

**Microbiology and Tumorbiology Center
Karolinska Institutet**

**Dysregulation of Receptor Induced Apoptosis
during Human Leishmaniasis:
A Possible Mechanism of Skin Ulceration.**

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**Karolinska
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Abstract

Leishmaniasis is endemic in 88 countries and 12 million people per year have been estimated to be at risk of infection. The causative agent, the protozoan *Leishmania*, is spread by sand-flies and infects macrophages in the mammalian host. Leishmaniasis in humans form a spectrum of clinical presentations. In cutaneous leishmaniasis (CL) caused by *L. major*, the infection is localised in the skin and manifests as one or several ulcers that typically spontaneously heal within one year after infection, often leaving marked scarring. In visceral leishmaniasis (VL) caused by *L. donovani*, infected macrophages are found in liver, spleen, bone marrow and lymph-nodes and the infection leads to hepatosplenomegaly, wasting, fever and if not treated, to death. Systemic T-cell deficiency occurs early during VL and leads to uncontrolled parasite replication. In general, solid immunity upon healing occurs after resolved VL and CL. In this thesis, the hypothesis that alterations of death receptor-mediated apoptosis have an impact on the pathogenesis of human leishmaniasis has been explored. During VL, dysregulation of the Fas/FasL pathway was investigated, both at one site of infection, the spleen, and on circulating lymphocytes from the peripheral blood. In the case of CL the hypothesis was that increased death receptor-mediated apoptosis in the microenvironment surrounding infected macrophages may induce bystander apoptosis of keratinocytes, leading to skin ulceration.

Dysregulation of the Fas/FasL pathway occurred during human VL and CL.

Levels of soluble Fas (sFas) and soluble Fas ligand (sFasL) were elevated in plasma of patients with active VL and individuals co-infected with VL-HIV-1 compared to healthy controls, and the levels of sFas and sFasL were normalized 6 months after successful treatment. During active VL, the expression of membrane bound Fas, and to a lower extent FasL, was up-regulated on spleen cells, where parasites multiply. In contrast, expression of Fas and FasL were not altered on peripheral blood mononuclear cells (PBMC) during VL. Furthermore, *in vitro* infection of macrophages with *L. donovani* results in up-regulation of Fas expression on the surface of infected cells and increases the levels of sFasL in supernatants from infected cultures. During active CL caused by *L. major*, a disease mainly localised to the skin, the Fas and FasL levels were not altered in serum or on PBMCs analysed *ex vivo*. However, when CL PBMCs were re-stimulated with *L. major*, Fas was up-regulated on effector T-cells and high levels of sFasL were detected in the supernatants as compared to control PBMCs.

Keratinocyte apoptosis is altered during CL.

Dysregulation of the Fas/FasL pathway in the microenvironment surrounding *L. major* infected macrophages under the skin was visualised in biopsies collected from CL patients. A substantial number of apoptotic keratinocytes were observed in the epidermis of morphologically active and healing CL skin samples. Fas expression was increased on the epidermis in active CL, whereas FasL expressing macrophages and T-cells were found in the subepidermal infiltrate during active disease. Supernatants from re-stimulated CL-PBMC cultures containing high levels of sFasL induced apoptosis in human keratinocyte cell line (HaCaT), and apoptosis could be inhibited in 2/3 supernatants by blocking Fas. A commercial apoptosis-specific microarray was used to assess alterations in keratinocyte RNA-expression during exposure to supernatants from *L. major* infected PBMCs. Fas and TRAIL mRNA and protein expression were significantly up-regulated compared to untreated keratinocytes. Supernatant induced apoptosis of keratinocytes was partly inhibited through blocking Fas or FasL, and more efficiently through inhibition of TRAIL by neutralising antibodies or soluble TRAIL-R. Furthermore, TRAIL expressing keratinocytes were detected in skin biopsies from CL cases.

Blocking the Fas/FasL pathway *in vivo* may reduce ulceration during murine CL.

In order to obtain the proof of the concept that Fas/FasL signalling is involved in keratinocyte-apoptosis leading to ulceration in the skin during CL, the Fas/FasL pathway was blocked in a murine model of CL by intraperitoneal treatment with FasL neutralising antibodies (MFL-4). Skin inflammation, skin ulceration and ulcer size were followed weekly and compared to infected, untreated mice. Our results suggests that blocking Fas/FasL signalling during murine CL lead to less apoptotic keratinocytes and diminished ulceration. During treatment, the number of IFN γ producing CD8⁺CD3⁺ cells was increased at the site of infection when FasL was neutralised which is suggestive of efficient parasite eradication. However, there was no reduction of parasite load at the site of infection or in draining lymph nodes and parasite replication was high upon discontinuation of anti-FasL treatment.

Conclusion

The Fas/FasL pathway was shown to be dysregulated both in human VL and CL. A possible mechanism of ulcer formation during CL was proposed by apoptotic death of keratinocytes through enhanced FasL and TRAIL signalling. This data was further strengthened in a treatment experiment in a murine model of CL, where blocking the Fas/FasL system reduced ulcer formation during *L. major* infection.

Key Words: Cutaneous leishmaniasis, visceral leishmaniasis, Death Receptors, Fas, FasL, Apoptosis

List of Papers

This thesis is based on the following original papers and manuscripts that will be referred to in the text by their roman numerals:

- I. **Eidsmo L**, Wolday D, Berhe N, Sabri F, Satti I, El Hassan AM, Sundar S, Chiodi F, Akuffo H
Alteration of Fas and Fas Ligand Expression during Human Visceral Leishmaniasis.
Clin Exp Immunol;130(2):307-13, 2002;
- II. **Eidsmo L**, Nylén S, Khamensipour A, Chiodi F, Akuffo H
The Contribution of the Fas/FasL Apoptotic Pathway in Ulcer Formation during *Leishmania major* Induced Cutaneous Leishmaniasis.
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- III. **Eidsmo L**, Fluor C, Eriksson Ygberg S, DeMilito A, Rethi B, Akuffo H, Chiodi F
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Submitted
- IV. **Eidsmo L**, Nylén Spoomaker S, Lieke T, Peters N, Yagita H, Sacks D, Akuffo H, Chiodi F
The effect of neutralisation of FasL on skin ulceration in murine models of cutaneous leishmaniasis.
Manuscript

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Paper II is Reprinted from Am J Pathol 2005 166: 1099-1108 with permission from the American Society for Investigative Pathology

Abbreviations

AICD	activation-induced cell death
AIDS	Acquired Immuno Deficiency Syndrome
CAD	caspase activated DNase
cDNA	complementary dioxy-ribonucleic acid
CL	cutaneous leishmaniasis
CTL	cytotoxic lymphocytes
CXCL	c-x-c chemokine ligand
DCL	diffuse cutaneous leishmaniasis
DR	death receptor
DD	death domain
DMSO	dimethyl sulfoxide
DNA	dioxy-ribonucleic acid
dNTP	2'deoynucleoside 5'triphosphate
EDAR	ectodysplasin A receptor
ELISA	enzyme-linked immunosorbent assay
FasL	Fas ligand
FACS	fluorescent activated cell sorter
FCS	foetal calf serum
FITC	fluoro-isothiocyanate
HAART	highly active anti-retroviral therapy
HIV	Human Immunodeficiency Virus
ICAM	intra cellular adhesion molecule
Ig	immunoglobuline
IFN	interferon
IL	Interleukin
IVIG	intravenous immunoglobulin
LCL	localised cutaneous leishmaniasis
LPG	lipophosphoglycan
LST	Leishmanin skin test
MCL	mucocutaneous leishmaniasis
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MNC	mononuclear cells
mRNA	messenger ribonucleic acid
NHS	normal human serum
NK cell	natural killer cell
NNN	Novy-Nicolle-McNeal medium
NGFR	nerve growth factor receptor
OPG	osteoprotegrin
PBMC	peripheral blood mononuclear cells
PE	phycoerytrin
PKDL	post kala-azar dermal leishmaniasis
PS	phosphatidyl serine
TEN	toxic epidermal necrolysis
TGF	transforming growth factor
TNF	tumour necrosis factor
TNFRSF	tumour necrosis factor receptor superfamily
TRAIL	TNF-receptor associated
TRAIL-R	TNF-related apoptosis-inducing ligand
TUNEL	TdT-mediated dUTP nick end labeling
VL	visceral leishmaniasis

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Introduction

The field of immunology is expanding at enormous speed, as is the knowledge on dysregulation of the immune system during disease. Targeting of specific immune mechanisms has proven to be a promising, new approach for therapy of diseases as diverse as cancer, autoimmune and possibly infectious diseases. Due to the vast amount of preclinical research and safety trials needed to create new drugs, most efforts in this field have been put into diseases affecting people in high-income countries, where investments in health-care are considerably larger as compared to low-income countries.

In this doctoral thesis, an attempt was made to investigate dysregulation of apoptosis, or programmed cell-death, as a mechanism of immunopathology during leishmaniasis. Leishmaniasis are a group of diseases affecting mainly people in low-income countries, and although considerable efforts are put into the search of new treatment approaches and vaccine development, some aspects of pathogenesis during these diseases remain unclear. In this thesis work, two different death receptor mediated apoptotic pathways, FasL and TRAIL induced apoptosis, were investigated in patient material and *in vitro* systems. An attempt to investigate the role of FasL during *in vivo* development of CL was approached through blocking FasL in a murine model of CL.

An Introduction to the Architecture and Function of the Skin

The skin is the largest organ in the body, consisting of 15-20% of the total body mass. Historically, the skin has been regarded as a mechanical shield, keeping electrolytes, heat and fluids on the inside of the body and keeping unwanted material such as micro-organisms and toxic agents on the outside but the skin also have important sensory, immunological and endocrine functions. The skin is built up by epidermis, dermis and hypodermis or subcutis. Epidermis is composed of stratified squamous epithelium created by keratinocytes organised in different cell layers. At the lower parts of the epidermis, adjacent to the dermis, in the one-layered stratum basale, basal keratinocyte stem cells divide and migrate outwards to stratum spinosum, granulosum and corneum (figure 1).

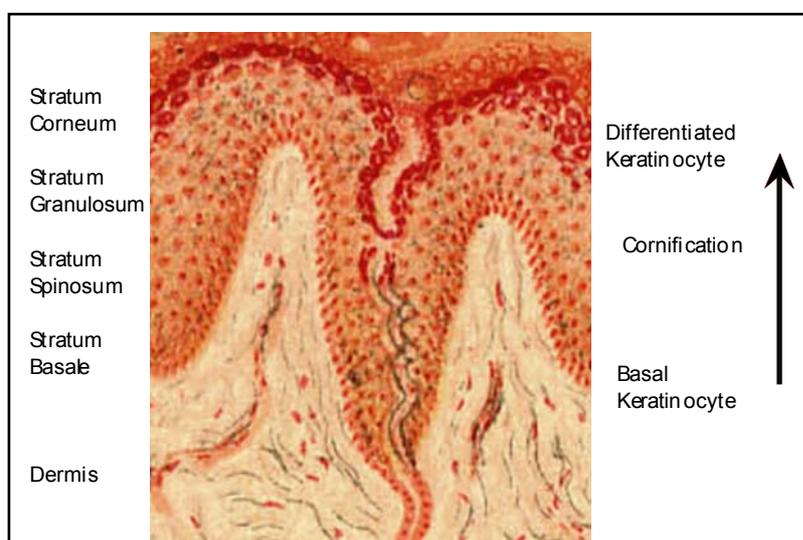


Figure 1) Morphology of the normal epidermis.

Epidermis is built by several layers of keratinocytes at different stages of maturation. The basal layer of keratinocytes are stem cells which also have inflammatory and antigen presenting properties. Within epidermis, Langerhan's cells and melanocytes reside. Detail from an illustration in *La Pratique Dermatologique*, Paris 1900¹ kindly provided by The Hagströmer Medico-Historical Library, Karolinska Institutet, Stockholm, Sweden.

During migration, the keratinocytes gradually mature and become more adapted to their function as a physical barrier to the external environment (a process called cornification) and keratinocytes become filled with keratin and decompose their organelles and nucleus. Mature keratinocytes in stratum corneum are tightly connected and surrounded by lipids and proteins which form a tough water-proof surface. The epidermis also harbours; melanocytes whose melanin protects from UV-radiation, Langerhan's cells, the antigen presenters of the skin and Merkel cells, functioning as sensory cells. The epidermis is connected by the basement membrane to underlying dermis (figure 1). The basement membrane of the skin forms a cell-free, glue-like matrix that allows passage of cells and nutrients. The dermis can be divided into two compartments. In the superficial papillary layer blood vessels, lymph vessels, nerves and a delicate network of collagens and connective fibres are found. The underlying reticular layer is characterized by thicker connective fibres and harsher collagen. The dermis is attached to the hypodermis (or subcutis) which consists of loose connective tissue and fat deposits².

In healthy skin constant immune surveillance is important for early detection of microbial invasion. Keratinocytes, Langerhan's cells, macrophages and dermal mast cells express a number of pattern recognition receptors, such as toll like receptors³, and upon proper stimulation release antimicrobial peptides and chemokines⁴ initiating inflammatory responses and recruitment of inflammatory cells. Memory T-cells constantly circulate the skin. Keratinocytes will upon initial stimulation produce for example interleukin 1 (IL-1 α) and tumour necrosis factor α (TNF α). Activated Langerhan's cells migrate to draining lymph nodes and evoke adaptive immune responses. T-cells activated by Langerhan's cells express molecules facilitating the homing to the skin, such as cutaneous lymphocyte antigen (CLA). About 30% of circulating memory T-cells express CLA. Activated T-cells secrete IFN γ in the skin, leading to further keratinocyte activation and secretion of C-X-C chemokine ligands (CXCL) 9-11, which in turn results in recruitment of more activated T-cells⁵. Thus keratinocytes are capable of both initiating and increasing the immune responses during inflammation.

Leishmaniasis

The leishmaniasis form a spectrum of diseases ranging from benign, self-healing skin ulcers to overwhelming lethal systemic infections. This broad spectrum of diseases caused by the same parasite is partly an effect of different sub-species of the parasite, but the immune system of the host also plays a role in the emergence of the diverse clinical manifestations of *Leishmania* infection. The majority of leishmaniasis cases fall within the group of cutaneous leishmaniasis (CL). CL can be further subdivided into localised cutaneous leishmaniasis (LCL), diffuse cutaneous leishmaniasis (DCL) and mucocutaneous leishmaniasis (MCL). The lethal, systemic form of visceral leishmaniasis (VL) presents initially with vague systemic symptoms such as fever, but in advanced stages is characterised by splenohepatomegaly, lymph node enlargement and wasting.

Leishmaniasis are old diseases that have been present in wide-spread parts of the world for a long time. The ulcerative form of CL was described in Iran around 1000 AD and in the Americas pre-Incan designs on pottery illustrations reminiscent of CL

clinical signs can be found. The first known documentation of visceral leishmaniasis (VL) available is dated 1824 in India. The parasite was visualised in tissue obtained from ulcers and spleens by several independent investigators around 1900 (Drs Borovsky and Shulgin in Tashkent, Wright in Armenia and Cunningham, Leishman and Donovan in India) ⁶⁻⁸ The protozoa was given its name by Leishman and Donovan, who in 1903 simultaneously reported finding of round intracellular bodies in tissue specimens from Indian CL and VL ⁹⁻¹².

