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# **A ROLE FOR NK CELLS IN INNATE IMMUNITY AGAINST HUMAN LEISHMANIASIS**

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Cover: Giemsa stained *in vitro* cultured *L. aethiopica* promastigotes.

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## ABSTRACT

Leishmaniasis is a group name for a spectrum of diseases caused by intracellular protozoa belonging to the family *Leishmania*. The parasites has been widely used as a tool to study the Th1 / Th2 paradigm of resistance and susceptibility in mice. A role of NK cells in leishmaniasis has been implicated, but not fully explored.

In this thesis work we have continued previous studies exploring the cellular, in particular NK cell, responses to *Leishmania* antigens in blood mononuclear cells from humans with no history of leishmaniasis. It is of importance to study the responses in unexposed donors since they a) represent a potential group to whom a vaccine will be given and b) will give information about the innate responses to *Leishmania*, which may be of importance in determining disease outcome and/or protection. The last part of this work investigated the contribution of NK cells in the development of human cutaneous leishmaniasis.

It has long been known that individuals who have healed from cutaneous leishmaniasis are protected against further disease. Thus, a vaccine against leishmaniasis would appear to be achievable. Vaccine studies performed on humans have shown that live *Leishmania* vaccine induces solid protection, while heat-killed *Leishmania* + BCG induce variable protection. We tested if the differences in the efficacy of the two types of vaccines were in part due to differential cellular responses initially induced by live and dead parasites. Results show clear differences in the type of responses evoked by live and dead parasites. Live promastigotes induced IFN $\gamma$  secretion in NK cells, while killed promastigotes tended to induce CD4<sup>+</sup> cells proliferation. Furthermore, we demonstrate that live promastigotes independent, of other cell subsets and IL-12, could induce NK cells to IFN $\gamma$  secretion. Suggesting that NK cells can contribute independently and very early on in the defence against pathogens.

A number of vaccine candidates against leishmaniasis, such as Leish-111f, LACK (*Leishmania* homologue of receptors for activated C kinase) and the amastigote antigens P-2, P-4 and P-8, have demonstrated encouraging results in mice but, are yet to prove themselves in humans. We tested the stimulating capacity of P-2, P-4 and P-8 in healthy donors and found P-2 to have most reactivity. A similar pattern of reactivity, but with enhanced magnitude was observed when cells were stimulated with LACK. Both LACK and P-2 stimulated cells to proliferation and secretion of IFN $\gamma$  and IL-10. Both T cells and NK cells were involved in these responses. Furthermore, we demonstrated that the induction of IFN $\gamma$  as well as proliferation to these vaccine candidates were MHC class II dependent, whereas IL-10 secretion tended to be enhanced by blocking MHC class II. Direct activation of NK cells could not be achieved by LACK or P-2 requiring antigen presenting cells for induction of NK responses.

NK cells have been implicated in protection and healing of cutaneous leishmaniasis. To follow up on these data and data from unexposed individuals we have evaluated the contribution of NK cells to IFN $\gamma$  response in cells from Iranian patients with active cutaneous leishmaniasis. Initial cross sectional studies indicated that purified NK cells from active cutaneous leishmaniasis patients had reduced ability to secrete IFN $\gamma$  compared to cells from healthy controls. Furthermore, in cured patients, CD4<sup>+</sup> cells appeared to downregulate the NK cell induced IFN $\gamma$ . However, when cytokines (IFN $\gamma$  and IL-13) were evaluated in a longitudinal study in individual donors before and after artificial infection we found that NK cells contributed significantly and equally to the cytokine response both before and nine months after infection, when most of the donors showed signs of disease. The choice of study group and infection dose may have contributed to these unexpected results.

The cumulative results of the studies continue to suggest a role for NK cells in the control of leishmaniasis.

## LIST OF PAPERS

- I. **Nylén S**, Mörtberg U, Kovalenko D, Satti I, Engström K, Bakhiet M, Akuffo H. Differential Induction of Cellular Responses by Live and Dead *Leishmania* Promastigotes in Healthy Donors. Clin Exp Immunol. 2001;124:43-53
- II. **Nylén S**, Maasho K, Söderström K, Ilg T, Akuffo H. Live *Leishmania* Promastigotes Can Directly Activate Primary Human Natural Killer Cells to Produce Interferon-gamma. Clin Exp Immunol. 2003;131:457-67
- III. Maasho K, Satti I, **Nylén S**, Guzmán G, Koning F, Akuffo H. A *Leishmania* Homologue of Receptors for Activated C-kinase (LACK) induces both IFN-gamma and Interleukin-10 in Natural Killer Cells of Healthy Blood Donors. J Infect Dis. 2000; 182:570-8
- IV. **Nylén S**, Maasho K, McMahon-Pratt D, Akuffo H. Leishmanial Amastigote Antigen P-2 Induces MHC class II Dependent NK Cell Reactivity in Cells from Healthy Donors (submitted)
- V. **Nylén S**, Eidsmo L, Mohammadi A, Khamesipour A, Akuffo H. NK Cell Responses In Patients With Cutaneous Leishmaniasis (manuscript)

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## CONTENTS

<b>INTRODUCTION</b>	1
<b>History</b>	1
<b>The Diseases</b>	3
<i>Epidemiology &amp; geographical distribution</i>	3
<i>Diagnosis and treatment</i>	5
<b>The Parasite</b>	6
<i>Life cycle of Leishmania</i>	7
<i>Surface structures</i>	7
<b>Immunology of Leishmaniasis</b>	8
<i>Animal models</i>	8
<i>Establishing an infection and surviving the immune response</i>	9
<i>The role of selected cell subsets in Leishmania infection</i>	10
<i>Neutrophils</i>	10
<i>Macrophages</i>	10
<i>Natural killer cells</i>	11
<i>Dendritic cells</i>	12
<i>CD4<sup>+</sup> T cells</i>	13
<i>CD8<sup>+</sup> T cells</i>	14
<i>B cells and immunoglobulins</i>	15
<i>Cytokines in leishmaniasis (list of selected cytokines)</i>	16
<b>Vaccines Against Leishmaniasis</b>	18
<i>Killed Leishmania promastigotes</i>	18
<i>Leish-111f</i>	19
<i>LACK</i>	19
<i>Amastigote antigens</i>	20
<b>AIMS OF THIS THESIS</b>	21
<b>ETHICAL CONSIDERATION</b>	21
<b>METHODOLOGY</b>	22
<b>Donors</b>	22
<i>Healthy controls</i>	22
<i>Leishmaniasis patients</i>	22
<i>Volunteers inoculated with live L. major promastigotes (leishmanization)</i>	23
<b>Preparation of Mononuclear cells</b>	23
<i>Freezing and transportation of cells</i>	23
<b>Antigens and Mitogens</b>	23
<i>Parasites</i>	23
<i>Whole promastigote antigens</i>	23
<i>Purified antigens</i>	24
<i>Amastigote antigens</i>	24
<i>LACK</i>	24
<i>Leish-111f</i>	24
<i>LPG</i>	24
<i>Other antigens and mitogens</i>	24
<b>Inhibition Assays</b>	25

<i>Blocking of IL-12</i>	25
<i>Blocking of MHC class II</i>	25
Proliferation Assay	25
FACScan	25
Cytokine Analysis	26
<i>Cytokine gene expression</i>	26
<u>mRNA isolation</u>	26
<u>RT-PCR</u>	26
<u>Semi-quantitative evaluation of mRNA</u>	27
<i>Measurements of secreted cytokines</i>	27
<u>ELISA</u>	27
<u>ELISpot</u>	27
Isolation and Separation of Cell Subsets	28
<i>Cytokine gene expression in positively selected cell subsets</i>	28
<i>Depletion of specific cell subsets</i>	28
<i>Isolation of NK cells by negative selection</i>	28
<i>Isolation of NK cells by flow assisted cell-sorting</i>	29
Cytotoxicity Assay	29
RESULTS AND DISCUSSION	30
CONCLUSIONS	48
CONCLUDING REMARKS	48
ACKNOWLEDGEMENT	49
REFERENCES	50

## Appendix (papers I-V)



## LIST OF ABBREVIATIONS

ACL	American cutaneous leishmaniasis
ALM	Autoclaved <i>Leishmania</i> major
APC	antigen presenting cell
C	complement
CD	cluster of differentiation
cDNA	complementary dioxy-ribonucleic acid
CR	complement receptor
CCR	CC chemokine receptor
CL	cutaneous leishmaniasis
CPM	counts per minute
CTL	cytotoxic lymphocytes
DAT	direct agglutination test
DC	dendritic cell
DNA	dioxy-ribonucleic acid
DCL	diffuse cutaneous leishmaniasis
dNTP	2'deoxy nucleoside 5'triphosphate
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescent activated cell sorter
FCS	foetal calf serum
FITC	fluoro-isothiocyanate
Ft-LAg	whole freeze-thawed <i>Leishmania</i> promastigotes
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	good manufacturing practices
Gp	glycoprotein
HK	heat killed
IC	isotype control
ICAM	intra cellular adhesion molecule
Ig	immunoglobulin
IFN $\gamma$	interferon-gamma
IL	interleukin
iNOS	inducible nitric oxide synthase
kDa	kilo dalton
LACK	<i>Leishmania</i> homologue for receptors for activated C-kinase
LC	Langerhans cell
LCL	localized cutaneous leishmaniasis
LPG	lipophosphoglycan
LST	leishmanin skin test
M $\phi$	macrophage / monocyte
MCL	mucocutaneous leishmaniasis
mAb	monoclonal antibody
MHC	major histocompatibility complex
MNC	mononuclear cell
mRNA	messenger ribonucleic acid
NHS	normal human serum
NK	natural killer cell

NNN	Novy-Nicolle-McNeal medium
NO	nitric oxide
PAC	plastic adherent cells
PALS	periarteriolar lymphoid sheath
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PdV5.2	hybridoma producing monoclonal IgG1 anti MHC class II
PE	phycoerythrin
PHA	phytohaemagglutinin
PKDL	post kala-azar dermal leishmaniasis
PMN	polymorphonuclear neutrophil granulocyte
PROM	promastigotes
PPD	purified protein derivate of <i>Mycobacterium tuberculosis</i>
RBC	red blood cell
RCL	Relapsing /recidivans cutaneous leishmaniasis
RT-PCR	reverse transcriptase polymerase chain reaction
SAM	sheep-anti-mouse antibody
SLA	soluble <i>Leishmania</i> antigen
MLA	insoluble, mainly membrane associated, <i>Leishmania</i> antigen
<i>spp</i>	species
TCR	T cell receptor
TDR	UNDP/ World Bank / WHO special programme
Th	T-helper
TNF $\alpha$	tumour necrosis factor-alpha
TGF $\beta$	transforming growth factor-beta
VL	visceral leishmaniasis
<i>wt</i>	wild type

## INTRODUCTION

Leishmaniasis is the collective name for a group of human diseases caused by parasitic protozoa of the genus *Leishmania*, which are all transmitted through the bite of infected sandflies. Most *Leishmania* species normally have rodent or canine hosts and infection of humans is in most instances accidental. The *Leishmania spp* cause disease in tropical and subtropical areas around the world. According to their geographical distribution, the Leishmanias may be divided into New World and Old World *Leishmania spp* (Table 1). In humans the disease presents itself in at least four different forms: cutaneous, diffuse cutaneous, mucocutaneous and visceral, some of which may have devastating consequences. Over the last ten years the leishmaniasis have become an increasing problem. This is thought in part to be due to man-made environmental changes, which have increased the human exposure to the sandfly vector. Leishmaniasis often claims its victims among the poorest and most vulnerable members of the community.

