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The Contribution of Epidermal Apoptosis to the Clinical Outcome of *Leishmania aethiopica* Induced Cutaneous Leishmaniasis and its Laboratory Diagnosis using Low Cost Culture Media

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Stockholm 2010

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"*Bantiiwwan waqaa gararraati ol ka'i, Yaa Waaqayyo! Ulfinni kees lafa hundumaa irratti oli hajedhu." Far: 57: 11*

"Be exalted, O God, above the heavens; let your glory be over all the earth." Psalm 57:11

To: Rahel Geremew Hailemariam
Oliyana Geremew
&
All my family

1 ABSTRACT

Background: Cutaneous leishmaniasis (CL) induced by *Leishmania aethiopica* has two clinical manifestations: ulcerating, self-healing CL and non-ulcerating, non-healing CL. The grossly disfiguring multiple nodules on the face and exterior surface of limbs during non-ulcerative CL are sometimes misdiagnosed as other skin infections. Thus the need for definitive and prompt laboratory diagnosis is required. Identifying *Leishmania* parasite by culture method is considered as a definitive method for initiation of treatment and as an effective component of leishmaniasis control methods. Recently the involvement of Fas (CD95) and Tumor Necrosis Factor (TNF) Related Apoptosis Inducing Ligand (TRAIL) induced apoptotic pathways were proposed to be involved in tissue destruction and ulceration during *L. major* induced CL.

Aims: 1) to develop an alternative culture media that could minimize the cost for culturing *Leishmania* from patient lesions.

2) to investigate if the expression of FasL and TRAIL differs in ulcerating and non-ulcerative CL.

Methods: GALF-1 media was formulated in our lab and compared to RPMI 1640 medium and conventional Locke's semi solid media (LSSM) which is one of the modifications of Novy-MacNeal-Nicolle (NNN) culture media. Amastigotes transformation, cryopreservation, recovery of parasites, cost and mass cultivation were analysed. Expression of Fas ligand (FasL), TRAIL and apoptosis were assessed by immunohistology in human skin biopsies from *L. aethiopica* induced ulcerative or non-ulcerative CL. FasL and TRAIL blocking experiments were performed in a murine model of CL.

Results and discussion: GALF-1 is cheap and its ingredients available in a low income country such as Ethiopia. GALF-1 was able to transform amastigotes from Ethiopian patients' samples and could be used to cultivate promastigotes in large quantities. Cost analysis showed 80% to 95 % decreased costs as compared to conventional media. Promastigotes cultured with GALF-1 could be cryopreserved in liquid nitrogen with comparable re-culture potential to conventional media. Affordability of diagnostic assays is a key issue for resource poor countries and the possibility to cut the cost of the efficient culture method for diagnosis through the use of inexpensive local formulated reagents could improve the diagnosis of leishmaniasis in low income endemic countries.More FasL expressing cells were detected in dermis of ulcerative CL as compared to non-ulcerative CL and controls. TRAIL expression was higher in ulcerative CL as compared to non-ulcerative CL and controls in both epidermis and dermis. Increased dermal expression of FasL and TRAIL was associated with ulcer formation during CL. This correlated with an inhibition of the ulcerative process in a murine CL model during FasL and TRAIL neutralisation.The mechanisms of the involvement of FasL and TRAIL in ulceration was not elucidated butand putative reason(s) for the difference in dysregulation of apoptosis are discussed.

2 LIST OF PUBLICATIONS

I. **Geremew Tasew**, Amha Kebede, Dawit Wolday, Endalamaw Gadisa, Sven Britton, Liv Eidsmo, Hannah Akuffo. **Low cost liquid medium for in vitro cultivation of *Leishmania* parasites in low-income countries,** Global Health Action 2009. DOI: 10.3402/gha.v2i0.2046

II. **Geremew Tasew**, Susanne Nylen , Thorsten Lieke , Befekadu Lemu, Hailu Meless, Nicolas Ruffin , Abraham Asseffa , Hideo Yagita, Sven Britton, Hannah Akuffo, Francesca Chiodi, Liv Eidsmo. **Systemic FasL and TRAIL neutralisation reduce leishmaniasis induced skin ulceration.** Manuscript, Submitted for publication

3 CONTENTS

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4 LIST OF ABBREVIATIONS

AICD	Activation induced cell death
APC	Antigen-presenting cells
Bcl_2	B cell lymphoma 2
CR	Complement receptors
CON	Healthy control
CL	Cutaneous leishmaniasis
DCL	Diffuse cutaneous leishmaniasis
DcR	Decoy receptor
DD	Death domain
DR	Death receptor
FADD	Fas associated death domain
Fas	CD95/APO-1
FasL	Fas ligand
IHC	Immunohoistochemistry
IL	Interleukin
LCL	Localized cutaneous leishmaniasis
LSSM	Locke's semi solid media
MCL	Mucocutaneous leishmaniasis
MHC	Major histocompatibility complex
MØ	Macrophage
MIP-1a	Macrophage inflammatory protein 1a
MIP-2	Macrophage inflammatory protein 2
mTRAIL	Membrane bound TRAIL
NNN medium	Novy-MacNeal-Nicolle (NNN) medium
PAF	Platelet activating factor
PCD	Programmed cell death
PE	Prostaglandin E2
Pos Con	Positive control
PS	Phosphatidyl serine
RPMI 1640	Roswell Park Memorial Institute-1640
sTRAIL	Soluble TRAIL
TGF3	Transforming growth factor 3
Th	T helper
Th1	T helper -1
Th2	T helper -2
TNF-R	Tissue Necrosis Factor Receptor
TNFα	Tumor Necrosis Factor alpha
TRAIL	Tumor Necrosis Factor (TNF)-related apoptosis Inducing ligand(APO-2 ligand)
TRAIL-R1	TRAIL-Receptor1
TRAIL-R2	TRAIL-Receptor 2
T reg	T- regulatory cells
TUNEL	Terminal deoxy nucleotide transferase- mediated d-UTP nick end labelling
VL	Visceral leishmaniasis

5 INTRODUCTION

Leishmaniasis is a group of complex diseases displaying clinical and epidemiological diversity. Cutaneous leishmaniasis (CL) clinically resembles many other skin diseases [1, 2] and the lesions due to CL are sometimes misdiagnosed as other skin diseases. Thus definitive diagnosis based on the demonstration of parasites in patient's samples is very important when CL is suspected. Confirmation of the disease is important since treatment of leishmaniasis remains expensive and the drugs of choice induce significant toxicity. It is therefore crucial to have tools that enable prompt definitive diagnosis of leishmaniasis in endemic areas to initiate appropriate treatment. It is believed that detection of DNA of parasite by polymerase chain reaction (PCR) is the most sensitive and specific tool[3], but it is unavailable in many low income countries because of its high cost. In Ethiopia, culturing *Leishmania* parasite from clinical samples is more sensitive than microscopic examination of stained smears or serology [4]. Because of the high cost of culture media, this method is limited only to the higher level laboratories. Therefore we developed a new low cost liquid medium for *Leishmania* culture designated as GALF-1. GALF-1 was supplemented with urine and was compared with RPMI1640 (Roswell Park Memorial Institute-1640) medium and conventional Locke's semisolid medium (LSSM), one of the modifications of Novy-MacNeal-Nicolle (NNN) medium. The comparison of these culturing media was done in terms of amastigotes to promastigotes transformation, recovery of parasites after cryopreservation, cost and mass cultivation.

CL in East Africa is induced mainly by *L. aethiopica* which results in ulcerating, self healing cutaneous leishmaniasis (ulcerative CL) or non-ulcerating, non-healing cutaneous leishmaniasis (non-ulcerative CL). The difference in theses two clinical manifestations caused by a single parasitic species is not fully known[5, 6]. Despite the development of science and technology, there is still a challenge in the development of *Leishmania* vaccine to prevent and effective treatment to cure leishmaniasis. Thus further knowledge of leishmaniasis immunology is crucial. In this thesis, an attempt was made to investigate if dysregulation of apoptosis inducing ligands were correlated to clinical manifestations of CL. Fas (CD95) ligand (FasL) and Tumor Necrosis Factor (TNF) Related Apoptosis Inducing ligand (TRAIL) induced apoptosis were investigated in human skin biopsies using in vitro experiments and in vivo blocking experiments in a murine model of ulcerative CL.

6 LEISHMANIASIS

Leishmaniasis is a parasitic infection caused by obligate intracellular protozoa of the genus *Leishmania*, usually transmitted by the bite of various species of sand-flies [7]. The clinical manifestation of infection with *Leishmania* varies from a chronic skin lesion (CL), to erosive mucosal membrane with progressive destruction of the nasopharynx and severe facial disfigurement during mucocutaneous leishmaniasis (MCL), and to a life-threatening systemic infection with hepato-spleenomegly during visceral leishmaniasis (VL). The pathology induced depends upon a complex interaction between the infecting species of *Leishmania* , the genetic and immunological status of the host [8]. In zoonotic leishmaniasis the reservoir hosts are wild or domestic animals while in the anthroponotic leishmaniasis the reservoir host is man. Controlling leishmaniasis is complicated by the fact that many species of sand-flies are potential vectors and some 100 species of animals could serve as a reservoir host[9].

In the Old World, leishmaniasis has a long history as descriptions of cutaneous leishmaniasis are found since the first century AD. Similarly in the New World, pottery from Ecuador and Peru dating from 400-900 AD illustrates faces afflicted with a process consistent with leishmaniasis as previously reviewed [10].The first description in English of a lesion resembling leishmaniasis was made in 1756 by Russell who described the "Aleppo evil" from Syria. In 1885, Cunningham observed organisms in macrophages (MØ) from lesions of "Delhi boil" in India. In 1903 Leishman published his finding of the parasite in the spleen of a patient who had died of Dumdum fever in Dumdum, India in 1900. A few months later Donovani described identical organism in a spleen aspirate from a living child. And later, Ross named the parasite as "*Leishmania donovani*". There were many other names for leishmaniasis include oriental sore, Aleppo evil, Delhi boil, Baghdad sore, Rose of Jericho, Chiclero's ulcer , uta, espunda (mucous form), forest yaws, Dumdum fever (Visceral form), Kala azar and black fever[10]. As reviewed by Oumeish, the first reported case of CL in African countries was in Tunisia in 1903 and Ethiopia in 1913 and the first case of VL was reported in Sudan in 1904 [11]. There was also additional evidence that indicated the first observed case of ulcerative CL in Ethiopia was in 1912 by Martoglio while Balazer and others reported the first non-ulcerative CL in 1960 from the highland area , and likewise the first report of VL in Ethiopia was from Omo Rate and Kelem at the North-West end of lake Turkana in 1926 as reviewed by Humber and colleagues [12]. The isoenzyme analysis of parasites isolated from non-

ulcerative CL patients in Ethiopia indicate that the same species (*L. aethiopica*) is involved in both non-ulcerative CL and ulcerative CL infections. This difference in the outcome of infection is claimed as a result from failure of cell mediated immunity to develop in the non-ulcerative CL patients [12].Variation in induction of different cytokines between *L .aethiopica* induced DCL and LCL antigen [13-15] and ultra structural difference in size and membrane structure [16] have been reported. However, a study using PCR-RFLP [17] was unable to distinguish LCL derived parasites from DCL derived parasites.

Although leishmaniasis is becoming more and more prevalent both in developed and developing countries, effective treatment and control measures for these diseases are currently not in place. The current control measures relay on chemotherapy to alleviate disease progression and on vector control to reduce transmission. To date there are no effective vaccines produced against leishmaniasis. In the longer term, however, there is an assumption that vaccines ought to become a major tool in the control of leishmaniasis. Unfortunately the development of vaccines has been hampered by significant antigenic diversity and the fact that the parasites have a digenetic life cycle in at least two hosts (Sand-fly vector and human, and/or an animal as a reservoir).The population most in need of protection may be the one which is normally unable to mount an appropriate innate or adaptive immune response and is therefore most susceptible to disease[18].

6.1 Life cycle of *Leishmania*

Infection by *Leishmania* parasite is initiated by introduction of promastigotes stages of the parasite (fig.1) into the skin of mammalian host during the blood meal by infected female Sand-fly vectors of the *Phlebotomus* species in the Old World, and *Lutzomia* species and rarely by *Psychodopygus*[10] in the New World.

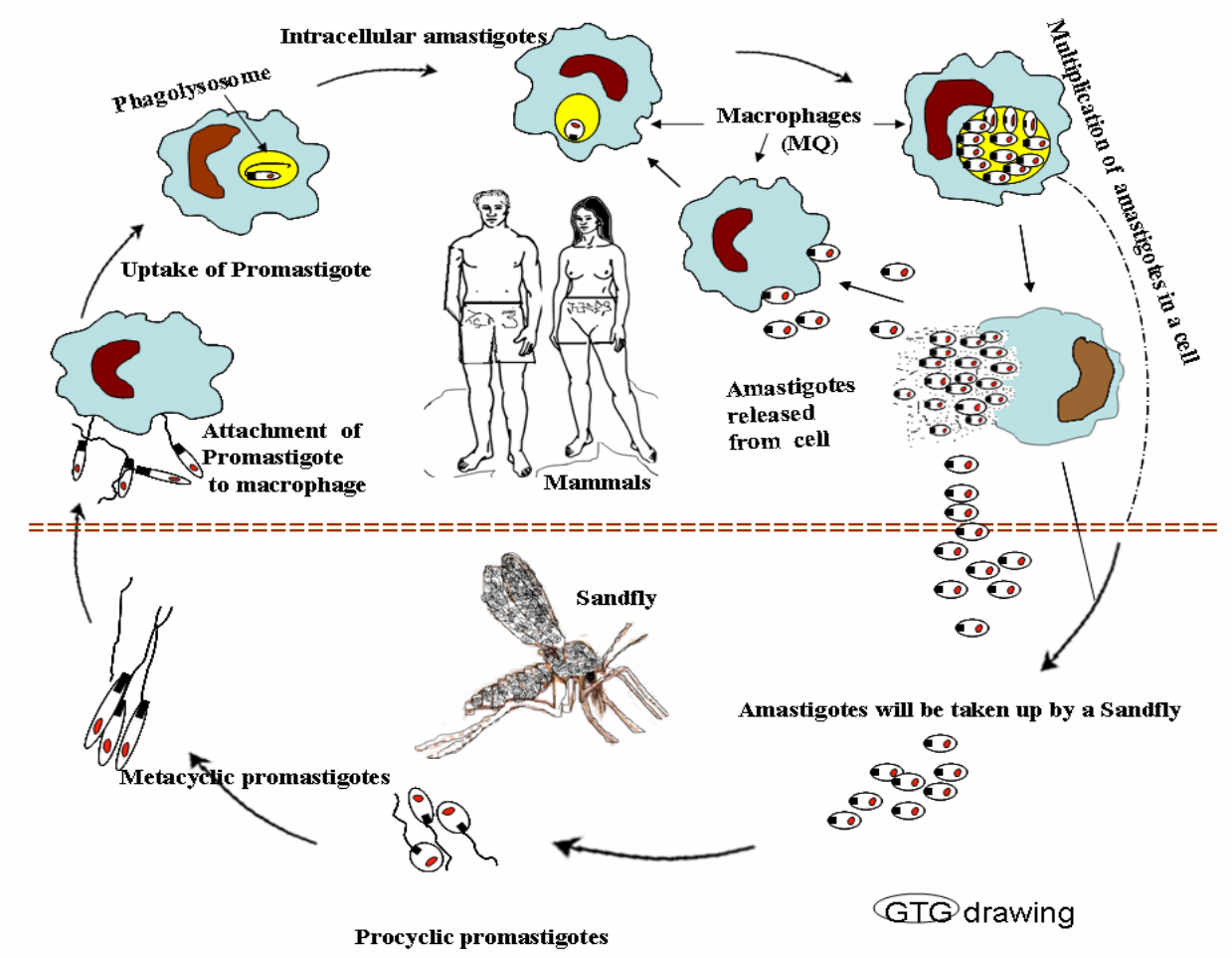

Figure.1 Leishmaniasis is transmitted between mammals and *Phlebotomine* sand-flies. The sandflies inject the infective stage, promastigotes, during blood meals. Promastigotes that reach the puncture wound are phagocytized by macrophages and transform into amastigotes. Amastigotes multiply in infected cells and affect different tissues, depending in part on the *Leishmania* species. This originates the clinical manifestations of leishmaniasis. Sandflies become infected during blood meals on an infected host when they ingest macrophages infected with amastigotes. In the sandfly's midgut, the parasites differentiate into promastigotes, which multiply and migrate to the proboscis. The promastigotes are inoculated into the potential host with sand-fly saliva, which enhances the infectivity of some species of *Leishmania* by increasing the survival rate in the vertebrate and increases the severity of cutaneous lesions. Hyraxes are reservoir hosts of *L. aethiopica* and human infection with *L. aethiopica* parasites only occurs near to hyrax colonies. There are different species of sandfly vectors that transmit different species of leishmaniasis. In Ethiopia and Kenya, Visceral leishmaniasis due to *L.donovani* is transmitted by *Phlebotomine celiae* sandfly species, while *P. orientalis* transmits visceral leishmaniasis due to *L. donovani* in Saudi Arabia, Yemen, Ethiopia and Sudan. CL due to *L. aethiopica* in Ethiopia and Kenya is transmitted by *P. longipes* species.