Epidemiology

Leishmaniasis is reported endemic in 88 countries in the world and the prevalence is estimated to be around 12 million cases world-wide. Although epidemiological surveillance is lacking in many endemic areas the documented yearly incidence of VL is 500 000 cases and CL is 1.5 million cases worldwide. It has been estimated that 90% of all CL cases occur in Afghanistan, Brazil, Iran, Peru, Saudi-Arabia and Syria, whereas 90% of VL cases are found in Bangladesh, Brazil, Sudan, India and Nepal [WHO]. Leishmaniasis is also reported in southern Europe, Central and South America. Leishmaniasis is mainly zoonotic, with gerbils, dogs and rodents as the main mammalian host and man as an accidental host. However, in endemic areas, both CL and VL can exist as anthroponotic infections, where man serves as the reservoir of infection and the main transmission occurs from man to man. Leishmaniasis, especially VL, can also be transmitted though intravenous transmission in areas of drug abuse, often when individuals are double-infected with HIV and VL ¹³ with an increased number of infected cells in peripheral blood.

The estimated global burden of disease is believed to be inaccurate due in part to the passive case detection data used to estimate the disease prevalence in many endemic countries. Like many other diseases prevalent in the tropics, the leishmaniasis in low endemic countries are related to economic development and man-made environmental changes which increase exposure to the sand fly vector. Many of the attempts to improve economic output result in enhancing the chances of contracting leishmaniasis. These include timber extraction, mining, building dams, widening areas under cultivation, irrigation schemes and road constructions in primary forest¹⁴. Epidemics of leishmaniasis are known to occur frequently at many foci but the strategy of government and health authorities is often to react rather than to anticipate and prevent ^{15,16}. During the nineteen ninties a VL epidemic was reported in Sudan with mortality rates as high as 30% in some areas was reported^{17,18}. In Europe the threat of leishmaniasis has been seen with the increase in overlapping of visceral leishmaniasis (VL) and HIV infection. *Leishmania*/HIV co-infection has been considered to be a real “emerging disease”, especially in Southern Europe ¹³.

The Parasite

About 20 different species of the protozoa *Leishmania* of the family *Trypanosomatidae* have been described to date. CL in the Old World, namely Europe, Asia, North Africa, and the Middle and Far East is caused by *L. major*, *L. tropica* or *L. aethiopica*. In the Americas, *L. amazoniensis*, *L. braziliensis*, *L. panamensis* frequently cause CL. VL is mainly induced by *L. donovani* and *L. infantum* and in the Americas by *L. chagasi*. The unicellular parasite has two life-

forms; the ovaloid, intra-cellular amastigotes reaching 2-6 μm in diameter and the slender, elongated, flagellated extra-cellular motile promastigote that is 10-15 μm long and about 2 μm wide^{6,8}. Both life forms contain nuclei and a kinetoplast which is the *Leishmania* equivalent of mitochondria. Asexual replication through cell division takes place both in the amastigotes and promastigote form.

Leishmania Life Cycle

Leishmania has a biphasic life-cycle that involves a mammalian host and a sand-fly vector (*Lutzomyia spp* in the Americas and *Phlebotomus spp.* in the rest of the world) which breed in moist soil, forest areas, caves or the burrows of rodents and feed from infected animal reservoir hosts or humans. In the mammalian host, infective metacyclic flagellated promastigotes are injected into the dermis, together with salivary components, by the bite of a feeding female sand-fly. The promastigotes rapidly enter macrophages, Langerhan's cells and neutrophils by receptor mediated and passive phagocytosis and reside intracellularly in phago-lysosomal cytosomal compartments, and transform there into non-flagellated amastigotes^{6,8,16}. In the phago-lysosomal vacuole rapid amastigote division occurs and it is suggested that the parasites eventually lyse the infected cells and infect surrounding cells. The parasite life-cycle is completed when a feeding sand-fly ingests amastigotes during a blood meal from infected skin. In the sand-fly, the amastigotes transform into promastigotes and attach to the epithelium covering the mid-gut of the fly, where promastigote division occurs. Upon maturation, the composition of the promastigote cell-membrane changes so that the infective (metacyclic¹⁹) promastigote detaches from the gut and travel towards the salivary glands of the sand-fly^{6,8}. As the infected sand-fly feeds again, and deposits promastigotes in the dermis, the life-cycle is completed.

Cutaneous Leishmaniasis

Several clinical features of CL in the Americas and the rest of the world show distinct differences, where the American CL tend to have a more rapid pathological development, wetter ulcers and different healing evolution. This thesis work has concentrated on *L. major* induced CL as presented in Iran where the majority of CL cases are caused by *L. major* and *L. tropica*. In rural areas, the disease is mainly zoonotic and caused by *L. major*, whereas in urban areas considerable anthroponotic spread of *L. tropica* exists. In endemic areas, children tend to encounter the infection early in life. Upon healing of the infection the majority of people carry life long protection to re-infection.

Clinical Picture of CL Caused by *L. major*

L. major induced CL represents the most benign form of CL, although even *L. major* induced ulcers may in some cases become very large, disfiguring and chronic. An itchy papule often arises upon a sand-fly bite, possibly due to the inflammatory components of the sand-fly saliva, and resolves within a few days. After a period of a few asymptomatic weeks (even up to several months or sometimes years) a small itchy papule re-appears on the skin. The papule evolves into a nodule and eventually the epidermis ulcerates and is covered by a dry crust. Ulcerated lesions present with a raised ridge of infiltrated skin and the ulceration is shallow, seldom more than 2-3

mm deep. Scaly crusts typically cover the ulcer, and secondary bacterial infections often exacerbate the ulcerative process of the skin. Spontaneous healing typically occurs around three months to one year after onset of disease, leaving often unsightly scarring. In most cases, one or a few ulcers will arise, but in some cases the number of lesions may be very large. During the field work to collect material for this thesis one case with more than 90 lesions was assessed. Patients with more than 200 ulcers have been described. Due to the number of ulcers and the placement of the ulcers, CL can cause disfiguration of the face, but also impaired use of joint-function due to extensive scar formation^{6,8} (figure 2). Anecdotal data suggest that in order to obtain solid, long-lasting immunity towards CL, a vigorous immune response with active ulceration is needed. However, subclinical *L. major* infection leading to subsequent immunity has been suggested in endemic areas²⁰.

In rare cases healing is interrupted upon *L. major* infection. The lesion may present as an ulcer, where the ulcerative process reverts, but erythematous plaques may linger on for up to 10-15 years. Clinicians in endemic areas claim that upon tissue sampling, injections, or mechanical trauma the ulcerative process may be re-initiated^{8,21}.



Figure 2) Picture of *L. major* induced CL on face and close to joint.

Active ulcers of typical clinical appearance and distribution are depicted. Photographs kindly provided by Dr T. Lieke, MTC, Karolinska Institutet.

Immunopathology during *L. major* Infection

The typical morphological changes in epidermis and dermis occurring during *L. major* induced CL natural infection are listed in table 1. The keratinocytes surrounding the ulcer upregulate the intracellular adhesion molecule 1(ICAM-1) and major histocompatibility complex (MHC II), express *Leishmania* antigens^{22,23} and thus possibly function as antigen presenting cells, driving homing of inflammatory cells to the skin. As the host responds to infection and immunity develops, the epidermis become increasingly hypertrophic with proliferation of keratinocytes in the middle layers of epidermis. Necrosis of the dermis precedes ulceration. Neutrophils in karyorrhexis, an old, pathological definition of cells with morphological resemblance to apoptosis with condensed nuclei, are prevalent in CL lesions¹⁶. Ulceration appears few weeks after lesion development. During early ulceration IL-4, IL-10 and

interferon gamma (IFN γ) are reported to be expressed in the central part of the ulcer²⁴, whereas the epidermis surrounding the ulcer is infiltrated with Langerhan's cells.

With time, epithelioid and Langerhan's giant cells granulomas are found, and parasites become scanty, eliminated by activated macrophages and active immune responses⁸. Granulomas are a typical feature of localised CL during healing and usually circumscribe necrotic material, infected and destructed macrophage, extracellular parasites and possibly control local spreading of infection by isolating infective material. In the material included in this thesis, no such granulomas were detected in active or healing biopsies. This is consistent with a histological study on *L. major* caused CL in Sudan where histological profiles showed several different pattern, where granulomas sometimes occurred with or without surrounding inflammatory infiltrate of T-, B- cells and histiocytes and lesions without granulomas were also described²⁴. In the same study, CD8⁺ T cells were present in the ulcer during active, but not healing disease. Local production of IFN γ , IL-12 and TNF α was correlated to healing and IL-4 and IL-10 to chronic ulcers²⁴. Healing typically lead to fibrosis in the dermis.

L. aethiopica induced CL is an intriguing example of how multi-faceted leishmaniasis is. *L. aethiopica*, endemic in the highlands of Ethiopia and Kenya either presents as an ulcerative, localised, self-healing disease or as non-ulcerative, infiltrative, chronic lesions (called diffuse cutaneous leishmaniasis (DCL). In DCL, an abundance of

	Epidermis	Dermis
Acute phase (hrs-2-3 days)	No changes in epidermis	Local inflammatory reaction. Histamine secretion and neutrophilic and monocytic infiltration. <i>L. major</i> rapidly infects local histiocytes
Silent phase (weeks-months)	No overt pathology. Atrophic changes in epidermis.	Rapid parasite replication and spreading. Parasites present in upper dermis, both intra- and extracellularly. Monocytes/macrophages dominate the inflammatory infiltrate.
Active phase	Keratinocyte up-regulation of ICAM and MHC II. Proliferation of keratinocytes in middle layers of epidermis. Epidermal infiltrate of Langerhan's cells.	Dermis show sign of necrosis containing neutrophils in apoptosis (karyorrhexis). Inflammatory infiltrate of T-cells (CD4 and CD8), B-cells (plasma cells), macrophages. a) Granulomas are formed by epithelioid cells, macrophages and Langerhan's cells and found adjacent to inflammatory cells b) Granulomas are found without other inflammation. c) Inflammation without granulomas
Ulcerative phase	Ulceration of epidermis, keratinocyte cell-death	
Healing phase	Re-epitheliation and keratinocyte proliferation.	Activated macrophages kill off parasites. Dermal fibrosis. Epithelioid and Langerhan's cell granulomas. Few remaining parasites.

Table 1: Immunopathology of the skin during CL.

Several different histological profiles of dermis have been described during the active phase of *L. major* induced CL, possibly due to 1) differential localisation of biopsies investigated or compared to distance to central ulcer, 2) samples were obtained during different phases of the infection or 3) inter-individual differences in immunopathological responses during CL^{6,16,24}.

parasites are found under atrophic, but intact, epidermis. This lends support to the possibility that strong immune-activation leading to ulceration may be necessary for parasite clearance. During DCL a clear zone without inflammation is present adjacent to the basement membrane⁸. The dermis is thickened and packed with macrophages containing a high number of amastigotes. One feature of DCL seems to be non-responsiveness to leishmanial antigens, both in skin and *in vitro* based assays²⁵⁻²⁷.

Visceral Leishmaniasis

In VL a firm nodule in some cases develops in the skin at the site of infection within weeks of the encounter with the parasite and, upon tissue sampling from such nodule, parasites can be detected. Onset of disease can be acute or chronic. Patients often develop disease symptoms within a year of infection, such as intermittent fever, wasting, hepatosplenomegaly and anaemia⁶. Subclinical disease has been described but not thoroughly investigated. In some cases, post kala azar dermal leishmaniasis (PKDL) occurs late in infection, presenting with non-ulcerative nodules of the skin. PKDL was first described in India, where incidences of 5-10 % of all VL cases have been reported. Recent indications are that these numbers are decreasing in India²⁸. PKDL is prevalent in up to 50% of VL cases upon discontinuation of VL treatment in Sudan²⁹.

Immunopathology during VL

The infection spreads from the skin through the lymph by infected circulating histiocytes and neutrophils and parasite replication takes place throughout the reticulo-endothelial cells in lymph-nodes, spleen, liver and bone-marrow⁶. Acute lymphopenia is noted in active disease, together with granulocytopenia⁶. VL is also characterized by depressed T-cell function as evidenced by diminished *Leishmania*-specific and/or non-*Leishmania*-specific T-cell responses^{30,31}. The T-cell deficiency is coupled with a progressive release of a number of cytokines with down-regulatory effects on macrophage function (e.g. IL-4, IL-10, transforming growth factor β (TGF β), and TNF α). This results in diverting the immune response from IFN γ producing T-helper 1 (Th-1) cells that are needed to clear the infection. In the spleen, the pathological consequences of VL include accumulation of macrophages containing the pathogen. In the bone-marrow, infected macrophages replace hematopoietic tissue, which provides a possible explanation to the pancytopenia described during the late stages of the disease^{6,8,16,32}. Interestingly during early infection hyperplasia and proliferation of the reticulo-endothelial system occurs⁶. Hyperglobulemia is a common feature in VL, a hallmark of unspecific activation of the immune system. During acute VL, patients show both *in vivo* and *in vitro* generalised T-cell unresponsiveness. During PKDL the dermis of affected areas is infiltrated with macrophages, lymphocytes and plasma cells and the cytokines IFN γ , IL-4 and IL-10 are expressed in the affected areas. Parasites may be present, but not in abundance³³.

Leishmaniasis and HIV

HIV-1 infection leads to suppression of T-cell numbers and functions and thus allows opportunistic infections in the host. HIV-1 infects T-cells and shares one target of infection, the macrophage, with *Leishmania*. Co-infection of VL and HIV-1 has been reported from 35 countries and it has been estimated that the risk of contracting VL when living in an endemic area increase 100-1000 folds in AIDS patients¹³. In cases

of Ethiopian co-infection of VL and HIV-1, effective VL treatment led to a decrease in HIV viral load. When HIV viral load was high prior to treatment, VL treatment was shown to be less effective³⁴. *L. donovani*, or part of the parasite, has furthermore been shown to increase HIV replication in cells of both monocytic and T-cell lineages, both in cell-lines and *ex vivo* material³⁵⁻³⁷.

Diagnosis

In areas where CL is endemic, diagnosis is frequently made on clinical grounds alone and a chronic skin lesion is suggestive of CL. To confirm the diagnosis microscopic detection of amastigotes in aspirates, scrapings and smears from the ulcers, stained with May Grünwald-Giemsa, Wrights³² or Romanovsky solution³⁸ is used. In early or late phases of CL parasites may be scanty and culture of tissue sample is necessary for parasite detection. Lately PCR has become an alternative quick and reliable diagnostic method, albeit not available in many endemic foci. Several methods are available to type the infective species, for example PCR, monoclonal antibodies against species-specific antigens and isoenzyme patterns of individual species¹⁶. Detection of antibodies in serum and skin testing with Leishmanin skin test (LST), evoke cell-mediated inflammatory responses and an induration of the skin. LST can be used in epidemiological trials as a sign of past or present exposure to *Leishmania*. The latter method however has no role in the diagnostic procedure in endemic countries as immune responses remains for years after the infections and may be the sign of earlier subclinical disease^{6,32}.