## History

Leishmaniasis has been present in the Americas for a long time. Designs on pre-Inca pottery and the existence of skulls dating back to the first century AD show signs typical of the disease. In Iran the disease is mentioned in writings of Abu Ali al-Husain ibn Abdallah ibn Sina (Avicenna) (981-1037 AD). The first reported outbreak of kala-azar or black fever (visceral leishmaniasis) was from Jessore, India in 1824. At this time the disease was thought to be a type of malaria. In 1885 Cunningham made the first scientific description of *Leishmania* when he reported numerous round and oval intracellular bodies in the tissue specimen of Delhi Boil. The histological description was later recognized as featuring cutaneous leishmaniasis. In 1903 Leishman published a paper about the findings of small bodies in the splenic smear of a soldier who died from a febrile disease contracted at Dum-Dum, India, (hence, the name dum-dum fever). At the same time Donovan reported that he had found similar organisms in patients with prolonged fever. These bodies were later referred to as Leishman-Donovan (LD) bodies and the organism that caused visceral leishmaniasis was named *Leishmania donovani* in recognition of the discoverers. When Rogers in 1904 succeeded in culturing the parasites it became clear that *Leishmania* was a haemoflagellate. In 1927 Adler and Theodor succeeded in transmitting *L. tropica* from artificially infected sandflies to humans and in 1942 Swaminath, Shortt and Andersen proved (in humans) that transmission of kala-azar (*L. donovani*) occurred through the bite of *Phelbotomus argentipes*, reviewed in [1, 2]

Table 1: A selection of *Leishmania spp* pathogenic to humans, their geographical distribution and reservoir animals

Species	Geographic distribution	Disease manifestations	Reservoirs
<b>Old World</b>			
<b><i>L. major</i><sup>#</sup></b>	Rural semi-deserts in: Middle East, Central Asia, Northern India, Pakistan, North Africa	CL	Great gerbil
	Sub-Saharan savanna, Sudan		Rodents esp. Nile rat and African gerbils
<b><i>L. tropica</i></b>	Towns in Middle East, Central Asia and eastern Mediterranean basin	CL, RCL*, (VL)	Humans Rats (?), dogs (?)
<b><i>L. aethiopica</i><sup>#</sup></b>	Highlands of Ethiopia and Kenya	CL, DCL	Rock hyraxes
<b><i>L. donovani</i><sup>#</sup></b>	North East India- South East Nepal, Bangladesh, Burma	VL, PKDL**	India: Humans
	Sudan, Kenya, Horn of Africa		Africa: uncertain, rodents / dogs /humans
<i>L. infantum</i>	Mediterranean basin, Middle East China, central Asia	VL	Dogs, foxes, jackals
<b>New World</b>			
<i>L. chagasi</i>	Central America, northern South America esp. Brazil and Venezuela	VL	Foxes, dogs, opossums
<b><i>L. mexicana</i><sup>#</sup></b>	Yucatan, Belize, Guatemala, Southern Texas	CL, DCL	Forest rodents
<i>L. pifanoi</i> <sup>#</sup>	Dominican republic Venezuela	CL, DCL	Forest rodents
<i>L. amazonensis</i>	Tropical forests of South America	CL, DCL, (VL)	Forest rodents
<i>L. venezuelensis</i>	Venezuela	CL, DCL	Forest rodents
<b><i>L. braziliensis</i></b>	Tropical forests of South and Central America	CL, MCL	Forest rodents, peridomestic animals
<i>L. peruivana</i>	West Andes of Peru, Argentinean highlands	CL	Dogs
<i>L. lansoni</i>	Amazon region, Sub-Andean regions of Peru and Bolivia	CL	Rodents (?)
<i>L. colombiense</i>	Colombia, Venezuela	CL, (VL)	Sloths (?), dogs (?)
<b><i>L. guyanensis</i></b>	Amazonian forests of Guyana, French Guiana, Surinam, Brazil and Colombia	CL, MCL	Sloths, tamandua (tree-living anteater)
<i>L. panamensis</i>	Panama, Costa Rica, Colombia	CL, MCL	Sloths

<sup>#</sup> *Leishmania spp* used in this thesis \* Leishmaniasis recidivans / lupoid leishmaniasis \*\* Post-kala-azar dermal leishmaniasis. Bold indicate *spp* complex.

## The Diseases

Leishmaniasis appears with a broad spectrum of clinical manifestations depending on the infectious species and the immunological competence of the host. Although, the division of the manifestations is not absolute, the leishmaniases can widely be classified into four different categories:

*Cutaneous leishmaniasis* (CL) is the simplest and most common form of leishmaniasis. The disease is known under many names (oriental sore, Baghdad Boil, Delhi Boil etc) in the Middle East and is characterized by a lesion that appears at the site of the sandfly bite. The lesion, that in most cases is self-healing, ulcerates after some time and scar formation takes place. The incubation period and the duration of lesion vary substantially depending on the infectious species. Leishmaniasis caused by *L. major* normally appears within four month after infection and heals within one year. African highland leishmaniasis, caused by *L. aethiopica* and urban CL caused by *L. tropica* have slower evolution of lesions and healing can take several years. The clinical picture of New World CL differs depending on the infecting species. In the Americas several different species cause CL. The most frequently reported sores are caused by *L. braziliensis*. These normally heal within a year, but may last much longer. The clinical features of *L. mexicana* complex vary, however; the lesions are often solitary and heal spontaneously within 6 months to a year.

*Diffuse cutaneous leishmaniasis* (DCL) is a rare form of CL caused by *L. aethiopica* in the Old World and *L. mexicana* complex in the New World. DCL is characterized by disseminated chronic skin lesions, which contain large numbers of amastigotes. The lesions resemble those of lepromatous leprosy. This form of leishmaniasis is often very difficult to treat.

*Mucocutaneous leishmaniasis* (MCL), sometimes known as espundia is believed to be a consequence of primary cutaneous leishmaniasis caused by *L. braziliensis* and *L. guyanensis* (although, other South American *spp* can also cause MCL). It is thought that parasites from the original skin ulcer spread to mucosal sites where they cause dreadful and massive tissue destruction, especially of the mouth and the nose. Mucosal lesions normally develop within two years but may appear as late as 30 years after the primary cutaneous lesion.

*Visceral leishmaniasis* (VL), or kala-azar, is the most severe form of leishmaniasis and if left untreated the mortality rate is almost 100%. The disease is characterized by irregular fever, anaemia, weight loss and swelling of the liver and spleen. The incubation period can be months or years and unlike other forms of leishmaniasis internal organs are prominent sites of infection. After treatment and recovery, VL patients may develop chronic cutaneous leishmaniasis, known as post-kala-azar dermal leishmaniasis (PKDL) that requires long-term treatment. VL may be caused by *L. donovani* and *L. infantum* in the Old World and by *L. chagasi* in the New World.

### **Epidemiology & geographical distribution**

*Leishmania spp* are estimated to cause disease in 12 million people living in 88 countries, of which 72 are developing countries (*figure 1*). The visceral form of leishmaniasis annually claims approximately 59 000 deaths (number of cases per year estimated to 500 000) [3].

The risk to acquire the infection is dependent on several factors including: proximity of residence to sandfly breeding and resting sites, working in such sites, type of housing, extent of exposure to sandfly bites, age, sex and natural resistance [4-8]. Immunity may be reduced by extrinsic factors such as nutrition and other infections [9-11].

The leishmaniasis are mainly zoonotic with small mammals being the main reservoirs. Zoonotic reservoirs usually provide a stable chronic source of infection and human contribution to the reservoir pool is often negligible. Some forms of leishmaniasis are anthroponotic e.g. VL caused by *L. donovani* in India and parts of China. Here, PKDL cases are believed to represent the stable inter-epidemic reservoir for the disease. The *Leishmania* species and their geographical distribution and reservoirs are presented in table 1.

*L. major* is the most common cause of zoonotic CL of the Old World. It is endemic throughout the flat hot semi-desert and dry-silt valleys of the Middle East and North Africa. In Iran zoonotic cutaneous *L. major* leishmaniasis is found in most parts of the country except the Caspian littoral area and Northwestern quarter of the Central plateau. There are at least 15 different foci in Iran of 3 different types depending on the animal reservoir (different gerbils) and sandfly vectors.

Another major causative agent of CL in the Old World is *L. tropica*. This form of leishmaniasis is mainly urban and humans are considered as the main reservoir. Although, the parasite has been isolated from viscera of rats and skin of dogs their role as reservoirs are uncertain. In Iran *L. tropica* CL is present with low incidence rate in most of the big cities throughout the country. In distinct highland areas of East Africa *L. aethiopica* is an important causative agent of CL.

In Central and south Americas all *Leishmania* spp. are zoonotic. *L. braziliensis* is the most common cause of both mild and severe CL. The other causative agents of CL in this part of the world belong to the *L. mexicana* or *L. guyanensis* complex [12].

*L. chagasi* is the cause of VL in the forested tropical parts of the Americas, with the main endemic area found in northeast Brazil. Boys and young male adults are most commonly affected. Both wild and peridomestic animals serve as reservoirs for the parasite.

In the Old World *L. infantum* is a causative agent of zoonotic VL. This parasite is present in a broad belt between 30°N-45°N from Portugal in the West to the coast of China in the East. As the name indicates it is mainly observed in young children. The other risk group is immuno-suppressed adults. The reservoirs for *L. infantum* are various canine species and foxes.

Visceral leishmaniasis caused by *L. donovani* is mainly anthroponotic, but in sub-Saharan Africa zoonotic transmission also occurs, with rodents being the most likely reservoirs. [12].

The majority of CL cases (>90%) are found in Afghanistan, Iran, Saudi Arabia, Syria and Brazil and more than 90% of all VL cases occur in Bangladesh, Brazil, India and Sudan. [3]. The patient studies described in this thesis are from Iran.

There is reason to believe that the numbers of leishmaniasis cases are increasing. Over the last 10 years there has been a sharp increase in the number of recorded cases and the number and extension of endemic regions have expanded. The spread is mainly due to development related factors such as massive rural-urban migration and agro-industrial projects that bring non-immune people into endemic rural areas. As an example CL caused by *L. tropica*, in Kabul, Afghanistan, has steadily increased in

number since first reported in 1964 with an estimate of more than 200 000 cases in a population of less than 2 million 1996 [2]. The high turnover of people in the city of Kabul had been suggested to be the reason why *L. tropica* remained endemic [13]. Political unrest, which affected the health system is likely to have aggravated this situation. Another contribution to the increase of leishmaniasis cases, in particular VL is the HIV/AIDS epidemic. As of 2001, 33 countries have reported co-infection with *Leishmania* and HIV. Co-infection has become an emerging disease, in particular in Southern Europe where 25-70% of all VL cases are related to HIV and as many as 9% of the AIDS cases suffer from VL. Intravenous drug users have been identified as the main population at risk in Southern Europe [2].



Figure 1: Distribution of leishmaniasis around the world. The lighter shade shows areas affected by CL and the darker shade those areas affected by VL.

### ***Diagnosis & treatment***

During the first half of the course of a simple CL lesion the possibility to isolate *Leishmania* is up to 80%. Once healing begins, parasitological diagnosis may become more difficult. In the most straightforward method, material scraped from the edge of a lesion is fixed and stained with Giemsa-stain. A positive identification is 100% specific, in the hands of skilled personnel. This method can be used on aspirates from spleen or bone marrow for diagnosis of VL. However, this method does not identify all positives and cannot distinguish between species. Culturing of the scraped material or aspirates onto a semi-liquid NNN (Novy-Nicolle-McNeal) -blood agar medium may be useful when LD bodies, due to low numbers, cannot be identified in smears or when propagation of the parasites is required for further analysis i.e. species identification.