These parasites are obligatory intracellular and invade neutrophils [19], MØ and dendritic cells (DC) soon after promastigotes stages entering into the host's dermis. In MØ, the promastigotes transform into amastigotes in a phagolysosome termed as parasitophorous vacuole. In this vacuole, the amastigotes reside and multiply by binary fission until they fill and rupture the cell. There is an

assumption that amastigotes also reside in the cytoplasm compartment may leave the cells through a mechanism that would resemble excoytosis [20]. Latter amastigotes will be taken up by the sand-fly along with the blood cells during her blood meal on an infected host.

In the sand-fly, amastigotes are transformed into procyclic promastigotes with in 48 hours, and then attach to the midgut epithelium of the fly and divide by binary fission [21]. These promastigotes then become differentiated into infective metacyclic promastigotes , which are able to detach from the gut and reach proboscis by fast-swimming towards the pharynx of the sand-fly [22-24]. The Sand-fly can then transmit the parasite to a new host during her blood meal by introducing metacyclic promastigotes into the dermis and the parasite life cycle will be completed. There is also a possibility of transmission of leishmaniasis through blood transfusion and it has been proposed that much of visceral leishmaniasis associated with human immunodeficiency virus (HIV) infection in southern Europe is transmitted by sharing contaminated needles and syringes during misuse of drugs [25].

6.2 Epidemiology of leishmaniasis

Leishmaniasis has important clinical and epidemiological diversity, and exists in its various forms in all continents except Australia and Antarctica [26]. There are about 88 leishmaniasis endemic countries of the world, of which 72 are developing countries. Globally an estimated 12-15 million people are affected and approximately more than 350 million people are at risk of infection. Reports from about 65 countries of the world indicated that there are about 500,000 new cases of VL and globally an estimate of 1 to 1.5 million new cases of CL are reported annually. However it is difficult to provide realistic estimates of those infected versus those at risk. Some assumptions indicated that there is still a great difference between the true number of cases and the reported number of cases due to several factors such as undiagnosed, misdiagnosed or unreported cases [27, 28]. The incidence of leishmaniasis continues to rise and new epidemics are largely attributable to changes in the habitat of the vector and leishmaniasis is considered as emerging disease.

There are over 20 species of *Leishmania* that have been recorded as causing human infections which are either zoonotic, or have recent zoonotic origins. The distribution of each species is determined by distribution of its vector, reservoir host, or both[25, 27]. Depending on virulence factors of *Leishmania* species and host immune responses, infection can be manifested as VL,

MCL or CL forms. CL is the most common clinical type and is still an important public health problem in the 21st century due to not only environmental risk factors such as massive migrations, urbanisation, deforestation, new irrigation schemes, but also to individual risk factors such as HIV, malnutrition, genetic disposition, and poverty. The disease still requires improved control tools and the WHO/TDR financed research for leishmaniasis has been more and more focusing on the development of new tools such as diagnostic tests, drugs and vaccines. The epidemiology and clinical features of the disease are highly variable due to the interplay of numerous factors in the parasites, vectors, hosts and environments involved[27].

6.3 The Taxonomy of *Leishmania*

Leishmania is one of protozoan parasites grouped in the order of Kinetoplastida and in the family of Trypanosomatidae (fig.2).

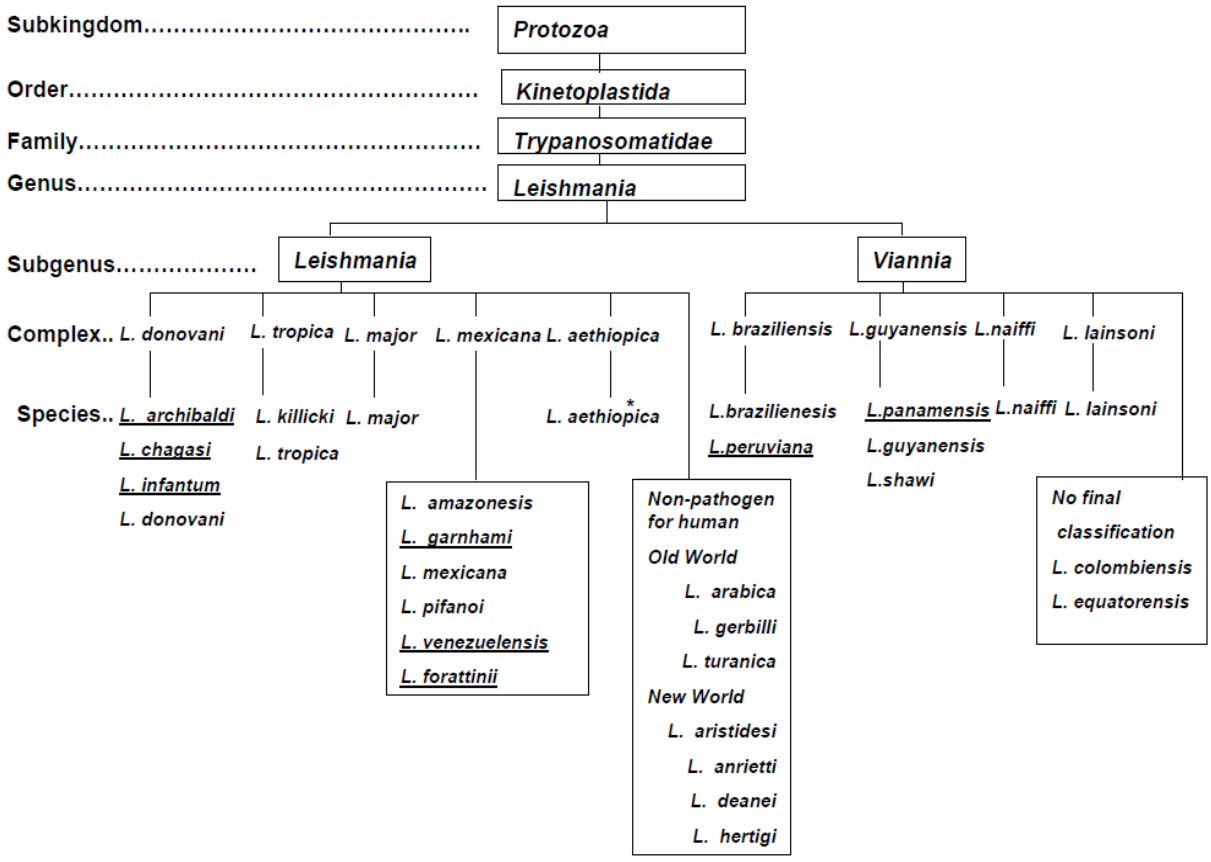

Figure.2 Taxonomy of *Leishmania* species: underlined species have been questioned and * Shows parasitic species to be discussed by this thesis work. This taxonomy of *Leishmania* species has been adopted from Banuls and Hide et al, 2007.

Members of the order Kinetoplasida are characterized by having a single large mitochondrion known as a kinetoplast from which the order name Kinetoplastidais drawn. The parasite contains a kinetoplast, a nucleus, and the mitochondrial DNA of the trypnosomes. Naturally all species of *Leishmania* have two different morphological stages, one in the vector and the other in the mammalian host. Promastigotes exist in insect vector or *in vitro* culture at 25^0C and are morphologically elongated, flagellated and measure about 10-15 µm. Amastigotes exist in mammalian host cell and are rounded, non-flagellated and measures 3-5 µm in size. Amastigotes predominately exist and multiply in the mononuclear cells such as MØ. Different species of *Leishmania* are morphologically similar and species have historically been classified according to clinical manifestation and geographical distribution as Old World and New World species. The agents of Old World CL are: *L. major, L. tropica* and *L. aethiopica* while agents of the New World CL and MCL are: *L. Mexicana , L.V. guyanensis* and *L.V. brazilienusis panamensis* [29].

6.4 Cutaneous leishmaniasis

The Old World CL is caused mainly by three species*: L. aethiopica, L. major,* and. *L. tropica,* whereas the New World CL is caused by several species and subspecies of *Leishmania*, most commonly by *L. braziliensis* complex and *L. mexicans* complex [7]. After inoculation of infective promastigotes and an average incubation period of about 1 week to 3 months, a typical cutaneous lesion begin as small erythematous papule which enlarges to a plaque or nodule which latter develops into an ulcer or disseminated nodules. The ulcer has a well circumscribed border and its base is granulomatous, crusted and the margins are hypertrophic but without extensive undermining[10]. Ulcers often appear on the face and extremities but also on other exposed areas of the body where infected sand-fly vectors have fed. Clinical presentations of CL are extremely diverse and depend on a variety of parasites and host factors that are poorly understood[7]. The Old World CL lesions are self healing in most cases, however when the lesions are multiple and disabling with disfiguring scars it creates a lifelong aesthetic stigma[27]. As previously reviewed some *Leishmania* species such as *L. aethiopica, L.mexicana* and *L.braziliensis* can produce two clinically different forms of CL[30]. From the Old World CL, infection caused by *L. aethiopica* is unique in that it either results in LCL (ulcerative CL) or DCL (non-ulcerative CL) in its clinical manifestation [25]. *L. aethiopica* infection is restricted to the East African highlands, where it depends on rock hyraxes as reservoir hosts [31].

6.5 Localized cutaneous leishmaniasis (LCL)

Old World ulcerative CL(also refered to as LCL) is a form of CL or Oriental sore that first appears as a persistent insect bite lesion without noticeable heat or pain[25]. The characteristic lesion(fig.3) appears as an erythematous papule at the site of parasite inoculation which increases in size and ulcerates [32]. Inflammatory satellite papules and subcutaneous indurations may develop near the initial lesion representing a reaction to local dissemination of the parasite or its antigenic products [33, 34]. Resolution of the lesion is correlated to extravasation of leucocytes into the infected area leading to inflammation, isolation, necrosis of the infected tissues and the formation of a healing granuloma in the floor of the lesion.

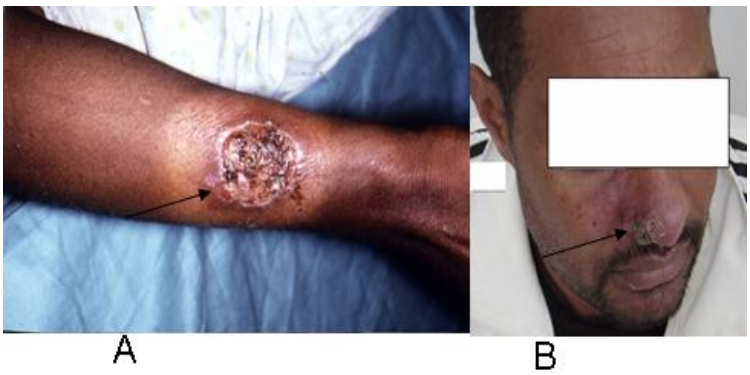

Figure.3 Pictures for *L. aethiopica* induced ulcerative CL patients from Ethiopia. The patients were from highlands of Ethiopia. A) Shows ulcerative CL on hand's of a patient (picture donated by Prof. Hannah Akuffo) and B) ulcerative lesion on a nose of a patient (picture donated by Mr. Endalamaw Gadisa). The black arrows are indicating the lesions.

The necrotic process may be rapid, causing a large and open wet sore especially by *L. major*, or may be more chronic ulceration by *L. tropica*, and *L. aethiopica*. Spontaneous cure without treatment is common, but disease duration varies greatly according to the subtype of parasite and the site of the lesion[25]. The ulcer can remain relatively dry with a central crust (dry form) [32]. Natural healing has been reported to be within 1 to 5 years [35].

6.6 Diffuse cutaneous leishmaniasis (DCL)

Non-ulcerative CL or DCL is a disfiguring and rare form of skin disease (fig.4) which has been described in few countries including Ethiopia. The primary cutaneous lesion begins as a non-ulcerative papule followed by migration of the organisms to other parts of the skin, resulting in disseminated nodules [25, 32]. Although painless, the lesions form papules or multiple nodules, especially on the face and extremities. These nodules are grossly disfiguring and sometimes misdiagnosed as lepromatous leprosy. Unlike lepromatous leprosy, during non-ulcerative CL there is no nerve involvement and the parasite does not invade internal organs.

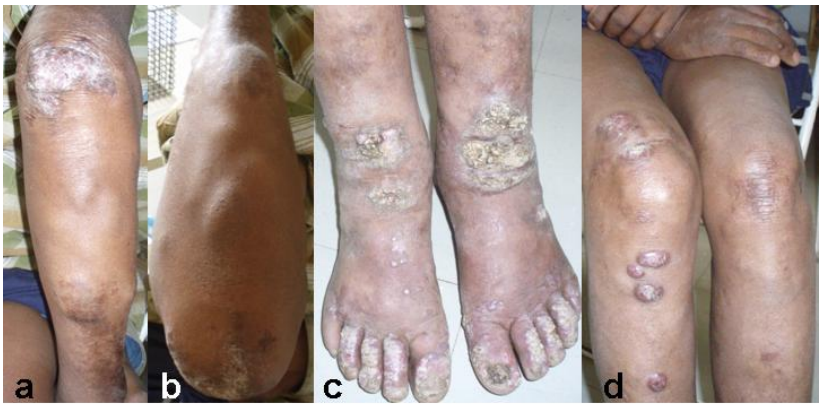

Figure.4 Nodules due to non-ulcerative CL induced by *L. aethiopica* existed over 20 years in a patient in Ethiopia a) Nodules and scares from previous treatment on a right hand. b) Nodules on the forearm looks like big mass c) In lower extremities, the feet were disfigured and were exposed to secondary bacterial and probably fungal infections d) Nodules on the knee right leg are from which silt skin smear and scraping for culture were taken. The samples were positive for both slit skin smear and NNN culture media. These pictures were taken from a single patient at AHRI, Addis Ababa, Ethiopia, 2008.

There is no ulceration or mucosal involvement in non-ulcerative CL. It does not heal spontaneously and responds only partially to treatment with frequent relapses [10]. This chronic skin disease is caused by *L. aethiopica* or *L. amazonensis* [25] or *L. mexicana* [32] which also cause ulcerating CL. The clinical manifestation of the disease is associated with a specific anergy or lack of antigen specific of cellular immune responses, and abundant heavily parasitized MØs in the dermis [5, 6, 32].