In VL visualisation of amastigotes in spleen or bone marrow aspirates has been widely used and in the hands of skilled personnel the method is safe³⁹. However, due to the risk of complications upon aspiration (mainly uncontrolled bleeding that may be fatal) there is a need for improved, less invasive diagnostic tools. In addition, in endemic areas, the occurrence of antibodies together with typical clinical presentation is sometimes enough for diagnosis and initiation of treatment. Several methods for sensitive and specific detection of VL antibodies and serum and urine are being evaluated⁴⁰. LST is positive during early VL and after healing, but due to the associated T-cell depression responses are often low or missing during active VL.

Treatment

In the case of self-healing CL the main decision is to treat or not to treat since chronic ulcers are understandably badly accepted by patients, however benign in nature. Often patients request treatment and a number of therapeutical approaches have been used aimed at reducing ulceration and disfiguring scarring. Local application of heat and cryotherapy has been used leading to local tissue destruction that may in turn evoke inflammatory responses and activate infected macrophages. Many treatment regimes used against CL have not been evaluated in controlled studies. The Iranian national protocol for *L. major* induced CL treatment based on WHO recommendations is performed in accordance with the patient's wishes. No treatment is recommended for single or few lesions, but intra-lesion injections of Glucantime (meglumine antimoniate) can be administered once or twice a week for up to 15 weeks. Multiple lesions, non-healing lesions or lesions close to vital organs, are treated with daily intramuscular injections of Glucantime (60 mg/kg body weight) for 14 days²¹.

While the treatment in CL mainly has cosmetic value, the treatment of VL is necessary for survival of the patient. Antimonials have been used in VL treatment

since 1910 and sodium stibogluconate since the 1950ies⁶ with decreasing leishmanicidal effect due to emergence of resistant parasite strains. Miltefosine, an alkylphosphocholine, is the first oral drug that has been approved for treatment against VL. Although efficient it has been shown to be teratogenic in rodents and has thus not been tested in women of child-bearing age. Amphotericin B, is a pentavalent compound interacting with the cellular membranes of the parasite, and in its liposomal preparation serves as a good and well tolerated anti-VL therapy, although prohibitively expensive and thus not available to the majority of VL patients. Paromycine, an aminoglycoside antibiotic, is being evaluated as an alternative oral drug due to its leishmanicidal effects in CL and VL and relatively low price as compared to miltefosine and liposomal amphotericin B.^{28,40}

Vaccines

Due to the clinical severity of VL and the often poor health facilities in endemic areas, there is a need for a safe and effective vaccine. The feasibility of such vaccine has been proposed to be easy due to several factors. In the majority of cases life-long protection towards future infection occurs upon healed leishmaniasis. Interestingly, a population in Sudan that moved from a *L. major* endemic area to a *L. donovani* endemic area was suggested to be protected against VL⁴¹. A number of vaccine candidates utilising variable approaches have been tested to find a safe and effective vaccine against leishmaniasis. Due to proposed cross reactive protection between *L. major* and *L. donovani* and as *L. major* infection is endemic in many areas and easy to detect, attempts to create effective vaccines against *L. major* infection have been based on approaches to develop vaccines against VL. Although highly refined DNA vaccines and immunomodulatory vaccines have been tried, and proven successful in animal models of CL, the most successful approach in man is still the inoculation of live promastigotes in selected parts of the body away from the face, a process commonly known as leishmanization⁴². This approach of live vaccination has been used for many centuries and is still used in clinical practise in Iran and Uzbekistan⁴³.

Animal Models of Leishmaniasis

L. major infection in mice of different genetic background has served as a great toolbox for immunologists interested in infectious diseases, and it was the first model in which different disease outcome due to Th1 or Th2 type immune responses could be shown^{44,45}, reviewed in⁴⁶. Typically, more than a million parasites have been injected subcutaneously into the mice and, depending on the genetic background of the mice, visceralising (Balb/c) or healing infections (C57BL/6 among others) have been monitored during different conditions. Although useful for basic immunological studies these models have not been suitable to study tissue reactions and pathology during *L. major* infection, since the natural course of infection has differed from leishmaniasis in man and symptoms such as ulceration have been absent. A murine disease model has been set up in both susceptible (Balb/c) and resistant (C57BL/6) mice where infection is introduced through intradermal injection of a low number of highly infectious (metacyclic) parasites (100-10000) in the presence or absence of salivary components from the vector. In this setting, *L. major* infection leads to a silent phase of a few weeks after which a lesion appears, ulcerates (in the case of Balb/c and in C57BL/6 in the presence of salivary gland components) and either heals (C57BL/6) or cause severe ulcerative processes in the infected tissue^{47,48}.

Apoptosis

The homeostasis of the human body is dependant on constant cell-renewal. As new cells are needed, a mechanism for removal of old or unwanted cells is essential yet without evoking inflammatory responses. Apoptosis, or programmed cell-death, provides a mechanism for removal of senescent cells, cells that have served their function or cells that may be dangerous for the organisms, such as cancerous, hyperactivated, autoimmune or infected cells. A plethora of different apoptotic pathways have been described to date, many of which overlap each other. At the molecular level, apoptosis is tightly regulated and is mainly orchestrated by the activation of the aspartate-specific cysteine protease (caspase) cascade. Apoptosis is initiated mainly through two pathways; the intrinsic apoptotic pathway (or the mitochondrial pathway) depends upon mitochondrial participation and is receptor-independent. The extrinsic or death receptor dependent pathway involves the interaction of a death receptor with its ligand. Apoptosis involves the entire cell machinery, and it is regulated by pro- and anti-apoptotic proteins. The end result of either pathway is caspase activation and cleavage of specific cellular substrates, resulting in the morphological and biochemical changes associated with the apoptotic phenotype which includes alterations of the mitochondrial membrane stability, leading to release of cytochrome c (reviewed in ⁴⁹) and nuclear events (reviewed in ⁵⁰), leading to DNA fragmentation. During the process, phosphatidyl serine (PS) is expressed on the cell-membrane, facilitating receptor mediated phagocytoses of apoptotic cells by, for example, macrophages. The process of apoptosis is completed when the apoptotic cells are removed by phagocytosis.

Although the main role of apoptosis has been considered to ensure homeostasis of multicellular organisms such as humans, several biochemical hallmarks of apoptotic death have also been described in unicellular organisms such as *Leishmania*, and these hallmarks include expression of PS, DNA fragmentation and changes in kinetoplastic membrane with release of *cytochrome c* ^{51,52}. It has been proposed that apoptosis of parasitic unicellular organisms may serve several functions. Firstly it may reduce the infective dose of the vector or the host thus ensuring survival of both the vector and the host and maximal chance of transmission of the parasite. In addition, unicellular organisms with signs of apoptosis such as PS-expression may not necessarily have to complete the apoptotic process but provide signals that enhance the survival of the entire parasitic population. In a model of *Trypanosoma cruzi* infection in macrophages, exposure of apoptotic cells to macrophages facilitated infection and intracellular growth of *T. cruzi* ⁵³. In the case of *Leishmania*, PS expression may facilitate receptor mediated entry into macrophages without evoking immune responses of the host. Apoptosis of unicellular organisms may also be a way to strengthen the fitness of the population by removing non-infectious organisms. This would be of use for parasitic survival for example in the vector, where removal of promastigotes failing to develop into metacyclics would increase the chances of establishing infection.

Morphology of Apoptosis

Independent of how apoptosis is initiated, typical morphological changes can be detected in apoptotic cells. The cell-membrane remains intact, so that no inflammatory intra-cellular components leak out into the surrounding tissue. Protein synthesis is stopped, the chromatin condensation and DNA fragmentation is initiated.

The cell shrinks and the nucleus is fragmented. The cell membrane changes its protein expression facilitating phagocytosis, and becomes uneven in shape (blebbing) and the cell is broken up into apoptotic bodies. Few apoptotic cells are found in healthy tissue, despite often constant homeostatic cell-turnover due to efficient phagocytosis⁵⁴.

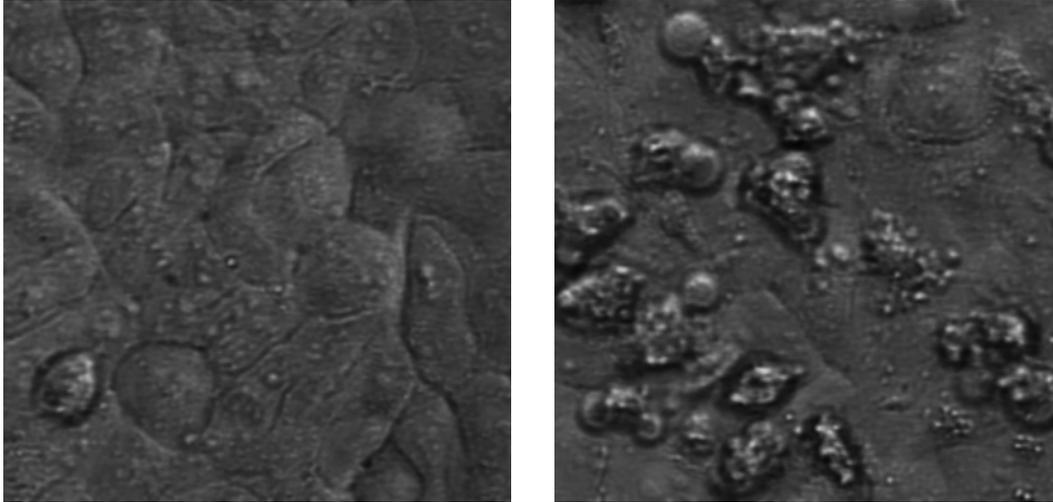


Figure 3) Cell morphology changes during apoptosis.

To the left healthy keratinocytes in culture and to the right apoptotic keratinocytes showing shrinking and blebbing.

Death Receptor Mediated Apoptosis

Extrinsic apoptosis can be initiated through activation of a number of membrane bound receptors. To date, eight death receptors have been characterized; namely Tumor Necrosis Factor Receptor 1 (TNFR-1; also designated TNFRSF1A, DR1, CD120a, p55 and p60) Fas (or TNFRSF6, CD95, APO-1 and DR2), DR-3 (or TNFRSF12, APO-3, LARD, TRAMP and WSL1), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1; or TNFRSF10A, DR4 and APO-2), TRAIL-R2 (or TNFRSF10A, DR5, KILLER and TRICK2), DR-6 (or TNFRSF21), ectodysplasin A receptor (EDA-R) and nerve growth factor receptor (NGF-R or TNFRSF16) (reviewed in^{55,56}). The nomenclature Fas and TRAIL-R1 and -R2 will be used for the death receptors investigated in this thesis. A common feature of death receptors is a cytoplasmic domain of approximately 80 residues termed the death domain (DD). Upon receptor activation, intracellular signalling is initiated after recruitment of several molecules to the DD. Four decoy receptors, binding death ligands but lacking DD and thus unable to initiating apoptosis, have been described, TRAIL-R3 (or TNFRSF10C, DcR1), TRAIL-R4 (TNFRSF10D, DcR2), DcR3 (TNFRSF6B) and osteoprotegerin (OPG, TNFRSF11B) (figure 4).

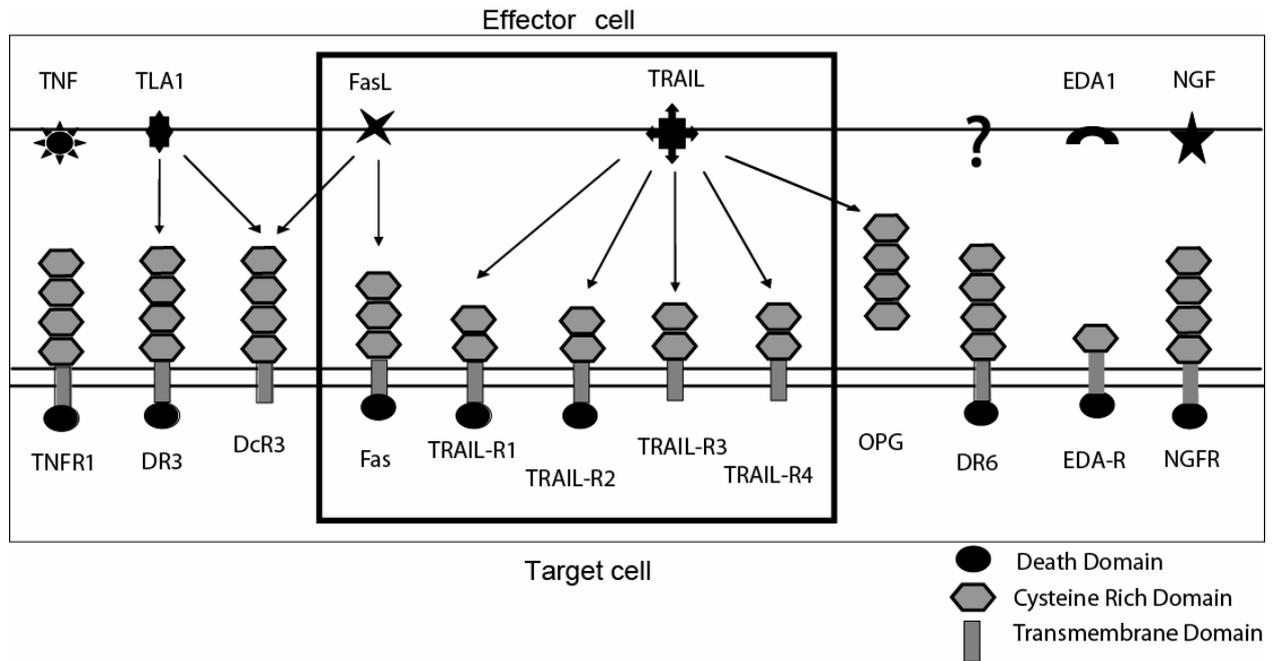


Figure 4) Death Receptor Mediated Apoptosis. Adapted from⁵⁷. Death Receptors investigated during this thesis work highlighted. The natural ligand for DR6 is yet to be determined.

FasL Induced Apoptosis

Fas Ligand (FasL) (also known as CD178) is a type II transmembrane receptor and was described in 1993 mainly expressed by activated T-cells, NK cells and macrophages^{58,59}. FasL can be induced in a number of cells, including keratinocytes^{60,61}. FasL has been implicated in cytotoxic T lymphocyte (CTL) and natural killer (NK) cell-mediated cytotoxicity and is stored in intracellular vesicles in cytotoxic cells, ensuring high levels of membrane bound FasL during cytotoxicity⁶². A soluble form of FasL, sFasL, is cleaved off FasL expressing cells by a metalloproteinase (MMP), possibly MMP-7⁶³. Fas was described by two independent groups in 1989^{64 65} and is expressed as homotrimer transmembrane receptor, but can also be produced in a soluble form (sFas) by alternative splicing of the full-length mRNA⁶⁶. Fas is expressed, at variable levels, on most cells types in the body. Already 1993-94 the first Fas (*lpr*) and FasL (*gld*) deficient mouse strains were available⁶⁷ which allowed extensive studies on the relevance of Fas mediated apoptosis in physiological and pathological contexts⁶⁸. Fas or FasL deficient mice develop generalised lymphoproliferative disease partly due to inefficient clearance of T-cells. Fas/FasL interactions have been proposed to be necessary to down-regulate the number of reactive cells during an immune response at the peak and contracting phase of immune responses by activation induced cell death (AICD)^{69,70}.

