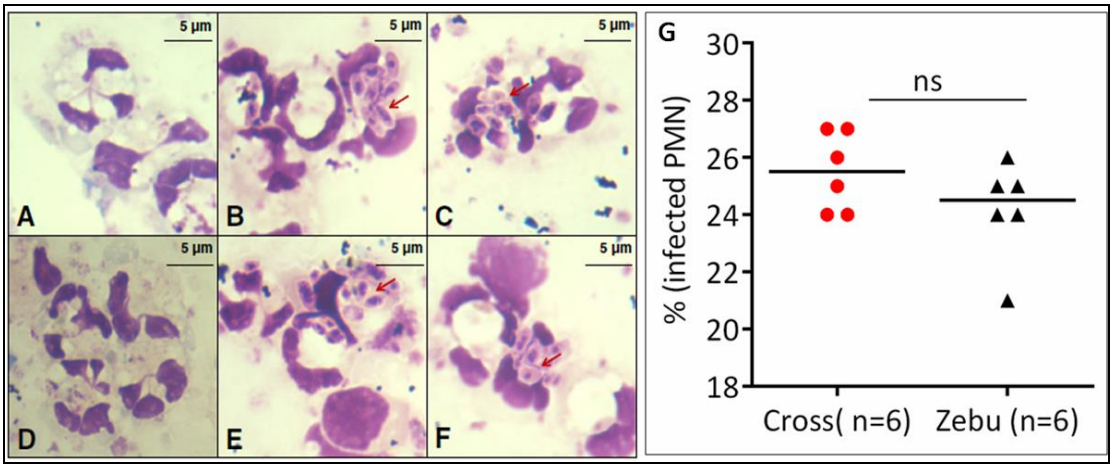

Figure 21. Infection of bovine neutrophils by *L. donovani* parasites.
Leishmania donovani stationary phase promastigotes ($1x10^6$) were added to 100 µL of whole blood of Zebu or Zebu-Holstein crossed cattle and co-incubated for 22 hours. Erythrocytes were lysed, and remaining cells were washed twice in PBS. Cytospin smears from cell suspensions were stained with Giemsa staining solution, and examined for PMN infection by microscopic observation and enumeration of intracellular amastigote like forms. PMN from Zebu-Holstein crossed animals are shown in panels (A-C) and from Zebu animals in panels (D-F). Panel A, D: uninfected PMN control; panels B, C, D, F: PMN infected with *L. donovani* parasites, designated by the red arrows, at 22 hours of culture. The original photomicrographs were taken at 1000× magnification. G) Ratio of *L. donovani* infected bovine PMN from Zebu and Zebu-Holstein crossbreeds. No statistically significant difference was observed ($p > 0.05$). Bars show the median ratio of infected neutrophils, ns: not significant.

Further microscopic examination of cytospin slides revealed no significant difference in *L. donovani* infection rates between PMN of the two cattle breeds (Figure 21 G). The infection rate in monocytes was less than 1 % (data not shown).

3.2.2. Bovine MDM were readily infected with *L. donovani*

Above, I have demonstrated that bovine PMN can readily be infected by *L. donovani* in the presence of all cellular elements of peripheral blood and plasma factors (Figure 21). Since macrophages are the final host cell for *Leishmania* parasites [80,267] and fewer than 1% of monocytes were observed to be infected with *L. donovani* under this conditions (data not shown), next I assessed bovine monocyte derived macrophage (MDM) infection by *L. donovani.* Monocytes were isolated from blood of Zebu and Zebu-Holstein cross and differentiated into MDM *in vitro*. These macrophages were counted and co-incubated with *L. donovani* promastigotes at 1:10 multiplicity of infection (one macrophage: ten promastigotes). After co-culture at 37^{o}C, cultures were washed carefully with warm PBS, air dried, fixed with methanol, stained with Giemsa solution and examined under the microscope. Results from this experiment show that after 3 hours of co-incubation, most *L. donovani* promastigotes were observed to be attached to MDM via their flagella, whereas only a few were in the posterior polar position (data not shown). The number of externally attached promastigotes to MDM decreased after 24 hours of co-incubation (data not shown). By microscopic evaluation, macrophages were observed which had engulfed promastigotes at 24 hours of incubation. Every day the culture was examined, and at day 5 post-infection, the infecting promastigotes had transformed morphologically to amastigotes forms (Figure 22).

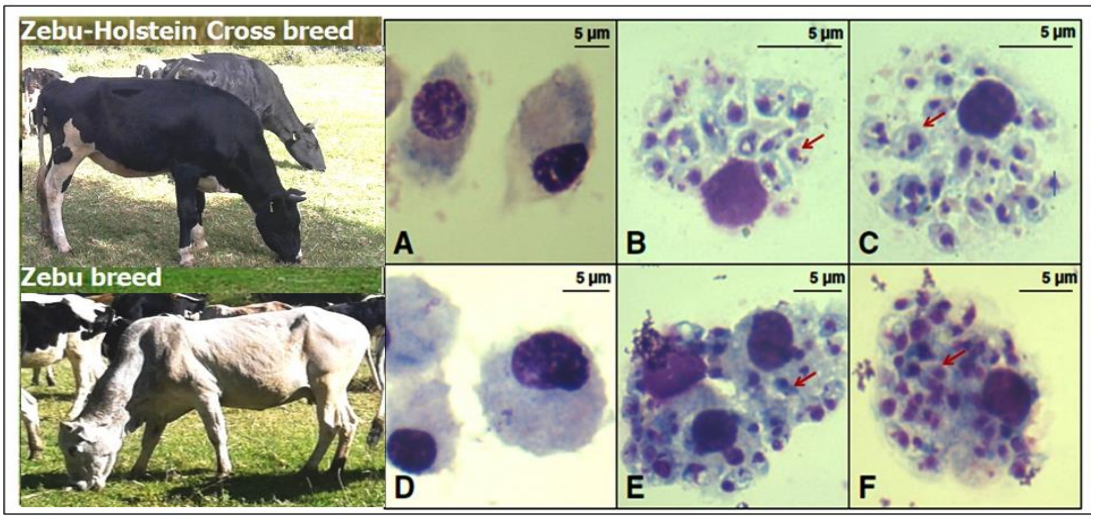

Figure 22. Bovine MDM infected with L. donovani promastigotes.
Bovine MDM was seeded in chambered slides containing completed RPMI 1640 culture medium. The culture was incubated at 37°C for 30 minutes, 5% CO_2 to allow cell adherence. Stationary promastigote stage of *L. donovani* was added to the culture at multiple of infection (MOI) of ten promastigotes to one MDM. The chambered slide was incubated for additional 90 minutes at 37°C , 5% CO_2, and washed carefully with warm sterile wash medium until extracellular promastigotes were removed. Then complete medium was added for further incubation. After day five, Chambered slide was washed carefully with warm PBS for two to three times and air dried. The slide was fixed with absolute methanol and stained with 10% Giemsa staining solution for 10-15 minutes and examined under light microscope. Microscopic examination revealed bovine MDM was infected with *L. donovani* stationary promastigotes at day 5 of co-culture at 37°C. MDM from either Zebu-Holstein cross breeds (A, B, C) or Zebu cattle (D, E, F) were either uninfected (panels A, D) or infected with *L. donovani* (panels B, C, E, and F). The original photomicrographs were taken at 1000× magnification.

Examination under light microscope confirmed that *L. donovani* can infect bovine MDM. Next, using eGFP expressing *L. donovani* promastigotes I investigated the infection rate by flow cytometry. Monocyte derived macrophages were infected with eGFP expressing *L. donovani* promastigotes as described above. Flow cytometry analysis of the cells revealed that MDM from both cattle breeds were infected with the parasites after co-incubation for 24 and 48 hours (Figure 23). MDM from the cross-breeds had a higher infection rate (73.9 % at 24 hours, and 77.8 % at 48 hours) than MDM from pure Zebu breed (47.16 % at 24 hours and 49.55 % at 48 hours) (Figure 23).

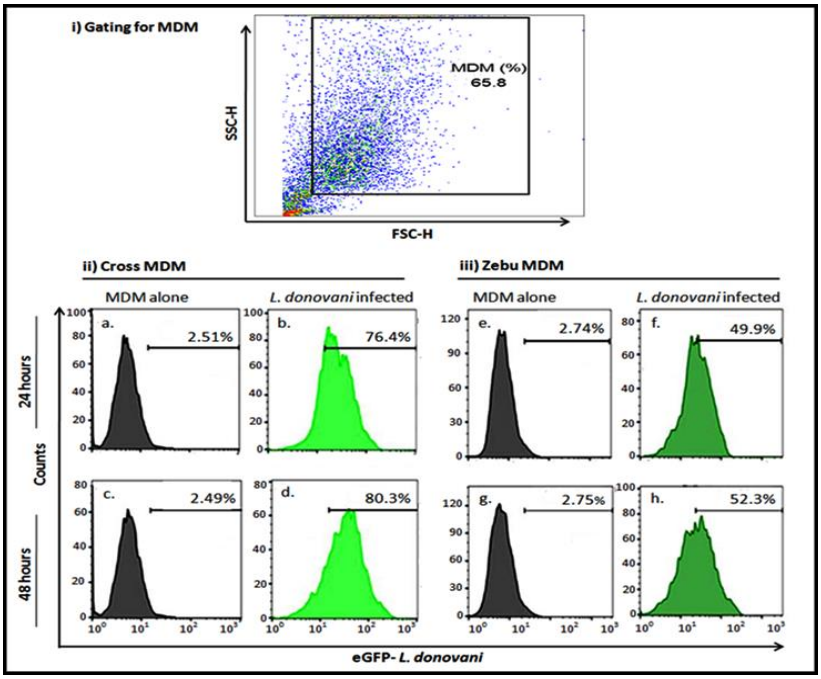

Figure 23. MDM infection with eGFP expressing *L. donovani*.
This is a representative data for a single experiment done per each cow, Cross (n=6) and Zebu (n=6). Peripheral blood mononuclear cell (PBMC) from blood of Zebu and Cross animal were isolated and incubated for plastic adherence to isolate monocytes. After the non-adherent cells were discarded, cells were washed with warm PBS containing 5% complete medium. Afterwards cells were re-suspended in complete medium and incubated at 37°C for 6-8 days for differentiation to macrophages, while *L. donovani* promastigotes expressing eGFP were cultured for 5-7 days at 26-27°C using complete mediums supplemented with hygromycin-B 30 µg/mL. Macrophages were co-incubated with eGFP *L. donovani* stationary promastigotes at the ratio of 1:10 at 37°C for 24 and 48 hours time points. At each time point, culture was washed carefully with sterile PBS and infection was measured using flow cytometry. i) A representative forward scatter (FSC) and sideward scatter (SSC) plot shows gating for cultured monocytes derived macrophages (MDM). ii) A representative infection assay result for eGFP *L. donovani* in Cross MDM at two time points of infection, iii) A representative infection assay result for eGFP *L. donovani* in Zebu MDM at two time points of infection. GFP fluorescence was evaluated among gated MDM from either cross-breed MDM (a-d) or Zebu cattle (e-h). Grey colored plots represent uninfected MDM, whereas light and dark green colored plots depict MDM infected with eGFP-expressing *L. donovani*. Cells were evaluated after 24 hours of infection (upper panels) or 48 hours of infection (lower panels).