6.7 Mucocutaneous leishmaniasis (MCL)

MCL also known as "espunda" is a skin disease which occurs at the site of a sand-fly bite and is characterized by an ulceration of the mucous membrane (fig 5). It is a sequelae of new world CL resulting from direct extension or lymphatic metastasis to the nasal or oral mucosa [36]. It is most commonly reported in the New World [37, 38]. The main etiologic agent of MCL is reported to be *L. braziliensis* [39]. MCL patients suffer from progressively destructive ulcerations of the mucosa, extending from the nose and mouth to the pharynx and larynx. These lesions are not self-healing and often appear months or years after a first episode of cutaneous leishmaniasis when the MØ of the naso-oropharyngeal mucosa become colonized [40].

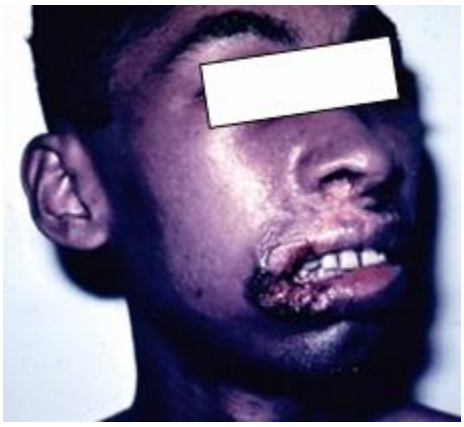

Figure.5 MCL patient with lesion on lips and nose due to *L. braziliensis* complex. This type of disease is reported from in various parts of South America. This picture was taken from: http://www.tulane.edu/~wiser/protozoology/notes/kinet.html**.**

Mucosal lesions develop within two years in 50% of patients and 90% within 10 years of infection. This

extensive tissue damage leads to secondary complications such as pneumonia which could be fatal [10]. Although it is not traditionally considered as MCL, *L. aethiopica* induced leishmaniasis can sometimes have mucus membrane involvement.

6.8 Visceral leishmaniasis (VL)

Kala azar or VL is a systematic disease caused by infection with *L. donovani*, *L. infantum* or *L. chagasi*. The infection is initiated by inoculation of infective promastigotes by the insect vector. The primary lesion of VL consists of a small erythematous papule, sometimes referred to as leishmanioma, is rarely seen [10]. The onset of the disease is gradual and after two to six months of incubation period, VL patients manifest symptoms and signs of persistent systematic infection including fever, fatigue, weakness, loss of appetite and weight loss. Parasitic invasion of blood and reticulo-endothelial system results in enlargement of lymph nodes, spleen and liver [40] leading to characteristic symptoms such as discomfort in the left hypochondrium, splenomegaly (fig 6), lymphadenopathy, emaciation, pancytopenia, and hypoglobulinemia [10].

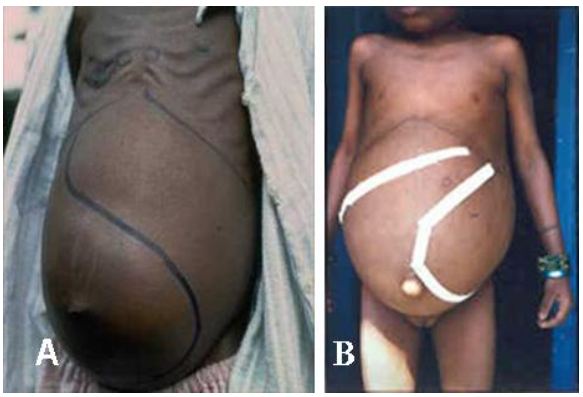

Figure. 6 Pictures (A and B) showing Visceral leishmaniasis patients with spleenomegaly and hepatomegaly. Most often this form of disease is caused by *L. donovani*, *L. infantum*. Fig. A was taken from: http://www.york.ac.uk/res/cii /people/Kaye -Paul. shtml and fig B was from: http://www.who.int/ leishmaniasis/surveillance/Slides manual/en/

Treatment of VL is usually successful in the absence of immunodeficiency but relapses have been reported [41]. A complication with skin involvement commonly known as post kala azar dermal leishmaniasis (PKDL) is mostly caused by *L. donovani*. It has been reported from Sudan and Indian that patients treated for visceral leishmaniasis [42, 43] most often develops PKDL during or within months after treatment [43]. PKDL is characterized by hypopigmented or erythematous macules on any part of the body or redness of the face which may later become popular or nodular lesions on the face. The chronic lesions consist of multiple nodular infiltration of the skin, usually without ulceration which may confuse with lesions of leprosy. In PKDL there is low concentration of the parasite however it may may affect the tongue or the lips and can ulcerate [1, 44]. Sub-clinical infection is reported in VL endemic areas [45].

6.9 Diagnosis of leishmaniasis

The clinical signs and symptoms of all forms of leishmaniasis are non-specific and diffuse. *Leishmania* infection during CL resembles many other skin diseases such as acute furunculosis, foreign-body granuloma, sarcoidosis, carcinoma of the skin, erythema, fungoid infections, tuberculosis and mycobacterial ulcers, tropical ulcer, myiasis, impetigo, lupus vulgaris, yaws, blastomycosis and leprosy [1, 2]. The early lesions of MCL resembles many other disease conditions like polymorphic reticulosis, Wegener's granulomatosis, lymphoma, histoplasmosis, yaws, tuberculosis, nasopharyngeal carcinoma, and other destructive lesions [36, 46, 47]. Similarly, the signs and symptoms during VL infection overlaps with many other diseases such as malaria, tropical splenomegaly syndrome, schistosomiasis, cirrhosis with portal hypertension, African trypanosomiasis, milliary tuberculosis, brucellosis , typhoid fever, bacterial endocarditis, histoplasmosis, malnutrition, lymphoma, leukaemia and others [2, 36]. Definitive diagnosis is mainly based on demonstration of parasites [1, 46]. However, health workers in endemic or epidemic areas often relay on clinical evaluation, which sometimes combined with simple, often inefficient tests to make diagnosis. Over-diagnosis of leishmaniasis may result in fatal consequences since the current drugs of choice for treatment of leishmaniasis have significant toxicity, and therefore treatment should not be given without justification and confirmation of the parasite[48].

Prompt and definitive diagnosis of leishmaniasis is important for initiating appropriate clinical management and treatment of the disease [46, 49]. The diagnosis of leishmaniasis is reliably made by the demonstration of the parasite. Other laboratory diagnosis include, immunological, and molecular methods [2, 36]. The commonly used parasitological methods for demonstration of *Leishmania* parasites in clinical samples are:

1) Microscopic examination: Microscopic examination of relevant tissues aspirates or biopsies such as bone marrow, spleen, lymph nodes or liver, skin slit smears or biopsies[2, 36] from leishmaniasis patients could reveal *Leishmania* amastigotes. The amastigotes are readily seen in smears or touch preparations of infected tissue stained with Giemsa's stain (pH 7.2) and sections of tissue stained more conventionally with haematoxyline and eosin (H & E) are much more difficult to interpret. To ensure that the visualized structures are amastigotes, rather than other "dot"-like structures (*e.g.*, *Histoplasma* spp¸ platelets), an experienced observer should look for the

characteristic size (2-4 mm in diameter), shape (round to oval), and internal organelles, the nucleus and kinetoplast. With Giemsa staining, the cytoplasm typically takes pale blue; the nucleus and kinetoplast take purple-pink colour.

2) Culture examination: Confirmation of the parasite causing leishmaniasis by the use of culture method is desirable to avoid misdiagnosis of leishmaniasis. In addition isolation of the parasite in culture is the most appropriate diagnostic method to preserve the organisms for long period of time to be used for future reference. The non-motile amastigote form of the parasite in a clinical samples transforms into a motile promastigote on solid NNN medium containing 20-30 % rabbit or sheep blood after 3 days of incubation at 22-26 ^{0}C [1, 2] . Various other liquid media including RPMI 1640, M199 or Tobies medium supplemented with 10 % foetal calf serum can be used [2, 9]. Recently human urine has been described to be used instead of foetal calf serum in *in vitro* culture of *L. donovani* [50]. Culture based diagnosis of MCL has low sensitivity as the organisms are often scanty [2].

3) Isolation in experimental animals: As an alternative method, inoculation of the clinical material obtained either into a susceptible BALB/c mouse or into a hamster footpad or nose may be used for cultivation of certain *Leishmania* parasite. Histopathologic evaluation of biopsy samples of animal lesions may be characteristic but is rarely specific enough to make a diagnosis without identification of the amastigotes [2].

4) Other immunological methods: Immunological method which could have value in the diagnosis of VL are: Direct agglutination test, Fluorescent antibody test, Enzyme linked immunosorbent assay (ELISA), an immunochromatography using a recombinant antigen (rK39), Immunoblotting, and Leishmanin skin test (LST) are some of the methods used. Despite the availability of large number of serological tests, no serological method is helpful for CL and MCL because antibodies tend to be undetectable or present in low titre due to poor humoral response [51, 52]. Molecular methods require expertise and expensive equipments but are increasingly becoming relevant to diagnosis infectious diseases. Polymerase chain reaction (PCR) is among the molecular methods used for diagnosis of clinical leishmaniasis and has been proved to be the most sensitive and specific diagnostic tool available [2].

6.10 Establishment of infection and intracellular survival

Leishmania are unicellular and obligatory intracellular organisms that live exclusively in monocytic cells (monocytes, histocytes, MØ and DC) in their mammalian hosts. Soon after initial inoculation of metacyclic promastigotes into dermis, the complement system as one of the innate immune defence mechanisms starts to clear this pathogen from the host cells. Metacyclic promastigotes may have the capacity to activate complement pathways [53]. They are also able to express protein kinases that phosphorylate C3, C5 and C9, which leads to inhibition of complement activation.

The two major surface molecules of *Leishmania*, lipophosphoglycan (LPG) and glycoprotein 63(gp63) are used for attachment to the host cell by mediating the binding of C3bi to the parasite surface. This attachment to host cell leads to activation of complement which results in an opsonization process. The opsonization process helps *Leishmania* parasites to escape smartly from the hostile extracellular environment by promoting phagocytosis through Complement receptors as previously reviewed [54].

Furthermore complement such as C3a and C5a serve as chemo-attractants for other cells[55, 56]. Along gradients of such complements and some chemokines (macrophage inflammatory protein (MIP)-1a, MIP-2), some inflammatory cells (such as monocytes and neutrophils) migrate to the site of invasion in the skin to counteract the infection. Infection of MØ with *Leishmania* via CR3 is a silent process and usually established without the knowledge of the immune system. The parasites within MØ start to replicate until the infected cell ruptures and more surrounding cells are infected[57].

The other important strategy for establishment of infection by *Leishmania* is to invade the immune system by entering into granulocytes [58]. Neutrophils are among the primary granulocytic leucocytes infected by *Leishmania.* Upon ingestion of *Leishmania* they secrete high levels of MIP-1β, which in turn attracts MØ. The attracted MØ now readily phagocytose infected neutrophils, thus *Leishmania* can also use neutrophils to silently enter into their final host cell [54]. These parasites have adapted different ways to avoid intracellular killing and are able to multiply in hostile environment of MØ. They have higher chance of survival within the cell by adjusting the pH [59, 60] and by inducing enzymes to detoxify oxygen metabolites and other leishmanicidal products produced by the infected cell [61]. Some evidence also suggest that there

is suppression of MHC (class I and II) expression on the infected cells, thus interfering with proper presentation of parasite peptide for T cell activation to produce IFNγ[62]. Promastigotes that do not penetrate monocytes are killed, probably by complement mediated lysis [1]. The chances that *Leishmania* will enter the target cells are increased because they bind to target cells at multiple receptor sites. Infected MØs have been shown to produce colony stimulating factor(CSF) which stimulate precursor cells there by providing new target cells for the parasite [1]. Delaying apoptosis in infected cells by *Leishmania* parasites [63] may also be another strategy to provide an intracellular environment for the pathogens by extending the life span of the host cells[63, 64].

Healing from *Leishmania* infection often leads to lifelong immunity against the same subspecies[54] . An immune response toward *Leishmania* infection begins with activation of the innate immune system through Toll like receptor-2 (TLR2) presented on MØ, DC and NK cells. TLR-2 recognize LPG expressed on the parasite surface and lead to production of proinflammatory cytokines such as TNFα, IFNγ and IL-12, as well as co-stimulatory molecules [65, 66]. Cell mediated immunity (CMI) rather than antibody mediated mechanism is important for clearing *Leishmania* infection since the parasite is an obligate intercellular organism residing in host cells. The elimination of *Leishmania* by CMI involves activation of MØ that lead to killing of *Leishmania* parasites. The MØs play a major role in this regard as they are host cells and effector cells whose leishmanicidal efficacy depends on the presence of activating cytokines such as IFNγ and TNFα [67]. These cytokines can partially be produced by the innate immune system during the initial inflammatory response; but a parasite-specific T cell response is necessary for clearance and determines the disease outcome [30]. The paradigms of T helper (Th)-subset involvement in infectious diseases are indicated, in large part, on the results of studies of resistance and susceptibility to *L. major* in inbred mice. In murine *L. major* infection , it has been established that a T helper -1(Th1) response leads to the cure of the disease, whereas a T helper -2(Th2) response leads to disease progression [68]. BALB/c mice responded to infection with preferential production of Th2-type cytokines, particularly interleukin (IL)-4 and IL-10,. These display susceptibility to *Leishmania* and non-healing infections. Recovery from infection by resistant mice (e.g. C57BL/6) has been linked to induction of a polarized Th1-type response, resulting in MØ activation and killing of the parasites [69]. As reviewed by Ruiz and Becker, in animal model, the immune system may control *Leishmania* through cytotoxicity by

$CD4^+$ T, $CD8^+$ T, and NKT cells, mainly through mechanisms involving antigen-dependent or independent apoptosis of target cells [30].

Two mechanisms of T-cells induced cytotoxicity have been proposed: either through the exocytosis of lytic granules that contain molecules such as perforin, which form pores in the lipid membrane of the target cells leading to collapse of the membrane potential allowing lytic granuels to enter the cell. The lytic granules of the cathepsin family, such as granzyme B, activate a signalling cascade leading to caspase-mediated apoptosis. The second cytotoxic mechanism is through the membrane expression of FasL/CD95L, which induces the trimerization of its receptor Fas/CD95 on target cells, initiating apoptosis through activation of caspase 8[70]. Moreover TNF and TRAIL also induce apoptosis through similar mechanisms as FasL [71]. The regulation of immunity against *Leishmania* infection appears to be very complex. Progress in current pathogenesis of leishmaniasis research has significantly contributed to a better understanding of protective immunity against *L. major* and the hope is that current research will form a basis for the development of an effective vaccine.