Upon Fas activation, two types of intracellular responses have been described. In type I cells, pro-caspase 8 and pro-caspase 10 are recruited to the DD through interactions with Fas Associated Death Domain (FADD) and form death inducing signalling complexes (DISCs), cleaving and activating caspase 8. A high number of DISCs are present in type I cells and through active caspase 8, caspase 3 is directly activated. In type II cells, DISC formation is low and caspase 8 signalling occurs through the Bcl-2 family protein Bid, which generate truncated Bid (tBid) that induces

cytochrome c release from mitochondria. *Cytochrome c* forms an apoptosome complex consisting of pro-caspase 9 and apoptosis activating factor-1, which will activate pro-caspase 3. Caspase 3 activates a number of proteins, eventually leading to DNA fragmentation and cell death. The distribution of type I and type II cells and their physiological role remain unclear. Cross-linking of Fas may also induce NF- κ B nuclear translocation^{71 72} (figure 5) which, amongst many functions, has been shown to be a potent inhibitor of apoptosis⁷³. Thus in cells resistant to Fas mediated apoptosis Fas signalling may lead to inflammatory responses and secretion of chemokines such as IL-8⁷⁴. However, NF- κ B is a transcription protein that is widely activated in response to proinflammatory cytokines during microbial infection and induces a vast number of cytokines and chemokines, where the resultant effect on cell activation and survival is difficult to predict.

Possible Role and Signalling Capability of sFasL

sFasL has been shown to both block and activate Fas in different experimental setups^{58,75-77} and the function of sFasL is still debated. Early studies suggested that the Fas activating capability was present in human, but not murine sFasL⁵⁸. Soluble trimeric FasL interacts with trimerized Fas but has been shown to fail to activate as⁷⁸. In contrast, hexameric soluble FasL or antibody crosslinked sFasL are sufficient to induce Fas signalling⁷⁹ (figure 5). Some findings suggest that the apoptosis inducing ability and toxic effect of sFasL is considerably enhanced by its binding to the extracellular matrix⁸⁰. When T-cells were exposed to short term incubation with fibronectin bound FasL followed by T cell activation through CD3; IFN γ secretion and late T-cell apoptosis (after 3 days) occurred. This indicate that FasL bound to extracellular matrix induce early inflammatory responses followed by late apoptosis in T-cells that have migrated to areas of inflammation⁷⁷. sFasL may act as a chemoattracting factor inducing migration of phagocytes⁸¹. sFasL can also under certain circumstances, such as serum starvation, induce IL-6 and IL-8 production in fibroblasts⁷² and it has been shown *in vivo* and *in vitro* in murine systems that macrophages can be stimulated by FasL (vesicle bound) to release macrophage inflammatory protein (MIP)-2 and induce macrophage RNA expression of IL-1 β , MIP-2, MIP-1 α , and MIP-1 β ⁷⁴.

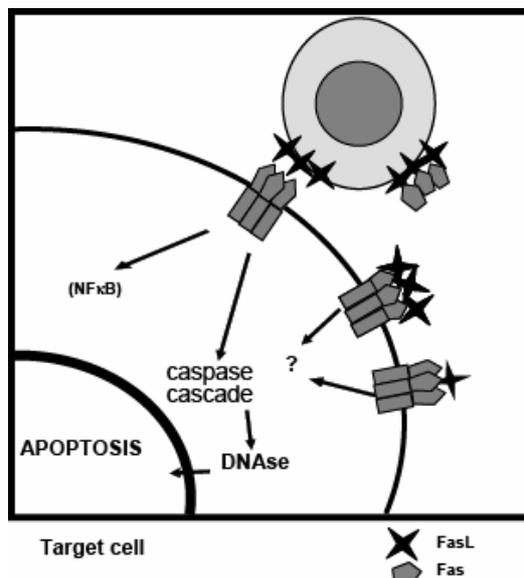


Figure 5) Overview of Fas/FasL signalling pathway.

Fas is expressed as a homotrimer, and upon FasL ligation caspase activation occurs, through DISC formation or tBid and mitochondria, leading to apoptosis. However, in Fas resistant cells such as resident macrophages, Fas ligation may lead to upregulation of NF- κ B and eventually secretion of inflammatory molecules such as chemokines. The role of sFas is probably to inhibit apoptosis. The function of sFasL is still debated; possibly the concentration of sFasL determines whether Fas ligation will lead to efficient signalling.

TRAIL Induced Apoptosis

TRAIL was characterised in 1995. TRAIL is a type II transmembrane protein showing structural and functional similarities to FasL. A soluble form of TRAIL is cleaved off TRAIL-expressing cells by proteolytic enzymes. Five known receptors bind TRAIL with similar affinity (fig 6). Activation of TRAIL-R1 and TRAIL-R2 lead to DISC formation and caspase 8 activation, which either directly activate caspase 3 (type I) or lead to mitochondrial release of cytochrome c (type II). TRAIL can trigger apoptosis independently through TRAIL-R1 or -R2 and in cells where both receptors are present they can form hetero complexes. TRAIL-R3 lacks intracellular signalling domain and functions as a decoy receptor. TRAIL-R4 lacks a DD and can activate NF- κ B through a truncated cytoplasmic domain thus inducing activation and proliferation in cells⁸².

TRAIL is widely expressed in many tissues although its role under physiological conditions is not well characterized. TRAIL has been suggested to form an additional pathway of T cell-mediated cytotoxicity⁸³ and that TRAIL is important in immune surveillance, by early detecting and killing of infected cells or cells during oncogenic transformation⁸⁴. This scenario has been suggested partly due to the ability of TRAIL to trigger apoptosis in a number of immortalised cell-lines. However, dysregulation of TRAIL has been described in HIV^{85,86} and listeriosis⁸⁷. TRAIL-deficient mice show impaired thymic apoptosis, as well as increased susceptibility to autoimmune diseases such as collagen induced arthritis⁸⁸.

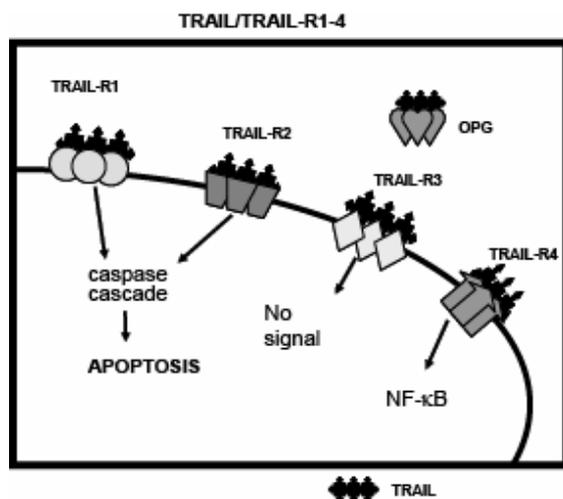


Figure 6) Effect of TRAIL interaction with TRAIL R1-4.

Apoptosis is initiated through TRAIL activation of TRAIL R1 and -R2. TRAIL-R3 is a decoy receptor and will not initiate intracellular signalling. TRAIL-R4, on the other hand, will lead to NF- κ B signalling and may thus protect cells from apoptosis. OPG is a soluble receptor binding TRAIL and its function has not been thoroughly described.

Life and Death in the Healthy and Inflamed Skin

Cornification of keratinocytes shows similarities to apoptotic death with degradation of the nucleus, organelle destruction and protease activation. Cornification of a keratinocyte takes approximately two weeks in comparison to the few hours needed for induction, execution of apoptosis and removal of apoptotic cells⁸⁹. Cornified keratinocytes are not removed but continue to fulfil their function of protecting the underlying tissues. Although DNA is degraded during cornification, TdT-mediated dUTP nick end labelling (TUNEL) positive cells are not commonly found in adult skin

and caspase 3 or caspase activated DNase (CAD) does not seem to be activated during cornification in the adult skin. It has been proposed that anti-apoptotic members of the bcl-2 family are expressed to protect mature keratinocytes from apoptosis by extrinsic pathways.

Fas is expressed by healthy skin⁹⁰ and dividing keratinocytes undergo apoptosis upon Fas-activation⁹¹ although some investigators have suggested that IFN γ pre-treatment is necessary to render keratinocytes susceptible to apoptosis^{90,92,93}. FasL expression has been proposed to take place in healthy skin⁹⁴ and *in vitro* experiments suggest FasL expression by subsets of primary keratinocytes and by the immortalised keratinocyte cell line HaCaT⁶⁰. FasL is shown to be upregulated on keratinocytes upon cytokine stimulation can induce apoptosis in an activated T-cell line (Jurkat). Apoptosis of Jurkat could be reversed in the presence of Fas-blocking antibodies⁶⁰. According to these latter findings a model of immuno-privilege of the skin or a method for skin to down-regulate inflammatory infiltration of the skin was proposed⁶⁰. However, the risk of paracrine killing of adjacent basal keratinocytes leading to skin pathology should also be considered in such scenario. Basal dividing keratinocytes are susceptible to Fas and TRAIL⁹⁵ induced apoptosis, and UVB irradiation can induce apoptosis in basal keratinocytes through clustering of Fas receptors⁹⁶, facilitating FasL mediated or FasL independent^{97,98} apoptosis. Fas mediated apoptosis plays a role in the pathogenesis of atopic dermatitis, where both Fas and IFN γ killing of keratinocytes by T-cells are found in acute exzematous lesions⁹⁹. Keratinocytes undergoing apoptosis release chemoattractants and thus a loop of activation is created during atopic dermatitis between infiltrating, FasL expressing T-cells and activated, Fas expressing keratinocytes¹⁰⁰. Keratinocytes activated by IFN γ also secrete IFN γ ⁹². Apoptosis by CLA expressing, activated Th1 cells lead to apoptosis in the skin and decline of CLA+ Th1 lymphocytes, which could explain the predominance of Th2 during dermal disease¹⁰¹.

TRAIL, as well as TRAIL-R1 and -R4, are expressed in normal skin¹⁰² and the knowledge on the expression pattern of TRAIL-R2 and -R3 in healthy skin is still limited. TRAIL-R1 and -R2 are expressed in the keratinocyte cell-line HaCaT^{92,103,104} and induce apoptosis upon stimulation. The role of TRAIL induced apoptosis in skin homeostasis remains to be fully evaluated although recent publications have proposed that TRAIL induces apoptosis in undifferentiated, but not differentiating, primary keratinocytes¹⁰⁵. TRAIL signalling through TRAIL-R1 in keratinocytes (HaCaT) may lead to NF- κ B activation, inflammatory responses and secretion of IL-8¹⁰⁴.

Alteration of Apoptosis during Leishmaniasis

Upon withdrawal of growth factors, murine monocytes/macrophages undergo spontaneous apoptosis and upon *in vitro* *L. donovani* infection apoptosis was inhibited, leading to enhanced macrophage survival and offering the parasites more time to replicate¹⁰⁶. This finding was confirmed in *L. major* infected macrophages *in vitro*¹⁰⁷, where inhibition of apoptosis was accompanied by inefficient *cytochrome c* release and decreased caspase 3 activation as compared to uninfected macrophages. Inhibition of spontaneous apoptosis by *L. major* has also been reported in *in vitro* infected human and murine neutrophils¹⁰⁸.

The role of Fas and FasL during experimental leishmaniasis and parasite clearance has been investigated in several well distinct experimental settings¹⁰⁹⁻¹¹³. Fas-mediated apoptosis of *Leishmania*-harboring macrophages was required for the resolution of lesions in mice infected with *L. major*. Mouse macrophages infected with *L. major in vitro* show up-regulated surface expression of Fas in response to IFN- γ , and as a result become susceptible to CD4+ T-cell-induced apoptosis¹¹⁰, although others report specific killing of *L. major* activated CD4-cells by FasL¹¹⁴. Inhibition of neutrophil apoptosis¹⁰⁸, during the early phase of infection led to a new route of silent entry of parasites into macrophages when infected, apoptotic neutrophils were ingested by resident macrophages¹¹⁵. When a high dose, subcutaneous infection with *L. major* was introduced in *gld*^{109,110} and, *lpr* mutant mice, the mutant mice were more susceptible to infection as compared to wild type mice¹¹². In the case of *gld*, the infection was efficiently cleared after systemic administration of sFasL¹¹⁰. It has been reported that TNF-R signalling (but in this setting not Fas signalling) is important for T-cell death leading to resolution of *L. major* induced inflammation in murine knock-outs of Fas or TRAIL¹¹⁶.

In a model of *L. donovani* induced VL in C57BL/6, where infection was introduced by intra venous injection of 20 million promastigotes, infection was more efficiently established in C57BL/6*gld* mice and the parasite burden was higher as compared to control C57BL/6¹¹⁷. AICD of T-cells was of similar magnitude in Fas deficient and competent mice, suggesting the several apoptotic pathways were activated during this process. During murine *L. infantum*, FasL expressing cytotoxic T-cells were purified from the spleen in infected mice¹¹⁸ and were proposed to take part in the pathogenesis of the spleen. Accumulating evidence thus exists in animal models of both CL and VL that apoptosis is altered during leishmaniasis.

Could Alterations of Death Receptors Offer New Therapeutic Approach in Human Disease?

The homeostasis of life and death has been ascribed a role in pathogenesis in a vast number of diseases. The field of apoptosis expanded during the 1990ies providing a large volume of data on different aspects of apoptosis. Cell-proliferation and lack of death of malignant cells leads to cancer. Aberrant cell-death of T-cells during HIV-1 infection leads to immunosuppression and susceptibility to opportunistic infections. Autoreactive T-cells not deleted during autoimmune disease harm healthy tissue. Increased understanding on apoptotic dysregulation during disease is leading to the development of novel therapies (reviewed in^{119,120}). It is however too early to evaluate if pharmacological fine tuning of apoptotic machinery may become an efficient approach to cure disease without unacceptable side-effects. Recent advances in our understanding of apoptosis have clearly shown that old pharmacological agents function through induction of apoptosis, for example a number of agents used in chemotherapy.

In vivo experiments in which mice and primates have received sTRAIL, did not lead to induction of apoptosis in liver and thymus⁸⁴, whereas systemic treatment with Fas-agonistic agents lead to liver failure and death⁶⁸. The TRAIL / TRAIL-R1-2 apoptotic pathway has been suggested to be pro-apoptotic mainly in malignant cells¹²¹. Thus hope has been laid on cancer-treatment with TRAIL agonistic agents and clinical trials with such drugs are underway (reviewed in^{56,122,123}). This preferential

involvement of TRAIL in apoptosis of malignant cells has been recently challenged by several reports showing that TRAIL can induce apoptosis in non-malignant cells such as hepatocytes¹²⁴ and primary keratinocytes¹⁰⁵. In skin, T-cells (both CD4 and CD8) infiltrating the skin and in circulation during atopic dermatitis¹²⁵ have been shown to express TRAIL. Albeit promising, administration of TRAIL agonistic agents in a context of cytokine driven activation and dysregulation of TRAIL mediated apoptosis, for example during a common influenza or arthritis must be evaluated.