3.2.3. Bovine MDM can be infected by *L. donovani* through infected blood leukocytes

During the natural course of infection, the female sand fly creates a blood pool in the dermis where it deploys its salvia content along with *Leishmania* promastigotes during

its blood meal. These inoculated promastigotes are immediately taken up primarily by blood PMN [81]. Macrophages, the final host cells of the parasites, become infected after ingesting infected neutrophils [80,267]. In order to mimic this natural infection, next I investigated whether bovine MDM can be infected up on co-incubation with infected bovine neutrophils. As described above (3.2.1) neutrophils were nearly exclusively infected after co-incubation of whole blood with the parasites. Thus, whole blood was first co-incubated with *L. donovani* promastigotes for 22 hours at 37^{o}C, and red blood cells were then lysed. Leukocytes were washed to remove non engulfed parasites and the washed leukocytes were re-suspended in complete RPMI 1640 medium and co- incubated with MDM in chamber slides. The culture was kept in humidified incubator at 37^{o}C, 5% CO_2 for up to 16 days. After the 16th day in culture, cells in the chamber were washed, stained with Giemsa staining solution and examined under microscope. The results obtained indicate that bovine MDMs can be infected upon co-incubation with infected blood leukocytes (Figure 24).

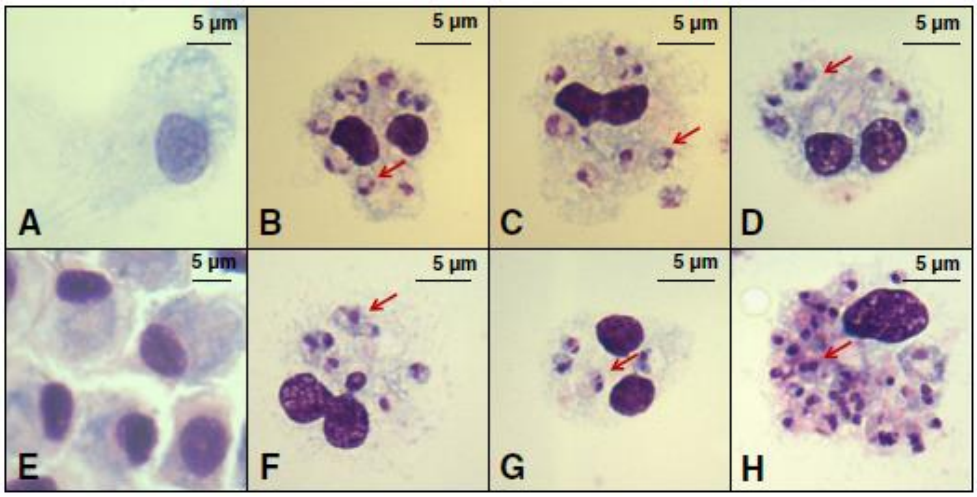

Figure 24. Co-incubation of infected cattle blood leukocytes with MDM led to *L. donovani* infection in the MDM.
Bovine whole blood was infected with *L. donovani* stationary promastigotes *in vitro* at 37 ^{o}C for 22 hours. Erythrocytes were lysed and the leukocytes were added to chamber slides containing bovine MDM. Monocyte derived macrophages were then cultured in a humidified incubator at 37^{o}C with 5% CO_2 for 16 days. The medium was then carefully removed and the slide was stained with 10% Giemsa staining solution, and examined under the microscope for infection. The result shows that MDM from Cross and Zebu breeds were infected through this route. MDM from cross-breeds (panels A-D) or Zebu cattle (E-H) and mixed with uninfected blood leukocytes (A, E) or blood leukocytes infected with *L. donovani* (B, C, D, F, G, H). The red arrows depict amastigotes within infected MDM.

3.2.4. Amastigote to promastigote transformation

During the natural infection cycle, amastigotes from mammalian host samples are transformed into promastigotes in the gut of the sand fly. This transformation occurs also *in vitro* if infected cells are cultured in biphasic NNN medium. In the above experiments (Figure 21-23), I showed that bovine PMN and MDM can be infected with *L. donovani*. Thus after this observation I aimed to investigate if the observed amastigotes could be transformed into promastigotes *in vitro* after reducing the temperature from 37 °C to 26°C. I observed that amastigotes in MDM transformed into motile promastigotes after three days in biphasic NNN medium. After three days parasites in the intact macrophages were seen to actively vibrate. After five days, promastigotes started to liberate from the MDM and further multiplied in culture. Promastigotes were sub-cultured into liquid media, harvested at log phase of the culture, and cryopreserved in liquid nitrogen. After one month, cryopreserved *Leishmania* stablates were thawed and sub-cultured in liquid medium.

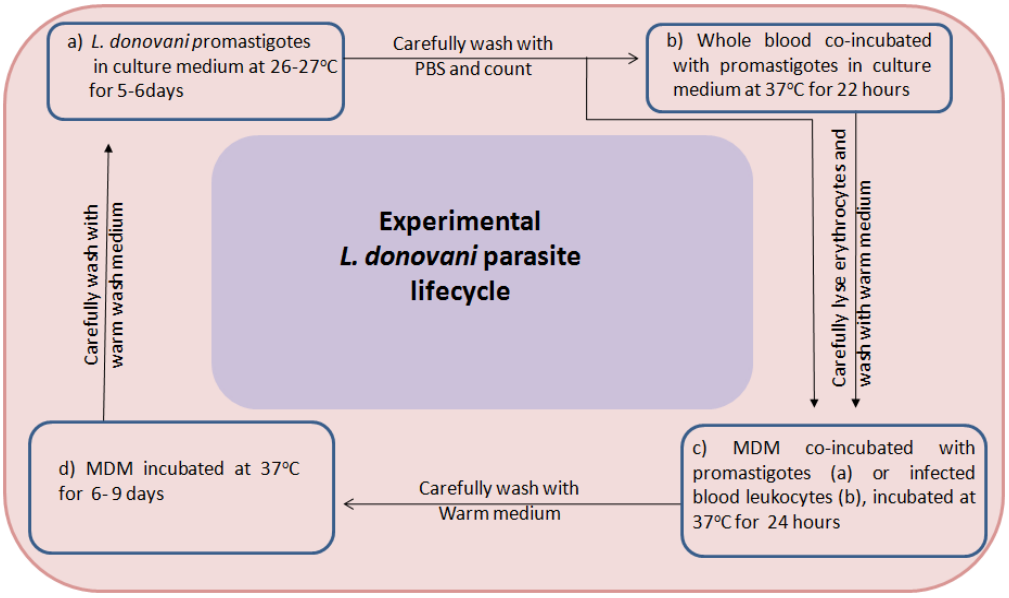

Figure 25. Schematic experimental in vitro lifecycle for *L. donovani* parasites using bovine blood cells.
Leishmania donovani promastigotes (a) in culture media co-incubated with bovine whole blood for 22 hours (b) or directly co-incubated with MDM (c) at 37°C. Infected bovine MDM (c) was washed carefully with warm medium (5% complete RPMI 1640 in PBS) and kept in culture complete medium for 6-9

days at 37°C (d) for amastigote development. In order to transform amastigotes to promastigotes the temperature of the culture was reduced to 26-27 °C with fresh medium replacement (a).

3.2.5. PCR-RFLP confirmed that *L. donovani* parasites infect bovine cells

It has been demonstrated above that *L .donovani* can infect bovine PMN and MDM. In order to proof that the infecting parasite was *L. donovani*, the parasites were characterized using polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP). This way I could exclude the possibility that the bovine cells contain or get infected with other morphologically similar organism like *Leptomonas* species [268]. Amplification of parasite DNA with ITS-1 primers resulted in a PCR product of 328 bp size (Figure 26a). The digestion of the PCR product with HhaI (Figure 26b) revealed for both amastigote and promastigote samples from MDM cultures; a *L. donovani* reference sample resulted in an identical band.

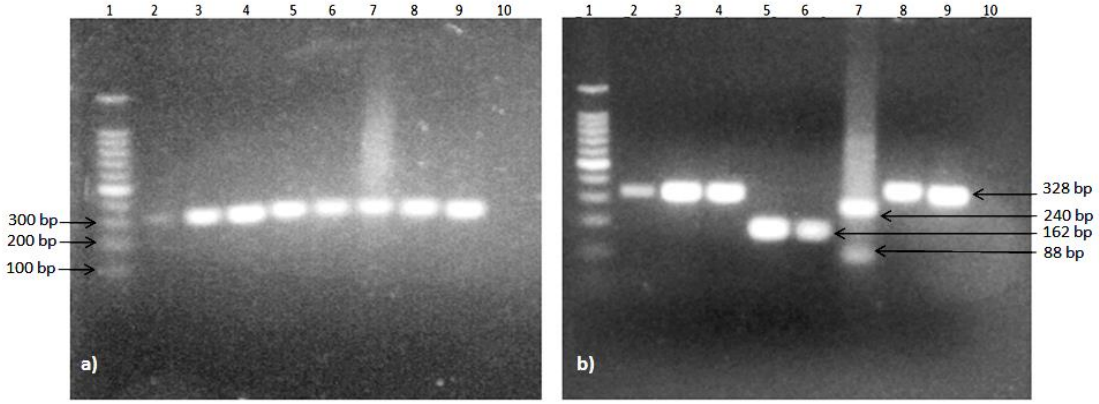

Figure 26. PCR products after ITS-1 primer amplification and HhaI digestion of promastigote and amastigote DNA.
Panel a) depicts primer amplification products as follows. Lane 1:100 bp Ladder; Lane 2: *L. donovani* reference strain; Lane 3: *L. donovani* promastigotes cultured from MDM; Lane 4: *L. donovani* amastigote from MDM; Lane 5: *L. aethiopica* reference strain; Lane 6: *L. aethiopica* culture promastigotes; Lane 7: *L. major*; Lane 8:L. tropica; Lane 9: *L. infantum*; Lane10: NC (negative control; TE buffer). Panel b) illustrates HhaI-digested ITS-1 amplicons as follows: Lane 1: 100 bp ladder; Lane 2: *L. donovani* reference strain; Lane 3: *L. donovani* cultured promastigotes; Lane 4: *L. donovani* cultured amastigotes; Lane 5: *L. aethiopica* reference strain; Lane 6: *L. aethiopica* clinical isolate culture promastigotes; Lane 7: *L. major*; Lane 8: *L. tropica*; Lane 9: *L. infantum*; Lane 10: NC (negative control; TAE buffer).

3.3. Part III- Biomarkers for VL clinical cure

Previous studies from other countries showed that serum profile of MMP-9 and sCD40L had an inverse correlation with spleen size and parasite load in VL, while IL-10 had positively correlated with the parasite load [251,252]. Currently there is an urgent need for reliable biomarkers for cure after treatment in human VL [2]. With this notion, serum levels of MMP-9, sCD40L and IL-10 were evaluated in VL patients before the start of treatment and after treatment with sodium stibogluconate (SSG) 20 mg/kg/day for 30 days. Forty nine male HIV negative VL patients and 30 male, HIV negative and apparently healthy endemic controls (EHC) were recruited in the study. Blood was collected from the VL patients before starting treatment and after the course of treatment was completed with initial cure from active VL. Another approach could have been to collect blood samples after definitive cure. However, according to VL treatment guideline by Federal Ministry of Health (FMoH) of Ethiopia, the response rate in HIV negative VL for SSG in Ethiopia is good and relapses can only occur below 5% of the treated patients in immune-competent individuals [11]. Therefore, in the present study patients after initial cure were investigated.