6.11 *Leishmania aethiopica*

Leishmania aethiopica is the main causative agent of CL and a zoonotic parasite transmitted to humans in the highlands of Ethiopia and Kenya at altitude between 1700-2700 meters [72] with rock hyraxes as reservoirs, and *Phlebotomus pedifer* and *Phlebotomus longipes* as vectors [35, 73]. Leishmaniasis caused by this parasite was also described in Israel due to migration of Falashas from Ethiopia to Israel [74]. *L. aethiopica* causes two different clinical manifestations: ulcerating, self healing often described as localised CL (LCL) and non-ulcerating, non self-healing and disseminated form known as diffuse CL (DCL) [5, 6]. Ulcerative CL display strong cell-mediated response and healing eventually results within 1 to 5 years [35] with characteristic depressed scar formation and apparent solid protection against re-infection. Non-ulcerative CL patients produce little or no cell-immunity against the infecting parasite and spontaneous healing does not occur. This condition is characterized by the formation of disfiguring nodules that contain large numbers of amastigotes and is very resistant to leishmanicidal treatment [5, 6]. Non-ulcerative CL is associated with antigen specific unresponsiveness [15, 75]. The same parasitic species (*L. aethiopica*) has been reported to cause both ulcerative CL and non-ulcerative clinical spectrum of skin diseases in Ethiopia[17]

6.12 *Leishmania* Hiv co-infection

Since 1998, in many parts of the world, leishmaniasis is considered and rising as the third most frequent opportunistic infection in patients with HIV/ADIS [76, 77]. It attacks the immune system, and worsens the patient's already immuno-compromised by HIV[76]. Leishmaniasis-HIV co-infection has been reported in about 34 countries around the global[78] . HIV-VL co- infection is considered as a major AIDS–defining illness with high relapse and mortality rates [79]**.** It is believed that chronic immune activation induced during parasite infection increases HIV viral load and enhances the progression of AIDS in HIV patient[80]. Such Immunological disorders due to HIV may provide favorable condition for the uncontrolled multiplication of the parasite during HIV co- infection[81]. Similarly some studies have shown that leishmaniasis amplifies an increase of serum HIV-1 viral load[82, 83], enhances the rapid progression of HIV infection to AIDS stage and reduces life expectancy in HIV-infected patients [49]. LPG of *Leishmania* promastigotes and its intra-membrane structural component in amastigotes were shown to have capability in promoting virus replication in T cells through complex biochemical pathways[83].In response to *Leishmania,* HIV-1 may modulate in vitro and ex vivo cytokine production[84] . Greater quantities of IL-4 and IL-10 were released from PBMC of *Leishmania* - HIV co-infected patients than those from HIV-positive patients but not infected with *Leishmania* [85]. HIV-1 infected cells secreted large amounts of protein called transcriptional trans-activator (Tat) to override the leishmanicidal effect of IFN-γ and enhance *Leishmania* replication in human macrophages [81]. T-cell immune response shifts to the Th2 type in response to *Leishmania* infection in patients with T-lymphocyte defects due to HIV-1[82]. It has been confirmed that HIV-1 inhibits the proliferative response to *L. donovani* [86].

7 THE SKIN AND ITS STRUCTURE

The skin is an ever-changing and the largest organ of the body with many different specialized cells and structures[87-89] . It functions as a protective barrier that interfaces with the hostile environment and is involved in maintaining the proper temperature for the body[87, 88]. The skin gathers sensory information from the environment, and plays an active role in the immune system protecting the body from pathogens[87]. Dendritic cells such as the Langerhan's cells of the epidermis and dermal dendritic cells of dermis steer adoptive immune responses. The skin also acts as a storage centre for lipids and water[88], and vitamin D is synthesised in parts of the skin exposed to sunlight[89]. The skin has got three layers (fig.7), the epidermis, the dermis and the hypodermis (subcutaneous adipose layer).

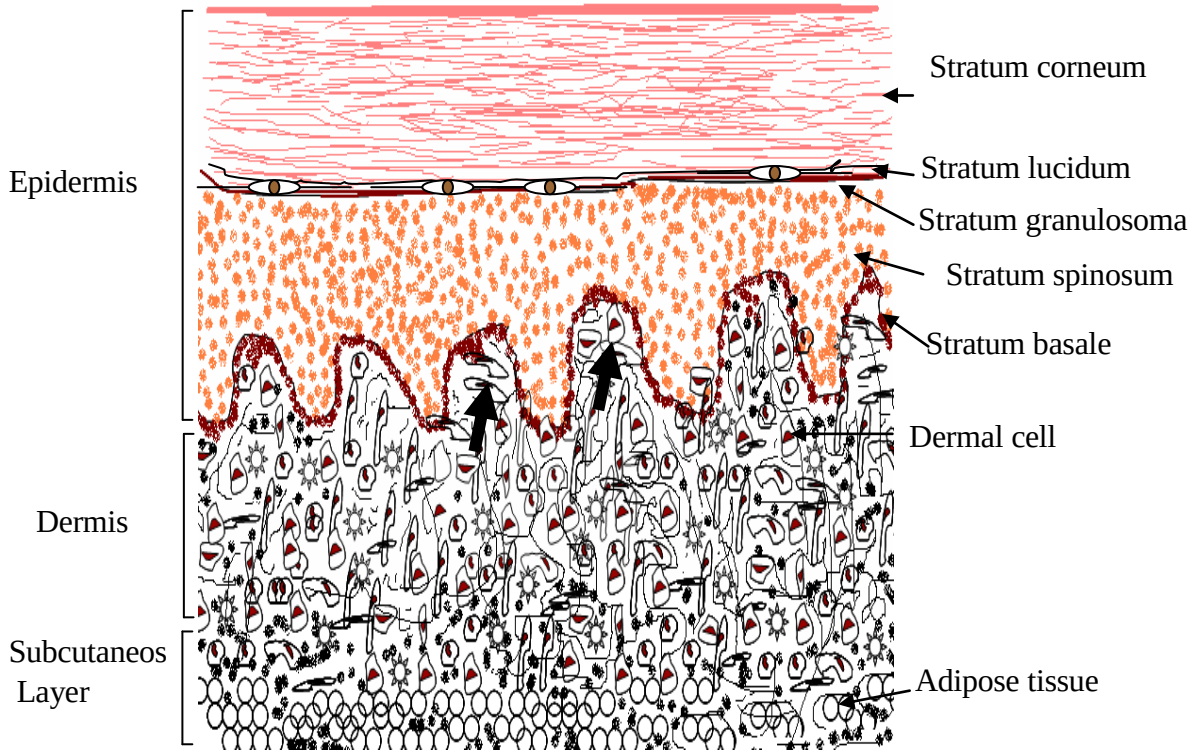

Figure.7 Schematic representation of skin structure with its three layers. The two black arrow heads show dermal papillae of dermis.

7.1 Epidermis

Epidermis is the outer most layer of the skin (fig.7) which provides waterproofing to the skin and serves as a physical barrier to infection. The thickness of the epidermis varies in different types of skin. It is composed of stratified squamous epithelium and contains four principal types of cells. From these cells, about 90% are keratinocytes which are weld to one another by anchoring junctions known as desmosomes[88, 90]. The second cell type in this layer is melanocytes which produce the pigment melanin, and comprise about 8% of the epidermal cells. Melanin is a brown-black pigment that contributes to skin colour and absorbs ultraviolet (UV) light [88, 91]. The third type of cell in the epidermis is Langerhan's cells or dendritic cells residing and renewing in the skin in homeostasis. They interact with helper T cells in immune responses. A fourth type of cell found in the epidermis is known as Merkel cells[88]. These cells are located in the deepest layer (stratum basale) of the epidermis of hairless skin, where they are attached to keratinocytes by desmosomes. The exact function of theses cells is not well know[87] but it seems to involve in light touch sensation[88]. In most regions of the body, the epidermis is about 0.1 mm thick except in the palms and soles where thickness is estimated to l to 2 mm[88, 89] due to exposure to friction is greater. The epidermis contains no blood vessels, and is nourished by diffusion of nutrients from the dermis. Epidermis is divided into layers at the innermost layers (stratum basale). The cells move up the strata changing shape and composition as they differentiate and become filled with keratin[88]. They eventually reach the top layer called stratum corneum and become sloughed off, or desquamated, a process known as keratinization and takes place within weeks. The 5 sub layers of epidermis are named from bottom to top as: stratum basale (germinativum), stratum spinosum, stratum granulosum, stratum licidum, and stratum corneum[87, 90]. The stratum basale, the bottom sub layer, has cells shaped like columns. In this layer the cells divide and push already formed cells into higher layers. As the cells move into the higher layers, they flatten and eventually die.

7.2 Dermis

The dermis is the second layer of the skin beneath the epidermis (fig.7). It is composed of connective tissue containing collagen and elastic fibres. It is very thick in the palms and soles, and very thin in the eyelids, penis and scrotum. Blood vessels, nerves, glands, and hair follicles are embedded in the dermis. The dermis is tightly connected to the epidermis by a basement

membrane and harbours many nerve endings that provide the sense of touch and heat. It contains the hair follicles, sweat glands, sebaceous glands, apocrine glands, lymphatic vessels and blood vessels [87, 88]. The blood vessels in the dermis provide nourishment and waste removal to its own cells as well as the stratum basale of the epidermis[92]. It contains specialized cells such as fibroblasts, mast cells , MØ and nerve cells., Structurally the dermis is divided into two areas: a superficial area adjacent to the epidermis, called the *papillary region*, and a deep thicker area known as the *reticular* region[87, 88]. The papillary region is composed of loose areolar connective tissue. It is named for its finger like projections called *papillae*, which extend toward the epidermis. The papillae provide the dermis with a "bumpy" surface that interdigitates with the epidermis strengthening the connection between the two layers of skin.

7.3 Hypodermis

The hypodermis lies below the dermis (fig.7) which attaches the skin to underlying bone.and muscle. It supplies the skin with blood vessels and nerves[87]. It consists of loose connective tissue and elastin. The main cell types existing in this layer are fibroblasts, MØ and adipocytes. The hypodermis contains 50% of the total body fat which serves as padding and insulation for the body.

8 PROGRAMMED CELL DEATH (PCD)

Programmed cell death (PCD) is a genetically regulated active process that follows intracellular or extracellular stimuli. It has been identified in all analysed multicellular organisms and in an increasing number of unicellular organisms. The most common types of PCD are apoptosis (type I PCD) and autophagy (type II PCD) which have clear differences in the molecular background and in the time course of biochemical and morphological events [64].

8.1 Apoptosis

Apoptosis is a process of controlled cell death usually seen in multi-cellular organisms. In contrast to necrosis it is an ordered and efficient process that prevents initiation of the inflammatory pathway, and therefore it confers advantages for the whole organism [64]. It plays an important role in the normal tissue development, in the pathogenesis of different infectious diseases and in the elimination of harmful or unnecessary cells from the body system [93]. The process of apoptosis is the orderly and immunologically silent process which dismantles off a cell into pieces following the activation of a family of cysteine proteases known as caspases (cysteine-aspartic acid proteases) [93, 94]. This process can be initiated by an extrinsic death receptor (DR) pathway and an intrinsic (mitochondrial or stress-induced) pathway. The final stage of apoptosis is associated with distinct morphological and biochemical changes, including cell shrinkage, membrane blebbing, altered plasma membrane permeability, exposure of phosphatidylserine (PS), loss of mitochondrial integrity, chromatin condensation with DNA fragmentation and protein cleavage [64]. It has a crucial role in the maturation of the immune system and the specific immune response. Infectious agents from viruses to parasites can either delay or induce apoptosis of different types of host cells. Apoptosis following lymphocyte polyclonal activation and stimulation of peripheral T lymphocytes, as a result of the engagement of specific counter receptor systems, is a good example for defining host immuno-competence and mechanism of immunopathology as reviewed in [95]. Activation by cleaving a set of proteins of proteases of the caspase family causes disassembly of the cell. This caspases mediated proteolytic cascade represents a central point in apoptotic response and therefore its initiation has to be well controlled. Among these regulatory factors DRs, B-cell lymphoma 2(Bcl-2) family proteins and the tumour suppressor protein (p53) are of central importance [96, 97]. The process of apoptosis does not harm adjacent cell since

cellular membrane remain intact during apoptotic cell death, preventing escape of cellular material from apoptotic bodies and reducing any onset of inflammatory cells [98].

8.2 Autophagy

Autophagy is a process by which cells digest parts of their own cytosolic material. This allows the recycling of molecules under conditions of nutritional limitation and remodelling of intracellular structure for cell differentiation. It includes sequestering of long-lived proteins and organelles in a double membrane vesicle (the autophagosome), which is delivered to the lysosome for protein degradation[99, 100]. Cells can undergo a delayed autophagic form of cell death as an alternative to apoptosis. Autophagy has initially been described in eukaryotic cells as a rescue mechanism that is induced upon starvation or oxidative stress [99]. Limited self-digestion of cell materials, including organelles, can help individual cells to provide energy and to facilitate survival for up to several days. However, if conditions do not improve, self-digestion continues and eventually results in autophagic cell death [99].

8.3 Necrosis

Necrosis is uncontrolled death of cells in tissues or organs, usually caused due to inflammation, cancer, injury or infections [101]. It takes place as a consequence of the interruption of cellular processes and this would be accompanied by the release of lysosomal enzymes [64]. In contrast to apoptosis, the process of necrosis usually involves groups of cells, loss of energy, normal cellular ion and water controls, cell swelling, extensive membrane and intercellular organelle disruption and lysis. The distribution of the lytic cellular enzymes into the surrounding tissue induces a massive leukocytic inflammatory response, which may be extremely destructive. Typically necrosis involves irreversible changes within the nuclei (such as Karyolysis), loss of cytoplasm, structure, dysfunction in various organelles (especially mitochondria) and finally cytolysis as a result of high amplitude swelling. The release of dying cells' contents into the intracellular space can cause further injury or even death of neighbouring cells and may result in inflammation or infiltration of proinflammatory cells into the lesion leading to further tissue damage [102].

8.4 Morphology of apoptosis

Apoptosis is morphologically defined by cellular and nuclear shirinkage (Pyknosis), chromatin condensation blebbing, nuclear fragmentation (Karyorrhexis) and the formation of apoptotic bodies [101]. Apoptosis involves a sequence of distinct morphological changes that remain as a reliable means of identification of apoptotic cells. Several biochemical means of identification of apoptosis of cells have been developed after the discovery that apoptotic cells were accompanied by double stranded cleavage of nuclear DNA at regular 123 base pairs interval [98].The morphological characteristic of apoptotic cells in histological haematoxylin eosin staining (*in vivo* experiment) exhibit dense, dark blue, nuclear chromatin fragments, and the condensed cytoplasm is eosinophilic (pink or orange). In an *in vitro* homogenous adherent cell culture in the absence of phagocytic cells, apoptotic cells tend to detach from the growth plate, showing rounding , loss of surface villi and blebbing of apoptotic body [98].

8.5 Death receptor mediated apoptosis

The extrinsic pathway originates at the membrane and engages cell surface involving several DRs including Fas, TNFR-1, DR3, DR4, DR5 and DR6. These death receptors contain an intracellular region of approximately 80 amino acids that is designated as "death domain" (DD). The DD is an important structure that plays a key role in the transduction of apoptotic signals. Each DR is independently activated via its respective ligand. The death ligands are related to cellular expressions that include TNFα, FasL and TRAIL [103]. Upon ligand binding, the activated DRs trimerize and recruit intracellular adaptor molecules as well as proximal caspases to transduce apoptotic signals [104, 105].

8.6 Activation Induced Apoptosis

Apoptosis was originally described as protective mechanism against disease by eliminating unwanted cells[106]. It plays a major role in many diseases and regulates cell turnover [107]. To mention a few diseases in which apoptosis are involved, acute as well as chronic asymptotic *P. falciparium* infections may be associated with a marked increase in the level of mononuclear cell apoptosis [108]. Inhibition to apoptosis of host cells by intracellular pathogens such as *Theileria* [109], *Toxoplasma* [110] or the agents of human granulocytic ehrlichiosis [111] could provide an intracellular environment for the pathogens. The general characteristics of apoptosis are well

established and occur through distinctive morphological and molecular characteristics including chromatin condensation, fragmentation of DNA into oligonucleosome size pieces, swelling and progressive cell degradation [106, 112, 113]. The molecular mechanism operating behind apoptotic events remains fully unknown. However one known route of induction of apoptosis is activation induced cell death (AICD) induced by pro-apoptotic FasL and TNF-α [114]. FasL induces programmed cell death or "apoptosis" in cells expressing its cognate receptor, Fas [115]. Fas is a molecule belonging to the TNF- receptor (TNFR) family, and can be expressed in trans- membrane or soluble form and mediates apoptosis upon engagement by its ligand, FasL [116, 117]. It is widely expressed in numerous different cell types through out the body, whereas FasL expression appears to be more restricted [115]. Following activation, different cell types within the immune system express FasL including T and B cells [115, 118]. FasL expression on T-cells induces contact mediated apoptosis of Fas positive cells [119]. Although the full spectrum of physiological roles for Fas-FasL system has yet to be determined, the receptor- ligand is best known for its role in immunoregulation [115]. Fas and FasL are co-expressed on the surface of activated lymphocytes, and Fas mediated autocrine 'suicide' or juxtacrine 'fratricide' (killing of neighbouring cells upon contact via mutual triggering of Fas by FasL expressed on both cells) of lymphocytes help to terminate immune response [120]. In addition to Fas/FasL apoptosis pathway, more recently a report from our lab indicated the role and involvement of TRAIL pathway in the keratinocytes apoptosis and skin ulceration during CL induced due to *L. major* [121]. TRAIL is a newly identified member of the Nerve Growth Factor (NGF) superfamily which exist as a type II membrane protein or as a soluble protein (41kDa) in culture supernatants[122]. Membrane bound TRAIL (mTRAIL) and soluble TRAIL (sTRAIL) were shown to induce apoptosis in susceptible cells upon trimerization of their receptors and after activation of the caspase cascade, leading to fragmentation of DNA [123]. TRAIL induces apoptosis selectively both *in vitro* in transformed cells and *in vivo* in tumor cells but not on most cells except in thymocytes, neural cells and hepatocytes. However the regulation of TRAIL induced apoptosis appears to be complex [124]. An experimental study conducted on $TRAIL^{-/-}$ mice have shown that TRAIL deficient mice not only have a defect in negative selection but also have an increased susceptibility to autoimmune disease [125].