An interesting example of the therapeutic potential of altering death receptor mediated apoptosis is the accumulating data from intravenous immunoglobulin (IVIG) treatment during toxic epidermal necrolysis (TEN or Lyell's disease). IVIG exhibit a broad spectrum of immunoregulatory activities *in vitro* and *in vivo*. Both Fas activating activities¹²⁶ and Fas blocking properties⁶¹ have been ascribed IVIG¹²⁷. Drug-induced toxic epidermal necrolysis (TEN) is a life-threatening disease characterized by extensive destruction of the epidermis (up to 90% of the body surface). Alteration of Fas and FasL in keratinocytes^{61 128} and in the effector T-cell compartment¹²⁸ leading to keratinocyte death and ulceration has been described in TEN^{61,128,129} and high serum levels of sFasL have been measured at the time of diagnosis. Systemic treatment with IVIG containing Fas-blocking antibodies limited the ulcerative process⁶¹ and reduced mortality in several multi-center analysis¹³⁰⁻¹³⁴. although conflicting data has been published¹³⁵, possibly due to the different levels of Fas blocking antibodies in different preparations of immunoglobulins. It would be beneficial to know the Fas blocking potential of individual IVIG batches before initiation of treatment. Short term tailored treatment schedules with purified Fas-blocking antibodies could be envisaged.

For diseases such as CL, which have a treatment regime of up to 30 intradermal injections of Glucantime, the results obtained in TEN could offer a new therapeutic approach. Adjuvant therapies like Fas blocking antibodies, could, if proven efficient in reducing ulceration during CL, present a feasible approach to reduce tissue damage.

Aims

This project was undertaken to investigate if dysregulation of apoptosis has a role in the pathogenesis of human leishmaniasis.

The following focused and feasible aims were phrased and investigated:

- To investigate dysregulation of Fas/FasL during human VL and CL.
- To investigate mechanisms of keratinocyte apoptosis during human CL.
- To block the Fas/FasL during murine CL and study the effect of this therapeutical approach on ulceration in this animal model.

Ethical Considerations

Ethical approval were sought and approved in Sweden, at the WHO and in all participating study sites in India, Iran, Sudan and Ethiopia. Informed consent was obtained from all sample donors for the usage of biological material. The experiments performed at the National Institutes of Health, Bethesda, USA, were performed under the ethical permission available in the laboratory of Dr David Sacks.

Volunteers, Material and Methods

Parasites

L. major (Friedlin) was used for animal experiments and *in vitro* infection of healthy PBMCs (generously provided by Dr D. Sacks, NIH, Bethesda).

L. major (MRHO/IR/76/ER) was used for restimulation of CL-PBMCs (generously provided from Dr A. Khamesipour, Center for Research and Training in Skin Diseases and Leprosy, Tehran, Iran).

L. donovani (MHOM/SD/93/BM1) was used for *in vitro* infections of spleen MNCs (obtained from a Sudanese patient and kindly provided by Dr Iman Satti, Khartoum, Sudan).

L. major (Friedlin V1) was cultured in Medium 199 with 20% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 40 mM HEPES, 0.1 mM adenine (in 50 mM HEPES), 5 mg/ml hemin (in 50% triethanolamine), and 1 mg/ml 6-biotin (M199/S). Infective-stage metacyclic promastigotes of *L. major* were isolated from stationary cultures (4–5 days old) by negative selection using peanut agglutinin (Vector Laboratories, Burlingame, CA, USA) as previously described¹⁹. *L. major* (MRHO/IR/76/ER), and *L. donovani* were cultured in RPMI (GibcoBRL) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (RPMI medium).

Patient Material

Mononuclear cells and serum Plasma and cell samples originated from Ethiopia, Sudan, India and Sweden. Spleen cells and PBMC were collected from patients with visceral leishmaniasis in Ethiopia. Control spleen cells were collected in Sweden. Serum and peripheral blood mononuclear cells (PBMC) or spleen mononuclear cells¹³⁶ obtained were separated as previously described¹³⁷. VL patients were treated with amphotericin B, miltefosine or sodium stibogluconate. All patients fully recovered with no amastigotes detected in the spleen aspirates.

Skin Biopsies were donated by CL patients and healthy Iranian volunteers. The CL patients were all male military recruits who moved from non-endemic areas to *L. major* hyperendemic foci before the onset of disease. CL subjects had a 1-7 months history of ulceration. Control skin was obtained from 3 healthy Iranian volunteers undergoing cosmetic surgery and processed in the same way as the biopsies from CL patients. Biopsies were taken under sterile conditions following local anaesthesia from the indurations lining the ulcers in 8 CL patients. The biopsies were split and either frozen in OCT (TissueTek, Zoeterwoude, Netherlands) or fixed in 4% formalin and paraffin embedded.

Diagnosis of VL, CL and HIV-1 VL was diagnosed by the demonstration of *Leishmania* parasites in Giemsa stained smears from spleen, lymph nodes or bone marrow aspirate materials and/or culture and by histopathology. Recovery from VL was assessed in terms of improved clinical status, negative spleen aspirations and improved hematological values. CL was diagnosed clinically and by detection of parasites in direct smears and/or culture of skin scrapings. Promastigotes were propagated by culture on NNN (Navy-Nicolle-McNeal) (from some of the smears and were identified as *L. major* by iso-enzyme technique and monoclonal antibodies¹³⁸. HIV infection was determined by two enzyme-immuno assays according to national medical standard procedures at the time of sample collection. None of the HIV-infected patients had received antiretroviral therapy at the time of the study. Phenotypic analysis for CD4 on lymphocytes was determined using FACScan (Becton Dickinson, USA)¹³⁹.

Mice

Female Balb/c and C57BL/6 (aged 6-8 weeks obtained from CRL [Wilmington, MA, USA] and TACONIC [Rockville, MD, USA]) received intradermal injection of 5×10^4 metacyclic *L. major* (Friedlin V1) in the distal part of both ears by injection with 27 G needle in approximately 5 µl PBS⁴⁸. In the case of C57BL/6, 1/5 salivary gland homogenate were injected with the promastigotes to induce ulcerative disease⁴⁷. 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). Erosion of ear tissue was defined as inflammatory /ulcerative process that led to complete destruction of dermis and epidermis on both the ventral and dorsal leaf of the ear. After euthanization both ears and retromaxillar lymph nodes were removed. The ears were washed in EtOH and

photographed prior to further processing. Ear and lymph node material were fixed in 4% paraformaldehyde in PBS and analysed histologically.

Treatment regimes: Balb/c: Ten Balb/c mice were treated with MFL-4 (0.5 mgs i.p. twice per week)^{140,141} and 9 mice with isotype matched IgG control (0.5 mgs i.p. twice per week) (Rockland, Gilbertsville, PA, US) week 1 – 4 after infection. Five and 4 mice from each treatment group were analysed at week 5 and week 8 post infection. **C57BL/6:** 6 animals were treated with MFL-4 (0.5 mgs i.p. twice per week) and 6 mice with isotype matched IgG control (Rockland) week 5-7 after infection. Three mice from each treatment regimen were analysed at week 8 and week 12 post infection

Estimation of parasite load in ear and retromaxillar lymph nodes Parasite titrations were performed as previously described^{48,142}. Using a pair of fine forceps, the ventral and dorsal dermal sheets individual infected ears were separated and immediately processed, deposited dermal side down in 1 ml of DMEM containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml Liberase CI enzyme blend (Boehringer Mannheim,) and incubated for 2 h at 37°C. The tissue and liberase solution were transferred to a Teflon-coated micro-tissue grinder (BD), processed for 3.5 minutes, filtered using a 70 µm cell strainer (BD) and collected in 10 ml DMEM containing 10 % FCS, 0.05% DNase I and 100 U/ml penicillin, 100 µg/ml streptomycin. After washing, the tissue homogenate was diluted in 1 ml M199 medium. 0.1 ml of the sample was serially diluted in 96-well flat-bottom microtiter plates containing biphasic medium prepared using 50 µl of NNN medium containing 30% of defibrinated rabbit blood overlaid with 50 µl of M199/S. The retromaxillar lymph nodes were recovered and mechanically dissociated using a pellet pestle and then serially diluted as described above. 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.

Cell Lines

HaCat, immortalised human keratinocytes¹⁴³, kindly provided by Prof. Fusenig, Heidelberg, Germany, was propagated in DMEM supplemented with 10% FCS supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (DMEM medium) and plated on sterile permanox culture slides (Nunc) or in 25 mm² cell culture flasks and incubated at 37° in air with 5% CO₂ until 100% (paper II) or 70-80% (paper III) confluence was reached (4-5 or 2-3 days after seeding). The FasL transfected murine cell line Balb/c FasL/L5178Y¹⁴⁴ (effector cell) and the Fas-expressing Balb/c cell-line A20 (target cell) were kindly obtained from Dr Hideo Yagita, Juntendo University School of Medicine, Japan and were propagated in RPMI medium.

Fas Ligand Neutralizing Antibody (MFL-4)

Hamster anti murine FasL (IgG) MFL-4 was obtained from H. Yagita, prepared as previously described¹⁴⁴. The blocking activity of MFL-4 was tested *in vitro* using the FasL transfected murine cell line Balb/c FasL/L5178Y¹⁴⁴ (effector cell) and the Fas-expressing Balb/c cell-line A20 (target cell)¹⁴⁵. The effector cell was mixed with target cells at a 1:1 ratio and incubated at 37C for three hours in 10% FCS in RPMI medium in the presence of 0,1 – 50 µg/ml of MFL-4 or control IgG (Rockland). After washing, AnnexinV / PI (BD, Stockholm, Sweden) staining was performed and analysed by FACS (BD). Control antibody, Hamster IgG, was obtained from Rockland.

ELISA Detection of sFas, sFasL and sTRAIL

Levels of sFas (Pharmingen, BD, Stockholm, Sweden in paper I, R&D, Amersham, UK paper II), sFasL (Nordic BioSite, Täby, Sweden, paper I-III) and sTRAIL (paper II, R&D, Amersham, UK) were assessed in duplicates in supernatants or serum samples using commercial ELISAs according to the manufacturer's instructions.

Histology

May Grünwald-Giemsa The morphology of tissue biopsies was evaluated in May Grünwald-Giemsa stained sections in duplicates. Paraffin embedded sections were deparaffinated and rehydrated in 100% ethanol, followed by 5 minutes incubation in 100% methanol, 3 minutes in May-Grünwald (Sigma-Aldrich, Stockholm, Sweden) diluted 1:2 in Giemsa buffer (Sigma-Aldrich) and methanol 1:1 and 11 minutes in Giemsa (Sigma-Aldrich) diluted 1:12 in Giemsa buffer.

Visualisation of Fas, FasL, TRAIL, TRAIL R1-4, Cytokeratin 18 in skin biopsies Paraffin embedded skin biopsies were sectioned in 5 μm sections. Deparaffination and rehydration were performed as previously described¹⁴⁶. Frozen biopsies were sectioned in 12 μm thick sections, briefly dried and fixed in acetone. Sections were incubated with primary antibodies at 12,5 – 40 $\mu\text{g}/\text{ml}$ in parallel to isotype control for 15 minutes at room temperature. Streptavidin-avidin enhancement was performed according to the manufacturer's instruction (DAKOPATTS, Stockholm, Sweden). The antigens were visualised with VIP (Vector, Burlingame, CA) or DAB (Vector) and hematoxylin (Sigma-Aldrich) counterstaining was performed. Frozen skin biopsies were sectioned in 12 μm thick sections, briefly dried and fixed in acetone. The sections were incubated in protein block (DAKOPATTS) for 10 minutes, followed by 1 hr incubation with the primary antibody at 40 $\mu\text{g}/\text{ml}$, followed by 1 hr incubation with Cy3 conjugated goat anti mouse (Jackson ImmunoResearch, West Grove, PA) at 1:400. Isotype antibody controls were used in parallel. The sections were washed, mounted in 20% DABCO (Sigma-Aldrich) in glycerol and analysed with x 25 ocular magnification in a Nikon Optiphot 2 UV microscope.

TdT-mediated dUTP nick end labelling (TUNEL) Apoptosis was assessed through visualizing fragmented DNA using TUNEL kit according to the manufacturer's instructions (Roche, Penzberg, Germany) Positive controls were obtained by addition of DNase (Life Technologies, Sweden) and negative controls by omission of the TdT-enzyme.

Evaluation of immunohistochemistry The sections were analyzed using a Nikon Optiphot 2 microscope. Fas was analyzed using a 6.5x or 10x lens, whereas FasL, M30 and TUNEL were analyzed using 40x or 100x oil immersion lens. Expression of Fas was assessed using an arbitrary scale from – to ++++. All sections were assessed blind, and independent of morphological designation. Levels of FasL expressing cells, apoptotic cells and caspase activity were quantified by counting the number of positive cells in 9-13 fields at x40 magnification. The number of cells per mm^2 was then calculated from the known diameter of the field at x40 magnification. Duplicates were performed at different time-points and differences between duplicates were typically less than 15%.

Induction and Measurement of Apoptosis in Human Keratinocytes

HaCat, immortalised human keratinocytes¹⁴³, kindly provided by Prof. Fusenig, Heidelberg, were plated on sterile culture slides (Nunc, Nalgene, Hereford, UK) and incubated at 37°C until confluence was reached (4-5 days). Apoptosis was assessed following 40 hrs incubation with 1 $\mu\text{g}/\text{ml}$ Fas activating monoclonal⁸⁸ (CH11, MBL, Nagoya, Japan^{65,147}) or supernatants obtained from PBMCs (diluted 1:4 in DMEM medium). In an attempt to block Fas and TRAIL mediated apoptosis of keratinocytes during these conditions, the following neutralising and blocking antibodies added to the cultures: the Fas blocking monoclonal antibody ZB4 (MBL) 1-2 $\mu\text{g}/\text{ml}$ ¹⁴⁷; the FasL blocking antibody (R&D, Amersham, UK) was added at 2.5 $\mu\text{g}/\text{ml}$; the of TRAIL blocking antibody 2E5 (Alexis, KeLab, Gothenburg, Sweden) was added at 2.5 $\mu\text{g}/\text{ml}$ and sTRAIL-R2 (Alexis) was added at 5 $\mu\text{g}/\text{ml}$. The samples were washed twice in Annexin V binding buffer and incubated in the dark for 25 minutes with AnnexinV/Propidium Iodide¹⁴⁸. Following incubation, the cells were fixed in 1% Paraformaldehyde (Sigma) for 10 minutes at room temperature. The cells were mounted with anti-fading solution (Vector) and immediately analysed.

Leishmania in vitro Re-Stimulation or Infection of Human Mononuclear Cells

Stationary phase cultures of *Leishmania* promastigotes (4–5 days old) were added at 1×10^6 cells /ml (cell:parasite 1:1) to PBMC or spleen cells in 6 well plates, 25 mm^2 flasks or in chamber slides (Nunc, Heresford, UK) and incubated over night at 33°C air with 5% CO_2 and then moved to 37°C air with 5% CO_2 in RPMI medium. Infectivity was assessed after 72 hr by staining adherent cells with 5 $\mu\text{g}/\text{ml}$ propidium iodide (Becton Dickinson, Stockholm, Sweden) and FITC conjugated anti CD68. Five to seven days after infection, cells and supernatants were harvested, centrifuged at 1200 x g for 10 minutes and either ultracentrifuged or aliquoted and immediately transferred into -70°C. Supernatants from two different donors were ultracentrifuged at 30 000 x g for 30 minutes, followed by 100 000 x g for 1 hour. The ultracentrifuged fractions were transferred to -70°C. *L. donovani* and *L. major* (Friedlin)

established infection in macrophages during in vitro conditions, whereas *L. major* (MRHO/IR/76/ER) could not be detected in macrophages after 1-5 days of infection.