PCR of lesions and aspirate specimen can be used to increase the sensitivity of detection. The method can be used for detection of *Leishmania* in general, but the most relevant application is species identification [14-16]. The advantage of the PCR method is that only small quantities of parasites are required to ascertain a correct diagnosis.

Hence only short term culturing of specimen is required. Although, PCR diagnostics are available the most widely used technique for species identification in many endemic countries is biochemical identification by isoenzyme typing. This method requires large amounts of parasites and requires culturing of specimens for up to 1 month. Antibody titers are often too low to be of practical value in diagnosis of CL. However, in MCL, DCL and VL antibodies are more consistent and readily detected and thus, antibodies based tests; ELISA, Western blot, indirect immuno-fluorescence antibody test (IFAT) and direct agglutination test (DAT) may be of use [17-21].

Leishmanin skin test (LST) / Montenegro skin test [22] can be used on its own, but is more frequently used together with serological methods, to diagnose leishmaniasis when parasites cannot be isolated. The test has also been used for early evaluation of *Leishmania* vaccines efficacy. Briefly, formalin or methiolate killed promastigotes are given by intradermal injection in the forearm and the induration of the skin is measured at 48 and 72 hours. The size of induration considered as positive has to be determined in each geographical setting (usually negative control +3 SD) [23]. A positive reaction is an indication of exposure, but not always indicative of on-going disease. Patients suffering from active VL are often negative in the LST test. However, after successful treatment most VL patients become LST positive.

Two pentavalent antimonial compounds commonly used against leishmaniasis are Pentostam (sodium stibogluconate) and Glucantime (meglumine antimonite). Both compounds act on the glycolysis and fatty acid oxidation pathway, which occur in all *Leishmania* species. The disadvantages of the antimonials are: the cost, the need for injection and the frequent side effects (anorexia, musculoskeletal pain and cardiac effects). Furthermore, when given systemically the drugs are not very effective in treatment of CL. This may in part be due to the fact that pentavalent antimonials are concentrated in the spleen and the liver, whereas very low levels are detected in the skin. Intra-lesional injection has been shown to accelerate healing and can be used to avoid disfiguration in CL. Other drugs that can be used as an alternative, or second line, or together with the antimonials are Amphotericin B (Fungizone) and pentamidine isothionate. These drugs are often given after failure of antimonial treatments due to the severe side effects. Liposomal forms of Amphotericin B have low toxicity, but are extremely expensive and not affordable in endemic areas. Treatment of CL can also be achieved by cryo-therapy and surgical excision of lesion [24].

## **The Parasite**

*Leishmania* belongs to the protozoan order *Kinetoplastida* and the family *Trypanosomatidae*. All species have two distinct morphological forms: In the sandfly, as well as at 25°C in *in vitro* cultures, the parasites assumes the elongated, flagellated free-living form termed promastigote (10-15µm). Within the mammalian host the rounded non-flagellated amastigote (3-5µm), living and dividing within macrophages, is found. Multiplication takes place in both the promastigote and amastigote stage. The parasite contains a nucleus and a kinetoplast, the mitochondrial DNA of the trypanosomes.



### ***Life cycle of Leishmania***

Transmission to humans is through blood-sucking sandfly vectors of the species *Phlebotomus* in the Old World and *Lutzomia* species in the New World. After inoculation by the bite of an infected female sandfly, the promastigotes are taken up by macrophages. The promastigotes change into amastigotes in a phagolysosome termed parasitophorous vacuole. Within the vacuole the amastigotes are believed to reside and multiply by binary fission until they rupture the cell. However, it has been suggested that amastigotes also reside in the cytoplasmic compartment and that they may leave the cells through a mechanism that would resemble exocytosis [25]. The released amastigotes are ingested by new macrophages and the infection is amplified. The life cycle continues when a sandfly ingests amastigotes following a blood meal. Within the sandfly, the amastigotes transform into promastigotes, which multiply in the midgut. After a few days the promastigotes differentiate into the infective metacyclic stage and move forward to the mouthparts of the sandfly [26]. When the sandfly feeds the cycle is completed (*figure 2*).

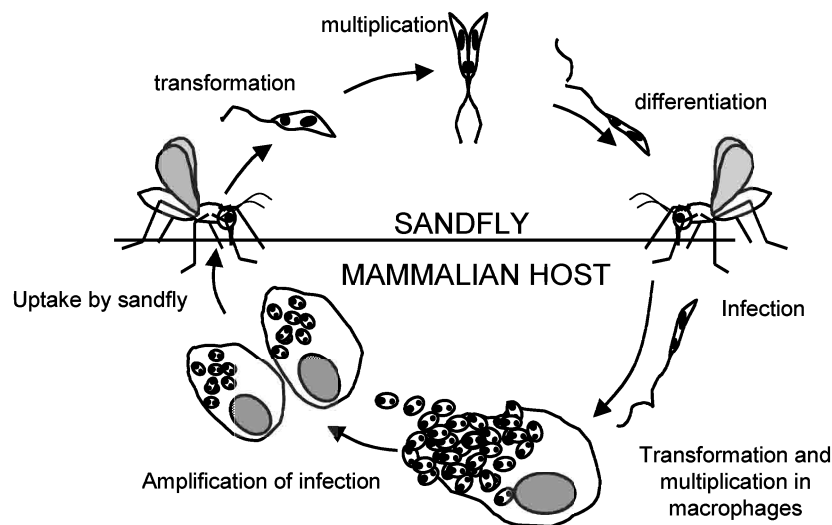


Figure 2: Life cycle of *Leishmania*

### ***Surface structures***

Leishmanial parasites express a unique family of glycoconjugates on their surface, the phosphoglycans [27, 28]. The major cell surface glycoconjugate is the lipophosphoglycan (LPG). Its abundances and location implies one or more important functions for the parasite [29, 30]. LPG consists of linear or branched, phosphorylated

disaccharide repeats of  $[(6\text{-Gal}\beta 1\text{-4Man}\alpha 1\text{-PO}_4)_n]$ . Although, all *Leishmania* species share a conserved LPG backbone structure, the length and the structure of side chains vary. When the promastigote develops the LPG is modified. The modifications of the LPG are involved in the stage specific attachment to and release from the sandfly midgut [31, 32]. When the non-infective pro-cyclic stage differentiate to the infective metacyclic stage the repeats per LPG molecule increase approximately two-fold concomitant with a decrease in the presentation of terminally exposed sugars [33, 34]. The conformational changes increases the parasites resistance to complement mediated lysis and have been suggested to be of importance for invasion and survival within the macrophage [35-39].

However, the importance or redundancy of LPG for invasion and survival has been questioned by Ilg and co-workers, who recently demonstrated that LPG deficient *L. mexicana* mutants could infect and survive within macrophages and were equally infectious compared to the wild type parasite in a mouse model [40, 41].

Amastigotes do not synthesize LPG, but contain several glycoconjugates related to LPG [42, 43] that may have immunomodulatory effects [44].

Gp63 (leishmanolysin) is the most abundant protein on the surface of promastigotes and is highly conserved among *Leishmania* species [45]. This 63 kDa protein, expresses protease activity and is suggested to be involved in attachment to and uptake by macrophages by binding to the complement receptor type 3 (CR3) [46, 47]. Gp63 is expressed throughout the life cycle, but is differentially processed and localized in the different stages [48, 49]. A recent study of gp63 null mutants demonstrated this protein to be a virulence factor [50].

## Immunology of Leishmaniasis

### *Animal models*

Immunology of *Leishmania* infection has mainly been studied in infection models of *L. major* in mice. Most strains of mice (C57BL/6, C3H, CBA) develop a self-limiting cutaneous disease and are considered as resistant. Others (notably Balb/c) cannot control the infection and develop a lethal systemic disease. The *L. major* mouse model has served as an excellent tool for explaining the Th1 / Th2 paradigm in relation to an intracellular infection, reviewed in [51]. However, when other strains of *Leishmania* are used the patterns of resistance and susceptibility may deviate from observation made using *L. major* [52, 53]. Although resistant to *L. major* CBA, C3H and C56BL/6 mice are susceptible to *L. amazonensis* infection. In CBA mice *L. amazonensis* lesions disseminate (resembling DCL) and in C3H and C57BL/6 chronic lesions are developed [53, 54]. In all the latter mouse strains the susceptibility appears to be due to the absence of Th1 cell response rather than to the presence of a Th2 cell response [55].

As may be anticipated, it appears to be more difficult to establish infections in mice with *Leishmania* species that do not have rodents as their main reservoir, compared to rodent adapted species. The susceptible Balb/c mouse is relatively resistant to infection with the *L. donovani*, *L. braziliensis* and *L. aethiopica* [56-58]. To establish systemic *L. donovani* infection in Balb/c mice very high doses of parasites ( $2 \times 10^7$ ) are given. Alternative routes of infection and the use of amastigotes instead of promastigotes may prolong the systemic infection [59]. However, in contrast to human VL, *L. donovani*

infection in Balb/c does not continuously progress. Thus, other animals like hamsters, that develop a progressive, lethal disease are sometimes used as model of VL [60, 61]. Experimental infections of dogs (natural reservoir for *L. donovani* complex) has also been used to study VL [62].

In most *L. major* and other “cutaneous” *Leishmania* mouse models the promastigotes are injected in the footpad and the infectious dose ( $1-2 \times 10^6$  promastigotes) given is more than a 1000 times higher than would be anticipated during a natural *Leishmania* infection. Thus, most studies should be considered as infectious models of *Leishmania* and not leishmaniasis disease models. However, a model for CL has been developed where the infectious dose, the route of infection and co-administration with sandfly saliva have been taken into consideration [63]. This model appears to better resemble the natural infection and can thus, be considered as a disease model.

### ***Establishing an infection and surviving the immune response***

To establish an infection the promastigotes injected by the sandfly has to survive the attack of the innate immune system. The first line of defence that the promastigote encounters constitutes of complement, antibodies and phagocytic cells, all of which can kill the parasite [64]. The interaction with the complement system is dependent on the species of the parasite and the developmental stage [65]. Resistance to serum increases as the promastigote develops into the infective metacyclic stage and further increases after transformation into the amastigote [26]. The surface coat, which is modified during metacyclogenesis seems to play an important role for this survival by restricting access of the lytic components to the parasite membrane [34]. Although, the complement system has the potential to lyse promastigotes, the parasite utilizes the same system to facilitate infection and gain entry into the macrophages. The activation of the alternative complement pathway, creates a C5a gradient, which acts as a chemoattractant for macrophages [66]. The surface deposition of complements on the promastigote enhances attachment to and uptake by phagocytic cells [67-69]. Binding of C3b to the surface of the parasite leads to rapid conversion into iC3b by factor 1 and gp63. Two complement receptors, CR1 and CR3, can mediate uptake of opsonized *Leishmania* [70, 71]. By utilizing CR1 oxidative burst can be avoided [72]. The mannose fucose and lectin like receptors have also been described as receptors for macrophage attachment [73]. The central cell for infection and replication of *Leishmania* is the macrophage, although, other cells have been found to harbour *Leishmania* parasites including dendritic cells (DC), fibroblasts and neutrophils [74-77].

To establish infection the promastigotes must enter a macrophage efficiently avoiding the potentially destructive mechanisms of this cell [68]. The macrophage microbicidal system includes oxygen metabolites, lysosomal hydrolases, low pH and cationic proteins. In addition to facilitating uptake of promastigotes, fixation of complement has been shown to increase the intracellular survival of *L. major in vitro* [67, 78]. Once inside the cell the parasite resides within a parasitophorous vacuole, which then fuses with secondary lysosomes to form a phagolysosome in which the parasite survives and replicates [79]. The *Leishmania* infected macrophages have been shown to have a functional lysosome, which can destroy other material ingested [80]. How the parasite survives in the central part of the macrophages defence machinery is

not clearly understood. The highly negatively charged LPG has been suggested to be important for the survival within the phagolysosome [30]. Uptake of amastigotes is facilitated by opsonization with antibodies and is in part mediated via Fc receptors [81-83].