3.3.1. Serum level of sCD40L and MMP-9 were significantly lower in VL before treatment than after treatment

Serum collected from VL patients before treatment demonstrated significantly lower serum level of sCD40L than serum collected from EHC individuals (Figure 27). After treatment with SSG for 30 days the serum levels of the patients normalized and were similar to EHC individuals (Figure 27a). In VL patients before treatment, the median serum level of MMP-9 was significantly lower compared to serum from EHC individuals. A somewhat enhanced serum MMP-9 level was observed after treatment. However, unlike sCD40L, the serum level of MMP-9 in the VL patients after treatment

did not normalize and remained significantly lower than in EHC individuals (Figure 27b).

3.3.2. Secretion of IL-10 was signifcantly upregulated during clinical VL and returned to normal level after treatment

In VL patients, serum collected before treatment showed significantly higher level of IL-10 than from EHC individuals. After treatment the serum IL-10 level returned to normal level and was similar to the IL-10 level found in EHC individuals (Figure 27c).

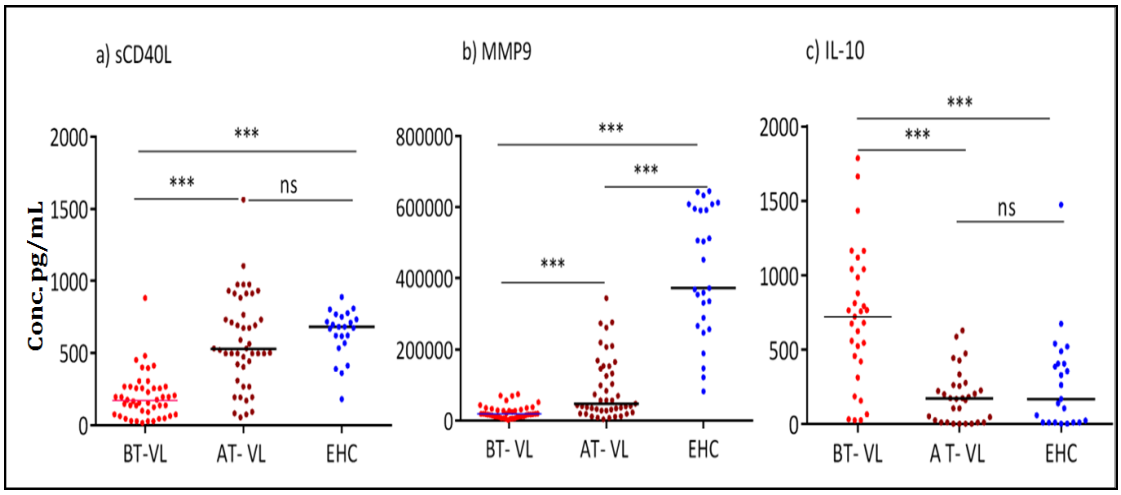

Figure 27. sCD40L, MMP 9 and IL-10 serum levels in VL before and after treatment with SSG.

Venous blood samples, before and after treatment from VL cases and from EHC was collected for serum separation. Then serum level of sCD40L [VL (n=46), EHC (n=23)], MMP-9 [VL (n=45), EHC(n=28)] and IL-10[VL(n=31), EHC(n=23)] was assessed by ELISA. Data was analyzed using GraphPad Prism and STATA version 11, and considered statistically significant when the paired t-test *p*-value <0.05. The median serum level of sCD40L, MMP 9 and IL-10 after clinical cure was compared to serum level before treatment and to EHC. BT-VL: VL cases before treatment, AT-VL: VL cases after treatment, EHC: Endemic healthy controls, ***p<0.0005, and 'ns' stands for ' not significant'.

To better visualize the impact of therapy on serum levels of these cytokines we depicted the levels within individual patients before and after treatment. sCD40L (Figure 28 a) and MMP9 (Figure 28 b) showed an increasing trend while IL-10 showed a decreasing trend (Figure 28 c) with therapy. In sum, the results show that the serum level of both sCD40L and IL-10 is normalized after treatment with SSG. However; the serum level of MMP-9, although clearly increasing after therapy, did not reach the levels of healthy individuals. Therefore sCD40L and IL-10 but not MMP-9 appears to be suitable markers for test of cure in VL.

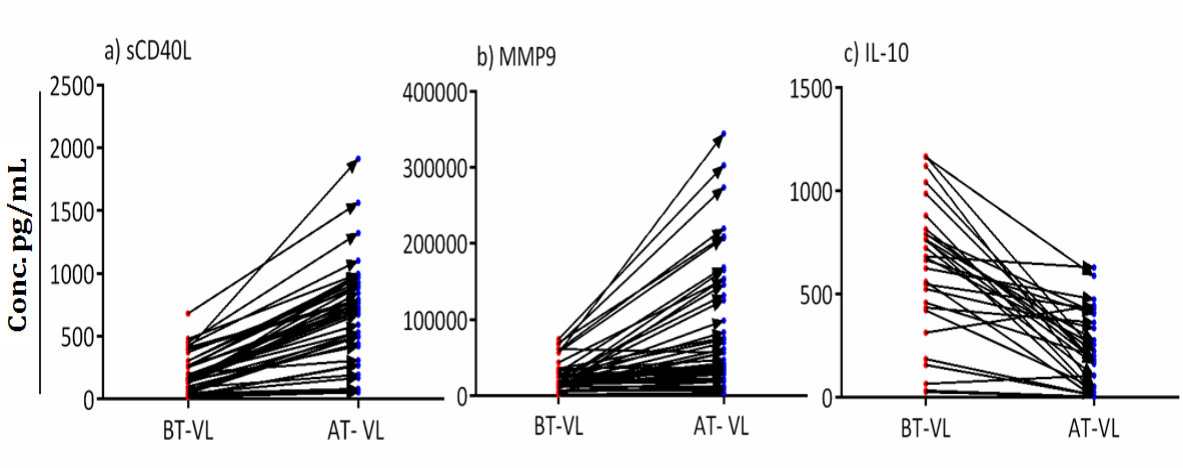

Figure 28.VL patients serum concentration of sCD40L, MMP-9 and IL-10 before and after treatment.

The trend showing serum level change of sCD40L (a), MMP-9(b) and IL-10 (c) after treatment of VL cases with generic sodium stibogluconate. The sample size used for each was sCD40L (n=46), MMP-9(n=45) and IL-10 (n=31). BT-V: VL cases before treatment, and AT-VL: VL cases after treatment.

4. DISCUSSION

Visceral leishmaniaisis or Kala-azar is caused by parasites of the *L. donovani* complex which are naturally transmitted through the bite of an infected sandfly. The major clinical symptoms of this life-threatening disease include anemia, fever, weight loss, splenomegaly, and hepatomegaly. In Ethiopia, VL is an endemic disease and an increasing public health concern since the 2005 outbreak of VL in Libo Kemkem (a highland area at Northwest of Ethiopia) claimed the lives of hundreds of Ethiopians. The innate immune system is the first line of defense defending the host from invading organisms in a non-specific manner. Innate immune mechanisms provide immediate defense against infection and controls the pathogen load during the early phase of infection. In contrast to the intensive research on adaptive immune responses, however, surprisingly sparse knowledge exists about the innate immune responses to *Leishmania* parasites. Innate immune responses are not only essential for effective early defense, but also shape the development of adaptive immune responses. Understanding the innate immune functions in leishmaniasis is essential to combat the disease and to develop immune intervention strategies.

4.1. Part -I. Innate immune cell functions

The control of *Leishmania* parasites within the host cell is believed to be mediated by innate and adaptive immune responses [2,269]. The innate immune response plays an initial and essential role during host resistance against intracellular parasitic infections. This response would act both in controlling pathogen growth during the early stages of infection as well as in driving the secretion of cytokine

microenvironment in which parasite-specific T-cells are primed as reviewed by Peruhype-Magalhaes et al [270]. Intracellular organisms like *Leishmania* parasites live inside host cells and their survival is dependent on coexistence with the host. The majority of people who become infected with *Leishmania* do not develop clinical disease. The factors that drive resistance and susceptibility are not clearly defined [269]. Previously most studies focused on the role of acquired immune functions. However, during natural course of infection *Leishmania* parasites first interact with innate immune cells such as neutrophils and monocytes. Therefore, the role of innate immune cell functions in VL cases was investigated in this work. The study area was Libo Kemkem district in South Gondar administration zone of Amhara regional state of Ethiopia, a highland area, which was not previously known as VL endemic area. Addis Zemen Health center, located in Addis Zemen town of Libo Kemkem district, is a specialized health center for the treatment and management of VL patients where suspected VL cases are referred to from different health institutions including hospitals. A recent study conducted at this health center to analyze trends from the year 2005–2011, showed that among 761 non-emigrant VL suspected children below five years of age, 49.7 % were sero-positive for VL [271]. This finding, together with the earlier outbreak report of VL in this area[15], suggests an established VL infection in this highland area.

The leading (96.5 %) sign and symptoms among VL patients was splenomegaly, which was similar to the findings of a previous study in the same area [263] and in Sudan [272]. My current observation of a high ratio (62%) of severe malnutrition in the VL patients was similar to the previous report [273]. This finding corroborates with the previous knowledge that VL infection depresses bone marrow functions and reduces the number of leukocytes, RBC, platelets and particularly reduces the number of PMN [2]. During clinical VL the lifespan of leukocytes and erythrocytes is reduced, causing granulocytopenia and anemia [2]. In our study, anemia was the most common health problem in VL patients. This finding was similar to a study conducted in the same region recruiting male VL patients [274]. It was also shown that anemia, leukopenia and lymphocytosis had significant relationship with *Leishmania* parasite load [275].

In my study, thrombocytopenia was prevalent in VL, which is similar with the results of other studies [2,263,274].

Neutrophils surface activation markers

Naturally, leishmaniasis is transmitted through female sand fly bites, which creates a blood pool in the host skin during blood meal. Therefore, the initial *Leishmania* interactions with the host immune cells take place in whole blood [94]. In this study, mimicking the *in vivo* events, the response of whole blood neutrophils and monocytes to *L. donovani* promastigotes and to the TLR agonist LPS, and MALP-2 was determined in whole-blood assays using flow cytometry.

Since CD62L is rapidly shed from neutrophils upon activation CD62L shedding was assessed as measure of neutrophil activation [94,231,276]. The expression of CD66b on neutrophils was measured as additional activation and degranulation marker since the cell surface expression of CD66b is enhanced on neutrophils after activation-dependent degranulation [277–280]. Assessment of both markers showed a clearly reduced response of VL neutrophils upon exposure to *L. donovani*. Although measuring CD62L shedding indicated a reduced response of neutrophils to LPS, a compromised response was not evident to LPS or MALP-2 when the CD66b expression was investigated. These findings indicate a strong dysfunction of VL neutrophils regarding their response to *L. donovani* but no or only a limited dysfunction to the TLR agonists LPS and MALP-2. Apparently, neutrophils in VL patients display a strongly compromised capacity to respond to *L. donovani*.

In agreement to my data presented here, a recent report from India has shown a decreased shedding of CD62L from neutrophils and monocytes upon exposure to *Leishmania* parasites in malnourished VL patients [281].

Co-incubation of neutrophils of healthy individuals with *L. major* promastigotes led also to activation of neutrophils as demonstrated by the loss of CD62L expression and increased expression of CD66b [231]. Therefore, activation of neutrophils by *Leishmania* appears to be the proper response of neutrophils to parasites causing both VL and CL.