8.7 FasL induced apoptosis

The contribution of FasL in the complete control of parasite replication was indicated on experimental study in mice with *L. major* [126]. Studies in a non-ulcerative mouse model using a high infectious dose of *L. major* proposed that Fas-FasL pathway is necessary in the elimination of parasites. Administration of soluble exogenous recombinant FasL to FasL deficient mice, resulted in the resolution of non-ulcerative cutaneous lesions caused by *L. major*. *In vitro* experiments have shown that MØ infected with *L. major* up-regulate their surface Fas expression in response to IFNγ and as a result become susceptible to CD4+ T cell- induced apoptotic death [127].

8.8 TRAIL induced apoptosis

TRAIL (also known as Apo2-Ligand) is a death ligand that belongs to TNF super-family [105, 128]. It mediates its apoptotic effects by binding to its death receptors as a homotrimer. Several TRAIL receptors have been discovered to date including TRAIL-R1(DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1) and TRAIL-R4(DcR2) [103]. TRAIL-R3 and TRAIL-4 are designated as anti-apoptotic receptors that antagonize TRAIL-induced apoptosis. TRAIL-R3 does not harbour the death domain while TRAIL-R4 contains a deleted death domain and therefore both of these receptors are not capable to induce apoptotic signals [128]. Both, however, compete with TRAIL-R1 and TRAIL-2 for TRAIL-binding and upon over expression interfere with TRAIL-induced apoptosis. Osteoprotegerin (OPG) is an additional TRAIL-interacting molecule [129] that exists in soluble form. TRAIL and its receptors are important components of the extrinsic pathway of apoptosis. Upon activation, both DR4 and DR5 interact with Fas associated death domain (FADD), an intracellular adaptor molecule that bridges their interactions with the initiator caspases. It is also important to notice that TRAIL-R4 may contribute to the NF-κB activation resulting in inflammation[130].

8.9 Alteration of apoptosis during leishmaniasis

Many intracellular microbial pathogens influence host cell apoptosis, either to increase their life span within infected cells[63] or to spread infection to other cells[131, 132]. On the contrary, the host immune response induces apoptosis of infected target cells in order to remove intracellular pathogens. Both host and pathogen induce lymphocyte apoptosis in the course of infection, which

could alter the quality of subsequent immune responses [95]. Infectious agents such as viruses and parasites cause vigorous polyclonal immune responses in the host. These responses, in turn, are subjected to apoptotic mechanisms of suppression and creating a complex balance for immunoregulation. Effector leucocytes are produced to cope with the infection and at the same time must be eliminated to avoid the immunopathology that results from their unwanted continual activity. When apoptosis based immunoregulation shortens effector cell activity before the pathogen is eliminated, chronic infection may develop [95]. The best understood PCD mechanisms, in the host immune system, are those involving mature (peripheral) T lymphocytes which has two largely distinct mechanisms of PCD. The first is passive cell death [133], where inappropriately stimulated T cells become committed to die. It has been demonstrated that T cells can be protected from passive cell death by exposure to IL-2, or by co-stimulatory signals delivered through B7/CD28 interactions, which up-regulate expression of the anti-apoptotic protein Bcl-xL [133]. The second mechanism is activation-induced cell death (AICD), in which chronically activated T cells initiate an active, single cell-dependent death response (suicide) or dependent on cell interaction (fratricide). AICD requires appropriate expression and function of cell surface death receptors and ligands, such as the Fas/ FasL counter-receptor system, which is the major molecular effector of AICD [133]. More recently, it became clear that Fas/FasL pathway is involved in PCD induced by a variety of other chemical and physical injuries. This may indicate that it can also be expressed by many cell types under conditions of stress or inflammation. Recognition and engulfment of apoptotic bodies are major functions of phagocytes, both in embryogenesis and during immune responses[134]. More importantly, phagocytosis of apoptotic bodies by MØ triggers an autocrine, and possibly also a paracrine, anti-inflammatory response mediated by released factors such as prostaglandin E2 (PGE2), transforming growth factor 3 (TGF3) and platelet activating factor (PAF) [135], which could have further consequences for the immune regulation of parasitic infections.

There are three main ways in which induction of host cell apoptosis could benefit parasites and thus confer a selective advantage. First, host cell apoptosis could assist in the spreading of infection as a virulence mechanism, when infected MØ or other tissue cells ruptured as well as by direct killing of uninfected leucocytes by parasite molecules[58]. Second, parasitic infections hyperstimulate host lymphocytes and take advantage of the marked immunoregulatory changes that follow polyclonal lymphocyte activation[136, 137]. Third, host cell apoptosis could stimulate

the production of essential growth factors for parasites [138]. However, at the same time, intracellular parasites also need to inhibit apoptosis in their host cells, in order to complete their intracellular life cycle and so it is not surprising that both anti- and pro-apoptotic molecules could exist in a single virulent intracellular pathogen [139] and as reviewed by Schaumburg, F.,et al [140]. It is likely that expressions are differentially regulated by the pathogen according to their needs. Several pathogenic parasites are potent inducers of host polyclonal lymphocyte activation, both *in vivo* and *in vitro.* For instance, potent polyclonal T cell responses *in vitro* to live parasites or parasite antigens have been described for *Trypanosoma cruzi, Toxoplasma gondii, Plasmodium* and *Leishmania* infections in humans. Although parasite mitogens cannot be excluded, the primary response usually requires antigen processing, and major histocompatibility complex (MHC) presentation [141, 142]. Protozoan parasites activate a large fraction of both naive and memory T cell repertoires directed against a large set of cross-reactive peptide-MHC complexes. Accordingly, several studies have demonstrated antigen-specific and polyclonal T cell unresponsiveness following initial polyclonal activation by the parasitic infection[95] .

9 RESEARCH QUESTION

L. aethiopica is a protozoan parasite infecting human skin mononuclear cells. It induces two different spectrums of diseases namely; ulcerative form known as localized or ulcerative CL which is a self-limited, and a more severe (a rare case), non ulcerative disseminated form known as diffuse or non-ulcerative CL in Ethiopia. The pathogenesis behind the two different manifestations of *L. aethiopica* induced CL is not known. *L. aethiopica* induced CL pathology resembles many other skin diseases and it is difficult to initiate treatment with clinical signs and symptoms alone. This is a challenge for low income countries where many common tropical skin diseases are overlapping in *Leishmania* endemic areas. Therefore this thesis is addressing:

1) Could it be possible to cut the cost of culture media by developing low cost culture media from inexpensive locally available ingredients, which may have contribution in improving the diagnosis of cutaneous leishmaniasis in low income country?

2) Are the different clinical manifestations of *L. aethiopica* induced CL associated with different expression of apoptosis-related molecules at the site of infection as a possible explanation to the clinical outcome?

10 AIMS

The main objectives of this thesis work is to find an alternative culture media that could minimize the cost of culturing *Leishmania* from patient's samples, and to investigate if differential dysregulation of apoptosis is displayed in different manifestations of *L. aethiopica* caused CL.

Specific aims:

1. To develop an alternative culture media that could minimize the cost for culturing *Leishmania* from patient lesions.

2. To compare the extent of apoptosis in skin lesions/ nodules from ulcerative CL versus non-ulcerative CL induced due to *L. aethiopica.*

3. To determine the magnitude of FasL and TRAIL expressions in non-ulcerative and ulcerative manifestations of *L. aethiopica* induced CL.

11 ETHICAL CONSIDERATION

The study was ethically reviewed and approved in Ethiopia and in Sweden before implementation. Informed consents were obtained from all sample donors for the usage of biological material.

12 VOLUNTEERS, MATERIAL AND METHOD

12.1 Preparation of *Leishmania* culture media

a) LSSM, a modification of NNN media, was prepared by dissolving 9.2 gm nutrient agar (Oxoid Ltd, England), 0.6gm D (+)-glucose (Sigma Chemical Co.), 2.4gm NaCl (East Anglia Chemicals, LRG) in 400ml-distilled water. After sterilized by autoclaving, aseptically collected de-fibrinated sheep blood was added to a final concentration of 15%-20 %(75-100ml) inside safety cabinet. Aliquot of the mixture was dispensed into the culture flasks and was kept in a sloped position until the agar has set. Locke's overlay was prepared by dissolving 9.0g NaCl (East Anglia Chemicals, LRG), 0.4g KCl (Sigma Chemical Co.), 0.2g $CaCl_2$ (E. Merck, Darmstadt), 0.2gm $NaHCO_3$ (Sigma chemical co.) 2.5g D (+)-glucose (Sigma chemical co.), in 1000ml distilled water, and was then autoclaved.

b) RPMI 1640 was prepared by dissolving 10.43gram RPMI1640 (Gibcobrl, life technology) in 1000ml distilled water and autoclaved for 30 minutes at 121 ºC.

c) GALF-1 was prepared by mixing three cheap commercially available products (Table 1). Each of the three products was purchased in Ethiopia already composed as an ionized solution, a powder mixture or a health supplement tablet. The two dry ingredients were dissolved in the ionized solution without the need for additional distilled water or autoclaving. GALF-1 was then supplemented with 20% of heat inactivated FBS (Hyclone, USA) and 2 %(v/v) of sterile human urine obtained from apparently healthy men.

All the three media were supplemented with 100U/ 100-µg/ml penicillin-streptomycin (Sigma Chemical Co.), 2 mM L-glutamine (Flow Laboratories, Irvine, Scotland). The pH of the media was then adjusted to 7.1 ± 1 using 1N HCl / NaOH and finally filtered through 0.22µm pore size filter, cellulose acetate membrane polystyrene filter (Falcon, Becton Dickinson, England) which were at 4 ^{0}C in a fridge until used.

Table.1 The composition of GALF -1 media. GALF-1 preparation is simple and the ingredients are composed of ionized solution, mixture of powders and ground nutritional supplement tablet. All ingredients were dissolved well in the ionized solution and did not require autoclaving, and extra distilled water.

GA-ionized solution	mg/L	GL- powder	mg/L	Compound in tablet form	mg/L
Ca^{++}	72	Glucose anhydrous	20000	Ascorbic acid	150
Cl^{-}	32.5	$C_6H_5Na_3O_7.2H_2O$	2900	calcium pantothenate	25
Fe, total	0.08	KCl	1500	cyanocobalamin	0.015
$HCO3^{-}$	1128	Lemon extract	800	Folic acid	1.5
K^{+}	35			Niacin amide	45
Mg^{++}	46			Nicotinic acid	15
Mn^{++}	0.15			Pyridoxine hydrochloride	3
Na^{+}	252			Riboflavin	10
$PO4^{---}$	0.06			Thiamine nitrate	10
$SO4^{--}$	0.77				

12.2 Skin amastigotes transformation to promastigotes in medium

Lesion scrapping samples from CL suspected patients were aseptically inoculated into culture flasks (Falcon, Becton Dickinson, England) containing RPMI 1640, LSSM or GALF-1 medium and were incubated at 26 ^{o}C. The cultures were inspected every 24 hour under an inverted microscope for parasite growth. The amastigote transformation time (ATT) is the day when promastigotes are first observed in the culture was recorder. Promastigotes originally cultured in RPMI 1640 medium were used for growth curve analysis in the three media.

12.3 Cryopreservation and re-culture potential

Seven isolates of promastigotes that appeared and grew in GALF-1 medium were sub cultured two to three times in GALF-1 and were cryopreserved in liquid nitrogen as previously described [143]. Promastigotes kept in liquid nitrogen were thawed and cultured after differen t time intervals of storage (1, 3, 6, 12, and 24 months) and were observed for motility and the results were recorded. Promastigotes originally cultured in RPMI 1640 medium were used for growth curve analysis in the three media.

12.4 Media cost analysis

For each 100ml of LSSM, RPMI 1640 and GALF-1 medium, approximate cost was calculated using the archived document from the EHNRI laboratory chemical store, Addis Ababa and the

reference catalogue (Sigma-Aldrich Chemie GmbH, Taufkirchen. Germany) for conventional media, while local market was used for cost estimation of the newly formulated GALF-1 medium ingredients.

12.5 Skin biopsies

A total of (n = 32) skin biopsies were collected under sterile condition from Ethiopian CL patients (24 males and 8 females) at the Armauer Hansen Research Institute/ All African Leprosy Eradication and Training Centre, (AHRI)/(ALERT), Addis Ababa, Ethiopia. To assess background apoptosis in healthy Ethiopian controls, a total of 8 skin biopsies were collected from apparently healthy Ethiopian donors at a) Ethiopian Health and Nutrition Research Institute (EHNRI) histopathology laboratory, Addis Ababa (n=3), and b) St. Paul Specialised Referral Hospital, Addis Ababa, Ethiopia from surgical patients (n=5). Skin biopsy samples of approximately 4 mm in size were collected from 19 ulcerative CL patients (13 males, 6 females), 13 non-ulcerative CL patients (11 males & 2 females) and 8 apparently healthy skin control donors (5 females and 3males). The biopsies were immediately fixed in 10%formalin, and subsequently processed for dehydration and embedded in paraffin block at AHRI histopathology laboratory. Initially, 20 paraffin embedded blocks (9 from non ulcerative CL, 8 from ulcerative CL and 3from healthy skin control donors) were sectioned at 5µm thickness and adhered to glass slides for staining at the department of Microbiology, Tumor and Cell Biology (MTC) histopathology lab, Karolinska institute, Sweden, and similarly another 20 paraffin blocks(4 from non ulcerative CL, 11 from ulcerative CL and 5 from healthy skin control donors) were processed at AHRI/ ALERT pathology laboratory and stained at EHNRI laboratory Ethiopia, Addis Ababa.

12.6 Diagnosis of CL as ulcerative CL and non-ulcerative CL

Ulcerative CL and non-ulcerative CL were diagnosed by clinical diagnosis, histological examination of paraffin-embedded biopsies and visualizing *Leishmania* amastigotes in Giemsa-May-Grunewald stained smears or by culturing scrapings from lesions. Due to lack of availability of GMP (good manufacturing practise) and produced Leishmanin skin test(LST) antigen suitable for *L. aethiopica* induced leishmaniasis [144] skin testing of patients could not be done. All included samples for CL were confirmed by demonstration of *Leishmania* parasite by two or more of these diagnostic techniques. Clinically non-ulcerative CL was diagnosed basing on clinical

history as it usually begins with an initial primary lesion and then latter disseminates to involve other areas of the skin. These lesions are non ulcerative, which are often scattered over the limbs, buttocks, and face which usually look like lepromatous leprosy. However unlike lepromatous leprosy, there is no nerve involvement and the disease does not invade internal organs[10] and there is no history of kala- azar. It responds only partially to treatment and often relapses, becoming chronic disease. Histologically the lesions in non-ulcerative CL contained numerous disorganized MØ laden with *Leishmania* amastigotes, with few lymphocytes and many plasma cells, while the lesion in ulcerative CL were characterized by granuloma composed of prominent infiltration of lymphocytes, epitheliod cells, and parasites as previously described [5] and reviewed by Handman [18].