FACScan

Detection of Fas, FasL, Apoptosis and phenotypic detection of spleen MNCs and PBMCs

Approximately 0.5×10^6 cells were incubated on ice with FITC conjugated mouse anti human Fas monoclonal IgG (DAKOPATTS), PE (Phycoerythrin) conjugated mouse anti-human FasL monoclonal IgG (Alexis, Sweden and R&D), FITC and PE conjugated mouse isotype control IgG (DAKOPATTS) in FACS buffer (PBS containing 2% bovine serum albumin) for 15 minutes on ice. The following antibodies were used to determine different cell subsets: CD56-PE or APC, CD3-APC, CD4-FITC or PE, CD8-Cy3 or PE, CD19-FITC or PE (BD). The AnnexinV assay was performed according to manufacturer's instructions on fresh, untreated or *L. major* stimulated PBMCs. Non-viable cells were excluded through scattering properties on forward/side scatter. Apoptosis was defined as AnnexinV positive, PI negative cells.

Detection of intracellular CD68 by FACS CD68 was used as a macrophage specific marker.

Approximately 0.5×10^6 detached adherent cells were fixed in 100 μ l of 4% formaldehyde in a coned bottomed 96 well plate (Nunc) on ice for 15 minutes. The samples were washed in FACS buffer (0.1% BSA [Sigma-Aldrich] in PBS) and the cells were resuspended in saponin buffer (0.1 % saponin (Sigma-Aldrich) in FACS buffer) and incubated for 10 minutes. The cells were centrifuged at 800 x g for 3 minutes and resuspended in saponin buffer containing mouse-anti-human FITC conjugated CD68 antibodies (DAKOPATTS) or FITC conjugated isotype (DAKOPATTS). The samples were incubated for 15 minutes on ice, washed once in saponin buffer and once in FACS buffer and acquisition was performed immediately on a Becton Dickinson FACS Scan (Becton Dickinson, Stockholm, Sweden).

Detection of membrane bound TRAIL, Fas and TRAIL R1-R4 on HaCaT HaCaT cells were incubated with supernatants from *L. major* infected or control PBMCs (supernatants: medium 1:1) in 6 well plates for 20 hours and thereafter washed with PBS and adherent cells detached with 0.05 % EDTA in PBS. After an additional wash with PBS containing 1.5% BSA (Sigma Aldrich)(FACS buffer) the samples were incubated with FITC conjugated mouse anti human Fas monoclonal IgG (DAKOPATTS), PE conjugated mouse anti-human TRAIL monoclonal IgG (R&D), FITC or PE conjugated mouse isotype control IgG (DAKOPATTS) in FACS buffer for 20 minutes. TRAIL R1-4 were stained for by 30 minutes incubation in mouse anti human TRAIL R1-R4 (20 μ g/ml) (kindly provided by Immunex) followed by a 20 minutes secondary incubation with goat anti mouse RPE (DAKOPATTS).

Analysis of IFN γ secretion and cell subsets in murine lymph nodes and ears Tissue homogenate / lymph node cells were pooled after parasite titration had been prepared and fixed immediately (BD) or after 4 hrs stimulation with PMA (1:200 000, stock solution 20 μ g/ml), Ionomycin (1:1000, stock solution 1 mg/ml) and Brefeldin A (1:2000, stock solution 10 mg/ml) followed by fixation. The samples were incubated with an anti-Fc III/II receptor (BD PharMingen, San Diego, CA) mAb in PBS containing 0.1% BSA. The lymphocytes were identified by characteristic size (forward light scatter -FSC) and granularity (side light scatter -SSC), in combination with anti-TCR β chain (H57-597, FITC conjugated; BD PharMingen) and anti-CD4 or anti-CD8 (CyChrome conjugated) surface staining. Neutrophils were characterised as Ly6^{high} CD11b^{positive}. Macrophages were characterised as Ly6^{low} or negative and CD11b^{positive}. For each lymph node sample, between $2-4 \times 10^5$ cells were analysed. In the case of ear cells, the entire sample was acquired (typically between 800 000 and 1 000 000 events) to facilitate estimation of total number of cell subsets per ear. The data were collected and analysed using CellQuest software and a FACSCalibur flow cytometer (BD Biosciences).

Analysis of FACS results was performed using the CellQuest Software (Paper I-IV) (Becton Dickinson, Stockholm, Sweden), Lysis II (Paper II, ex vivo PBMC).

cDNA expression array

Spleen MNCs were infected with *L. donovani* and T cells were separated using positive CD3⁺ selection by MACS (Miltenyi Biotec, GTF, Gothenborg, Sweden) according to the manufacturer's instructions, with a purity of more than 95% as measured by FACS. Adherent cells were washed thoroughly and more than 99% of adherent cells were CD68positive and designated macrophages after 3 and 5 days. RNA was isolated from T cells and macrophages using Rneasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Approximately 2×10^6 HaCaT cells in a 70-80% confluent monolayer covering 25 mm², was incubated with 5 ml of DMEM (Gibco) supplemented with 10% FCS, penicillin streptomycin and L-glutamine (Gibco) (DMEM medium) or DMEM medium diluted 1:1 with infected or control supernatants for 6 or 24 hours. Non-adherent, apoptotic cells were washed off with PBS and RNA isolated using Rneasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Approximately 40-50µg of RNA was collected from each sample. Size chromatography of RNA was performed with an Agilent 2100 Bioanalyser. cDNA expression microarray analysis was performed using non-radioactive Human Apoptosis GEarray Q Series (SuperArray Inc, Bethesda, MD), according to the manufacturer's instructions. Briefly, 1.5µg of total RNA was reverse-transcribed with reverse transcriptase (Invitrogen AB, Stockholm, Sweden) and GEprimer mix (Invitrogen) in the presence of biotin-16-dUTP (Roche, Penzberg, Germany). Amplification of cDNA was performed during 30 cycles using LPR Amplolabeling kit (SuperArray). The biotinylated cDNA probes were denatured and added to the hybridisation solution. GEArray Q Series membranes dotted with tetra-spot cDNA fragments of 96 human apoptosis genes were prehybridized at 60°C for 2 h and then hybridised overnight with the cDNA probes. Membranes were then washed, blocked and incubated with alkaline phosphatase-conjugated streptavidin. The labelled biotin on the membrane was detected by chemiluminescent method. RNAs extracted from HaCaT cultures incubated with three sets of infected or control supernatants from three different PBMC donors were used.

Analysis of cDNA expression array analysis The luminescence intensities of hybridised cDNA probes were analysed by GeArray software (SuperArray). Local background was subtracted for each point. Each signal intensity was normalised against the house-keeping gene Ribosomal protein L13a. The ratio value of expression was obtained from the intensity value of each gene from each treatment and donor relative to its median intensity in RNA obtained from untreated HaCaT. For the analysis of gene expression changes after incubation with supernatants, the ratio of relative expression between samples incubated with supernatants or HaCaT alone was analysed. Genes were selected if the median ratio was at least 2 fold different in all three samples analysed for the different conditions described as compared to untreated HaCaT.

Statistical Analysis

Statistical significance was calculated using the Mann-Whitney test and correlation using Pearson's correlation coefficient. The SPSS (SPSS Inc., Chicago, USA) and the Prophet statistical software (Abtech Co, NIH, USA) were used (paper I). Mann-Whitney Signed or Unsigned Rank Test, the Wilcoxon Signed Rank test or linear regression test using Statview 2.3 was applied for statistical analysis (paper II-IV).

Results and Discussion

Serum Levels of sFas and sFasL are High during VL, and Normalise after Successful Treatment (papers I and II).

Elevated levels of Fas and FasL can be measured in serum during HIV-1 infection¹⁴⁹⁻¹⁵¹ and some investigators claim that Fas upregulation on T-cells is linked to the loss of CD4+ T-cell^{149,152,153}. As T-cell suppression occurs in both HIV-1 and VL, we measured the serum levels of sFas and sFasL in individuals with CL, VL, HIV-1 and VL-HIV-1 by ELISA and compared these levels to the ones found in healthy controls from the same endemic areas. The VL serum samples originated from patients in India, Sudan and Ethiopia. The CL samples originated from Ethiopia and Iran. In 13 VL patients, the effect of successful treatment on serum levels of sFas and sFasL were also investigated.

The levels of sFas and sFasL were elevated in plasma from patients with active VL and individuals co-infected with VL-HIV-1 compared to healthy controls and comparable with levels measured during HIV infection (figure 7). No difference in sFas or sFasL levels was detected when the patient groups from different endemic foci of VL were compared and the levels of sFas and sFasL normalised six months after successful treatment. Although higher levels of sFas were measured during CL as compared to healthy controls, the levels of sFas during CL were considerably lower than during VL or HIV-1. The serum levels of sFasL were not up-regulated during CL.

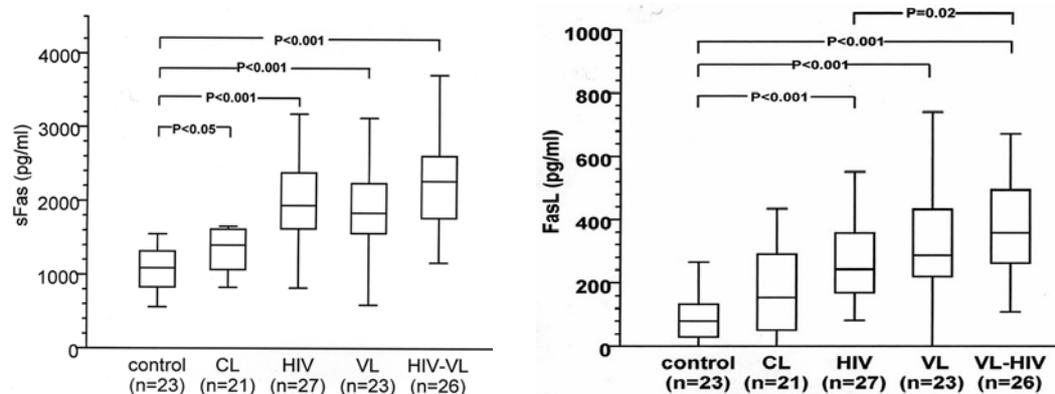


Figure 7) Fas and FasL are up-regulated during VL but not CL. Box plot representation of plasma levels of sFas (a) and sFasL (b) in patients and controls. The horizontal and vertical lines in each box represent the median and the range (95% confidence interval) respectively. P-values are indicated between groups where differences between medians were significant.

Thus systemic *L. donovani* infection induced enhanced sFas and sFasL levels akin to those observed during HIV infection. The high levels of sFas and sFasL measured during active infection may be an unspecific effect of immune activation. It may also be a sign of *Leishmania* induced dysregulation of FasL induced apoptosis and, biologically active sFasL could possibly lead to enhanced and premature killing of Fas expressing, *L. donovani* activated T-cells. In such scenario, sFasL could participate in the diminished T-cell response through deletion of *L. donovani*

activated T-cell clones at the site of infection. Interestingly, the levels of both sFas and sFasL normalised 6 months after successful VL treatment leading to killing of the majority of infecting parasites throughout the reticuloendothelial system. A retrospective study on levels of sFas in HIV-1 infection during highly active antiretroviral therapy (HAART) showed that the plasma levels of sFas failed to normalise after 1 year of therapy¹⁵⁴. In contrast to VL, the HIV infection is not cleared during treatment. Patients co-infected with both VL and HIV-1 deteriorates faster in both diseases as compared to patients infected with either VL or HIV-1. As the HIV status is improved upon effective VL treatment³⁴, it would have been interesting to follow co-infected patients receiving VL treatment to evaluate whether the improvement in HIV status also leads to normalised or decreased levels of sFas and FasL. This has not been possible within this thesis work however remains an interesting line of research for future students.

Fas and FasL are Up-Regulated on Spleen Cells but not on PBMCs during VL (paper I)

To investigate the possible origin of sFasL and sFas measured in serum during VL, membrane bound Fas and FasL were measured on mononuclear cells from blood and spleen of VL patients and compared to cells from healthy persons. The spleen cells from VL were compared to spleen cells from Swedish healthy controls. The control PBMCs were obtained from healthy Ethiopian controls, as it has been shown that the T-cell pool in Ethiopians show signs of increased activation as compared to a Dutch study population¹⁵⁵. In our hands, however, no differences of Fas or FasL expression could be measured in Swedish and Ethiopian PBMCs. In VL patients, the expression of membrane bound Fas, and to a lower extent FasL, was up-regulated on *L. donovani* infected spleen cells, the site of parasite multiplication (figure 8). The expression of Fas and FasL on VL-PBMCs was within normal range. The numbers of VL spleen MNCs were too small for phenotypic analysis. One spleen sample typically consisted of 500 000 cells. The small sample size did not allow evaluation of apoptosis, but in a study on PBMCs from Sicilian VL cases, apoptosis was increased in monocytes and T-cells *ex vivo* and after *in vitro* re-stimulation¹⁵⁶. Fas expression was not altered *ex vivo*, in line with the results obtained in our laboratory, however, upon re-stimulation the expression of Fas and FasL on T-cells increased significantly.

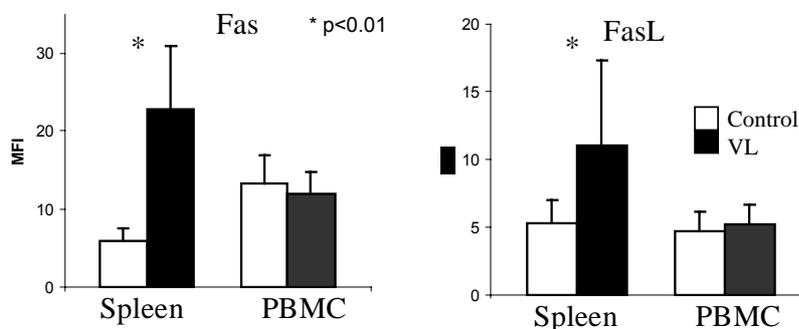


Figure 8) Fas and FasL were up-regulated on VL-spleen cells but not on VL-PBMCs as compared to healthy controls. Histograms showing mean values of Fas and FasL MFI. Error bars show standard deviation. Material from healthy controls is depicted by white bars and from VL patients by black bars. Eight Swedish control spleen MNCs were compared to 12 VL spleens MNCs and six healthy PBMC samples were compared to 5 VL PBMC samples.

healthy spleen cells would have to be performed. This proved to be impossible due to the low number of donors available.

2. To obtain a good quality cDNA hybridisation in this setting, 1.5 µg of pure RNA was needed requiring up to 10 million of *in vitro* stimulated T-cells. From one spleen aspirate, typically 1-3 million cells and in total 0.5 – 2 µg of RNA could be obtained, of which approximately 50% would be T-cells¹⁵⁷. Due to the inter-individual differences in gene expression found *in vitro* the validity of pooling RNA from several different patients would be debatable.

The methodology of *in vitro* infection and measurement of expression of apoptosis-related genes developed in this project were proven to be useful in the study of gene expression alterations in a keratinocyte cell-line upon exposure to *L. major* infected supernatants.