In the present study VL patients with active disease before the initiation of any treatment were included. From the results, I cannot conclude whether the *Leishmania*-specific dysfunction was a cause of disease development or the dysfunction is the result of the disease. Studying neutrophil response to *Leishmania* after clinical cure could clarify this issue. If the neutrophil response recovers after cure, it would clearly argue against an intrinsic neutrophil dysfunction to *L. donovani*.

Phagocytic capacity of neutrophils and monocytes in VL

Innate immune effector cells are naturally programmed to eradicate engulfed foreign bodies and facilitate the development of adaptive immunity. Neutrophils and monocytes are key cells of the innate immune system which migrate rapidly to sites of infection and become activated to initiate a cascade of defense mechanisms. This activation will enable neutrophils to recognize and engulf microorganisms by a process known as phagocytosis [282,283]. Phagocytosis, a receptor based function of innate immune cells, is the process of ingestion and destruction of microbes by activated phagocytic cells to clear invading microorganisms. In the present study *S. aureus* particles were incubated with VL and EHC whole blood, in the presence of all cellular and soluble constituents of the blood. A generally reduced response of neutrophils and monocytes from VL patient to all applied activating stimuli, namely *L. donovani* promastigote, the TLR-4 agonist LPS, the TLR-2/6 agonist MALP-2 and the TLR-3 agonist poly I: C was observed. Since the ROS production by VL neutrophils was also found to be compromised, the presented data regarding the compromised response to stimulating stimuli and decreased phagocytic capacity strongly suggest a

decreased ability of VL patient's leukocytes to kill the parasites. Again, from these results I cannot conclude whether the observed dysfunction was a cause of disease development or the dysfunction is the result of the disease. Repeated measurement of phagocytic capacity after clinical cure could clarify this issue. Another possible reason for the low phagocytic activity is that the blood of VL patients contains factors that inhibit phagocytosis or lacks factors that are necessary, possibly as opsonins, for efficient phagocytosis. Experiments with "washed" blood could prove or rule out this possibility.

Taken together my experimental findings indicate a severely impaired response of neutrophils from VL patients to *L. donovani* and to TLR ligands. Moreover, both neutrophils and monocytes from VL patients display a compromise phagocytic capacity to *L. donovani* and to TLR ligands. These observations suggest an innate immune dysfunction during VL infection. Whether this dysfunction is a cause of disease development or the dysfunction is the result of the disease remains to be clarified.

Generation of reactive oxygen species (ROS) by neutrophils and monocytes

The phagocytic cells, neutrophils and monocytes/macrophages, which are activated upon contact to microbes, generate reactive oxygen species (ROS) for oxidative attack on the phagocytosed microbes [132,142,284]. Generation of ROS by phagocytic cells is among the most potent effector functions used to kill the intracellular pathogens. In this study the generation of ROS by VL whole blood neutrophils and monocytes was significantly down regulated upon co-incubation with *L. donovani* promastigotes lysate, LPS, and MALP-2 compared to neutrophils and monocytes from control individuals. Similarly to this findings, in a previous study from India VL patients had severely compromised PMN and monocyte ROS production which could not be

recovered by stimulation with *L. donovani* [281]**.** In line with this findings, a previous study has showed that the marked impairment of ROS generation by monocytes was accompanied by a lower level of TLR4 ^{positive} monocytes [284]. The attenuation of oxidative burst within monocytes was considered as a direct modulation by the parasite [285]. The reduced capacity of the monocytes to produce ROS may be due to low level of NADH oxidase, NADPH oxidase and myeloperoxidase activity in monocytes of patients with active VL [132].

Secretion of cytokines and chemokines

A series of proinflammatory cytokines and chemokines are secreted following microbial infections. These cytokines have dynamic effects in stimulating innate immune cells like monocytes, macrophages, and neutrophils to react against, or bind to micro-organisms, and to summon other immune cells to the site of infection [204,286].

In my present work, whole blood from VL patients with active disease was collected before start of any treatment and EHC (sex and age matched) was incubated without any stimulant or with *L. donovani* lysate, and the TLR agonists LPS, MALP-2 and poly I: C. After 22 hours of blood culture, the level of several cytokines and chemokines was measured in the supernatants. The base line secretion of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 was significantly lower in VL patients compared to EHC. In contrary, in a previous study in Sudan significantly increased levels of pro-inflammatory cytokines were measured in VL patients compared to healthy individuals [287]. The discrepancy may be attributed to method difference in that my experiment used blood culture incubated at 37^{o}C for 22 hours, where as in the Sudan study direct separation of plasma after blood collection was employed.

It is generally accepted that *L. donovani* infection induces antigen specific immune dysfunction as evaluated by *in vitro* studies [288,289]. In support of this view, in my study *L. donovani* lysate did not stimulate VL patients' whole blood cells to produce TNF-α, IL-6, IL-17 and IL-12p70 as compared to controls. In line of these findings, reduced levels of TNF-α^+ and IL-12^+ monocytes were reported during active VL [270]. In the current study, the secretion of IL-12 p70 by whole blood cells of VL patients after stimulation with *L. donovani* lysate, LPS, and MALP-2 was significantly reduced as compared to EHC. My findings support the findings of Peruhype-Magalhaes et al, who demonstrated whole blood cells from active VL cases stimulated with *L. chagasi* lysate had a decreased IL-12 secretion.

In response to *L. donovani* lysate and LPS, the secretion of IL-6 in whole blood supernatants of VL patients was significantly lower than in control supernatants. However in contrary to my results, a recent publication showed high levels of IL-6 in sera of VL patients [270]. This difference might be attributed to parasite species and clinical or host difference in both studies. Interleukin 6 is a pleiotropic cytokine and has both pro and anti- inflammatory functions that affect immune response, tissue repair and metabolism. Interleukin 6 promotes fever, production of acute phase proteins from liver, differentiation of B cells into plasma cells, activation of cytotoxic T-cells, and regulates bone homeostasis [270]. IL-6 recruits monocytes [270], inhibits T-cell apoptosis and supports T-reg development [290].

In response to *L. donovani* promastigote lysate, the secretion of IL-17 in VL supernatants was significantly reduced compared to control supernatants. This observation supports the findings of a study in Eastern Sudan, which indicated that stimulation with *L. donovani* induces significantly less IL-17 in isolated PBMCs of VL subjects than in control subjects. The same study revealed that analysis of cytokine responses by cultured PBMCs from individuals who developed VL or who were protected from VL infection showed that IL-17 was strongly and independently associated with protection against VL [286]. Along with Th1 cytokines, IL-17 and IL-22

play complementary roles in human protection against VL and showed that a defect in Th17 induction may increase the risk of VL [286].

In my present study, when whole blood cells from VL patients were stimulated with *L. donovani* lysate, the secretion of IL-10 was significantly enhanced compared to blood cells of EHCs. This finding is in line with the previous showing an increased number of IL-10 positive monocytes in active VL patients after stimulation with *L. chagasi* lysate [270]. Similarly, a recent report indicated that IL-10 was elevated in sera of VL patients [291]. Interleukin-10 in VL patients was reported to enhance parasite replication in human macrophages [292].

Upon infection with *Leishmania* parasites, mononuclear phagocytes produce various chemokines, which are known to attract neutrophils to the site of infection as reviewed in Oghumu et al [293]. In my present study, when stimulated with *L. donovani* promastigote lysate, whole blood cells from VL patients produced significantly lower IL-8 and IP-10 than EHCs. Interleukin-8 is known as a potent chemo-attractant for neutrophils, which also induces degranulation and morphological changes [294]. When stimulated with *L. donovani* lysate, lower secretion of MIP-1β was observed in whole blood cells of VL patients than EHCs. Similarly, in a previous study, secretion of MIP-1α, a chemokines closely related MIP-1β, in response to *L. donovani* was reduced in VL patients [281]. Therefore, both studies indicate a reduced response of leukocytes from VL patients to *L. donovani in vitro*. However, another study showed elevated MIP-1β level in sera from VL patients [295]. The reason for the discrepancies between the studies requires further clarification.

Blood cells of VL patients stimulated with *L. donovani* had a tendency of lower MCP-1 production than EHCs, though the difference was not significant. In a murine VL model treatment with MCP-1 restricted parasitic burden via induction of superoxide anion and also restored the impaired protein kinase C (PKC) expression and activity [240]. During the healing process of localized cutaneous leishmaniasis (CL) Ritter and colleagues demonstrated that MCP-1 stimulates the killing of *Leishmania* parasites by

macrophages and promotes healing [296]. Similarly, observation from *in vitro* infected human macrophages with *L. infantum* demonstrated that MCP-1 and MIP-1α mediate macrophage activation for nitric oxide production and subsequent parasite clearance [297].

In conclusion, results of ROS and cytokine/chemokine production demonstrate that the alteration of innate immune function in clinical VL may be due to innate immune cell dysfunction. However if this impairment of innate immune cell function is resulted due to the *L. donovani* infection or this impairment is the reason for development of clinical disease is not clear so far. However, further studies aiming to analyse the alteration of innate immune function during VL would be required for the development of immunotherapeutic and/or prognostics methods.

4.2. Part -II. Infection of bovine phagocytes with *L. donovani*

Transmission of VL in Ethiopia is regarded as anthroponotic [2]. However, recent studies have reported the probable involvement of domestic animals such as cows, dogs, donkeys, sheep and goats in the transmission of *L. donovani* [50,51]. These studies showed detected antibodies to *L. donovani* and/or DNA of *L. donovani* in samples from these animals. So far no study confirmed the presence of viable *L. donovani* parasites in cells of these animals or showed if *L. donovani* parasites could infect the neutrophils and macrophages of these animals. In order to address these issues, a whole blood assay was carried out to investigate whether *L. donovani* promastigotes can infect bovine neutrophils and macrophages *ex vivo*. I show for the first time that bovine neutrophils and MDM can be infected when co-incubated with *L. donovani* promastigotes. These findings support the hypothesis that cattle may serve as reservoir host for *L. donovani* parasites.

It has been well documented that neutrophils are the first cells to infiltrate the site of *Leishmania* promastigote infection [298]. Isolated neutrophils from peritoneal cavities of C57BL/6 mice were shown to efficiently internalize both amastigotes and

promastigotes of *L. amazonensis* parasite [298]. In the present work, I showed that *L. donovani* promastigotes could infect bovine MDM from both Zebu and Zebu- Holstein Friesian cross breed cattle, in which cells they transformed into amastigotes *in vitro*.

The amastigotes survived in bovine MDM in culture for up to 16 days. It is an established fact that in their preferred mammalian host cell, the macrophages, *Leishmania* parasites survive and multiply [299]. Experimentally I demonstrated two scenarios of MDM infection by *L. donovani:* 1) direct infection by stationary phase promastigotes and 2) infection by co-culturing of MDM with infected leukocytes obtained after infection of whole blood with *L. donovani* promastigotes. With human cell, a similar scenario has been documented in *L. major* infection where infected neutrophils served as Trojan horses to infect macrophages [267].