### ***The role of selected cell subsets in Leishmania infection***

#### **Neutrophils**

Polymorphonuclear neutrophil granulocytes (PMN) have been shown in a mouse model to be recruited to the site of infection within hours [84]. These cells have been reported to exert anti-leishmanial activity by phagocytosis and killing of promastigotes [85, 86]. Both opsonin dependent and independent uptake mechanisms occur. The opsonin mediated uptake of the parasite appears to activate the PMN to parasite killing, whereas the non-opsonic phagocytosis appears to confer parasite survival [77]. Little is known about the role of PMN as host cells for the *Leishmania* parasite, but these cells could provide an intracellular niche for the parasites within the first hours of infection. *L. major* infection of PMN has been shown to delay the spontaneous apoptosis in these cells. It was hypothesized that infected neutrophils, once they become apoptotic can serve as “silent” delivery system for parasites into the macrophages. [87]. There is limited information about the role of PMN in human leishmaniasis. However, they have been detected adjacent to macrophages in CL lesions [88].

#### **Macrophages**

Skin macrophages could serve as immediate host cells for the parasites, however most macrophages are recruited and have been shown in experimental mouse models to appear at the site of infection within 1-2 days [84]. Macrophages are as well as being the central cell in the *Leishmania* infection also one of the important initiators of the immune response. In the early defence macrophages can secrete a number of cytokines and inflammatory mediators including, IL-1, IL-6, IL-8, IL-12, TNF $\alpha$ , type-1 interferons and IFN $\gamma$  [89] and thereby recruit and activate other cells. The role of various cytokines in leishmaniasis is summarized in the last part the “Immunology of Leishmaniasis” section.

To clear the infection the macrophage has to kill the amastigotes. T cell dependent IFN $\gamma$  production appears to be required for activation of the macrophage and clearance of the established infection [90, 91]. Mouse models have shown that upon activation by IFN $\gamma$ , iNOS is induced followed by enhanced NO production that is anticipated to lead to killing of the amastigotes [92-94]. When exposed to IFN $\gamma$ , macrophages upregulate MHC class II expression, which facilitates the presentation of parasite antigens to T cells.

*Leishmania* species have evolved strategies to silently enter the macrophages and thereby avoid activation and killing, reviewed in [68, 78]. Expression of MHC and co-stimulatory molecules is down-modulated on macrophages harbouring thriving amastigotes, making them poor antigen presenting cells that will avoid recognition by T cells [95-97]. It has been shown that efficient antigen presentation to T cells by macrophages requires killing and degradation of amastigotes [98]. Furthermore, *Leishmania* parasites also interfere with several other macrophage functions [99], amongst those are inhibition of the Th1 inducing cytokine IL-12 [100, 101] and interference with NF $\kappa$ B transcription [102, 103]. If IL-12 suppression is NF $\kappa$ B

dependent is unclear. However, it was recently reported that NF $\kappa$ B is required for optimal CD40 induced IL-12 production in macrophages [104].

#### NK cells

NK cells play an important role as mediators of innate resistance to many pathogens and cancer. Together with phagocytes they represent the first line of cellular defence against infection. The principal mechanisms that NK cells utilize are cytolytic destruction of infected cells and secretion of the pro-inflammatory cytokine IFN $\gamma$ . However, these cells can also secrete a number of other cytokines including: GM-CSF, Lymphotoxin (LT) TNF $\alpha$ , TNF $\beta$ , IL-5 IL-8, IL-10, IL-13 and TGF $\beta$  [105-107].

It has been shown that human NK cells could, depending on the presence of IL-12 or IL-4, differentiate into distinct subtypes, NK1 and NK2. NK cells differentiated in an IL-12 environment were skewed to secrete IFN $\gamma$  and IL-10 whereas IL-4 differentiated NK cells tended to produce IL-5 and IL-13 [108]. These subtypes may be of importance when describing the role of NK cells in an established inflammation / infection. An alternate classification of human NK is the division of freshly isolated NK cells into CD56<sup>bright</sup> the more cytokine secreting cell type, which spontaneously secrete IFN $\gamma$  and the CD56<sup>dim</sup>, the more cytotoxic type [109]. This classification is probably more relevant when assessing NK cells in the early innate cytokine response. Furthermore, the CD56<sup>bright</sup> subset expresses CCR7 and can be found in T cell areas of human lymph nodes. Endogenous T cell derived IL-2 was found to be co-stimulatory for IFN $\gamma$  production in these cells indicating that immune regulation of NK cells by adaptive T cell exist [110].

During many parasitic infections, such as *Toxoplasma gondii*, *L. major* and *Trypanosoma cruzi* there is an initial peak of NK activity [111-113]. It has been shown that NK cells appear at the site of *Leishmania* infection within 24 hours [84]. The early production of IFN $\gamma$  by NK cells was shown to be important in the outcome of *Leishmania* infection since, antibody mediated depletion of NK cell reduced early IFN $\gamma$  secretion in resistant C3H/HeN mice and rendered them susceptible to *L. major* infection [114]. NK cells have also been suggested to participate in parasite elimination later in the infection [115].

However, the importance of NK cells in control of *Leishmania* infection has been questioned since NK deficient C57Bl/6 mice have been demonstrated to control *L. major* infection [116]. The differences between the results may be due to the different mouse strains used. NK cell infiltration into the site of injection has been reported to differ between C57 and C3H mice, with C57Bl lacking the early NK infiltration found in C3H mice [114]. The more important cause for the different results is probably that different methods were used to assess the role of NK cells. In the first model normal mice were depleted of NK cells using antibodies and in the latter the effects were studied in NK and T cells deficient mice transplanted with bone marrow cells, that resulted in normal T cell development but no (very few) NK cells. The cumulative results suggest that NK cells have an important function in the normal mouse, but that their function can be taken over by other mechanisms in the “engineered” mouse.

Although, NK deficient mice can control infection NK cells may still play an important role in the early phase of infection. Susceptible Balb/c mice lacking NK cells develop lesions faster than normal wild type mice and harbours more parasites in the skin lesion compared to wild type mice [117].

The general consensus is that IL-12 is required to induce NK activation [118, 119] [120]. However, IL-12 independent activation of purified human NK cells has been achieved with *Leishmania* promastigotes [121]. Purified human NK cells have also been reported to secrete cytokine in response to stimulation with mitogens [107]. Several *Leishmania* species have been shown to downregulate IL-12 production by macrophages [100, 122, 123], thus, avoiding NK cell activation by macrophages. At least two cytokines, IL-10 and TGF $\beta$ , are known to be involved in inhibition of NK responses during parasitic infections [124].

In human leishmaniasis NK cells have been associated with protection and cure of CL [125]. Furthermore, NK activity in VL patients has been shown to be impaired and PBMC from VL patients have been demonstrated to down-modulate NK cell cytotoxicity *in vitro* [126, 127].

In the context of VL it can be noted that malnutrition is found to be a risk factor for human VL [4, 128] and NK cells are considered to be affected by malnutrition [129] [130]. A decrease in both number of NK cells and NK cell function (measured by cytotoxic activity and IFN $\gamma$ ) has been reported in both humans and mice after experimental diets (protein, energy and vitamin A restriction), whereas T cell numbers and function were not significantly affected [131-133]. Malnutrition has recently been reported to affect the innate immune responses and increase early visceralization in a *Leishmania* infection model [9]. However the effect on NK cells was not assessed in this study and has neither been assessed in other studies of *Leishmania* in malnourished mice.

#### Dendritic Cells

Dendritic cells (DC) are today considered to be key cells orchestrating the adaptive immune responses. They may determine the magnitude of the immune response, the quality of the response, contribute to tolerance and can maintain memory, reviewed in [134, 135]. Knowledge about receptors on DC that are involved in recognition of pathogen has only recently emerged and include Toll-like receptors and C-type lectins. Recently, a C-type lectin receptor, DC specific ICAM-3 grabbing nonintegrin (SIGN/CD209), was demonstrated to be a receptor for *L. mexicana* amastigotes [136]. DC seems to preferentially take up and be activated by amastigotes [137]. It has been suggested the Fc receptors participate in the uptake of amastigotes but not the uptake of promastigotes by DC [138]. Langerhans cells (skin DC) have the unique ability to transport viable *Leishmania* parasites from the site of infection to the draining lymph node [139]. In the lymph node, the Langerhans cell differentiates into professional antigen presenting dendritic cells, which constitutively express large amounts of MHC class II and co-stimulatory molecule B7.1 [140, 141]. Viable parasites have been shown to persist in DC of apparently cured mice for long periods of time (9 months) [142]. The persistence of antigen in these cells is thought to support the maintenance of T cell memory. Failure of DC migration to periarticular lymphoid sheaths (PALS) has been demonstrated in a *L. donovani* mouse model and was associated with defective expression of CCR7 [143].

DC have an essential role in priming of T cells (*figure 3*). These cells can efficiently present antigens to and prime both CD8 and CD4 T cells. It has been shown that IL-12 producing DC are needed for the initial priming of Th1 cells and in the absence of IL-12 Th2 priming is favoured [144]. Both murine and human derived DC have been shown to produce IL-12 in responses to *L. major* infection [123, 137, 145]. Inability to

release IL-12 has been associated with failure of DC to mediate protection against *L. major* [146]. Recent experimental *Leishmania* vaccine studies have clearly demonstrated the importance of DC in the induction of protective immunity, in particular in the initiation of the CD8<sup>+</sup> T cell mediated response [147, 148].

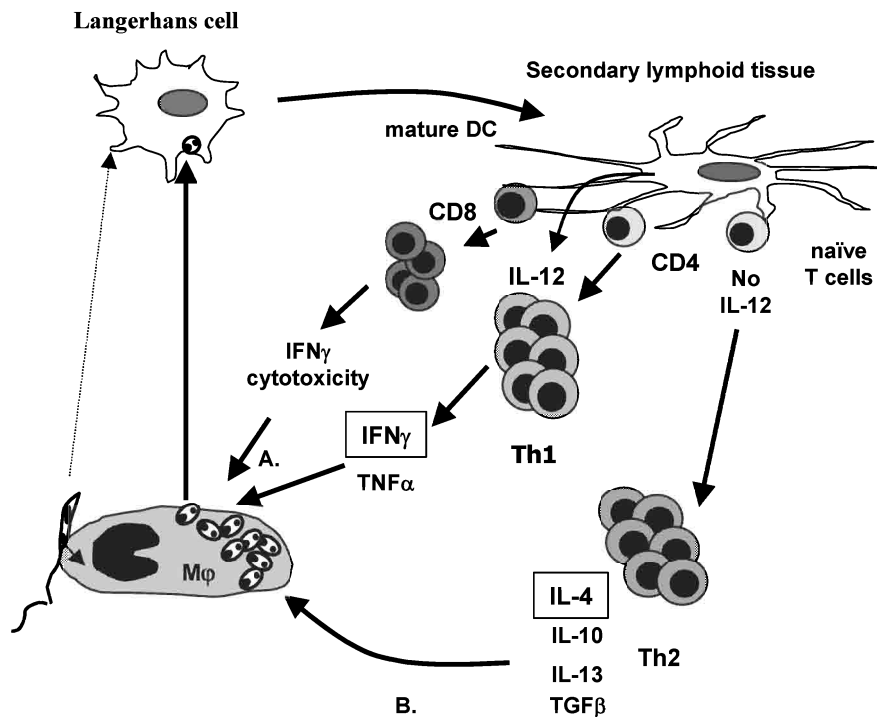


Figure 3: Influence of dendritic cells (DC) on development of T cell immune responses. A) If DC are activated to secretion of IL-12, CD4<sup>+</sup> T cells will differentiate into the IFN $\gamma$  secreting Th1 type and cells and expansion of cytotoxic and IFN $\gamma$  secreting CD8<sup>+</sup> cells will be favoured. These responses are associated with macrophage killing of the amastigotes and induction of protective immunity. B) If no IL-12 is produced this will lead to differentiation into the Th2 type CD4<sup>+</sup> T cells, secreting IL-4 and other cytokines which inhibit Th1 responses and have down modulatory effect on macrophages. Thus, this will lead to exacerbation of disease.