12.7 Detection and evaluation of apoptotic cells in paraffin embedded skin

In situ terminal deoxy nucleotide transferase- mediated d-UTP nick end labelling (TUNEL) (Roche Diagnostics GmbH, Mannheim, Germany) was performed according to the manufacturer's instruction. Positive controls for the tests were obtained by addition of DNase (Life Technologies, Sweden) and negative controls by omission of the TdT enzyme during the experiment. Evaluation of TUNEL was performed using a Leica fluorescent microscope and Olympus Optical BX51 fitted with Olympus U-CMAD3 Camera (Tokyo, Japan) with CC-12 soft imaging system and green filter. Apoptotic cells were counted in epidermis and dermis under x40 objectives per 10 fields.

12.8 Immunohistochemistry of skin biopsies

FasL and TRAIL were visualized in formalin fixed tissue as previously described[121, 145] and evaluated in Leica fluorescent microscope and Olympus Optical BX51 fitted with Olympus U-CMAD3 Camera (Tokyo, Japan) with CC-12 soft imaging system. Apoptosis was assessed through visualizing fragmented DNA using TUNEL (TdT-mediated dUTP nick end labeling) kit according to the manufacturer's instructions (Roche, Penzberg, Germany). The numbers of FasL expressing cells were counted in x25 objectives and apoptotic cells were counted in x40 objectives, more than ten fields were evaluated per sample. The expressions of TRAIL in tissue sections were graded using an arbitrary scale from (0) to (++++) depending on the intensity of brown colour produced in epidermis, dermis, inflammatory areas of dermis, and fibrous tissue area of dermis. The arbitrary scale was defined as: 0 = no staining (staining similar to isotype control), 1+ = weak

or minimal but widespread staining, 2+ = small areas of moderate staining, 3+ = large areas of moderate to diffuse staining and 4+ = moderate to strong staining. This arbitrary scale was later converted to numerical number: 0 to 0, + to 1, ++ to 2, +++ to 3 and ++++ to 4 for manipulating the data. In the case of FasL, levels of FasL-expressing cells were quantified by counting the number of positive cells in 10 fields per section at x 250 (x25objective, x10 eyepieces) magnifications.

12.9 Peripheral blood mononuclear cells (PBMC) preparation

Whole blood samples were collected from 7 apparently healthy Ethiopian individuals (3 females and 4 males), 28-44 years of age with no present or past history of infection with leishmaniasis or visiting of *Leishmania* endemic areas. All blood samples were tested for HIV –1/2 according to the present Ethio- Netherlands AIDS Research (ENARP) HIV test algorithms. Cases such as pregnancy, cinfection with diseases and any case which might have an impact on the study were not included during recruiting blood donors for whole blood collection. PBMCs were separated from peripheral blood on Ficoll (Amersham Biosciences, Sweden) as previously described [146].

12.10 Stimulation of peripheral blood mononuclear cells (PBMC) with live promastigotes

Leishmania promastigotes were confirmed as *L. aethiopica* by isoenzyme and PCR for 6 ulcerative CL and 3 non-ulcerative CL were sub-cultured in RPMI 1640 liquid media supplemented with 100-U/100-µg/ml penicillin-streptomycin (Sigma chemical co.) and 2mM L-glutamine (Flow Laboratories, Irvine, Scotland). Before co-incubation with mononuclear cells the promastigotes were harvested and washed 3 times with 40-50 ml PBS by centrifugation at 3000 rpm at 4^{o}C for 10 minutes. Thawed PBMC samples were suspended in complete RPMI 1640 counted using haemocytometer and plated at $5x10^{5}$ cells/ well in Lab-Tek II Chamber Slide of 8-wells (Nalge Nunc International, USA) well of 0.5ml working volume. Harvested stationary phase of *L. aethiopica* promastigotes (washed 3 times in PBS at 3000 rpm for 10 minutes) were added at concentration of 5 x 10^{5}/ well making 1:1 MNC to parasite ratio. Similar incubations were also done in the culture flasks at volume of 25 mm^{3} with 1:1 PBMC to Parasite ratio. Supernatants were collected on ice and immediately were frozen at –70-80 ^{o}C after incubation of the cultures in an incubator at 37 ^{o}C with 5% CO_2 atmosphere on the 5-7^{th} days.

12.11 Keratinocytes cell line (HaCaT)

HaCaT, immortalized human keratinocytes [147] kindly provided by Professor N. E. Fusenig (Heidelberg, Germany), were propagated in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% FCS, 100U/100 μg/ml penicillin- streptomycin, and 2 mM L-glutamine (DMEM medium) and plated on sterile permanox culture slides (Nunc) or in 25 mm^2 cell culture flasks and incubated at 37 ^oC in air with 5% CO_2 until 70-80% confluence was reached 2-3 days after seeding.

12.12 Induction of Keratinocyte apoptosis in vitro

Leishmania promastigotes propagated from ulcerative and non-ulcerative lesions were used to stimulate healthy PBMCs for 7 days at 1:1 ratio. The supernatants were collected and cryopreserved in -80^0C. Supernatants were added to cultures of the keratinocyte cell-line HaCaT [147] for 20 hrs and early apoptotic cells were assessed by AnnexinV/Propidium Iodide staining by microscopy.

12.13 In vivo model of murine ulcerative leishmaniasis

The infective-stage, metacyclic promastigotes of *L. major* (Friedlin V1), were isolated from stationary cultures (4–5 days old) by negative selection using peanut agglutinin (Vector Laboratories, Burlingame, CA, USA) as previously described [148].

12.14 Mice as animal model

Female Balb/c aged 6-8 weeks were infected intradermally with 5 x 10^4 metacyclic *L. major* (Friedlin V1)[57]. MFL-4 (monoclonal antibody to Fas Ligand)(0.5 mgs i.p. twice per week), anti TRAIL (N2B2) or isotype matched IgG control (Rockland) were injected i.p. at week 1 – 4 after infection. The evolution of the lesion was monitored weekly by measuring the diameter of the indurations of the ear lesion with a direct-reading vernier caliper (Thomas Scientific, Swedesboro, NJ, USA). After euthanization both ears and retromaxillar lymph nodes were removed. Groups of five mice were infected at three (MFL/4 and isotype control) and two different times (N2B2).

12.15 Estimation of parasite load in ear and retromaxillar lymph nodes

Parasite titrations were performed as previously described [57]. The number of viable parasites in each sample was defined as the highest dilution at which promastigotes could be grown out after 7 days of incubation at 26°C.

12.16 Visualization of FasL and TRAIL

Sections adhered to glass slides were de-waxed at 58 °C for 1 hour and immersed in xylene for 30 minute and then sequentially in absolute, 95%, 90%, 80%, and 70% ethanol for 5 minutes and each immersion followed by 5 minutes in deionised water. Re-hydrated sections were incubated with hydrogen peroxide for 5 minute to quench endogenous peroxidase activity and then incubated for 5 minute with protein blocking (Dako, Denmark) reagent. For FasL avidin biotin complex signal amplification system, for TRAIL biotin free tyramide signal amplification system (Dako, Denmark) were used as vectors to stain sections. Sections for FasL staining were incubated with mouse anti-FasL monoclonal (BD, Stockholm, and Sweden) at 20 μg/ml, or isotype controls (Dakopatts, Stockholm, Sweden) at 20 μg/ml. Similarly sections for TRAIL was incubated with monoclonal anti-human TRAIL (R&D systems, USA) at 10 μg/ml, or mouse isotype controls (Dako, Denmark) at 10 μg/ml for 15 minutes at room temperature. Streptavidin-avidin enhancements for FasL and signal amplification for TRAIL were performed according to the manufacturer's instruction (Dakopatts). Staining was then completed by 3 to 5 minutes incubation with 3-3' diaminobenzidine tetra hydro -chloride (DAB) that results in a brown coloured precipitate (DAKO Cop, Code No.K1500) and counterstained with Meyer's haematoxylin. Two sections were analysed for mouse negative control and FasL on a single slide and similarly two sections were analysed for mouse isotype control and TRAIL.

12.17 Statistical analysis

Statistical significance was calculated using the Mann-Whitney's U-test and student t-test. This was used at appropriate condition using Prism Graph Pad Software (Inc. Oberlin Drive, San Diego, USA) for statistical analysis.

13 RESULTS AND DISCUSSION

GALF -1 is low cost liquid medium for cultivation of *Leishmania* parasites (paper-I)

GALF -1 can transform skin amastigotes to promastigotes

GALF-1 medium could be used to grow *Leishmania* promastigotes from clinically suspected CL patients at culture temperature of 26 ^{o}C. Out of 15 skin-scraping samples collected from different clinically suspected CL patients, 14 were found to be positive by smear and culture medium. Amastigotes transformation time (ATT) [149] was evaluated for three media (RPMI 1640, LSSM and GALF-1) compared, and the median ATT from the individual lesion sample was similar, with median ATT of GALF-1 observed after 6 days, LSSM after 6.5 days and RPMI 1640 after 6.5 days (Fig.8).

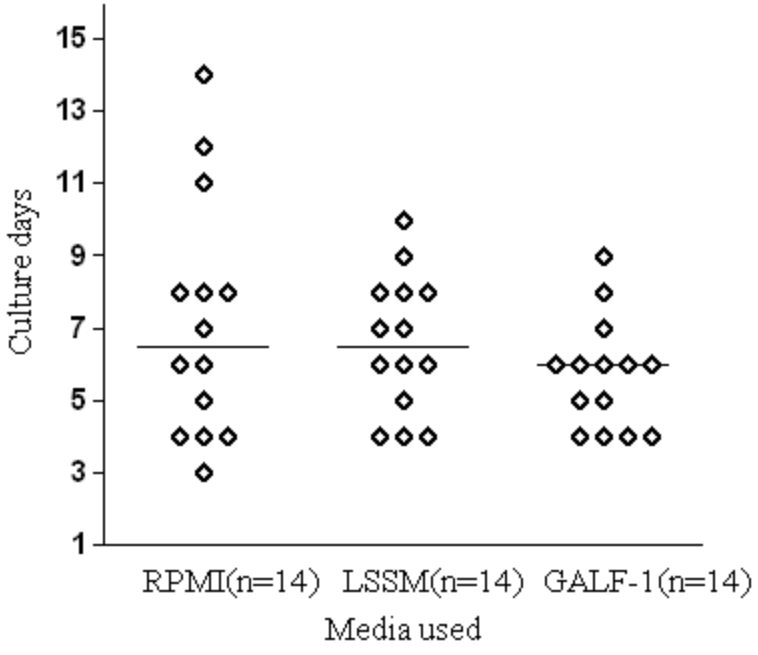

Figure.8 A graph showing different culture time in days (24 hours) at which the transformation of amastigotes to promastigotes was observed in different media. The solid line in each scatter plot shows median time in days for amastigotes transformation to promastigotes directly from CL patients' sample.

The shortest ATT during culturing with GALF-1 was 4 days and the longest period for promastigotes to appear in a culture was 9 days (Fig.8), while the shortest ATT for LSSM was 4 days and the longest time was 10 days, and the shortest ATT for RPMI was 3 days and the longest was 14 days. Demonstrating *Leishmania* parasites in the patients' samples by culture is considered as the gold standard in the diagnosis of leishmaniasis [150]. We show that GALF-1 media was able to transform amastigotes to promastigotes, allowing its use for primary isolation for the diagnosis of leishmaniasis from skin lesions.

GALF-1 is an alternative medium for mass cultivation of promastigotes

Promastigotes inoculated into GALF-1 reached stationary phase at (~ 1.5 x 10^6 /ml) within an average of 6 to 8 culture days, and declining phase at(~ 4.7x10^5 /ml) after 9 to 12 culture days. Similarly promastigotes in RPMI 1640 medium reached stationary phase at (~7.1x10^6/ml) within an average of 4 to 5 days followed by a declining phase at (~5.6x10^5 /ml) between the 6th and 9th day while in LSSM reached stationary phase at (~ 1.1x 10^6 /ml) after 6 to 8 days and declining phase at (~2.4x10^5 /ml) after 9 to 12 days of culture (Fig. 9).

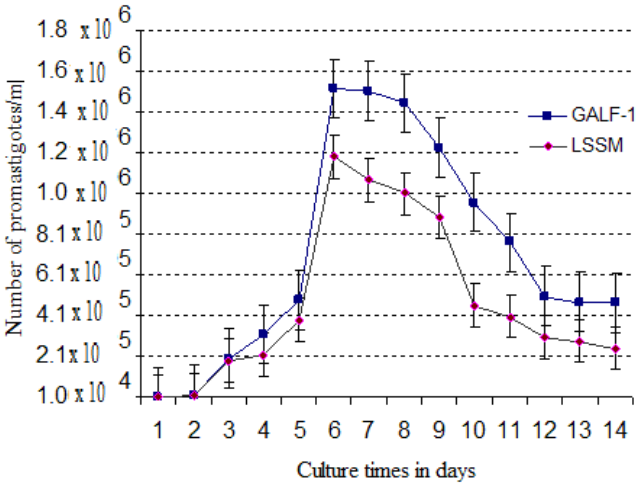

Figure.9 The growth curve, a representative of three independent experiments in triplicate, obtained from one *Leishmania* parasite isolate. The three experiments for growth curve used three different *Leishmania* parasites isolates. The growth curve was obtained for LSSM and GALF-1, and the error bars in the curves show the standard error of the mean of triplicate cultures. Promastigotes cultured in RPMI1640 medium were used for the analysis of growth curve and were pre- washed twice with PBS which was then suspended in the respective media solution and counted. Initially 1x10^4 Promastigotes /ml was inoculated into all three medium (RPMI, LSSM, and GALF-1) in triplicate in a total volume of 2ml and aliquots counted using haemocytometer at every 24 hours. Parasites inoculated into RPMI 1640 medium reached stationary phase (~7.1x10^6/ml) within an average of 4 to 5 days followed by a declining phase (~5.6x10^5 /ml) between the 6th and 9th day.

Parasites cultured in RPMI1640 media showed very short lag phase and rapid growth phase compared to parasites cultured both in LSSM and GALF-1. RPMI 1640 showed similar growth rate compared to GALF-1 media (P=0.051) and significantly faster growth rate than the conventional LSSM media (P=0.038). Although GALF-1 showed apparently faster growth rate (fig.9) than the conventional LSSM medium there was no significant difference (P=0.1544) observed. GALF-1 could be an alternative media between LSSM and RPMI 1640 to be used for culturing *Leishmani*a parasites. It can be used for mass cultivation of the parasites at moderate parasite density with long viability of promastigotes in culture. This may have great value for those who want to collect culture samples at field based studies where sub culturing is not frequently possible. For mass cultivation and fast growth of promastigotes, RPMI 1640 with 10% FBS is still the liquid media of choice but due to its unavailability and high cost, except in specialized research institutes, it is not practical for use at peripheral health service.

GALF-1 grown promastigotes recover well following cryopreservation

GALF-1 cultured promastigotes recovered equally well after several months of cryofreezing as promastigotes cultured in the standard medium RPMI 1640 (table.2). We confirmed that the recovery of promastigotes grown in GALF-1 medium does not be affected by cryopreservation. In addition, promastigotes cultured in GALF-1 medium could be kept cryopreserved for at least up to 2 years. It is important to note that one of the advantages of culturing *Leishmania* parasite is to preserve the parasite for future reference [151].