Despite the reports indicating seropositivity [300] and isolation of parasitic DNA from domestic animals living in VL endemic areas, as well as increased human risk to leishmaniasis to those living in close proximity to domestic animals [50,51], there is no evidence for the presence of viable parasites in cells of these animals. Cases of CL in cattle were reported in Switzerland [301] and in a horse in Germany [302]. However, in both cases the isolated parasites were not *Leishmania* known to cause human disease but identified as *Leishmania sp. siamensis.* Similarly, a non-typed *Leishmania* like organism was found in the skin of a naturally infected calf in Zimbabwe [303]**.** Equine cutaneous leishmaniasis in two horses was reported from North America and Puerto Rico [304] and a confirmed case of cutaneous leishmaniasis caused by *L. infantum* in a horse was reported from southern Germany [305].

Leishmania parasites are obligate intracellular parasites having dimorphic forms, the motile promastigotes in the female vector, and the non-motile intracellular amastigote replicating within the macrophages of the mammalian hosts [2]. In my experiments, parasites in the amastigote stage in infected bovine MDM transformed into motile

promastigotes when the temperature of the cultures was shifted to 26^{o}C for 3-5 days. This is the first report to demonstrate the transformation of amastigotes to promastigotes and vice versa using bovine cells.

Macrophages are known to synthesize natural resistance associated macrophage protein (Nramp), an integral membrane phosphoglycoproteins which is expressed in the lysosomal compartment of macrophages and is rapidly recruited to the membrane of microbe-containing phagosomes formed in these cells [306]. At that site, Nramp functions as a pH-dependent efflux pump for Fe2+ and Mn2-, restricting the availability of these essential metals for the engulfed microorganisms and contributing to the antimicrobial defenses of macrophages [307]. In mice, the naturally occurring glycine to aspartic acid mutation at position 169 (G169D) or experimentally induced mutations in Nramp cause susceptibility to a number of infections including *Leishmania* [308], suggesting Nramp plays an essential role in host defense against intracellular pathogens. Genetic polymorphisms in the gene encoding this protein have been reported to contribute to the resistance of African Zebu cattle to bovine tuberculosis [309]. Moreover, a study done by Ameni et al.[310] with Zebu, Zebu-Holstein crosses, and Holstein cattle under identical field husbandry conditions in Ethiopia showed both a higher prevalence and severity of bovine TB in the Holstein cattle compared with the Zebu or Zebu-Holstein crosses. However, in the current study with *L. donovani*, no statistically significance differences were observed in susceptibility to infection of MDM among these genetically disparate cattle breeds, although it is possible that the observed minor differences could have reached statistical significance with a larger sample size. Further studies are thus required to ascertain the contribution of genetics in susceptibility of cattle to *L. donovani* infection, as has been observed with *M. bovis* [311].

I was able to show that bovine PMN and MDM can be infected by *L. donovani.* Moreover, I have shown that MDM can be infected through co-incubation with infected bovine blood leukocytes. Although I did not use isolated neutrophils in my assays,

since after co-incubation of bovine whole blood with *L. donovani* promastigotes nearly all infected cells were neutrophils. Previous studies with human leukocytes demonstrated that *Leishmania* uses infected neutrophils as Trojan horses to infect macrophages [80,267].

In conclusion, *L. donovani* promastigotes can infect bovine PMNs and MDM *in vitro*. Inside bovine leukocytes the promastigotes can transform to amastigotes which in turn can transform into promastigotes upon MDM lysis. These findings further strengthen the hypothesis that domestic animals in Ethiopia can serve as reservoir of *L. donovani*. This is an important issue given the high number of Ethiopians residing in VL endemic foci that live in close association with their cattle. A proof of this hypothesis could represent a significant step towards understanding the mechanism of spread of the disease in Ethiopia.

4.3. Part -III. Biomarkers for VL initial cure

Human VL is characterized by clinical signs and symptoms like fever, splenomegaly, anemia, leukopenia, and weight loss and usually associated with a marked impairment of *Leishmania* specific Th1 response[289]. Moreover, sera of VL patients contain high levels of the cytokine IL-10, which can inhibit the activity of antiparasitic proinflammatory cytokines such as IFNγ and TNF-α [312]. The serum level of IL-10 in VL patients correlates with the parasite load, while there is inverse correlations between the serum levels of soluble CD40 ligand (sCD40L) and MMP-9 [254].

In Ethiopia currently there is no test of cure for the evaluation of treatment success. The applicability of sCD40L, MMP-9 and IL-10 serum levels as biomarkers for cure has not been investigated in Ethiopia so far. In my present work I evaluated the use of these molecules for their use as test of cure in VL in Ethiopia. CD40L is a member of the tumor necrosis factor (TNF) family of cell surface expressed mainly on $CD4^{+}$T-cell

subset shortly after T-cell activation and represents an early activation marker of T lymphocytes [313]. Its receptor, CD40, is constitutively expressed mainly on B cells, macrophages, and dendritic cells [314]. CD40L is cleaved from the cell surface of activated T cells by a matrix metalloproteinase and can be detected in the circulation as sCD40L[313]. Soluble CD40L appears to retain the ability to bind and activate CD40 on antigen presenting cells [315,316]. In a study on VL caused by *L. infantum* a gradual increase of serum sCD40L level was observed after treatment and the level of serum sCD40L negatively correlated with spleen size and parasite load [251].

In my present study VL patients' sera contained significantly lower sCD40L levels than sera of healthy controls. After treatment and clinical cure the serum level of sCD40L increased significantly reaching a level similar to that of the endemic health controls. This finding is in agreement with the previous report that successful treatment increases serum level of sCD40L in VL from Bangladesh and Brazil[255]. The growth of *L. infantum* in macrophages of asymptomatically infected patients was shown to be limited by sCD40L probably by enhancing the microbicidal mechanisms of infected macrophages (277). This suggests a protective role of sCD40L in VL. Our data show that a low serum level of sCD40L is also a characteristic of Ethiopian clinical VL caused by *L. donovani.* Since the serum level of sCD40L is elevated in most VL patients after clinical cure serum sCD40L is a candidate marker for successful treatment of VL in Ethiopia.

Macrophages and other cell types, including lymphocytes, endothelial cells are associated with tissue remodeling and modulation of inflammation by producing MMP-9[317]. MMP-9 regulates pathological remodeling processes that involve inflammation and directly degrades extracellular matrix (ECM) proteins and activates cytokines /chemokines that regulate tissue remodeling [318]. In a previous study the serum level of MMP-9 in VL was observed to negatively correlate with the parasite load [254]. In my present study serum MMP-9 level of patients with active VL patients was significantly lower as compared to healthy controls. After treatment and clinical cure serum MMP-9 level significantly increased, however, the level after treatment did not reach the level observed in endemic healthy controls. Since in most VL patients the

serum level of MMP-9 did not normalize after clinical cure, MMP-9 serum level does not appear to be a suitable marker of successful treatment in Ethiopian VL.

IL-10 is produced by macrophages, dendritic cells (DC), B cells, and various subsets of $CD4^+$ T and $CD8^+$T cells. IL-10 inhibits MHC class II and co-stimulatory molecule expression on monocytes and macrophages and limits the production of proinflammatory cytokines and chemokines [319]. From different geographic settings and causative *Leishmania* species, mixed Th1/Th2 responses were reported in VL patients (333,334). In previous studies, the serum level of IL-10 in VL patients was correlated to the parasite load [254,255], being at high level in patients with active clinical VL which declined upon successful treatment [320,321]. In the present study I showed a high serum level of IL-10 in Ethiopian clinical VL caused by *L. donovani*. The present study has also demonstrated a significant reduction in the serum level of IL-10 at clinical cure when compared to the level measured before treatment. After treatment, the level of serum IL-10 was comparable to that of the healthy endemic controls. It has been reported previously that IL-10 was among the mediators that reduce CD40L expression on $CD4^+$ T cells [313]. The observed decline of IL-10, therefore, may be in functional association with the increase of sCD40L serum level after clinical cure. Since the serum level of IL-10 normalized after clinical cure, IL-10 serum level appears to be a promising candidate marker for successful treatment of VL in Ethiopia. The present data suggested that sCD40L and IL-10 are potential serological biomarkers to monitor VL treatment in Ethiopia. However, the correlation of serum level of sCD40L, and IL-10 in VL patients after treatment with parasite load should deserve further studies in Ethiopia.

5. SUMMARY

Visceral leishmaniasis is caused by *Leishmania* (*L.) donovani* infecting phagocytic cells of the innate immune system such as neutrophils and monocytes/macrophages. However, little is known about the primary interaction of *L. donovani* with cells of the innate immune system. I hypothesized that *L. donovani* targets and inhibits innate immune cell functions which are detrimental for its survival. Therefore, one objective of this dissertation was to identify dysfunctions of innate immune cell function in VL patients. Flow cytometry based whole blood assays were employed to assess activation, degranulation, phagocytic capacity, reactive oxygen species (ROS) and cytokine production of whole blood cells upon exposure to *L. donovani* and to selected TLR ligands. After exposure to *L. donovani* VL neutrophils showed lower level of activation and degranulation, lower phagocytic capacity and lower ROS production than control neutrophils. Similarly, VL monocytes displayed less phagocytic capacity and release less ROS than control monocytes. Exposure of whole blood cells to *L. donovani* induced significantly less proinflammatory cytokines in VL patients than in controls. However the secretion of IL-10 was higher in VL patients. In conclusion, these results suggest a diminished antimicrobial/pro-inflammatory function of neutrophils and monocytes in VL. Whether the observed dysfunction was a cause of disease development or this dysfunction is the result of the infection, remains to be clarified. The transmission of VL is considered anthroponotic in Ethiopia. However, since recent studies demonstrated *L. donovani* DNA and antibodies to *L. donovani* in domestic animals in Ethiopia I hypothesized that phagocytic cells of cattle can be infected by *L. donovani*. My experiments aiming to investigate this issue demonstrated that bovine neutrophils and macrophages are permissive for infection with the parasite. This finding further supports the view that domestic animals such as cattle can serve as reservoir of *L. donovani* in Ethiopia. Currently, there is no practicable test of cure for VL treated patients in Ethiopia. The identification of prognostic biomarkers for VL treatment would be essential to monitor successful treatment. As potential biomarker candidates I assessed the serum levels of sCD40L, MMP-9 and IL-10 in VL

patients before and after treatment. Before treatment sCD40L, MMP-9 levels were significantly lower; the IL-10 level was significantly higher in VL patients than in control individuals. Since serum levels of sCD40 and IL-10 normalized after clinical cure, serum level of sCD40L and IL-10 are potential serological biomarkers to monitor VL treatment in Ethiopia. The correlation of serum level of sCD40L, and IL-10 in VL patients after treatment with parasite load should deserve further studies in Ethiopia.

6. ZUSAMMENFASSUNG

Viszerale Leishmaniose (VL oder Kala-Azar) gehört zu den schwersten parasitären Erkrankungen, die ohne Behandlung tödlich endet. VL ist eine durch Vektoren übertragene vernachlässigte Tropenkrankheit, die in Äthiopien endemisch ist und durch *Leishmania* (*L.*) *donovani* verursacht wird. Diese Arbeit konzentriert sich auf die VL-Erkrankung in Äthiopien und umfasst drei Teile: I) die angeborenen Immunzellfunktionen während der klinischen viszeralen Leishmaniose, II) die mögliche Rolle von Rindern bei der Übertragung von *L. donovani* und III) Biomarker für die Therapieerfolg von VL nach Behandlung.