Fas and FasL are Up-Regulated at the Site of Infection during CL (Paper II)

Patient material from CL patients was collected in a group of Iranian soldiers that had been recently transferred into a hyperendemic area of *L. major* transmission. The expression of Fas, FasL and the number of apoptotic cells were analysed in skin biopsies and in re-stimulated blood mononuclear cells from patients with 1-7 months history of *L. major* induced CL. A substantial number of apoptotic keratinocytes were observed, mainly in the superficial epidermis of morphologically active and healing CL skin samples. Fas expression was increased in the epidermis during active CL and FasL expressing macrophages and T-cells were found in subepidermal infiltrate, mainly during active disease. The number of dermal FasL expressing macrophages and T-cells correlated to the number of epidermal apoptotic keratinocytes. However, FasL was not expressed in the epidermis during CL. It has been shown that keratinocytes express low levels of FasL and that FasL expression can be enhanced by stimulation with proinflammatory cytokines such as IL-1β, TNF-α, IFNγ and IL-15⁶⁰, and that FasL expressing keratinocytes are competent for killing Fas expressing, activated T-cells. Expression by FasL has been proposed in healthy skin^{94,158}. In our accumulated data from healthy skin and mRNA expression in keratinocyte cultures no expression of FasL was detected. The reason as to why I could not detect the expression of FasL in skin may be due to different antibodies used¹⁴⁶. FasL have been shown on epitheloid granulomas during mycobacterial infection¹⁵⁹. In the same study, biopsies from four cases of *L. aethiopicum* induced CL were included as controls. In these biopsies, FasL expressing macrophages were present in the biopsies investigated, although not organised in granulomas. Up to 30% apoptotic T-cells (both CD4+ and CD8+ positive) were described in cells extracted from *L. braziliensis* induced ulcers and the level of apoptosis was suggested to decrease during healing¹⁶⁰, suggesting that at the site of infection, T-cells undergo AICD in established ulcers.

Although no alterations of Fas or FasL were measured *ex vivo* in serum or on PBMCs during CL, restimulation of PBMCs led to upregulation of Fas on the surface of PBMCs and high levels of sFasL in supernatants from restimulated cultures. In *L. major* re-stimulated CL PBMCs, the levels of sFasL in supernatants were more than 10 times higher compared to unstimulated CL PBMCs. Although interesting, it can be debated how well these results reflect the role of Fas/FasL interactions in ulceration

during CL as the biopsies obtained were taken at the borders of active or healing ulcers during established CL.

The Possible Apoptotic Role of sFasL during CL (paper II)

To evaluate the role of FasL in keratinocyte apoptosis during CL, sFasL containing supernatants from CL PBMCs were incubated with a human keratinocyte cell-line (HaCaT). Three of six supernatants from *L. major* stimulated CL PBMCs induced substantial levels of apoptosis in HaCaT after 40 hrs incubation and the levels of apoptosis correlated with the levels of sFasL in the supernatants (figure 10). Adding Fas blocking antibodies (ZB4) to supernatants from stimulated PBMCs and HaCaT blocked keratinocyte apoptosis in two out of three analysed supernatants. Supernatants from unstimulated CL supernatants did not induce apoptosis in HaCaT. Thus we had indications that sFasL in this setting could play a role for keratinocyte death and ulceration during CL.

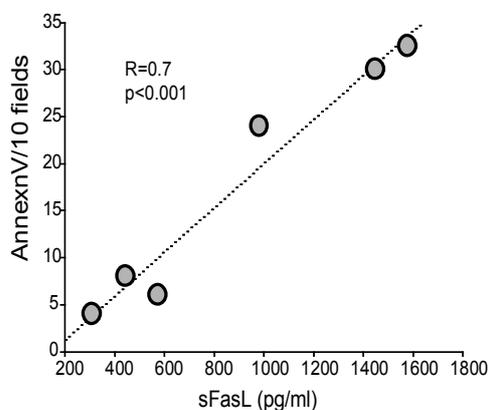
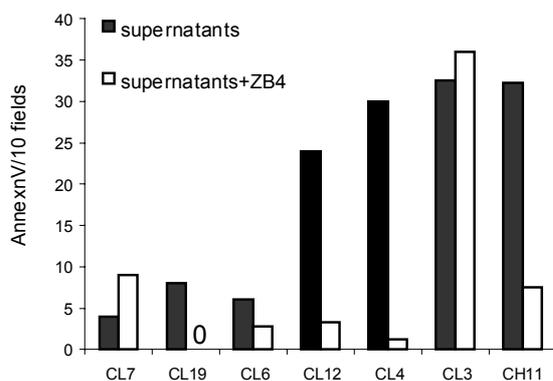


Figure 10) sFasL secreted by *L. major* stimulated CL PBMCs may induce apoptosis in Fas expressing keratinocytes

Upper graph Levels of sFasL in supernatants from stimulated CL PBMCs in comparison to the level of apoptosis induced in keratinocytes. Supernatants from six different CL PBMCs were incubated with HaCaT. All experiments were performed in duplicates. There was a strong correlation between the levels of sFasL in the supernatants used (x-axis) and the observed levels of apoptosis in keratinocytes (y-axes) ($r=0.7$, $p<0.001$).

Lower histogram Adding Fas blocking antibodies (ZB4, open bars) to stimulated supernatants and HaCaT blocked keratinocyte apoptosis for two out of three supernatants (CL 12 and CL4) containing high levels of sFasL. In one case, CL3, apoptosis was not blocked. The positive control CH11, IgM monoclonal antibody, induced apoptosis at $1\mu\text{g/ml}$. CH11 was effectively blocked in the presence of ZB4 at $1\mu\text{g/ml}$.



The function and apoptosis inducing abilities of sFasL is a matter of controversy. It has been proposed that membrane bound FasL, yet not sFasL, can induce Fas-mediated apoptosis^{76,78} although a number of studies have previously shown apoptotic activity by sFasL^{58,75}. One hypothesis is that in order to activate Fas, a high concentration of the ligand is needed and this is more easily obtained by membrane interactions as compared to soluble ligands^{78,79}. Some interesting data have been published in favour of this hypothesis^{80,77} proposing that sFasL deposited in the connective tissue induce apoptosis effectively. In our studies of keratinocyte

apoptosis in the skin, where FasL positive cells are present in the connective tissue, we propose that high amounts of FasL, soluble or membrane bound, are secreted in the microenvironment surrounding *L. major* infected macrophages, and accordingly a model of possible mechanisms of keratinocyte death during CL was proposed (figure 11). In the case of keratinocytes, it was proposed, in both HaCaT cultures and in organ cultures, that IFN γ was necessary to allow Fas-mediated apoptosis and that blocking FasL would not inhibit apoptosis completely, whereas blocking IFN γ would⁹². One could envisage a situation where sFasL locally binds to extracellular tissue at the site of production and thus acts as an apoptosis inducer. Activated lymphocytes up-regulate their expression of Fas¹⁶¹ whereby T-cells aiming at clearing infected macrophages may interact with deposited sFasL and undergo apoptosis. This may in turn lead to a reduction in Ag specific T-cells, and to the process of T-cell unresponsiveness, which was suggested in the case of atopic dermatitis¹⁰¹.

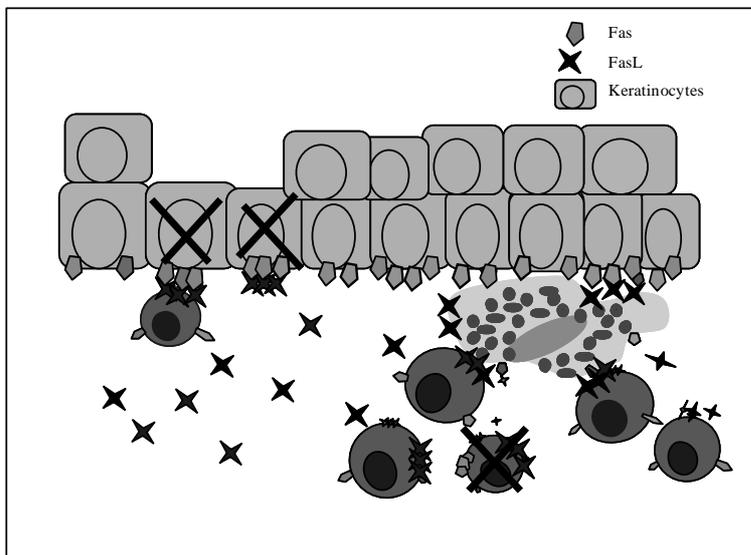


Figure 11) Possible ulcerative effect of up-regulation of Fas and FasL during CL.

Keratinocytes up-regulate Fas as a result of the inflammatory reaction surrounding *L. major* infected macrophages. FasL expressing T-cells and macrophages accumulate at the site of *L. major* infection. The Fas-FasL or Fas-sFasL interaction may lead to keratinocyte apoptosis and ulceration as well as to activation-induced T-cells apoptosis.

Further Characterisation of Keratinocyte Apoptosis in the Context of *L. major* Infection Indicated Alterations of the TRAIL Pathway (paper III)

As we were able to block keratinocyte apoptosis in 2/3 sFasL containing supernatants, and in the light of different apoptotic patterns noted in the microarray experiments performed on *L. donovani* infected macrophages, we investigated possible alterations of apoptotic pathways in keratinocytes during CL. We chose an approach where we through *in vitro* infection of human PBMCs with *L. major* obtained large amounts of sFasL containing supernatants. Supernatants from infected or uninfected PBMC cultures were co-incubated with the human keratinocyte cell-line HaCaT; a commercially available apoptosis-specific microarray was used to assess alterations in keratinocyte RNA-expression. The increased mRNA expression of Fas, TRAIL R1-, R2 and R4 was detected in untreated HaCaT. Upon exposure to supernatants from *L. major* infected PBMCs, the mRNAs for Fas and TRAIL were up-regulated in human keratinocytes, both at 6 and 24 hours post-exposure, and the pro-apoptotic TRAIL-R2 at 6 but not at 24 hours. TRAIL-R2 mRNA was also up-regulated in two out of three samples exposed to uninfected supernatants from

human PBMCs. The levels of TRAIL-R1 mRNA were high in all samples investigated. Interestingly, a transient up-regulation of the expression of caspase 8 mRNA was detected at 6 hours, but not at 24 hours, indicating that several components of the apoptotic pathway are affected by the infected supernatants from *L. major* infected PBMC cultures.

Up-regulation of Fas and TRAIL upon exposure to supernatants from infected cultures was verified at the protein level by cell-surface staining of HaCaT. TRAIL expressing keratinocytes were detected in skin biopsies from CL cases, mainly in the basal layers of epidermis, and TRAIL-R2 could be detected in some cells in epidermis surrounding CL ulcers. These results suggested that additional mechanism(s) could be operating in the skin in addition to Fas mediated apoptosis where FasL expressing cells were found in dermis adjacent to Fas expressing epidermis. In the case of TRAIL, both the ligand and the receptor were expressed by the same cells, the keratinocytes, and thus an autocrine / paracrine route of killing could be envisaged during inflammatory responses in the skin.

To further elucidate patterns of keratinocyte cell-death in HaCaT cultures exposed to infected supernatants, Fas and TRAIL were blocked alone or in synergy (figure 12). Apoptosis of keratinocytes was partly blocked through blocking Fas or FasL, and more efficiently through blocking of TRAIL by neutralising antibodies or soluble TRAIL-R.

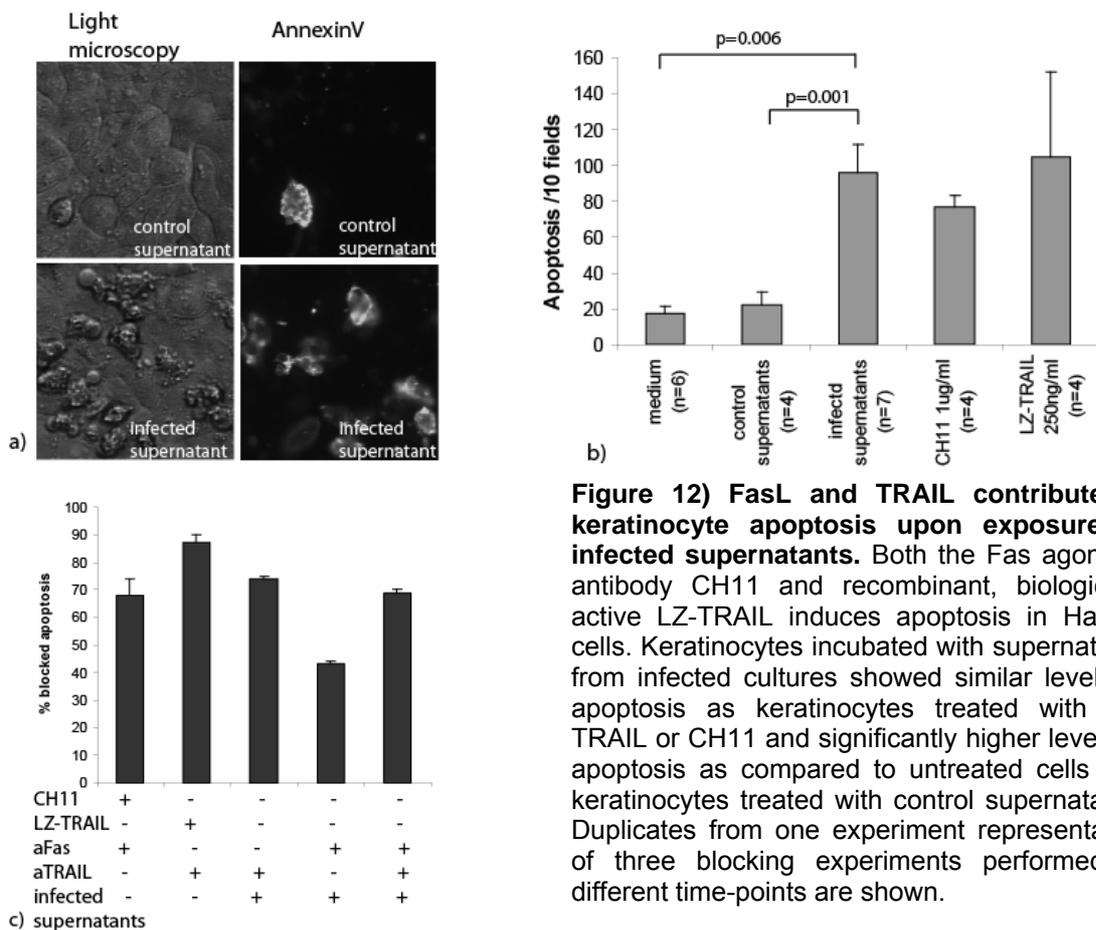


Figure 12) FasL and TRAIL contribute to keratinocyte apoptosis upon exposure to infected supernatants. Both the Fas agonistic antibody CH11 and recombinant, biologically active LZ-TRAIL induces apoptosis in HaCaT cells. Keratinocytes incubated with supernatants from infected cultures showed similar levels of apoptosis as keratinocytes treated with LZ-TRAIL or CH11 and significantly higher levels of apoptosis as compared to untreated cells and keratinocytes treated with control supernatants. Duplicates from one experiment representative of three blocking experiments performed at different time-points are shown.