Table.2 Promastigotes cultivated in GALF-1 were cryopreserved in liquid nitrogen and evaluated at different time intervals by thawing and growing at 26 ^{o}C. These were compared to promastigotes grown in RPMI 1640 medium and cryopreserved under similar condition. Arbitrarily grading (+: moderate, ++: good and +++: very good growth) was given to the densities of multiplying promastigotes in the culture when assessed under inverted microscope after 24 hours overnight incubation of frozen promastigotes. Seven *Leishmania* isolates (Leish-1 to Leish-7) were used for evaluation of cropreservation and five of the isolates with equal amount of aliquots (1.8ml) were cryopreserved both in GALF-1 and RPMI 1640 media. RPMI1640 has shown good parasite recovery in all *Leishmania* isolates in this experiment at all times and the last two rows of this table show the results of two isolates for comparison.

	Months of cryopreservation					
Parasite	**1**	**3**	**6**	**12**	**24**	**Media**
Leish-1	++	+++	++	++	++	GALF-1
Leish-2	+++	+++	+++	+++	+++	GALF-1
Leish-3	+++	+++	++	++	++	GALF-1
Leish-4	+++	+++	+++	++	++	GALF-1
Leish-5	+++	+++	+++	++	+++	GALF-1
Leish-6	+++	+++	+++	+++	+++	RPMI 1640
Leish-7	+++	+++	+++	+++	+++	RPMI 1640

GALF -1 medium is simpler and cheaper to use for culturing *Leishmania*

The main disadvantages of most media are their high cost to use for routine purpose especially in resource poor countries. In our study, we formulated our media from simple, cheap, easy accessible ingredients which performed comparably to commercially available RPMI-1640 and LSSM media. Excluding human power and equipment service, the economical assessment result showed that the cost for preparing 100ml of GALF-1 is ~ 0.2 USD, LSSM is ~ 1.0 USD and RPMI 1640 is ~ 4.5 USD (table.3). Thus GALF-1 is the cheapest media of the three tested which may reduce the cost of culture by ~ 95.6 % compared to RPMI 1640 and by ~ 80 % compared to LSSM. Similarly using LSSM reduces the cost by 78 % compared to RPMI 1640. Table 3 summarises advantages and disadvantages of the three media.

Table.3 Comparison of RPMI1640, LSSM and GALF-1 media. The data for comparison was collected from local shops, pharmacy and laboratories.

Comparison points	RPMI 1640	LSSM	GALF-1
Distilled water	Required	Required	Not required
Sheep blood	Not required	Required	Not required
Autoclaving	Required	Required	Not required
Filtration through 0.22µm	Required	Required	Required
Accessibility	Most Difficult	More Difficult	Easy
Production of ingredients	Developed nation	Developed nation	Could be in developing nation
Steps for preparation	Easy	Takes steps	More easy
Type of media	Liquid	Semisolid	Liquid
Cost/100ml	$ 4.5 USD	$1.0USD	$ 0.2 USD
Shelf life of prepared media	More than a month	A week	More than a month
Storage of ingredients	Requires fridge	Room temperature	Room temperature

Different liquid and semi solid media such as LSSM used for culturing *Leishmania* require many steps including autoclaving during their preparation [152-154]. The preparation of GALF-1, however, does not require collection of animal blood, autoclaving and distilled water for preparation. The ionized solution is easy available and can be used instead of distilled water for preparation of this media. However, ingredients used for LSSM preparation is expensive since it uses pure chemicals such as NaCl, KCl, $CaCl_2$, $NaHCO_3$ and glucose. LSSM medium is only to be used for primary isolation and parasites cultured in it should be sub-cultured into another liquid media when mass cultivation is required. The incorporation of 2% urine in the new formula helped to enhance *Leishmania* growth in culture. The addition of 1-5% urine to Schneider's Drosophila medium containing 10% foetal calf serum was reported to enhance the growth of 11 *Leishmania* strains representing 8 different taxonomic groups including *L. donovani* and *L. braziliensis* [149, 155, 156]. The active molecule in human urine that enhances *Leishmania* growth in culture has been shown to be xanthine [156]. The supplementation of urine/ xanthine to the media may be of great value for the primary culture of new isolates in routine culture[155, 157, 158] since culture could be established from lesions with as few as 10 amastigotes/ml as a result of urine additive[155]. The addition of 20% FBS enables enough supplement of hemin, which is essential for protein synthesis and proliferation of promastigotes in culture [159].

To our knowledge there is no GALF-1 like media which is cheaper and simply prepared from easy available ingredients to culture *Leishmania*. The costs of two liquid media previously compared by Limoncu and colleagues [150] ranged from 1.2 to 1.8 USD per 100ml each. But the cost for the

same amount of our new liquid media is 0.2 USD which is about 5.7 times less cost than Limoncu and colleagues liquid media. GALF-1 is by far the cheapest compared to the cost for the rapid test rk39 (1USD per test), direct agglutination test (DAT) 3 USD per test, and latex agglutination in urine 1USD per single test [27]. A total of 100ml of GALF-1 could be used for 20- 33 tests at a cost of 0.2 USD altogether. It may be helpful to cut the cost of the efficient culture medium for diagnosis through the use of inexpensive locally formulated media that could improve the diagnosis of leishmaniasis in country like Ethiopia, where affordability of diagnostic assays is a key issue.

Systemic FasL and TRAIL neutralisation reduce leishmaniasis induced skin ulceration (manuscript-II)

Dermal infiltration of FasL and TRAIL expressing cells were more prominent in ulcerative CL.

In *L. major* induced ulcerative CL , infiltration of FasL expressing T cells and MØs is present in dermis [145] and this finding was verified in ulcerative *L. aethiopica* infection (fig.10B). FasL expressing cells were detected in non-ulcerative CL but at significantly lower levels as compared to ulcerative lesions (fig. 10B and 10D). In healthy skin FasL was not expressed (fig.10B), however low or moderate TRAIL expression was detected in healthy epidermis. The levels of epidermal TRAIL expression was increased during *L. major* induced ulcerative CL [121].TRAIL expressing cells were present in dermis of both ulcerative and non-ulcerative *L. aethiopica* induced CL with significantly higher expression in dermal inflammatory areas in ulcerative as compared to non-ulcerative lesions (fig.10C and 10E).

As previously shown [121], TRAIL expression was increased in epidermis of both ulcerative CL and non-ulcerative CL as compared to healthy controls with no significant difference in TRAIL expression between non-ulcerative CL and ulcerative CL (results not shown).

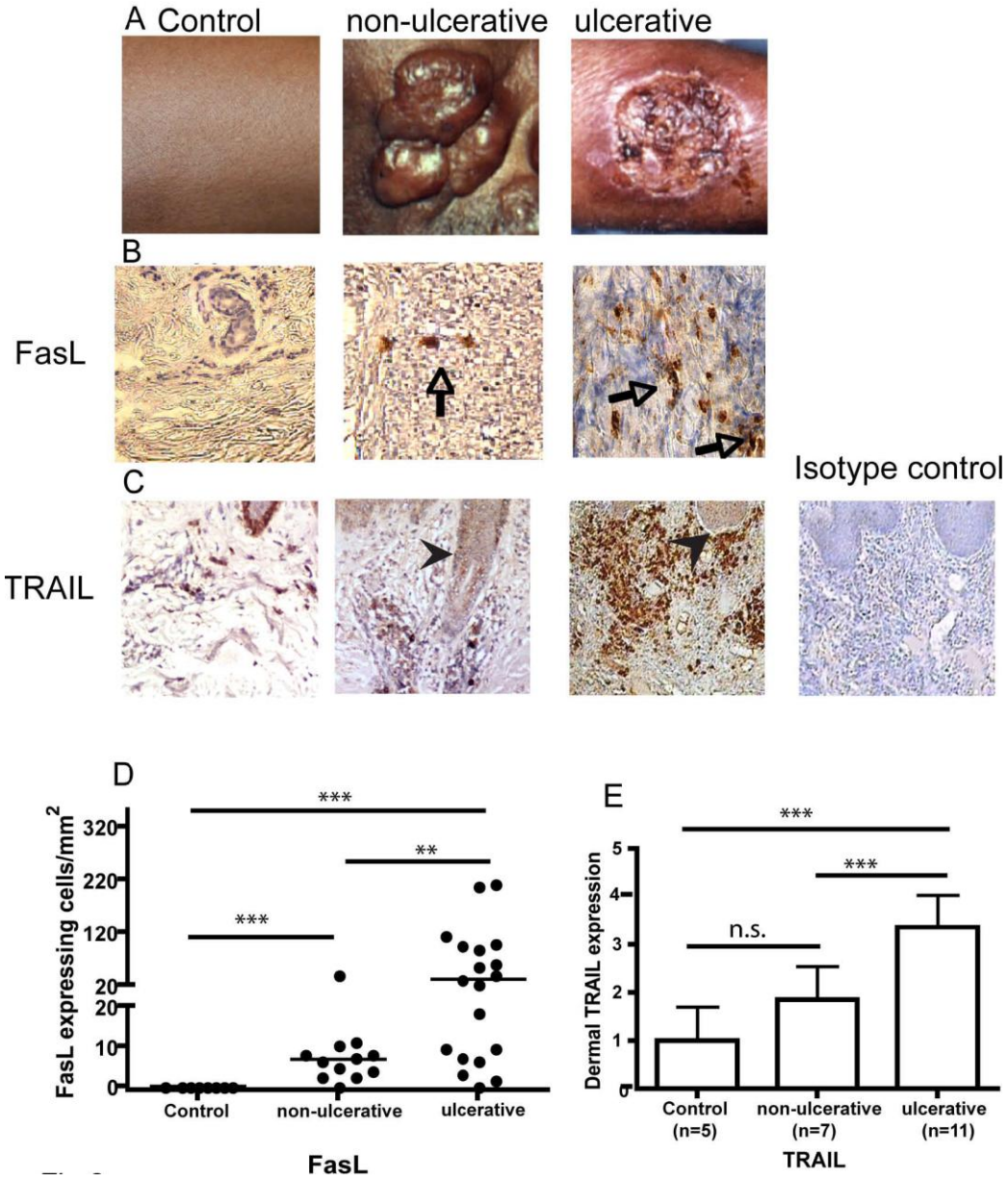

Figure.10 FasL and TRAIL expressing cells infiltrate dermis of ulcerative CL. (A) Photographs of representative non-ulcerative (center) and ulcerative (right) lesions of CL caused by *L. aethiopica*. Control skin (left) was obtained from a fore arm, non-ulcerative from a facial lesion and the ulcerative lesion from a fore arm. (B) FasL expressing cells (brown) in healthy and lesional dermis depicted at x63 magnification and counterstained with hematoxilin (blue). Arrows are marking FasL expressing cells. (C) TRAIL expressing cells in dermis (brown) counterstained with hematoxilin (blue). TRAIL expression is noted in epidermis (arrowhead) but mainly in dermal inflammation. Isotype control is depicted to the far right. (D) Enumeration of dermal FasL+ cells in controls (n=8), non-ulcerative CL (n=13) and ulcerative CL(n=19) by counting of 10-20 fields per sample at x 25 magnification. (E) Dermal TRAIL expression was assessed using an arbitrary scale where 0 represented no signal and 5 represented maximal signal. ***p>0.001, **p>0.01, *p>0.05, ns= non significant.

Variable levels of epidermal apoptosis was detected in lesion biopsies

In Previously study, TUNEL staining on human epidermis showed the same pattern of staining as caspase-cleaved cytokeratin 18, verifying that TUNEL can be used as a marker of apoptosis in *Leishmania* infected skin [145]. In the present study to determine if expression of TRAIL and FasL were correlated to keratinocyte apoptosis *ex vivo*, TUNEL staining was performed on biopsies from ulcerative and non-ulcerative CL patients. The result reveals that the number of epidermal apoptotic cells showed great inter-individual variation in all groups examined (fig.11 A-B). Ulcerative lesions did not contain significantly higher numbers of epidermal apoptotic cells as compared to non-ulcerative lesions and healthy skin. However there was a trend to a higher number of apoptotic keratinocytes in the ulcerative group. The previous study result from the same laboratory has shown an increase in the number of apoptotic epidermal cells in *L. major* caused ulcerative disease as compared to healthy skin in a cohort of young military recruits with a history of ulcerative leishmaniasis of less than three months upon transfer into hyperendemic *Leishmania* foci [121]. In the present hospital based study, the patient material was collected from a heterogeneous group of patients from endemic areas and the median duration of the disease at the time of tissue collection was longer.

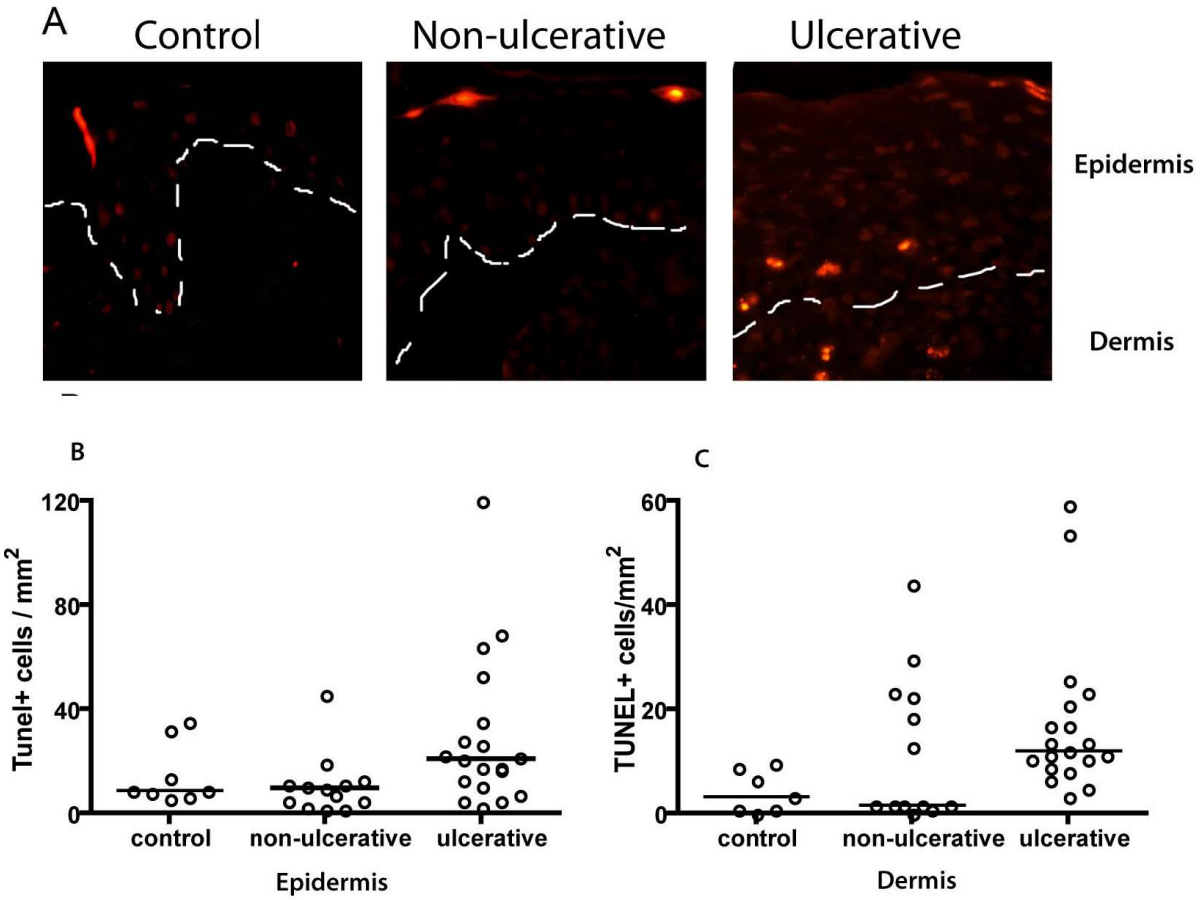

Figure.11 Apoptosis in skin biopsies from ulcerative and non-ulcerative CL**.** (A) TUNEL staining depicting apoptotic cells (orange) in healthy (left), non-ulcerative (central) and ulcerative (right) CL. The basal membrane separating epidermis and dermis is marked with a dashed white line. (B) Dot plot showing the number of epidermal and (C) dermal apoptotic cells in controls (n=8), non-ulcerative (n=13) and ulcerative (n=19) CL skins. The horizontal bar represents the median. No significant differences in number of apoptotic cells were found in the groups investigated.