Teil I. Ausgewählte angeborene Immunzellfunktionen während der klinischen viszeralen Leishmaniose

Der Parasit *L. donovani* wird während des Blutmahls durch den Biss weiblicher Sandfliegen übertragen. Der Biss der Sandfliege erzeugt eine Blutlache in der Dermis des Säugetierwirts, die die Promastigotenform des Parasiten enthält. An dieser Inokulationsstelle treffen Leishmania-Parasiten zuerst auf angeborene Immunzellen und infizieren phagozytotische Zellen des angeborenen Immunsystems wie Neutrophile und Monozyten / Makrophagen. Im Makrophagen transformiert sich die Promastigotenform des Parasiten in die Amastigotenform und vermehrt sich in den Phagolysosomen der Makrophagen. Über die primäre Interaktion von *L. donovani* mit Zellen des angeborenen Immunsystems ist jedoch wenig bekannt.
Bekannt ist, dass nur wenige Individuen, die in endemischen VL-Gebieten leben, VL entwickeln, obwohl wahrscheinlich die meisten/alle Menschen Bissen infizierter Sandfliegen ausgesetzt sind. Da die angeborene Immunantwort das Wachstum der Krankheitserreger früh nach der Infektion unter Kontrolle hält, stellte ich die Hypothese auf, dass eine Fehlfunktion der angeborenen Immunantwort der Grund für die Entwicklung von Krankheiten nach einer Infektion mit *L. donovani* ist. Daher habe ich in diesem Teil der Arbeit ausgewählte zelluläre angeborene Immunfunktionen bei

VL-Patienten und endemischen gesunden Kontrollen (EHC) untersucht. Vollblut-Assays wurden durchgeführt, um die Bedingungen der Zellen in der Zirkulation zu simulieren. Auf diese Weise können die zellulären Funktionen in Anwesenheit von allen zellulären Elementen und löslichen Faktoren des Blutes untersucht werden. Bevor ich mit dem eigentlichen Versuchen in Äthiopien begann, optimierte ich alle geplanten experimentellen Assays zuerst in Deutschland, im Institut für Medizinische Mikrobiologie und Hygiene der Universität zu Lübeck, und nach meiner Rückkehr nach Äthiopien, am Armauer Hansen Forschungsinstitut (AHRI), Addis Abeba.

Vollblut von VL-Patienten (n = 29) wurde im Gesundheitszentrum von Addis Zemen gewonnen. Diese Blutproben und Blutproben von gesunden Kontrollpersonen aus dem Endemiegebiet (EHC, n = 26) wurden für die Analyse ins Bahir Dar Regionales Zentrum für Gesundheitsforschung (BRHRLC), Bahir Dar, transportiert. Vollblutproben wurden unmittelbar nach der Ankunft im Labor verarbeitet, und Vollblut-Assays auf der Basis von Durchflusszytometrie wurden durchgeführt, um die Aktivierung, Degranulation, phagozytische Kapazität und die Produktion von reaktiven Sauerstoffspezies (ROS) zu messen. Dabei wurden die Proben mit den aktivierenden Stimuli *L. donovani*-Lysat, LPS, MALP -2 und Poly I: C behandelt. Die Analyse der Zytokinproduktion von Blutzellen nach Stimulation mit *L. donovani* und ausgewählten TLR-Liganden erfolgte mittels ELISA.

Nach Stimulation mit *L. donovani*-Lysat und dem TLR-2/6-Agonisten MALP-2 war die Aktivierung, gemessen an CD62L-*shedding* von VL-Neutrophilen signifikant niedriger als bei EHC-Neutrophilen. Die Stimulation von VL-Neutrophilen mit *L. donovani*-Lysat führte zu einer signifikant geringeren Degranulation, gemessen an erhöhte CD66b-Expression, als bei EHC-Neutrophilen. Wichtiger Befund war, dass im Gegensatz zu EHC-Neutrophilen, die Stimulation mit *L. donovani*-Lysat und TLR-Agonisten die phagozytotische Kapazität von Neutrophilen und Monozyten von VL-Patienten nicht erhöhte. In Bezug auf die ROS-Produktion reagierten VL-Neutrophile und Monozyten, im Gegensatz zu EHC, nicht auf fMLP, *L. donovani* und TLR-Agonisten.

Ohne zusätzliche Stimulation war die Freisetzung der pro-entzündlichen Zytokine IL-1β, TNF-α und IL-6 in VL-Blut signifikant niedriger als in EHC-Vollblutkulturen. Die Erhöhung der Freisetzung dieser proinflammatorischen Zytokine nach Stimulation mit *L. donovani* war in VL-Blut, im Vergleich zu EHC, signifikant niedriger. Als Reaktion auf *L. donovani* sekretierten VL-Vollblut-Leukozyten signifikant niedrigere Mengen an

Chemokinen wie IL-8 und IP-10 als EHC- Vollblut-Leukozyten. Im Gegensatz war der Gehalt an IL-10 nach Stimulation mit *L. donovani* in VL-Blutkulturen signifikant höher als in EHC-Blutkulturen.

Zusammenfassend lässt sich sagen, dass neutrophile Granulozyten von VL-Patienten eine stark reduzierte Fähigkeit besitzen, auf aktivierende Stimuli mit erhöhter Phagozytose und erhöhter Produktion von ROS und pro-entzündlichen Mediatoren zu reagieren. Diese Befunde deuten auf einen Funktionsdefekt von neutrophilen Granulozyten in VL hin. Ob diese funktionelle Beeinträchtigung die Ursache oder die Konsequenz der Krankheitsentwicklung ist, lässt sich nicht beantworten. Weitere Studien, die darauf abzielen, die Zeitkinetik der Veränderung der angeborenen Immunfunktion während der VL-Krankheitsentwicklung zu analysieren, könnten diese Frage klären.

Teil II. Die mögliche Rolle von Rindern bei der Übertragung von *L. donovani*

Die Rolle von Tieren als Reservoir für *L. donovani* ist noch nicht ausreichend geklärt. Die Übertragung von *L. donovani* in Äthiopien wird in der Regel als anthroponotisch angesehen. Jedoch fand eine kürzlich durchgeführte Studie in einem Endemiegebiet in Äthiopien in Rindern *L. donovani* DNA und Antikörper gegen *L. donovani* und gegen die Sandmücke *P. orientalis*. In Ostafrika gilt *P. orientalis* als der wichtigste Insekten-Vektor für die Übertragung von VL. Bisher wurden jedoch keine lebendigen *L. donovani*-Parasiten *in vivo* oder *in vitro* in Rinderzellen nachgewiesen. Ziel dieser Studie war es daher, zu untersuchen ob *L. donovani*-Parasiten in der Lage sind Neutrophile und Makrophagen von Rindern zu infizieren. Vollblut wurde aus Zebu (*Bos indicus*, n = 6) und deren Kreuzung mit Holstein Fries Rindern (n = 6) gewonnen.

Mittels Vollblut-Assay konnte ich zum ersten Mal zeigen, dass Rinder-Neutrophile und -Makrophagen infiziert werden können, wenn sie mit *L. donovani*-Promastigoten koinkubiert werden. In der vorliegenden Arbeit habe ich in *in-vitro*-Versuchen gezeigt, dass *L. donovani* Promastigoten in Rinder-Makrophagen zu Amastigoten transformiert werden. Die Amastigoten überlebten in Rinder-Makrophagen für bis zu 16 Tage. Diese Ergebnisse verstärken die Hypothese, dass Haustiere in Äthiopien als Reservoir für *L. donovani* dienen können. Dies ist ein wichtiges Thema angesichts der hohen Anzahl von Menschen, die in VL-Endemiegebieten in enger Verbindung mit ihren Rindern

leben. Ein Beweis dieser Hypothese könnte einen wichtigen Schritt zum Verständnis des Ausbreitungsmechanismus der Krankheit in Äthiopien darstellen.

Teil III. Biomarker für die Therapieerfolg von VL nach Behandlung

Nach dem Aufruf der Weltgesundheitsorganisation (WHO) besteht derzeit ein dringender Bedarf an verlässlichen Biomarkern für die Heilung nach der Behandlung von VL. Ergebnisse aus anderen Ländern zeigen, dass während des klinischen Verlaufs der VL-Infektion die Aktivierung oder Regulation sowohl des angeborenen als auch adaptiven Immunsystems zur Produktion von Biomolekülen führt, die potentielle Biomarker für die klinische VL-Heilung haben könnten. So wurde gezeigt, dass das Serumprofil von MMP-9 und sCD40L eine umgekehrte Korrelation mit der Milzgröße und der Parasitenlast in VL aufwies, während der IL-10-Serumspiegel positiv mit der Parasitenlast korreliert. Aufgrund dieser Beobachtung stellte ich die Hypothese auf, dass diese Biomoleküle (sCD40L, MMP-9 und IL-10) auch als Biomarkerkandidaten für die klinische VL-Heilung in Äthiopien verwendet werden können. In meiner Arbeit habe ich den Serumspiegel von sCD40L, MMP-9 und IL-10 in VL-Patienten vor und nach Therapie gemessen. Blut wurde von den VL - Patienten (n = 49) vor Beginn der Behandlung und nach einer Behandlung mit Natriumstibogluconat (SSG) 20 mg / kg / Tag für 30 Tage gesammelt. Unter Verwendung der ELISA-Methode wurde der Serumspiegel von MMP-9, sCD40L und IL-10 in VL-Patienten ausgewertet und mit EHC verglichen. Gefunden wurde, dass Seren von VL-Patienten signifikant niedrigere sCD40L-Spiegel aufweisen, als Seren von EHCs. Nach der Behandlung und der klinischen Heilung stieg der Serumspiegel von sCD40L signifikant an und erreichte ein Niveau ähnlich dem des EHC. Auch der MMP-9-Spiegel von aktiven VL-Patienten war niedrig im Vergleich zu EHC.

Nach der Behandlung stieg das Niveau von MMP-9 auch signifikant an, erreichte jedoch nicht das in EHC beobachtete Niveau. Vor der Behandlung wurde ein hoher Serumspiegel von IL-10 in klinischen VL beobachtet. Nach der Behandlung wurde eine signifikante Verringerung des Serumspiegels von IL-10 im Vergleich zu dem vor der Behandlung und EHC gemessenen Spiegel beobachtet. Zusammenfassend deuten die vorliegenden Daten darauf hin, dass sCD40L und IL-10 potentielle serologische Biomarker für die Überwachung der VL-Behandlung in Äthiopien sind.

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8. LIST OF TABLES

9. LIST OF FIGURES

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11. LIST OF PUBLICATIONS

1. **Geremew T.**, Endalamaw G., Adugna A., Menberework C., Markos A., Abraham A., Uwe R., Tamas L. **Alteration of innate immune cell function during clinical visceral leishmaniasis** *[Manuscript].*

2. **Geremew T.**, Endalamaw G., Adugna A., Menberework C., Markos A., Abraham A., Tamás L. **Dysfunction of blood neutrophils and monocytes in clinical visceral leishmaniasis** *[Manuscript].*

3. Ketema T., Teresa K., Adugna A., **Geremew T.**, Shimelis *M. ,* Solomon S., Legesse T., and Gilman K. H. Siu. **Treatment Outcomes of Tuberculosis at Asella Teaching hospital, Ethiopia: Ten Years' retrospective aggregated Data.** *Frontiers in medicine. 2018;5:38.*

4. Endalamaw G., **Geremew T.**, Adugna A., Woyneshet G., Menberework C., Markos A., Tamás L., Abraham A. **Serological signatures of clinical cure following successful treatment with sodium stibogluconate in Ethiopian visceral leishmaniasis**. *Cytokine, 91 (2017) 6–9.*

5. Adugna A., **Geremew T.**, Abay D., Mulusew A., Abate M., Abraham A., Enadalaw G. **Visceral leishmaniasis from an area previously not Known to be endemic; Dangur, Benshangul-Gumuz, Regional State, Northwest Ethiopia***: A Case Report. Ethiop Med J. 2016 Jan; 54(1):33-6.*

6. Adugna A., **Geremew T.**, Teshome T., Asfaw K., Abate M., Dagimlidet W., Abraham A., and Endalamaw G. **Visceral Leishmaniasis in Benishangul-Gumuz Regional State, Western Ethiopia: Reemerging or Emerging?** *Am J Trop Med Hyg. 2016, Jul 6; 95(1):104-8.*

7. **Geremew T.**, Endalamaw G., Adugna A., Aboma Zewude., Menberework C., Abraham A., Markos A., Uwe R., Ger van Z., Tamás L., and Ketema T. ***In vitro* permissiveness of bovine neutrophils and monocyte derived macrophages to Leishmania donovani of Ethiopian isolate.** *Parasites & Vectors,* 2016, 9:218.