The supernatants obtained from *in vitro* infected healthy PBMCs contained more sFasL, induced more apoptosis in keratinocytes and were only partially blocked by the addition of Fas or FasL blocking antibodies when compared to the supernatants obtained from *L. major* re-stimulated CL-PBMCs. The supernatants obtained from restimulated CL-PBMCs were added to a confluent monolayer of HaCaT. It has been previously proposed that HaCaT respond differently to receptor induced apoptosis depending on the maturation stage of the cultures, and that HaCaT grown to confluence show properties ascribed maturing keratinocytes, whereas HaCaT grown to 70-80% confluence better resembles basal keratinocytes^{105 95}. A differential maturation stage of keratinocytes could also be reflected in the expression of Fas and TRAIL in skin biopsies from CL cases, where Fas was mainly up-regulated in the superficial layer of epidermis, reflecting mature and fully differentiated keratinocytes, and TRAIL was mainly found in the still dividing and not matured keratinocytes in the basal layer of epidermis.

Several different scenarios can be envisaged from the observation that two receptor mediated apoptotic pathways are upregulated in the skin during CL. The Fas/FasL pathway, which is activated during immune responses, is important to contract and terminate immune responses. In this context, FasL killing of keratinocytes by activated infiltrating cells may be accidental as suggested by the fact that infected macrophages are found in the vicinity of the epidermis. Fas upregulation is considered to signal cell activation and keratinocytes adjacent to *Leishmania* infected macrophages show increased expression of HLA-DR and ICAM-1 as further proof of their activated state^{24,162}. Activated keratinocytes are capable of secreting high levels of chemoattractants and cytokines and thus, FasL killing of keratinocytes may be important to dampen local immune activation. TRAIL is expressed on normal keratinocytes in combination with TRAIL-R1 and high level of TRAIL-R3. During CL both TRAIL and TRAIL-R2 were up-regulated and it has been suggested that signalling through TRAIL-R2 is more efficient in inducing apoptosis as compared to TRAIL-R1 signalling¹⁶³. Activated keratinocytes could up-regulate the expression of both the ligand and its pro-apoptotic receptor (TRAIL-R2) in order to dampen local inflammatory responses. On the other hand, TRAIL-R4 signalling has been suggested to lead to activation of NF κ B and inflammatory responses. It could also be envisaged that the expression of TRAIL-receptors during the course of an inflammatory response decides the fate of the keratinocyte in such a manner that during early phases a majority of cells will express TRAIL-R3-4. During contraction of the inflammatory reaction receptor expression could be switched to the pro-apoptotic TRAIL-R1-2.

Blocking the Fas/FasL Pathway *in vivo* May Reduce Ulceration during Murine CL (paper IV).

Ex vivo and *in vitro* data suggested a possible role for Fas/FasL signalling in keratinocyte apoptosis and ulceration during CL. To validate this possibility, *in vivo* studies were required. We chose to block Fas/FasL interactions through short term systemic treatment with FasL blocking antibodies¹⁴⁴ during *L. major* infection^{47,48} in an otherwise immunocompetent murine model of CL, using both susceptible (Balb/c) and resistant (C57BL/6) mouse strains. Skin inflammation, skin ulceration and ulcer size were followed weekly and compared to infected, untreated mice. Cells were recovered from *L. major* infected ears and from draining lymph nodes and analysis of

cell subsets, IFN γ and parasitic load were performed. Apoptosis and Fas/FasL expression were investigated by immunohistochemistry in ears and lymph-nodes. Preliminary results suggest that blocking Fas/FasL signalling during murine CL (both Balb/c and C57) leads to a reduced number of apoptotic keratinocytes and diminished ulceration but not to a reduction of parasite load at the site of infection or in draining lymph nodes. During treatment the number of IFN γ producing CD8⁺CD3⁺ cells was increased at the site of infection when FasL was neutralised suggesting a more marked inflammatory process, with more IFN γ produced locally. IFN γ is important for parasite clearance during *Leishmania* infection through activation of macrophages which will kill intracellular parasites. High levels of IFN γ up-regulate Fas and TRAIL R1-2, and may facilitate TRAIL-killing of keratinocytes.

In the disease model used, the parasite load in ear tissue and draining lymph nodes is low after healing in C57BL/6 mice, whereas in the non-healing Balb/c mice the parasite load remains high throughout the course of the infection. During MFL-4 treatment no alteration of parasitic load was found. However, in C57BL/6 mice, parasite clearance seemed defective four weeks after discontinuation of MFL-4 treatment. Studies performed in Fas or FasL knock-out mice infected with a high number of *L.major* have proposed that intact Fas/FasL signalling is needed for effective parasitic clearance^{109,110,112}. Others have shown that effective parasite

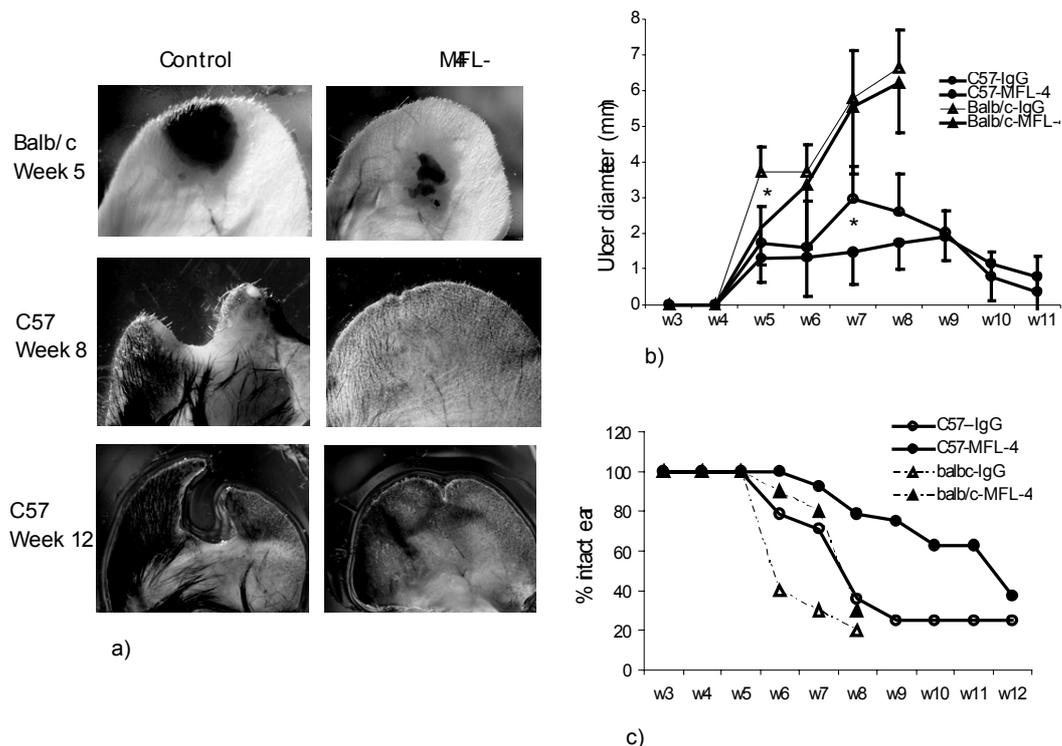


Figure 13) MFL-4 treatment during experimental CL lead to diminished ulcer and scar formation. a) Gross pathology in terms of ulceration during experimental CL in Balb/c and C57BL/6 mice treated with either control IgG or MFL-4. b) Pathology in terms of ulceration developed slower during MFL-4 treatment. At week 5 in Balb/c and at week 8 in C57BL/6, there was a statistically significant difference in ulcer size comparing MFL-4 treated samples with controls (* p<0.05). c) MFL-4 treatment prolonged the time until complete destruction of dermis and epidermis ulcer-free time after infection, both in Balb/c but more pronounced in C57BL/6.

clearance in FasL deficient mouse-models of *L. major* infection¹⁶⁴ and FasL has been proposed to be necessary for successful establishment of *L. major* infection in mice¹¹³. In *L. donovani* infection of FasL deficient mice the parasitic load was higher in FasL deficient as compared to FasL competent mice, although the number of IFN γ producing CD4 T-cells was increased¹¹⁷.

We wanted to study if a short time treatment with FasL blocking antibodies would allow effective parasite clearance. The results obtained suggest that MFL-4 treatment may inhibit tissue damage but, may also cause inefficient clearance of the parasites. It remains unknown whether clearance is inhibited or delayed and additional studies after discontinuation of treatment in MFL-4 treated C57BL/6 mice are needed to address this question. Furthermore, as MFL-4 treatment leads to increased local IFN γ production, and a scenario could be envisaged where the parasite clearing effects could be increased during MFL-4 treatment. This effect has not been noticed during the short treatment schedule used in this study. If a short term inhibition of FasL led to less overt ulceration and scarring, slower parasite clearance may be acceptable.

Although the results obtained are interesting, the effect on reduction of ulceration during this treatment regime was not complete. The most promising result was that in the case of C57BL/6 loss of ear tissue (figure 13 a) was prevented during MFL-4 treatment. In the light of the interesting data obtained on the TRAIL mediated pathway in tissue from CL in man and HaCaT cell line *in vitro*, we plan further experiments to explore the effect of administration of TRAIL neutralising antibodies¹⁶⁵ during murine CL, in the presence or absence of MFL-4 anti FasL antibody.

Neutrophils accumulate around *L. major* infections¹⁶⁶⁻¹⁶⁸ and may have a role in tissue damage leading to ulcerative processes during CL. In both C57BL/6 and Balb/c, the number of infiltrating neutrophils was much lower during MFL-4 treatment than in untreated mice. One plausible explanation may be that FasL stimulates a proinflammatory loop at the site of infection, leading to local secretion of chemoattractants such as IL-8. We plan to investigate this aspect further in future experiments.

FasL is Expressed on T-cells and Macrophages during CL in Man and Mouse (paper II and IV)

The cellular origin of FasL during ulceration was investigated in murine and human CL, by immunohistochemistry and in the mouse experiments also by FACS analysis. In skin biopsies from active CL, FasL expressing macrophages and T-cells were found in the inflamed dermis adjacent to ulcers. In murine CL, more FasL was expressed in the susceptible mouse strain Balb/c as compared to the resistant C57 during active ulceration. FACS analysis of ear tissue from C57BL/6 showed that FasL was expressed by 4-10 % of T-cells (both CD8 and CD4), 4% of macrophages and neutrophils during active ulceration. Upon healing, the number of FasL expressing cells was reduced. More FasL expressing cells were present during active disease than during healing disease. It has been suggested that FasL expression by *L. major* infected macrophages would lead to apoptosis of *L. major* activated, Fas expressing T-cells¹¹⁴. On the other hand, cytotoxic cells use FasL in parallel to the perforin/granzyme system to induce apoptosis in infected cells^{62,169}, and it has been

shown that CD8 cells are important in clearing *L. major* infection, probably through secretion of IFN γ ¹⁷⁰ and stimulation of CD4 cells, but also possibly through direct cytotoxic killing of *L. major* infected macrophages. To determine the resultant effect of FasL in this context during CL, further experiments would be necessary.

Could Pharmacological Alterations of Apoptosis during Leishmaniasis Serve as an Adjuvant to Existing Therapies?

Currently VL therapies are expensive, toxic or teratogenic. There are however a number of treatment regimes evolving that are promising and may reduce hospitalisation of patients whilst allowing effective parasite clearance²⁸. In the case of CL local injections of glucantime may be administered up to 15 weeks to shorten the ulcerative phase and limit scar formation. New therapeutic approaches would be welcomed by patients and clinicians. An adjuvant therapy with, for example, Fas blocking antibodies, that could be administered locally, could in theory reduce the ulcerative process and reduce scar formation. Several concerns would have to be considered before embarking on such studies. If the aim was to reduce ulceration and consequent scar formation, an obvious problem is that most patients seek medical advice when the ulcerative process is already chronic. The effects of blocking death receptors that are believed to be important in immune surveillance to eradicate oncogenic cells would also have to be considered as there would be a theoretic chance of allowing cancerous cells to survive and proliferate during treatment. Increased parasite replication during inhibition of FasL required further investigation, although this may be overcome with simultaneous administration of leishmanicidal agents. Finally the cost of a novel therapeutic approach targeting death receptors-ligands interactions would initially be high which is problematic in a disease that mainly affects people in low-income countries.

Conclusions and Future Perspectives

In this thesis the hypothesis that apoptotic pathways are altered during human leishmaniasis and that such alterations may have a role in pathogenesis of leishmaniasis was tested. In the case of VL high levels of sFas and sFasL were detected in serum from infected patients and normalised upon successful treatment. Membrane bound Fas and FasL were expressed on spleen cells, but not PBMCs, during VL as compared to healthy controls.

Fas was found to be up-regulated on epidermis surrounding *L. major* induced ulcers during CL while FasL expressing T- cells and macrophages were found in underlying dermis. The number of apoptotic cells in epidermis were increased during CL and correlated to the number of FasL expressing cells in dermis. Supernatants from restimulated CL-PBMCs were found to induce apoptosis in a human keratinocyte cell-line (HaCaT); this pro-apoptotic activity of supernatants could be blocked in 2/3 cases by the administration of Fas blocking antibodies. Systemic treatment with anti-FasL antibodies during murine CL reduced ulcer formation suggesting that Fas/FasL signalling is involved in pathogenesis of ulcers during CL. Alterations of a second death receptor pathway, the TRAIL-induced pathway, were detected in HaCaT exposed to supernatants from *in vitro* *L. major* infected PBMCs. These results were verified by detection of TRAIL up-regulation on HaCaT and skin biopsies from CL cases as compared to healthy controls.

A number of opportunities for further studies have become obvious as a result of this thesis work. In the case of VL, it would be interesting to follow serum levels of sFas and sFasL during HIV-1 and VL co-infection during treatment of the respective diseases, in view of the data available in literature indicating that control of one of the infections may affect the clinical outcome also for the second infection. It would be of value to evaluate the rate of apoptosis, TRAIL and TRAIL-R1-4 expression by immunohistochemistry on spleen cells obtained for diagnosis during active and healing VL. In such project, the cellular origin of enhanced expression of Fas and FasL could be determined.

In the case of CL, we plan to continue the investigation of the role of TRAIL and FasL mediated apoptosis during ulcer formation to better understand the role of these pathways during ulceration in CL. In this setting, TRAIL and FasL will be blocked, together and separately, during different treatment regimes. In this study ulceration, parasitic loads and inflammation will be followed. *In vitro* systems of co-incubation of murine keratinocyte with *L. major* infected cells will also be applied in this context.

The data obtained within the frame of the present thesis suggest that at least two death receptor mediated pathways, FasL and TRAIL mediated apoptosis,

are altered during CL. Although FasL mediated apoptosis is the death receptor pathway that have been most thoroughly investigated, accumulating data on TRAIL mediated apoptosis suggest that these pathways operate in parallel in a number of situations, such as during cytotoxicity. It is not surprising that a system as important to the homeostasis of an organism as apoptosis is controlled through several parallel pathways to ensure maintenance of homeostatic processes. Although tempting as therapeutic targets, it may be more complex to alter these apoptotic pathways in man as compared to inbred animal models.

The knowledge of the involvement of death receptor pathways in the pathogenesis of leishmaniasis needs to be consolidated through additional experiments with the hope to contribute to new therapeutical principles in a field neglected by large pharmaceutical companies. The murine CL model is a useful system to study the effects of manipulations of the apoptotic pathways during a microbial infection.

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