### *T cell responses*

Acquired T cell mediated immunity determines both resistance and susceptibility to *Leishmania*. CD4<sup>+</sup> T cells are undoubtedly the most important cells in mediating protective immunity to leishmaniasis. However several studies also indicate a crucial role for CD8<sup>+</sup> T cells.

#### CD4<sup>+</sup> T cells

The main functions of activated CD4<sup>+</sup> T cells are differentiation into cytokine secreting effector T cell and long-lived memory T cells. Effector T cells, based on the

cytokines they secrete, are divided into either Th1 (IFN $\gamma$ ) or Th2 (IL-4). The importance of CD4<sup>+</sup> T cells in *Leishmania* infection was initially demonstrated by showing that transfer of CD4<sup>+</sup> T cells from resistant mice that controlled primary infection protected normally susceptible mice from infection [91, 149, 150]. Furthermore, transfer of CD4<sup>+</sup> cells into highly susceptible T cell deficient nude mice made them relatively resistant [151, 152]. The effect of the CD4<sup>+</sup> T cells on the resolution of leishmaniasis in mice was shown to be a consequence of macrophage activating cytokine IFN $\gamma$  [153]. In humans the important role of CD4<sup>+</sup> T-cells is illustrated in the CD4<sup>+</sup> T cell deficient AIDS patients. These individuals are at greater risk of developing visceral leishmaniasis and the HIV virus accelerates the course of infection [2]. The study of CD4<sup>+</sup> T cell immune response in resistant and susceptible mice lead to the discovery of functionally separate CD4<sup>+</sup> T cell subpopulations defined by their ability to secrete different cytokines [154, 155]. Since then the *Leishmania* parasite has played a major role in the defining the Th1-Th2 paradigm of resistance and susceptibility to intracellular infections and the regulation thereof, reviewed in [51, 156]. Resistance to leishmaniasis in the mouse model characterized by induction of Th1 type responses and secretion IFN $\gamma$  and IL-2 by CD4<sup>+</sup> cells [154, 157]. IL-12 is considered to be the key cytokine for Th1 development and appears to be required for both induction and maintenance of protective responses against murine *L. major* infection [118, 158]. Susceptibility has been characterized by induction of Th2 cytokine IL-4, IL-13 and to some degree IL-10 [157, 159-161]. In line with the *L. major* mouse model IFN $\gamma$  secreting CD4<sup>+</sup> T cells from humans have been correlated to immunity against *L. major* [162, 163, 164] whereas IL-4 secreting CD4<sup>+</sup> T cells have been correlated to non-healing CL [164]. A similar, but not identical trend is seen when stimulating cells from VL patients. This form of leishmaniasis is associated with loss of specific CD4<sup>+</sup> T cells and impairment of Th1 responses [165, 166] together with an induction of a Th2 pattern, where IL-10 and not IL-4 appear to be the predominant Th2 cytokine [167-169]. In individuals that have cured *L. donovani* VL responding CD4<sup>+</sup> T cell were reported to be of three types: Th1 (IFN $\gamma$  secreting), Th2 (IL-4 secreting) and T cell clones secreting both IFN $\gamma$  and IL-10 [170]. In VL patients, cured of *L. infantum*, *Leishmania* specific CD4<sup>+</sup> T cells that secreted both IFN $\gamma$  and IL-5 were associated with control [171].

#### CD8<sup>+</sup> T cells

The main function of activated CD8<sup>+</sup> T cell is currently believed to be killing of virally infected cells. However, the IFN $\gamma$  secreting and cytolytic properties of these cells appear also to be relevant in resistance against parasitic infection. Priming of protective CD8<sup>+</sup> T cells responses appear to require the co-stimulatory properties provided by DC [172, 173], where IL-12 secretion will contribute to development of potentially protective CD8<sup>+</sup> T cells responses [174]. In this context naïve human T cells have been demonstrated to develop into cytotoxic CD8<sup>+</sup> T cells when primed in the presence of IL-12 and *Leishmania* promastigotes [175].

The importance of CD8<sup>+</sup> T cells in resistance to *Leishmania* and other intracellular parasites is becoming increasingly evident. However, already more than 15 years ago there were indications that CD8<sup>+</sup> cell were involved in resistance to *Leishmania* infection. It was noted that resistant mice had three times as many *Leishmania* specific CD8<sup>+</sup> T cells in the draining lymph node compared to susceptible mice [176]. Transfer of these cells mediated specific delayed type hypersensitivity reaction in naïve recipient



mice. Furthermore, the data indicated that CD8<sup>+</sup> cells were involved in control of the lesion. However, the importance of CD8<sup>+</sup> cells in resistance to *Leishmania* infection was questioned since, CD8<sup>+</sup> T cell alone could not induce a protective immune response [177] and CD8 deficient mice were found to be able to control infection and mount a protective immune response [178]. These latter results have been contradicted by a recent study, which demonstrated that; when a low dose of promastigotes are given by intradermal injection, CD8<sup>+</sup> cells in addition to CD4<sup>+</sup> cells are required to control the primary infection in the skin of mice [148]. Furthermore, recent evaluations of *Leishmania* candidate vaccines also indicate an important role for CD8<sup>+</sup> T cells in protective immunity in mice [179, 180]. In addition *in vitro* data have shown that *L. amazonensis* infected macrophages can present leishmanial antigens to CD8<sup>+</sup> cells via classical MHC class I pathway [181] and that human CD8<sup>+</sup> T cells can lyse autologous *Leishmania* infected macrophages [182].

In humans with CL, CD8<sup>+</sup> T cells have been associated with cure. While CD4<sup>+</sup> T cells were the dominant cell type responding in cultures of cells from patients with active disease, caused by *L. braziliensis*, an increase in responding CD8<sup>+</sup> T cells was observed after treatment and cure [183, 184]. In line with this finding CD8<sup>+</sup> cell responses have been reported to correlate with protection and healing of Ethiopian CL [125].

CD8<sup>+</sup> T cells responses have also been implicated to participate in the pathology of leishmaniasis. In the low dose intra dermal challenge *L. major* mouse model, CD8<sup>+</sup> T cells were reported to mediate dermal pathology [148]. The association to pathology has also been made based on the findings of CD8<sup>+</sup> cells accumulation in granulomas of ACL and post kala-azar dermal leishmaniasis patients, [185-187].

#### B cells and immunoglobulins

Upon stimulation B cells can differentiate into immunoglobulin secreting plasma cells and memory B cells. Clinical human VL is associated with polyclonal B cell activation and production of large amounts of both *Leishmania* specific and non-specific antibodies [188, 189]. However, B cells are considered to play a minor role in the host defence against *Leishmania* [190, 191]. However, B cells or their immunoglobulins have been implicated in the pathogenic process. B cell deficient Balb/c mice have been shown to have, enhanced resistance to *Leishmania* [192]. Moreover, antibodies have in genetically altered Balb/c mice been associated with *L. mexicana* complex pathogenesis [193] and a critical role for immunoglobulin G has been demonstrated for the maintenance of *L. mexicana* complex infection [194]. It was suggested that the role of antibodies in the pathogenesis was to mediate parasite uptake and regulate the immune response at the local cutaneous site of infection. It is known that amastigotes opsonized by antibodies can utilize the Fc receptor to gain entry into macrophages [83]. Amastigotes have further been shown to exploit IgG mediated FcRγ ligation and induce IL-10 [195]. A regulatory role for B cells would be supported by results from a *L. donovani* study where B cells were suggested to inhibit tissue destruction by neutrophils [196]. Although it was interpreted differently at the time, earlier studies have also indicated that B cell-deficient C57Bl/6 mice have a more severe pathology [197].

### ***Cytokines in Leishmaniasis***

The functions of the various cytokines often overlap and are sometimes mutually synergistic and other times antagonistic. They can be responsible for both beneficial and harmful effects during the course of various diseases. A list of selected cytokines found to be of importance in *Leishmania* infection is presented in this section, with an attempt to focus on what is known about these cytokines in relation to human leishmaniasis.

Interferon (IFN) $\gamma$  is mainly produced by Th1 cells and NK cells, but macrophages have recently been described to be an early source of this cytokine [198]. IFN $\gamma$  induces macrophage activation, enhances antigen processing, increases the expression of MHC class I and II molecules and is known to suppress Th2 type responses. The central role for IFN $\gamma$  in control of intracellular parasites, such as *Leishmania* is well established in experimental models. Active VL in humans has been characterized by impaired IFN $\gamma$  production, which was restored after cure [165]. Furthermore, administration of exogenous IFN $\gamma$  has been shown to promote cure of CL in humans [199]; however, limited effect was seen when used in VL patients [200]. This may be explained by down regulation of IFN $\gamma$  receptor expression as has been observed on PBMC from VL patients [201]. However, IFN $\gamma$  can be a two edged sword, if high levels of IFN $\gamma$  are constitutively produced it causes tissue destruction as seen in muco-cutaneous leishmaniasis [202, 203].

IL-2 was originally designated as T cell growth factor since its primary function is to drive T cell proliferation. Furthermore, it promotes growth and cytolytic activity of NK cells.

Cells from VL patients appear to have reduced ability to produce IL-2 [165]. DCL patients have a specific loss of *Leishmania* reactive T cells [204]. Administration of IL-2 was shown to reduce the local parasite load in these patients [205].

IL-4 is mainly produced by Th2 cells, but mast cells, basophils and NK-T are also known to produce this cytokine. This pleotropic cytokine inhibits macrophage activation, stimulates mast cell growth and activates B cells upregulation of MHC class II expression and production of IgG1 and IgE. It is also a T cell growth and survival factor. IL-4 is thought to be an antagonist of Th1 induced inflammatory responses, but recent data indicate that this cytokine early in murine (Balb/c) *L. major* infection may promote Th1 responses [206].

However, IL-4 production is clearly associated with susceptibility and Th2 development in the *L. major* mouse model. In humans IL-4 responses have been associated with non-healing CL [164] and to some degree VL [207]. However, this cytokine may be difficult to detect in human cells after *Leishmania* stimulation [208-210].

IL-10 is a potent suppressor of macrophage cytokine release and effector functions. It has down regulatory effects on IFN $\gamma$  secretion by T and NK cells and inhibits Th1 responses. IL-10 is mainly produced by CD4<sup>+</sup> T cells and macrophages, but can also be produced by NK cells. Induction of IL-10 has been shown to promote progression of *Leishmania* infection in the mouse model [195]. In humans IL-10 production has been

associated with the down regulation of cell mediated immunity observed in VL and DCL patients [167, 211].

IL-12 is produced by B cells, macrophages dendritic cells and neutrophils. Both human and murine myeloid cells have been shown to harbour pre-formed IL-12 that can be released within minutes after contact with an intracellular pathogen (*L. donovani*) [212]. This cytokine stimulates the CD4<sup>+</sup> T cell differentiation into the IFN $\gamma$  secreting Th1 type and favours generation of cytotoxic CD8<sup>+</sup> T cells [213]. Furthermore, it is the most potent inducer of IFN $\gamma$  secretion by NK cell and was originally called “NK cell stimulatory factor”. In the *L. major* mouse model secretion of IL-12 is crucial for induction of protective immunity and sustenance of the Th1 response [118, 158, 214, 215]. However, when another *Leishmania* species (*L. mexicana*) was used it was shown that control of infection could occur through an IL-12 independent pathway [216].