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10. Yohannis M H., Dawit W., Yohannes M., Aster T., Rawley CH., Ermias H., Nick A., **Geremew T**. and Tsehaynesh M. **Immunological Profile: CD4, CD8, HIV**

Cofactors and Viral Load in HIV Discordant Couples when Compared with Concordant Couples. *J Clin Cell Immunol .2016; 7: 468.*

11. Kidist Zealiyas, **Geremew T.,** Yonas W., Asfaw D., Kissi M., Getachew Ad., Abraham A., Asrat H., Beyene P., and Amha K. ***In vitro*** **activity of *Albizia gummifera* (J.F. Gmel.) C.A. Sm. seed extract against promastigote stages of five *Leishmania* species known to cause human leishmaniasis**. *Ethio J Pub Hlth Nutr. 2016 Sept.01(1):44-47.*

12. Adugna A., **Geremew T.,** Teshome T., Asfaw K., Abate M., Dagimlidet W.,Abraham A., Endalamaw G .**Visceral leishmaniasis in Benishangul-Gumuz Regional State, Western Ethiopia: Re-emerging or Emerging?** *Am J Trop Med Hyg 2016 Jul 2;95(1):104-8.*

13. Adugna A., **Geremew T.,** Abay D., et al. **Visceral leishmaniasis from an area previously not known to be endemic; dangur, benshangul-gumuz, regional state, Northwest Ethiopia: a case report** . *Ethiop Med J. 2016 Jan; 54(1):33-6.*

14. Rajamanickam V., Adugna W., **Geremew T.,** Dereje N., Jimma L. Biruk M. **Hepatoprotective effect of indigenous Foeniculum vulgare in ethanol intoxicated male and female albino rats**. *Am Jour Biol and Pharm Rese. 2016; 3(2): 56-64.*

15. Dereje N., **Geremew T.,** Eyasu M., et al. ***In-vitro*** **Investigation of Fractionated Extracts of *Albizia gummifera* Seed Against *Leishmania donovani* Amastigote Stage**. *J Clin Cell Immunol* .2015; *6: 373.*

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19. **Geremew T.,** Amaha K., Dawit W., Endalamaw G, Sven B., Liv E., & Hannah A. **Low-cost liquid medium for *in vitro* cultivation of Leishmania parasites in low-income countries**. *Global Health Action 2009.*

20. Woldemichael Tilahun W., Asfaw D., Dawit A., **Geremew T.,** Tsigereda B., Yared M., Firehiwot T , Daniel M. **Screening of some medicinal plants of Ethiopia for their molluscicidal activities and Photochemical Constituents**. *Pharmacol. 2006; 3:245-258.*

21. Kebede A., Verweij J.J., Endashaw T., Messele T., **Geremew T.,** Petros B., And A.M.Polderman. **The use of real-time PCR to identify Entamoeba histolytica and E.dispar infections in prisoners and primary- school children in Ethiopia**. *Annalsof Medicine and Parasitology, (2004) Vol.98, No.143-48.*

22. Gebertsadik A, Kebede A, Mezemer M and **Geremew T. Detection and differentiation of two morphologically identical species of Entamoeba**. *Ethiop.J.Health Dev.4: 18(2): 121-124.*

12. ORAL TALKS AND POSTER PRESENTATIONS

1. **Innate immunity and adaptive responses in leishmaniasis:** a collaborative German-Ethiopian research project on neglected infectious diseases. Oral and poster presentations, Hamburg, Germany, May 17-21, 2017.

2. ***In Vitro* Permissiveness of Bovine Neutrophils and Monocytes Derived Macrophages to *Leishmania donovani* of Ethiopian Isolate,** Oral talk on Ambo University,5th Conference, May-9-10, 2017, Ambo, Ethiopia

3. **Infection of bovine neutrophils and monocyte derived macrophages by *Leishmania donovani of* Ethiopian isolate**. Oral talk, 09-13 January 2017, **Armauer Hansen Research Institute *Scientific Advisory Board*** (SAB) annual conferences:, Addis Ababa, Ethiopia.

4. **Innate Immunity in Visceral Leishmaniasis**, Oral talk and poster presentation, **Armauer Hansen Research Institute *Scientific Advisory Board*** (SAB) annual conferences: **11-15 January 2016, Addis Ababa, Ethiopia.**

5. **Innate Immunity in Leishmaniasis**, Oral talk and poster presentation June 11, 2015, DFG -Conferences, Würzburg, Germany.

6. ***In vitro* infection of bovine neutrophils and monocytes derived macrophages to *Leishmania donovani* of Ethiopian isolate. Ethiopian Medical association annual conference, at United nations commission conference hall, Addis Ababa, Ethiopia.** Oral talk-February 12-13: 2016, UNCC, Addis Ababa.

7. **Experimental infection of bovine neutrophils and monocyte derived macrophages by *Leishmania donovani of* Ethiopian isolate.** Oral talk at third Ethiopian Public Health congress, October: 2016, Addis Ababa, Ethiopia.

8. ***In Vitro* Infection of Bovine Neutrophils And Monocyte Derived Macrophages By *Leishmania Donovani* of Ethiopian Isolate.** Oral talk at 1st Annual National Research Conference of Arsi University , 24 - 25 June 2016, Asella, Ethiopia.

9. **Innate immune Response in Human Visceral Leishmaniasis . Armauer Hansen Research Institute, *Scientific Advisory Board*** (SAB) annual conferences: Oral talk and poster presentation, **2015, Addis Ababa, Ethiopia.**

13. CURRICULUM VITAE

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Marital State: Married, father of two kids
Nationality: Ethiopian
Current positions: Researcher, PhD Student

Education

2012- present: Doctoral student at University of Lübeck, Germany
2010: Licentiate (MSc.) Degree in Infection Biology- Department of Microbiology, Tumor & Cell Biology (MTC) Karolinska Institute, Stockholm, Sweden.
1999: B.Sc. Degree in Medical Lab technology from former Jimma Institutes of Health sciences, Jimma, Ethiopia
1993: Diploma in Medical laboratory technology from former National Research Institute of Health, Addis Ababa, Ethiopia

Work experience

2000 – Present: Ethiopian Public Health Institute- as associate researcher
1999 – 2000: Nekemte Regional Public Health Laboratory, as laboratory technologist
1994 – 1999: Nedjo Health Center - as medical laboratory technician
1993 –1994: Gambela Hospital- as medical laboratory technician

Experience in some of Laboratory Techniques:

- FACScan analysis , ELISA, ELISPOT, PCR and conventional gel electrophoresis
- Immunological assays: Phagocytosis, cell surface marker staining and ROS assays
- Isolation and stimulation of peripheral blood mononuclear cells (PBMC)
- Culturing cell lines, PBMC and *Leishmania* parasites
- Immunohistochemistry staining and evaluation

- Immunofluorescent techniques (e.g, TUNEL, Annex V/PI)
- Rapid diagnostic test and direct agglutination test (DAT) for leishmniasis
- Identifying *Leishmania* bodies in Giemsa stained samples
- Ziehl–Neelsen staining for examination acid fast bacilli microorganisms
- Hematological and bacteriological laboratory examination
- Liver function test and renal function test
- Stool culture for Hookworms and *Strongyloides,* direct , concentration techniques and Kato katz techniques for stool examination
- Thick and thin blood film staining with Giemsa stain and identification hemoparasites

Awards

2013 Federal Democratic Republic of Ethiopia Ministry of Science and Technology. Innovative research grant to "Construction of hybrid Cytocentrifuge through Integration of Cytospin functions into conventional centrifuge to have complete function of both Cytospine and Centrifuge".

2010 Tore Godal Gold Medal Prize award winner of the year 2010 for excellence in biomedical research in Ethiopia, November 25, AHRI/ALERT, and Addis Ababa, Ethiopia.

2010 Global health Travel award for young Researchers, to attend a conference new Approaches to Understanding Host-Parasite Interactions (F1)- Colorado- USA

2009 World Health organization (WHO) research grant award on investigation of leishmaniacidal activity of some Ethiopian Medicinal plants used by traditional healer to treat leishmaniasis: for project number AF/ETH/ AAC /000 /RB/08 AMS CODE 2123883.

2009 Global health Travel award for young Researchers to attend Drug Discovery for Protozoan Parasites (D2)- Colorado- USA

2008 Global health Travel award for young Researchers to attend Translating New Technologies to improve Public Health in Africa (E1)-Uganda

2006 UNICEF/UNDP/World Bank/WHO/TDR training grant award, for up keeping Postgraduate study at Karolinska Institute, Sweden

.

14. ANNEX

14.1. Questionnaire used to capture patient data

AHRI/DFG- innate Immunity in Visceral Leishmaniasis project- 2012

Date: _____/_____/_____
(DD/MM/YYYY)
Patient Code: _____

I. Demography

1.1. Age_____ Sex 1. Male _____ 2. Female_____
1.2. Address: Region_____ Woreda_____ Kebele _____
House number_____ Phone umber_____
Ethnicity: _____ Religion:_____
1.3. Previous travel history to VL endemic area: Yes:_____, No_____;
If yes, indicate: Region_____ Woreda_____ Kebele_____
Duration of stay _____
1.4. Period of onset of symptoms in days: 1. before travel _____
2. after return _____
If the onset of symptoms was after returns specify the gap (in days) _____

2. Clinical signs and symptoms:

2.1. Duration of fever: _____weeks.
2.2. Weight: _____ (kg)
2.3. Height: _____ (cm)
2.4. Tick all that are applicable to clinical symptoms below:

S.No.	Clinical symptoms	Yes	NO
1	Past history of VL		
2	History of bleeding		
3	Bleeding signs		
4	Skin pallor		
5	Jaundice		
6	Oedema		
7	Ascites		
8	Hepatomegaly		
9	Splenomegaly		
10	Lymph nodes enlarged		

3. Types of clinical samples: Amount

A) Whole blood: _____ _____
B) Lymph node aspirate: _____ _____
C) Bone marrow aspirate: _____ _____
D) Spleen aspirate: _____ _____

4. Final diagnosis by clinician**:** Confirmed kala-azar _____

PKDL _____ other: _____, if other, what was the evidence:
_____
5. Final decision on the sample**:** A) included into study B) Not included into the study.
General comments:-
_____
_____
_____
_____