The level of apoptotic cells detected in *ex vivo* biopsies may not reflect the amount of cell death taking place in the tissue,due to the immediate and efficient clearance of apoptotic cells *in vivo* by phagocytic cells such as tissue MØs. Thus we utilised an in vitro experimental set-up in which keratinocytes were exposed to *Leishmania* derived supernatants in the absence of phagocytic cells was tested.

Keratinocyte apoptosis upon exposure to supernatants collected from PBMCs stimulated with parasites from CL

It has been hypothesized that distinct subtypes of *L. aethiopica* induce ulcerative and non-ulcerative diseases. To investigate the apoptotic inducing effect of parasites derived from ulcerative and non-ulcerative lesions, parasites from clinical lesions of these different clinical manifestations of CL were obtained and propagated *in vitro.* Infective promastigotes were used to stimulate PBMCs from healthy

individuals for six to seven days and supernatants from such cultures were added to an immortalised keratinocyte-cell line sensitive to FasL and TRAIL induced killing (fig.12A, B) in which Fas blocking and TRAIL neutralising antibodies completely inhibits apoptosis (fig. 12D).

Supernatants collected from PBMCs stimulated with parasites derived from ulcerative CL induced significantly more keratinocyte apoptosis as compared to unstimulated PBMCs or PBMCs stimulated with non-ulcerative CL derived parasites (fig.12A and C). Furthermore, keratinocyte apoptosis induced by supernatants from PBMCs stimulated with parasites derived from ulcerative CL could be inhibited by the addition of Fas blocking (fig.12E) or TRAIL blocking antibodies (fig.12F). No synergistic effect was noted when FasL and TRAIL were inhibited simultaneously (fig. 12G).

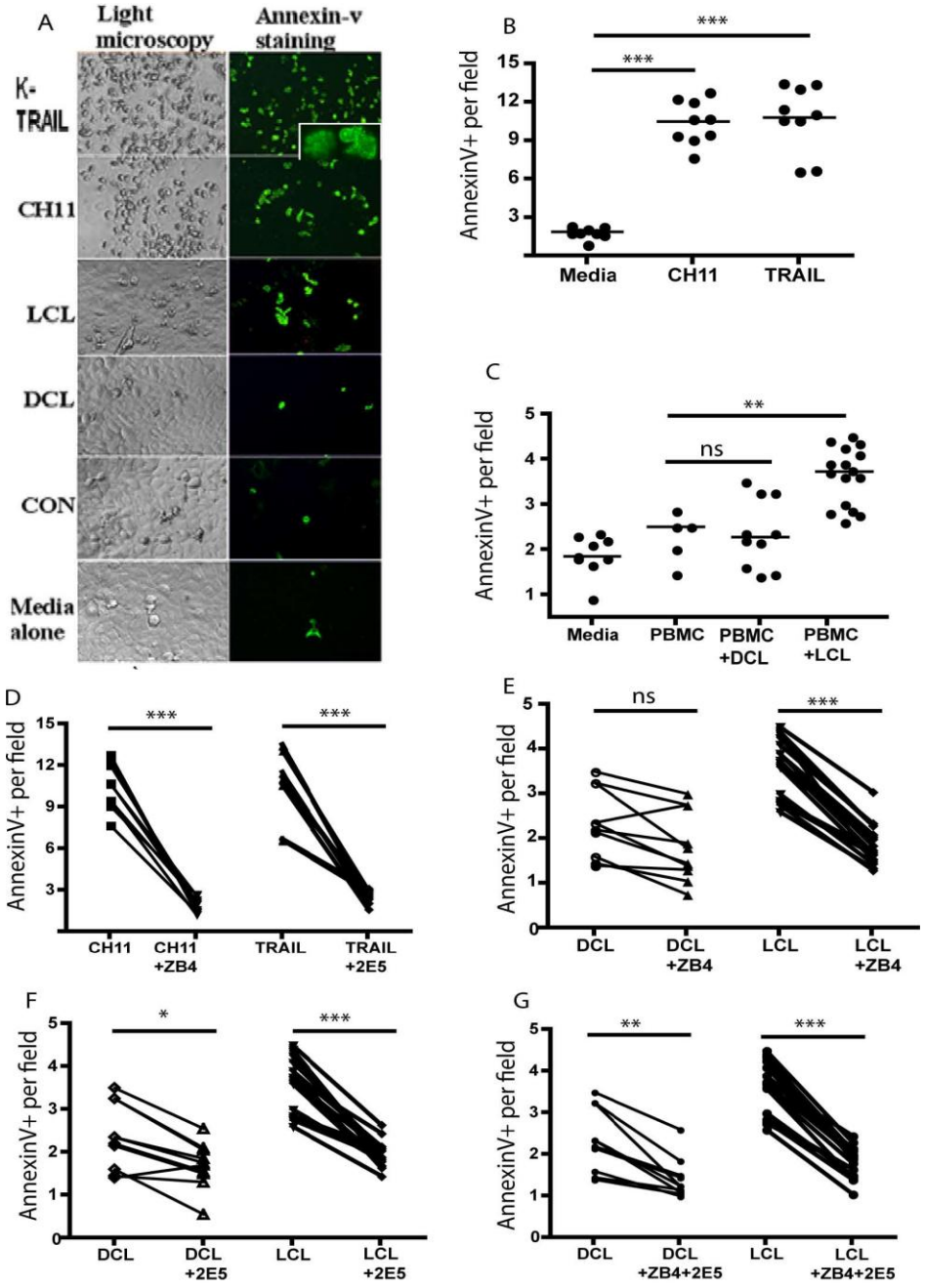

Figure.12 Supernatants from PBMCs stimulated with parasites from ulcerative CL patients induce more apoptosis in keratinocytes through FasL and TRAIL signaling than non-ulcerative CL patients. (A) Bright phase photographs (left panel) and AnnexinV(green)/ Propidium Iodide (red) staining (right panel) of monolayers of keratinocytes 20 hrs after addition of anti-Fas or TRAIL or supernatants from PBMCs stimulated with promastigotes propagated from ulcerative CL or non-ulcerative CL lesions. CON represents supernatants from un-stimulated PBMC incubated with "media alone". (B) Dot plot showing induction of keratinocyte apoptosis by addition of Fas-activating antibody CH11 or recombinant TRAIL. (C)Dot plot showing induction of keratinocyte apoptosis by addition of supernatants from parasite stimulated PBMC cultures. The horizontal bar represents the median value. (D)Plots showing the inhibitory effect of the Fas blocking antibody ZB4 (1-2 µg/ml) and the TRAIL blocking antibody 2E5 (2.5 µg/ml) on CH11 (1 µg/ml) and TRAIL (LZ-TRAIL 250 ng/ml) induced apoptosis (n=9). (E-G) Blocking effect of ZB4 and 2E5 on apoptosis induced by DCL (n=10) and LCL (n=16) cultures. ***p>0.001, **p>0.01, *p>0.05, ns= non significant.

In a murine model of ulcerative CL, short-term inhibition of FasL and TRAIL signalling decreased pathology without effect on the infectious burden

The current treatment alternatives during active CL are aimed at parasite eradication [35] and have little effect on tissue destruction. Treatment regimes result in exacerbation of inflammation leading to increased tissue destruction and scarring. Targeting specific immune mechanisms has proven to be a promising new approach for the therapy of cancer and autoimmune diseases. We were interested to investigate if such approach could be used to decrease the pathology caused by a protozoan infection such as *Leishmania* infection. The effect of systemic treatment with FasL and TRAIL neutralising antibodies during the ulcerative process of CL was investigated. *L. aethiopica* inoculation in mice did not lead to productive infection or ulcerative disease. Thus, a well characterised model of ulcerative CL using a low number of *L. major* metacyclics promastigotes injected intradermally into the ear of Balb/c was chosen [160]. Systemic treatment with FasL [161] or TRAIL[162] blocking antibodies was given twice weekly. A clear reduction in the development of ulcers was noted in the treated animals as compared to control treatment (fig. 13A-C). Blocking FasL or TRAIL was not sufficient to completely inhibit ulcer formation and no synergistic effect was noted by the simultaneous administration of FasL and TRAIL neutralizing antibodies (data not shown).

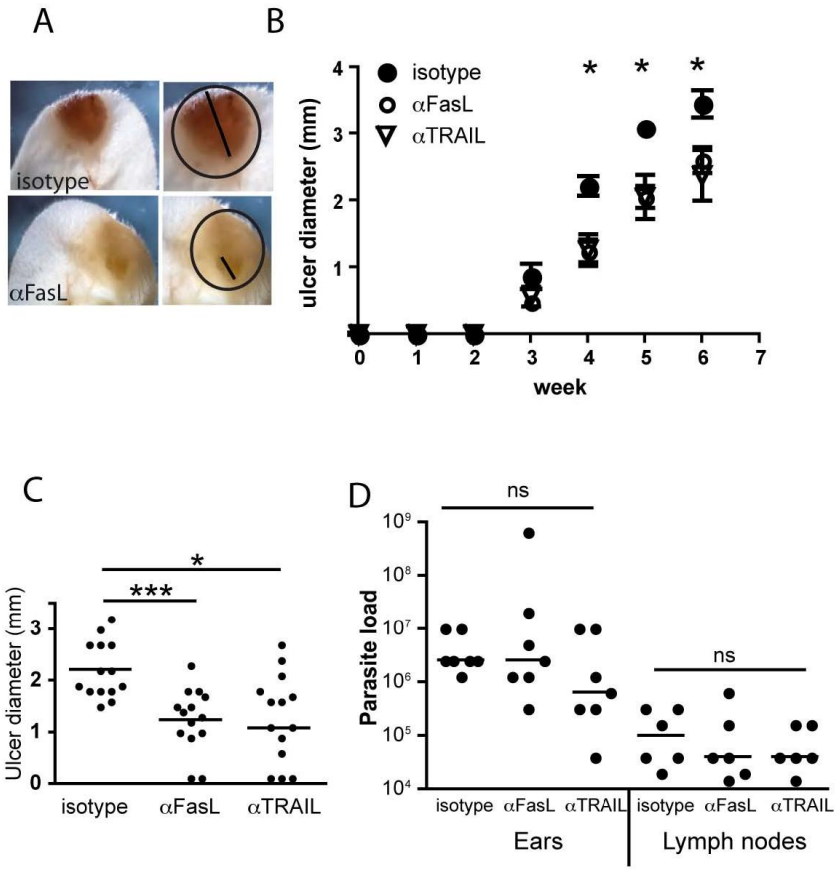

Figure.13 Blocking FasL and TRAIL reduce ulceration during experimental CL. (A) Photograph of *L. major* infected ears 4 weeks post infection treated with isotype controls (upper row) or anti-FasL neutralising Abs (MFL-4, lower row). The right panels depict the diameter of the ulcerated area (line) and the outline of the inflamed, lesional area (circle). (B) Development of ulcer in Balb/c following intradermal *L. major* infection was followed over time. The experiment was repeated twice and the pooled results of a total of 14 samples per treatment regime are shown. Mean and standard error of the mean is depicted. (C) Dot plot diagram showing individual measurements of ulcer size at four weeks after infection. (D) Dot plot diagram showing parasite loads from ears and lymph nodes at four weeks after the infection. Six samples from a representative experiment repeated two times with similar results are depicted. ***p>0.001, *p>0.05, ns= non significant.

It has previously been shown in Fas and FasL deficient mice that insufficient clearance of *L. major* infection and the treatment strategy used could potentially lead to uncontrolled parasite replication. Impaired control of parasite replication has been shown in Fas and FasL deficient knock-out mice[126, 127, 163] and systemic administration of exogenous recombinant FasL to FasL deficient *(gld)* mice led to elimination of parasites and resolution of non-ulcerative cutaneous lesions. *In vitro* studies have shown that MØs infected with *L. major* up-regulate their surface Fas expression in response to IFNγ and as a result become susceptible to CD4+ T cell- induced apoptotic death [127]. No data is available on the evolution of *Leishmania* induced pathology in TRAIL deficient mice. Based on the previous studies in FasL deficient mice, there is a potential risk to exacerbate parasite replication through inhibition of FasL during ulceration. In the model of ulcerative leishmaniasis used in these studies, parasite replication is not controlled and thus the effect of short-term inhibition of death ligands could not be investigated in a situation of self-healing leishmaniasis. However, in the model we have used systemic inhibition did not lead to increased

infectious loads at the primary site of infection. The infectious load at the draining lymph node was not altered during treatment, suggesting that dissemination of the infection is not enhanced by short-term inhibition of Fas/FasL and TRAIL-Rs/TRAIL when pathology is induced by infection with a low infective dose.

Fas-FasL interactions have been implicated in the pathogenesis of drug-induced toxic epidermal necrolysis (TEN), a life-threatening disease characterized by extensive destruction of the epidermal keratinocytes[127, 163]. Systemic treatment with intravenous immunoglobulins containing Fas-blocking antibodies limited the ulcerative process during TEN[163] and reduced mortality in several multi-centre analysis. In the case of CL we propose that an adjuvant therapy inhibiting FasL or TRAIL signaling in combination with leishmanicidals could reduce the ulcerative process and subsequent scar formation.

14 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis tried to introduce laboratory formulated low cost liquid medium that could be used for *in vitro* cultivation of *Leishmania* parasites. The main disadvantages of most media are their high cost to use for routine purpose especially in resource poor countries. The new formulated GALF-1 media from simple, cheap, easy accessible ingredients, which could perform comparably to commercially available conventional medium, can reduce cost significantly. Affordability of diagnostic assays is a key issue for endemic resource poor countries and the possibility to cut the cost of the efficient culture method for diagnosis through the use of inexpensive locally formulated media could improve the diagnosis of leishmaniasis in resource poor countries like Ethiopia. Further evaluation on the newly formulated media at large scale in field bases and in different laboratories setup is recommended to validate the media before to utilising it in peripheral laboratories. In this thesis we also tried to test the hypothesis that expression of death ligands leading to epidermal apoptosis differed in skin biopsies from ulcerative and non-ulcerative cutaneous leishmaniasis. Expression of death ligands and subsequent epidermal apoptosis may play a role in the clinical out-come of human cutaneous leishmaniasis due to *L. aethiopica* infection. We proposed that the presence of large numbers of infected MØs during non-ulcerative CL is due to reduced apoptosis in MØs as compared to ulcerative disease. Thus the up-regulation of Fas/FasL expression in the skin during ulcerative CL may enhance apoptosis in *Leishmania* infected MØs and keratinocytes contributing to the ulcer formation obtained. Based on our current data, we suggest that there is dysregulation of apoptosis in epidermis of CL induced due to *L. aethiopica*. Dysregulation of apoptosis may be one of the factors that may explain why two clinical forms of CL occur due to *L. aethiopica*, as non-ulcerative CL and ulcerative CL. The existence of significantly higher FasL count and up-regulation of TRAIL in ulcerative CL form compared to the non-ulcerative CL form further strengthens the possibility that apoptosis may be involved in the ulcer formation during CL due to *L. aethiopica*. We reported that supernatants from PBMC stimulated with *L. aethiopica* parasite, derived from both non-ulcerative CL and ulcerative CL, have shown dysregulation of apoptosis in immortalised human keratinocytes. Supernatants obtained from stimulation of PBMC by parasite derived from ulcerative CL were found to induce higher level of apoptosis in HaCaT than that of parasite derived from non-ulcerative CL. Further investigations have to be

conducted to supplement the present finding and to understand reason of dysregulation of apoptosis and its role during clinical presentation of CL due to *L. aethiopica* is required.

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