Addition of IL-12 to PBMC cultures of VL patients has been shown to induce IFN $\gamma$  and cytotoxic responses [217, 218].

IL-13 shares many biological functions with IL-4, but cannot on its own drive a Th2 differentiation since T cells lack functional IL-13 receptors [219]. This cytokine is mainly produced by activated T cells but can also be produced by other cells e.g. NK cells. IL-13 inhibits macrophage inflammatory cytokine production and contributes to a Th2 environment. In murine *L. major* infection IL-13 has been demonstrated to be a susceptibility factor [161]. IL-13 has also been shown to render *Leishmania* specific human T cells unresponsive to IL-12 by inhibiting expression of IL-12R $\beta$ 2 [210]. Furthermore, this cytokine was found to be the primary Th2 cytokine in American cutaneous leishmaniasis (ACL) lesions [210]. When IL-4 responses are not easily detected, IL-13 has been suggested as an alternative marker of Th2 responses [208, 210].

IL-18, also known as interferon- $\gamma$  inducing factor, is produced by activated macrophages and Kupffer cells of the liver. This cytokine induces IFN $\gamma$  production by T-cells and NK cells. IL-18 favours initially Th1 responses, but later in the immune response induction of Th2 cells. Although, IL-18 deficiency has been associated with susceptibility [220], this cytokine appears not to be required for induction of a Th1 responses and resolution of *L. major* infection in mice [221]. Cells from VL patients have been reported to have, impaired IL-18 responses [222].

Tumour necrosis factor (TNF) $\alpha$  as the name indicates has the ability to shrink some tumours by attacking their blood supply. TNF $\alpha$  is primarily produced by activated monocytes and macrophages, but also by T cells and NK cells. The main effect of TNF $\alpha$  is to cause local inflammation and endothelial cell activation. Moreover, TNF $\alpha$  activates macrophages and induces nitric oxide (NO) production. This cytokine is associated with protection of and immunity to leishmaniasis in both mouse and man [163, 223, 224]. However, high levels of TNF $\alpha$  have been suggested to contribute to the pathology associated with VL [225] and MCL [203].

Transforming growth factor (TGF) $\beta$  is an important anti-inflammatory cytokine that is mainly produced by chondrocytes, monocytes and T cells. TGF $\beta$  actions include

inhibition of cell growth and macrophage activation. Furthermore it is a potent IL-12 antagonist and can inhibit NK IFN $\gamma$  production and cytotoxic activity [124]. TGF $\beta$  has been found to be involved in immunosuppression in an experimental visceral leishmaniasis model [226]. Moreover, *L. amazonensis* and *L. chagasi* infected human macrophages were found to produce this cytokine [227, 228].

### **Vaccines Against Leishmaniasis**

It is believed that people who recover from leishmaniasis develop life-long protection against subsequent infection. Hence, a vaccine against leishmaniasis would appear to be feasible.

In the Middle East artificial inoculation with parasites derived from active lesions has been the traditional practice to avoid possible facial scars due to naturally acquired cutaneous leishmaniasis. This ancient vaccination was later modified to use cultured parasites as the inoculum. The method is known as leishmanization and was frequently used on soldiers in the Middle East, including during the Iran-Iraq war in the 1980s [229]. However, the side effects of leishmanization created problems: non-healing lesion, dissemination of lesions, secondary infections and low responses to other vaccines, reviewed in [230, 231]. Hence, the use of leishmanization was interrupted in many countries, but not all. In Uzbekistan routine leishmanization was never interrupted and is still practiced on soldiers and other people that are moving into *Leishmania* endemic areas. To date leishmanization is the only form of vaccination that has actually been successful in humans and leishmanization is asked for in CL endemic areas since, it is known to induce a solid protection and no other vaccine is currently available. However, leishmanization has been restricted to *L. major* which tends to cause benign forms of cutaneous leishmaniasis although even the use of this form of *Leishmania* for leishmanization is questioned. This is particularly pertinent in areas with high prevalence of HIV, since there is limited information about HIV-*L. major* co-infection available and dissemination of CL may occur [232]. There are no vaccines available against the more severe forms of leishmaniasis. A limited number of chemotherapies against *Leishmania* exist, however, most of them are expensive, require daily painful intramuscular injections or expensive monitoring when given intravenously and are associated with severe side effects. In addition drug-resistant *Leishmania* are becoming an increasing problem. Thus there is a need for vaccines against leishmaniasis.

Numerous anti-*Leishmania* vaccines / vaccine candidates have been developed reviewed in [233, 234]. Some of these are in the pipeline of being or are currently tested in humans. Among these are:

#### ***Killed Leishmania promastigotes***

ALM (Autoclaved *L. major*) + BCG (Bacillus Calmette Guérin ) belongs to the so-called “first generation vaccines” i.e. crude preparations of *Leishmania* antigens using whole killed promastigotes. These vaccines have shown variable degrees of protection against CL and VL when evaluated in humans [235, 236]. Alum precipitated ALM + BCG has been demonstrated to confer protection in a *L. donovani* monkey model [237]. In a recent vaccine study in Iran the effect of different doses of ALM +BCG+

alum as adjuvant was evaluated using leishmanization as challenge. The results of the vaccine study are not yet reported.

### ***Leish-111f***

The Leish-111f vaccine, produced by the Corixa Corporation, is the first molecularly defined anti-leishmaniasis vaccine to be evaluated in human clinical trials. A Double blind Phase 1 trial, initiated in January 2003 to evaluate safety and immunogenicity, will soon be completed.

The tri-fusion recombinant protein Leish-111f consists of three immuno-dominant protein antigens: TSA (homologue to eucaryotic thiol-specific antioxidant protein) [238], LmSTI1 (*Leishmania major* stress-inducible protein 1) [239] both derived from *L. major* and LeIF (*Leishmania* elongation initiation factor) [240] derived from *L. braziliensis*.

The antigens were selected based on their abilities to elicit robust T cell and antibody responses in infected humans and Balb/c mice [241]. The mix of TSA and LmSTI1 was demonstrated to induce protection in both mice and primate models of CL [242, 243]. The third component, LeIF, was shown to confer partial protection in Balb/c mice as a therapeutic vaccine, a property attributed to its Th1 promoting, IL-12 mediated, adjuvant activity [244]. LeIF was also demonstrated to be a potent stimulator of the innate immune system [240]. For practical reasons a single recombinant polypeptide (Leish-111f) comprising the sequences of all three open reading frames (ORF) genetically linked in tandem in the order TSA-LmSTI1-Leif was generated [241]. Leish-111f has on its own a relatively low inherent immunogenicity and requires a strong adjuvant to prime a specific immune response [245]. When administered together with IL-12 Leish-111f was demonstrated to protect Balb/c mice at a magnitude superior or equal to that seen with any of the individual components or soluble *Leishmania* lysate (SLA) [241]. IL-12 is not approved for use in humans as adjuvant, thus other adjuvant systems were explored. It was found that Leish-111f administered together with a naturally derived disaccharide adjuvant of *Salmonella minnesota* monophosphoryl lipid A and squalene, (MLP-SE) elicited protective immune response against *L. major* infection [245, 241]. This adjuvant combination may be suitable for human use [246, 247].

Pilot studies have shown that vaccination with a cocktail of the recombinant proteins in Leish-111f in combination with GM-CSF may be effective in treatment of patients suffering from *L. braziliensis* [248].

### ***Leishmania* homologue of receptors for activated C-kinase (LACK)**

LACK is a 36 kDa protein that was isolated using expression cloning technique [249], allowing recombinant proteins to be captured by macrophages for MHC class II restricted presentation to a *Leishmania* specific protective T cell clone (9.1-2). This CD4<sup>+</sup> T cell clone of Th1 phenotype was induced by SLA (*L. major*) vaccination and had previously been shown to confer protection in a mouse model [250]. The isolated protein, LACK, was shown to be conserved among related *Leishmania* species and was believed to be expressed in the cytosol [251]. Initial studies showed that LACK, when given alone, was a strong inducer of Th2 (IL-4 secreting) *L. major* specific CD4<sup>+</sup> T cells. However, when given with IL-12 as adjuvant LACK was demonstrated to be protective [249]. Furthermore, Balb/c mice made tolerant to LACK were protected

against *L. major* infection [252]. LACK DNA vaccine has been shown to be protective against *L. major* [253], but not *L. donovani* infection in mice [254]. In cells from patients with CL, LACK has been shown to induce both Th1 and Th2 type cytokines [255, 256].

### ***Amastigote antigens***

In the search for vaccines against leishmaniasis most work has been focused on promastigote molecules, and less on amastigotes. In view of the fact that, the amastigote stage is the developmental form found in the mammalian host, antigens specific for this stage should be considered as potential vaccines against leishmaniasis.

P-2, P-4 and P-8 are stage specific antigens derived from *in vitro* cultivated *Leishmania pifanoi* amastigotes [257]. P-2 is a cystein proteinase primarily associated with the internal megasome organelle, although some surface localization has been reported [258]. Cystein proteinases are considered as attractive vaccine targets [259] and other amastigote molecules with cystein proteinase activity have been shown to confer protection in mice [260, 261]. Furthermore, infection studies of cystein proteinase-deficient *L. mexicana* mutants in mice have implicated cystein proteinases as both virulence factors and modulators of the immune response [262]. P-4 is a protein associated with the endoplasmic membrane of the amastigote. P-8 is a glycolipid protein complex of the external membrane of the amastigote [258]. While P-2 induced minimal protection in mouse models both P-4 and P-8 induced significant cross species protection using *Cornebacterium parvum* as adjuvant [257].

In PBMC of patients suffering from American CL proliferative and cytokine responses were mainly induced by P-4 and P-8 [263], whereas patients infected with *L. aethiopica* primarily respond to P-2 and P-8 [264]. Of the three antigens, recent work has focused on P-8 as vaccine candidate. Protection against *L. amazonensis* infection mediated by this antigen was found to be CD8<sup>+</sup> T cell mediated and dependent on perforin and IFN $\gamma$  [180].

Other defined antigens that are considered as main vaccine candidates, but not reviewed here include: PSA-2 [265, 266], *L. donovani* A2 antigen [267] and gp63 [268, 269].

## AIMS OF THIS THESIS

Evaluation and characterization of cellular responses to *Leishmania* promastigotes and *Leishmania* vaccine candidate molecules in healthy individuals with no history of leishmaniasis

Evaluation of the fate of NK cells during the course of human cutaneous *Leishmania* (*L. major*) infection to explore if NK cell responses can contribute as markers of immunity to leishmaniasis in humans.

## ETHICAL CONSIDERATIONS

Ethical approval for the use of human material was obtained from the regional and local ethical committees at Karolinska Institutet. For samples obtained outside Sweden approval was given by local ethical committees and the ethical committee at TDR/WHO.

## METHODOLOGY

### Donors

#### *Healthy controls*

Buffy coat cells, prepared from citrate treated blood, were obtained from the blood bank of Karolinska Hospital, Stockholm, Sweden. Blood donors cannot have visited tropical or other developing countries 3-6 months prior to giving blood nor lived in these areas for an extended period of time.