Questionnaire Filled by_____Date___/____/_____ (dd/mm/yyyy)

Sample Collection by _____Date___/_____/_____ (dd/mm/yyyy)

14.2. Laboratory screening tests

Laboratory slip

AHRI/DFG-visceral leishmaniasis-innate immunity project

Date: _____/_____/_____ (DD/MM/YYYY), Patient Code: _____

Laboratory investigations

HIV 1/2 test: A) reactive _____ B) non-reactive _____
Total Leukocyte count: _____
Haemoglobin(g dl−1): _____
Diff. count (%): Neut_____, Mon_____,Eosin_____ Lymp_____
Platelets count: _____
Thick smear for malaria: Positive_____ Negative _____
Not done _____
Bone marrow aspiration: positive for LD bodies;
Grading (1–6): _____ Negative for LD bodies_____ Not done _____.
Spleen aspiration: Positive for LD bodies;
Grading (1–6): _____,
Negative for LD bodies, _____, Not done _____.
Direct agglutination test (DAT): positive_____ negative_____
Not done _____
Serological test (rK39): positive _____ negative _____
Not done _____
Other tests results:----------

Result filled by_____Date___/____/_____ (dd/mm/yyyy)
Aproved by_____Date___/____/_____ (dd/mm/yyyy)

14.3. Consent form- Amharic version

<u>የፈቃደኝነት ቅፅ በሁፈጥሮ የሰዉነት መከላከያ ጥናት በቦለ አዛር በሽታ ጊዜ</u>

የጤና ጣቢያ/ሆስፒታል ቁጥር ——————————

የአህሪ ቁጥር——————————————————

(የለጋሹ ኮድ#)

እኛ የአርማወር ሃንሰን የምርምር ተቋም ተመራማሪዎች፣ የሰዉነታችንን የተፈጥሮ መከላከያ ሕዋሳት (Innate Immunity Cells) ተግባር የካላዘር ሌሽማኒያሲስ/ በሽታን ለመቆጣጠር ያላቸውን አስተዋጽኦ ለማጥናት አቅደናል። ከዚህ ጥናት የሚገኘዉ ዉጤት በሽታዉን ለመቆጣጠር የሚደረገዉን ጥረት ይደግፋል ተብሎ ይታመናል። በመሆኑም ከእርሶ ለጥናቱ የሚያስፈልገው 5 ሚሊ ሊትር የደም ናሙና ከክንደምት ላይ በሰለጠነ ባለሞያ ይወሰዳል። የካላዘር አና የኤች አይቪ ኤድስ የምክር አገልግሎት ከተሰጠዎት በኋላ ምርምራ ይደረግሎታል። የምርመራ ውጤቶም በሚስጥር ይያዛል፣ማወቅ ከፈለጉ ብቻ ይነገሮታል። የድህረ ምርመራ ምክር አገልግሎትም ይሰጠዎታል። ቫይረሱ በደምዎ ውስጥ ካለ በጥናቱ ውስጥ አይካተቱም። ሆኖም ማን በጥናቱ ቢሳተፉም ባይሳተፉም አስፈላጊውን የምክርና ለማኛዉም ህሙማን የሚሰጠውን አገልግሎት በአቅራቢያዎ ካለው ሆስፒታል ወይም ጤና ጣቢያ ያገኛሉ። የካላዘር ጥገኛ ህዋስ በደምዎ ውስጥ ካለ አስፈላጊዉን ሕክምናም ያገኛሉ።

በዚህ ጥናት መሳተፍ ወይም ያለመሳተፍ እንዲሁም በፈለጉ ጊዜ ከጥናቱ የመዉጣት መብት አለዎት ። ስለጥናቱ ውጤት መረጃ ማማኘት ከፈለጉ ማማኘት ይችላሉ። ግልጽ ከሆነለዎ እና በጥናቱ ላይ ለመሳተፍ ፈቃደኛ ከሆኑ በፊረማዎ መስማማቶትን ያረገግጡ።

"አዎ፣ ተረድቻለሁ በጥናቱ ላይ ለመሳተፍ ዝግጁ ነኝ"

የሕመምተኛው ስም ——————————ፊርማ ——————ቀን: ———

የሃኪሙ ስም ——————————ፊርማ——————ቀን: ——

የአማኙ ስም—————————— ፊርማ ——————ቀን: ——

14.4. Consent form-English version

Health center/Hospital code:----------

AHRI number ----------

(Donor's code#)

We, researchers at Armauer Hansen Researcher Institute (AHRI), need to investigate the role of our body innate immune cell functions in controlling infection during kala azar disease. We believe that the result to be obtained from this study will support the attempt to control the disease. Therefore we need to collect 5 mL of whole blood from your vain. The blood will be drawn by educated professionals. After counseling, Kala azar and HIV-1/-2 screening test will be done for you. The result of these screening tests are strictly confidential and only disclosed to you upon your request through your post counseling doctors. If you are positive for HIV-1/-2, you will not be included into the study. If you are tested as Kala azar positive, you will get all necessary treatment according to leishmaniasis diagnosis and treatment guideline by Federal Ministry Health of Ethiopia. In addition you have the right to participate or withdraw from the study any time. Whether you are willing to participate or not to participate in the study, it will not affect your right to have all necessary services and counseling that are provided to other patients. If you feel that you need to have more information about the intended study, you can ask to understand all about the study. If you feel that you understand well and need to participate in the study by providing your blood please sign on the consent form on the space provided below.

"Yes, I am clear and agree to participate''

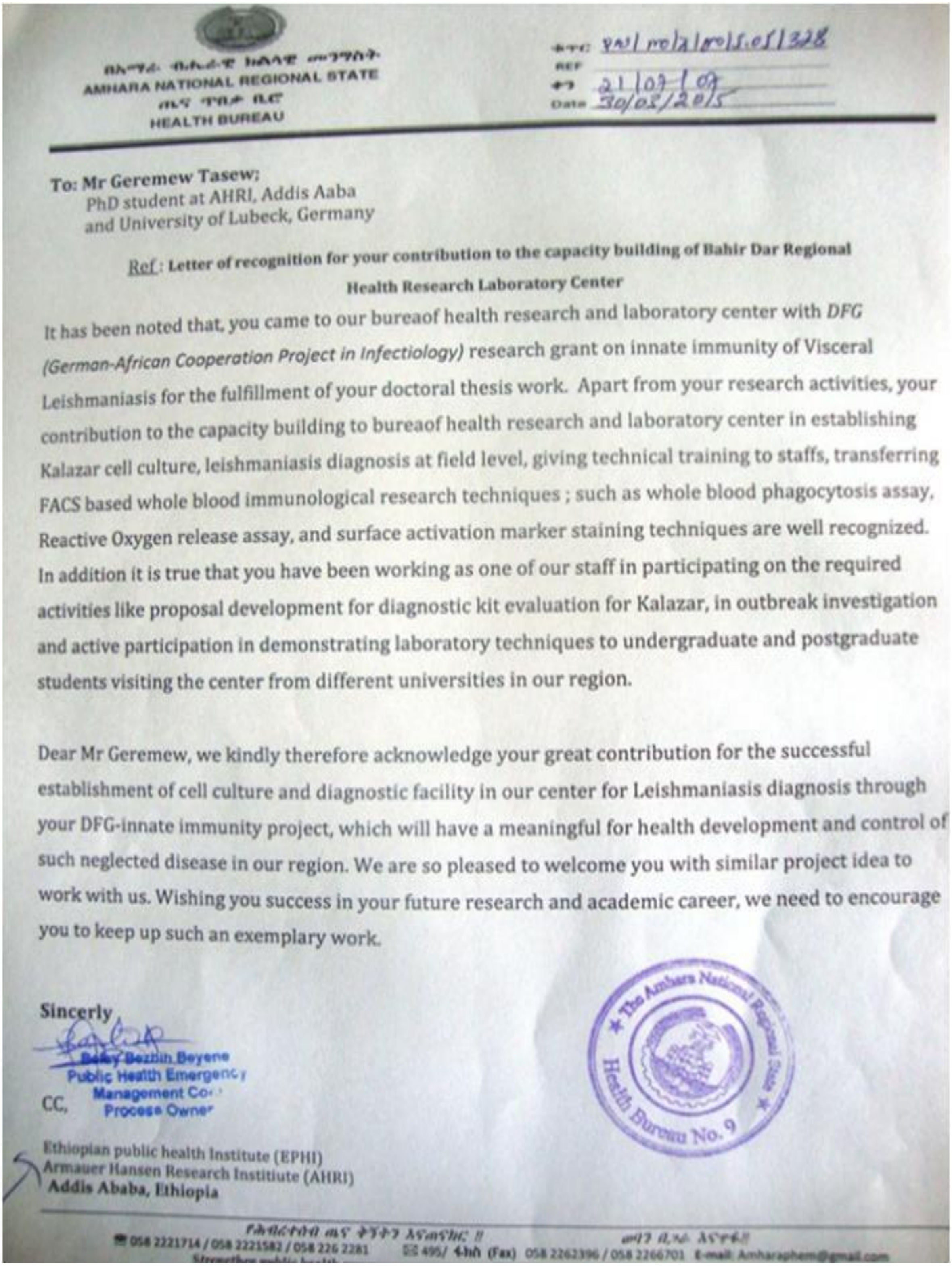
Patient's name …….. signature…….date…….

Doctor's name …….. signature…….. date …….

Witness's name ……signature ……date…….

14.5. Certificate of recognition

1. Letter of recognition for establishment of laboratory



AMHARA NATIONAL REGIONAL STATE
HEALTH BUREAU

REF
Date 30/08/2015

To: Mr Geremew Tasew;
PhD student at AHRI, Addis Aaba
and University of Lubeck, Germany

Ref: **Letter of recognition for your contribution to the capacity building of Bahir Dar Regional Health Research Laboratory Center**

It has been noted that, you came to our bureaof health research and laboratory center with *DFG (German-African Cooperation Project in Infectiology)* research grant on innate immunity of Visceral Leishmaniasis for the fulfillment of your doctoral thesis work. Apart from your research activities, your contribution to the capacity building to bureaof health research and laboratory center in establishing Kalazar cell culture, leishmaniasis diagnosis at field level, giving technical training to staffs, transferring FACS based whole blood immunological research techniques ; such as whole blood phagocytosis assay, Reactive Oxygen release assay, and surface activation marker staining techniques are well recognized. In addition it is true that you have been working as one of our staff in participating on the required activities like proposal development for diagnostic kit evaluation for Kalazar, in outbreak investigation and active participation in demonstrating laboratory techniques to undergraduate and postgraduate students visiting the center from different universities in our region.

Dear Mr Geremew, we kindly therefore acknowledge your great contribution for the successful establishment of cell culture and diagnostic facility in our center for Leishmaniasis diagnosis through your DFG-innate immunity project, which will have a meaningful for health development and control of such neglected disease in our region. We are so pleased to welcome you with similar project idea to work with us. Wishing you success in your future research and academic career, we need to encourage you to keep up such an exemplary work.

Sincerly

Public Health Emergency
Management Co
Process Owner

The Amhara National Regional State
Health Bureau No. 9

CC,

Ethiopian public health Institute (EPHI)
Armauer Hansen Research Institiute (AHRI)
Addis Ababa, Ethiopia

058 2221714 / 058 2221582 / 058 226 2281 (Fax) 058 2262396 / 058 2266701 E-mail: Amharaphem@gmail.com

2. Certificate of Tore Godal award