Cells from laboratory workers were prepared from defibrinated blood. The donors used in these studies had all lived in Sweden for more than 10 years, most all their life (with exception of donor D16 paper I). Several regularly donate blood to the blood bank. None of these donors have a history or evidence of past or present leishmaniasis. *Leishmania* is not transmitted in Sweden. Thus, the only possible exposure that could have occurred is during travelling or through exposure in the lab e.g. possible skin contact. No accidental injections had occurred in these donors, with the exception of two donors tested (D3, D16 paper I). We have referred to these adult donors as laboratory workers rather than naïve or unexposed, since we cannot completely exclude exposure. However, possible exposure to *Leishmania* in this group is considered to be very limited or absent. Furthermore, early studies from this group demonstrated that cellular responses (proliferation and IFN $\gamma$  secretion) could be detected in cell from donors that had never travelled outside Sweden and accordingly never could have been exposed [270].

Cord blood cells of healthy infants delivered by natural birth were collected from the umbilical cord vein and treated with citrate as anticoagulant.

Cells from Iranian healthy controls were obtained from defibrinated or heparinized blood. The main selection criteria for healthy controls were no history or evidence of present or past leishmaniasis. The responses of Iranian healthy control donors did not significantly differ from responses measured in cells from donors living in a non-endemic country (Sweden).

#### *Leishmaniasis patients*

Cells from Iranian cutaneous leishmaniasis cases were obtained from defibrinated or heparinized blood. All patients were male conscripts and all contracted the infection in a highly *L. major* endemic area of Iran (Mehran) when serving in the military. The duration of lesion varied from 1-7 months.

Cells from cured donors were obtained from defibrinated blood. The selection criterion of the donors was documented history of cutaneous leishmaniasis due to *L. major* or *L. tropica*.



### ***Volunteers inoculated with live L. major promastigotes (leishmanization)***

Cells were collected from heparinized blood of the same donors pre- and 9 months post leishmanization<sup>1</sup>. Inclusion criteria for the study were: skin test (LST) negative, no history and no evidence (scar) of leishmaniasis. All volunteers were living in a *L. major* endemic area (Ardestan, Iran). Thus, it is most likely that these individuals have been repeatedly exposed to the *L. major* parasite.

### **Preparation of Mononuclear Cells**

Peripheral blood mononuclear cells (PBMC) were separated from peripheral blood on a ficoll (Amersham-Pharmacia Biotec, Uppsala, Sweden) gradient [271], washed and resuspended, to appropriate concentration, in RPMI (Life Technologies /Gibco, BRL, Paisly, Scotland, UK) containing 10% heat inactivated normal Swedish AB<sup>+</sup> serum (NHS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). Cells were used fresh or frozen until ready for use.

### ***Freezing and transportation of cells***

Cells were frozen in 1.5 ml vials at approximately 10x10<sup>6</sup> cells /ml /vial in 10% DMSO in foetal calf serum (FCS) using Nalge freezing-containers (Nalge-Nunc, Rochester, NY, USA) according to manufactures instructions. After over-night freezing at -70°C the cells were transferred to liquid nitrogen tanks. For transportation cells were packed in Styrofoam boxes on dry ice cooled with liquid nitrogen and transported by air as checked-in luggage.

### **Antigens and Mitogens**

#### ***Parasites***

The following *Leishmania* species were used:

*L. aethiopica* isolates (confirmed by London School of Hygiene & Tropical Medicine to be *L. aethiopica* by isoenzyme typing) obtained from Ethiopian patients with localized CL.

*L. donovani* (MHOM/SD/93/BM1) obtained from a Sudanese VL patient.

*L. mexicana* wild type and mutants were obtained in collaboration with Dr T, Ilg, Max Planck Institut für Biologie, Tübingen, Germany.

*L. major* (MRHO/IR/76/ER) was a kind gift from Dr A. Khamesipour, Center for Research and Training in Skin Disease and Leprosy, Tehran, Iran.

#### ***Whole promastigote antigens***

*L. aethiopica*, *L. major* and *L. donovani* promastigotes were propagated as previously described and used at stationary growth phase [272]. *L. mexicana* wild type and LPG deficient mutants [41] were propagated in medium 199 supplemented with 5% FCS 100 U / 100µg/ml penicillin / streptomycin. The parasites were resuspended in PBS and then split into two portions. One portion was used directly for stimulation

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<sup>1</sup> Intradermal injection of viable metacyclic promastigotes as a form of vaccination against disfiguring CL [229]

(live-PROM) the other portion was killed by boiling for 10 minutes (HK-PROM). Live and heat killed parasites were prepared fresh on each occasion when cells were tested. Killing of parasites was also achieved by repeated freeze thawing (ft-LAg) as previously described [272]. If not otherwise stated, the final concentration of promastigotes was  $1.25 \times 10^6$  promastigotes/ml in all assays.

Soluble *Leishmania* antigen (SLA) was prepared essentially according to Scott *et al.* [273]. Furthermore, the membrane-rich pellets from two rounds of centrifugation (high speed + ultra centrifugation), containing non-soluble *Leishmania* antigens, were pooled, dialysed against PBS and finally resuspended in PBS and used as membrane-rich *Leishmania* antigen (MLA).

### ***Purified antigens***

#### **Amastigote antigens**

P-2, P-4 and P-8 [257, 274] derived from axenically cultured *L. pifanoi* were obtained in collaboration with Prof. D McMahon-Pratt, Dept. of Epidemiology Yale University, USA.

#### **LACK**

The *L. major* derived recombinant protein LACK (gift from Dr. N. Glaichenhaus) [249] was pre-treated with polymyxin B and used at a final concentration of 12.5, 25 and 50 µg/ml. If not otherwise stated the data presented are those where 25 µg/ml has been used.

Polymyxin B (Sigma, St Louis, MO, USA) was used to neutralize possible presence of endotoxin in the protein [275]. LACK (200 µg/ml) was treated with 25 µg / ml polymyxin B for 2 hours on ice prior to stimulation of cells. In addition we sent the LACK to be tested (performed by the Water section at Swedish Institute of Infectious Disease Control) for the presence of endotoxin using the standard Limulus Amoebae Lysate (LAL) test. Endotoxin levels in LACK preparations were 24-40 and 280 fold lower than levels accepted for use in human subjects by biomedical companies (30-50 EU/dose/70 kg) and the federal drug administration (FDA) (350EU/dose/70 kg). Initial experiments showed that polymyxin B did not affect cell viability.

#### **Leish-111f**

The vaccine candidate Leish-111f (CRXA-111f) [241] was produced under good manufacturing practices (GMP) and was a gift from the Corixa Corp., USA. The Leish-111f was reconstituted in PBS and used at a final concentration of 25 µg/ml.

#### **LPG**

Purified LPG derived from *L. donovani* [276] was a gift from Dr. S. Turco (University of Kentucky, USA). LPG was used at a final concentration of 12.5 µg/ml.

#### **Other antigens and mitogens used**

Purified protein derivative of *Mycobacteria* (PPD) was used as a non-*Leishmania* derived specific antigen control at a final concentration of 12.5 µg/ml

As positive controls in our assays we used the T cell mitogen Phytohaemagglutinin (PHA) at a final concentration of 12.5 µg/ml

## **Inhibition Assays**

### **Blocking of IL-12**

The blocking effect of  $\alpha$ -IL-12 antibodies on IFN $\gamma$  secretion by *Leishmania* stimulated cells was assessed in PBMC cultures stimulated with  $1.26 \times 10^6$  live promastigotes  $\pm 5 / 0.5 / 0.05$   $\mu\text{g/ml}$  polyclonal goat-  $\alpha$ -human IL-12 (R&D Systems, Minneapolis, MN, USA). Isotype control antibody (goat IgG) at the same concentrations was used as control of the assay. IFN $\gamma$  secretion induced by  $5 \mu\text{g/ml}$  recombinant human (rh) IL-12 alone was blocked by addition of  $5 \mu\text{g/ml}$   $\alpha$ -human IL-12 but not addition of  $5 \mu\text{g/ml}$  isotype control antibody.

### **Blocking of MHC class II**

Supernatants from the hybridoma, PdV5.2 producing monoclonal IgG1 with broad class II specificity [277] was used to block MHC class II dependent antigen presentation [278]. This antibody has previously been shown not to bind MHC class I. A mouse IgG1 monoclonal antibody of irrelevant specificity was used as control.

## **Proliferation assay**

Cells were plated at  $2 \times 10^5$  cells/well in 96 well flat-bottomed tissue culture plates. Each of the stimuli was tested in triplicate, as previously described [279]. Control cultures contained cell culture medium alone or live parasites alone. Cells were cultured in 5% CO $_2$  in air at 37°C for six days in total, if not otherwise stated. After three days of cell culture the supernatants were collected (60% of the medium volume) and stored at -20°C. The removed supernatant was replaced with an equivalent volume of fresh cell culture medium. This procedure has no appreciable effect on the magnitude of the proliferative response.  $^3\text{H}$ -thymidine (1  $\mu\text{Ci/well}$ ) was added to the culture 18 hours prior to harvest. Cells were harvested onto glass fibre filtermat (Wallac, Turku, Finland) and proliferation was assessed as counts per minute (CPM) using a 1450-microbeta counter (Wallac). Measurement of parasite proliferation was carried out in the same way as described for the cells, however the parasites were cultured at  $1.25 \times 10^4 / 10^5 / 10^6$  promastigotes /ml at 27°C or 37°C.

## **FACScan**

Mononuclear cells cultured for 6 days as described above were prepared for surface marker phenotype analysis as previously described [125]. Phenotype analysis for CD4 $^+$  and CD8 $^+$  (T-helper and cytotoxic), CD3 $^+$  and CD16 $^+$ /56 $^+$  (pan-T and NK cells respectively) stained with FITC and PE respectively and FITC stained  $\gamma\delta$ -TCR were carried out. Control IgG1 and IgG2 stained with FITC and PE respectively was used to determine the negative population. All antibodies were obtained from Becton Dickinson. Cells were analysed on a FACScan<sup>TM</sup> (Becton Dickinson, Mountain View CA, USA). Proliferation was determined by the enrichment of large blast cells after stimulation. Determined by forward light scatter, smaller cells with the least light scatter as seen in most unstimulated cultures were gated in region (R) 1, and the area

outside this was gated R2. In response to antigens, blast cells undergoing activation were scattered forward-most away from the R1 cell population into the R2 (figure 4).

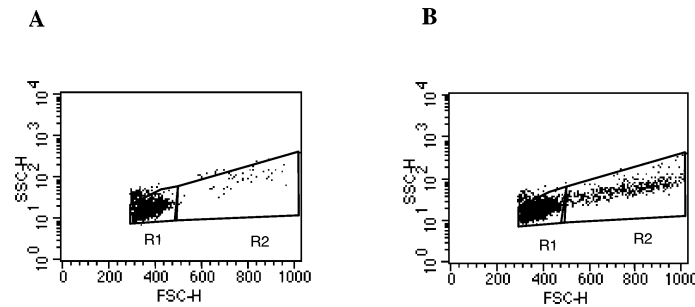


Figure 4: Assessment of proliferation by sorting of blast cells: Dot blots of A) unstimulated (RPMI) and B) *Leishmania* (HK-PROM) stimulated PBMC. Unstimulated cells are mostly gated in region (R) 1. When cells undergo activation they move forward (FSC-H) into R2. Thus, the differences in the proportion of blast cells observed in unstimulated and stimulated cultures can be used to assess proliferation.

The proportional increase (PI) of specific cell subsets was calculated using the following formula as previously described [125]:

$$\frac{[(\% \text{ large cells (R2) of total} \times \% \text{ large CD}^+ \text{ cells (R2) of stimulated culture}) + 1]}{[(\% \text{ large cells (R2) of total} \times \% \text{ large CD}^+ \text{ cells (R2) of unstimulated culture}) + 1]}$$

The correction factor +1/+1 was necessary since in some instances the % of large cells of a particular phenotype in the unstimulated cultures was zero.

## Cytokine Analysis

### *Cytokine gene expression*

#### mRNA isolation

For isolation of mRNA  $1 \times 10^6$  cultured cells were pelleted and lysed in 0.3 ml of lysis-binding buffer (Dyna, Oslo, Norway). The samples were stored at  $-70^\circ\text{C}$  until mRNA was isolated. Messenger RNA was isolated using Dynabeads mRNA direct kit (Dyna) according to the manufacturer's instructions for small-scale mRNA isolation. The volume of the mRNA solution was adjusted with water to 20  $\mu\text{l}$  in all samples.

#### RT-PCR

First strand DNA synthesis was carried out as follows: mRNA was denatured for 5 min. at  $70^\circ\text{C}$  and then immediately chilled on ice. The mRNA (20  $\mu\text{l}$ ) was added to a mixture consisting of 8  $\mu\text{l}$  5X first strand buffer (Life Technologies / Gibco), 3  $\mu\text{l}$  100 mM dithiothreitol; 4  $\mu\text{l}$ , 5 mM, dNTPs (dATP, dGTP, dCTP, dTTP); 2  $\mu\text{l}$ , 100 mM, random hexamer primers (Pharmacia, Uppsala, Sweden); 2  $\mu\text{l}$ , 200 units/ $\mu\text{l}$ , Moloney murine leukaemia virus reverse transcriptase (Gibco BRL) and 1  $\mu\text{l}$  RNase inhibitor

(RNasin, Promega, Madison, WI, USA). First strand cDNA synthesis was performed at 40°C for 50 min. The samples were thereafter heated to 95°C.

To check the quality of the cDNA and that the amount of mRNA used was comparable between samples, 1 µl cDNA (= mRNA from 25 000 PBMC) in 2 µl 10X PCR buffer with MgCl<sub>2</sub> (Perkin Elmer, USA); 0.75 µl 5 mM dNTPs; 2 µl of each primer (5 mM) and 0.1 µl Taq polymerase (Amplitaq, Perkin Elmer) was amplified with  $\beta_2$ -microglobulin specific primers [280] for 25 cycles. The temperatures and times used were as follows: Denaturation 94°C, 30s; annealing 58°C 30s; primer extension 72°C 1 min. cDNA amplification with cytokine specific primers was performed with 2 µl cDNA using the same PCR mixture and the same temperature profile as described above, with exception for IL-18 mRNA where the annealing temperature was 62°C and IL-10 mRNA where the PCR buffer contained 20% less MgCl<sub>2</sub>. Amplification of cytokine specific cDNA was carried out for 35 cycles. PCR products were separated by electrophoresis on ethidium bromide stained 1.5% agarose (Propanose, Kemila, Sollentuna, Sweden) gels and visualised by UV-light.

All primers used in this study were cDNA specific using custom made or previously described sequences as follows  $\beta_2$ -microglobulin [280], IFN $\gamma$ , TNF $\alpha$ , IL-2 and IL-4 [281], IL-12; sense: CAG CAG TTG GTC ATC TCT TG, antisense: CCA GCA GGT GAA ACG TCC A, (manufactured for Pharmacia Biotech, Uppsala, Sweden by Genoysys Biotechnologies Inc.), IL-10; sense: GCC TAA CAT GCT TCG AGA TC, antisense: TGA TGT CGT GGT CTT GGT TC and IL-18 [282] (Scandinavian Gene Synthesis, Köping, Sweden).

#### Semi-quantitative evaluation of cytokine mRNA

Gel pictures taken with Polaroid camera were analysed for arbitrary band intensity using the Bio Image Whole Band Analyzer software (Bio Image, Ann Arbor, MI, USA).

The samples were normalised by comparing the intensity of the  $\beta_2$  microglobulin bands.

#### ***Measurements of secreted cytokines***

##### ELISA

The levels of IFN $\gamma$  and IL-10 were measured in 72-hour supernatants using commercial ELISA kit (Mabtech, Stockholm, Sweden) according to the manufacturer's instructions. Cytokine levels were calculated in reference to the standard curve of known amount of recombinant IFN $\gamma$  and IL-10 respectively using linear regression analysis. The limits of detection for the IFN $\gamma$  and IL-10 assays were 27 pg / ml and 9.8 U / ml respectively.

##### ELISpot

IFN $\gamma$  and IL-10 ELISpot assays were performed using commercial ELISpot kits following the manufacturer's instructions (Mabtech, Stockholm, Sweden). PBMC were plated at  $5 \times 10^4$  (IFN $\gamma$ ) or  $2 \times 10^5$  (IL-10) cells/well with or without stimulation. The T cell mitogen PHA used at 12.5 µg/ml acted as positive control, while wells containing cell culture medium alone or cell culture medium + live promastigotes, were negative controls of the assay. The cells were cultured for 24, 40 or 48 hours in 5% CO<sub>2</sub> in air at 37°C. The number of spot forming units (SFU) was counted under a dissection

microscope (Leica MZ6, Heerbrugg, Switzerland) or using computerized ELIspot counting system (AID, Strassberg, Germany). One SFU theoretically corresponds to one cytokine-producing cell.

## **Isolation and Separation of Cells**

### ***Cytokine gene expression in positively selected cell subsets***

In order to evaluate which cell types were involved in the cytokine induction in the bulk cultures, cells cultured as above were harvested at 72 hours and positively selected for CD16/56<sup>+</sup> NK cells (1<sup>st</sup> step incubation with CD16/56 antibodies, 2<sup>nd</sup> step sheep-anti-mouse magnetic beads), CD4<sup>+</sup> or CD8<sup>+</sup> T cells. Positively selected cells, as well as plastic adherent cells (PAC), were then lysed using lysis buffer (Dyna, Oslo, Norway) and mRNA isolated using Dynabeads mRNA DIRECT kit (Dyna,) following the manufacturer's protocol. Semi-quantitative RT-PCR for IFN $\gamma$  and IL-10 gene expression was carried out as described above.

### ***Depletion of specific cell subsets***

PBMC were diluted to  $10 \times 10^6$  cells /ml. T cells (CD4 and CD8) were depleted using antibody coated magnetic beads (Dyna, Oslo, Norway) according to the manufacturer's instructions. NK cells were depleted by coating the cells with 15  $\mu$ g/ml mouse- $\alpha$ - human CD56 antibodies, (Pharmingen, San Diego, CA, USA) in 2% FCS on ice for 15 min, followed by 2x washing with PBS, and finally incubation with sheep- $\alpha$ -mouse (SAM) coated magnetic beads in 2% FCS. The antibody coated cells were allowed to bind to the beads under slow rotation for 30 min, 4°C. The cells attached to the beads were removed and the supernatant containing the depleted cells was collected. As control for the depletion we used an isotype control antibody (IC) (Pharmingen). Plastic adherent cells (PAC) (i.e. mainly monocytes) were removed by allowing these cells to bind to plastic for one hour in RPMI containing 10% NHS at 37°C 5% CO<sub>2</sub>. Whole PBMC were diluted to a concentration of  $2 \times 10^5$  cells / well. The depleted cells were used at a concentration that would corresponded to  $2 \times 10^5$  whole PBMC / well before depletion (i.e.  $2 \times 10^5$  cells minus specific cell subset).

### ***Isolation of NK cells by negative selection***

Isolation of NK cells was performed according to the protocol supplied by the manufacturer (Dyna), with slight modification. Briefly, to reduce red blood cell (RBC) contamination, PBMC were resuspended in 1-2 ml sterile water for approximately 20 seconds after the first of three washes. The isolated PBMC were first incubated with CD19 coated beads (Dyna), with rotation for 30 minutes at +4°C. Beads were removed with a magnet and the supernatant containing the depleted cells was collected and further incubated 30 minutes with CD3 coated beads (Dyna) followed by 40 minutes incubation with CD14 coated beads (Dyna) as described above. To remove as much as possible of remaining antigen presenting cell, depleted cells were allowed to adhere to plastic for 1-2 hours at 37°C, 5% CO<sub>2</sub> in air. Unbound cells were collected as NK cells. To assess the efficacy of the depletion, the purified NK cells were stained with CD4; no cells were found to be CD4 positive. In the gate containing viable lymphocytes 90%

were CD16/56 positive. The main contaminants using this assay are RBC, which were enriched for by this method.

#### ***Isolation of NK cells by flow assisted cell-sorting***

PBMC depleted of plastic adherent cells for 1 hour were diluted to a concentration of  $10 \times 10^6$  cells/ml in 2% FCS-PBS and stained with  $\alpha$ -CD3 (Pharmingen) and  $\alpha$ -CD14 (DAKO, Copenhagen, Denmark) conjugated with fluorescein isothiocyanate (FITC) and  $\alpha$ -CD56 conjugated with phyco-erythrin (PE) (Pharmingen) for 30 minutes on ice, 10  $\mu$ l of each antibody was used per  $10^6$  cells. The stained cells were washed with 2% FCS-PBS and resuspended to  $10 \times 10^6$  cells/ml in 2% FCS-PBS. PBMC were sorted on a FACS<sup>TM</sup> vantage SE (Becton Dickinson, Mountain View, CA, USA). From the cells gated as lymphocytes, cells positive for CD56 and negative for CD3 and CD14 ( $CD56^+$ ,  $CD3^-$ ,  $CD14^-$ ) were collected as NK cells.

#### **Cytotoxicity Assay**

NK cells purified by depletion from PBMC were stimulated for 24 hours with live- or HK-PROM and used as effector cells. Cytotoxicity against 721.221, a MHC class I negative B lymphoblastoid cell was measured using a standard 4-hour  $^{51}\text{Cr}$ -release assay [283].

## RESULTS AND DISCUSSION

### Relevance of Studying Responses to *Leishmania* in Naïve Donors

Innate immune responses can influence the ability of an infectious agent to establish an infection and participate in the initiation of the acquired immune response. Non-specific responses, being innate or cross-reactive, would be expected to influence and/or to be influenced by vaccination. Thus, we find it interesting to describe and characterise such responses further.

Early studies from this group demonstrated that cells from unexposed donors (never travelled outside Sweden) could proliferate and secrete IFN $\gamma$  when stimulated with *Leishmania* promastigotes. The magnitudes of the response were sometimes as high as responses observed in patients with active disease or individuals who had cured from leishmaniasis [270, 284]. Furthermore, it was shown that supernatants from *in vitro* stimulated cells of non-exposed donors were able to inhibit amastigote invasion of macrophages [270]. Subsequent studies targeted NK cells as being an important cell type in these responses [279].

### Live and Dead *Leishmania* Promastigotes Induce Differential Responses in Donors with No History of Leishmaniasis

Infection with live *Leishmania* promastigotes is believed to induce solid protection against subsequent disease. Unpublished observations by Khamesipour *et al.* showed that individuals that developed lesion after leishmanization were protected when re-challenged with live *L. major* (personal communication). Attempts have been made to vaccinate against leishmaniasis with killed promastigotes (+BCG). However, the killed vaccines have had variable degree of efficacy and are less protective than live vaccination [229, 235, 236, 285]. It is apparent that the live promastigote vaccination can establish an infection and provide the immune system with a more continuous stimulus compared to killed vaccines. Persistence of parasites has been shown in mouse models and is probably the most important feature for inducing long lasting immunity [142, 286]. However, we speculated that a part of the explanation why live promastigotes induce the better protection also lies in the differential immune responses initially evoked by live versus dead promastigotes. Thus, we evaluated the ability of live versus dead promastigotes to initiate cytokine production and proliferation in cells from healthy donors with no history of leishmaniasis (**paper I**). Our results show that live and dead promastigotes significantly differed in the responses evoked in healthy donors. Th1 related cytokine gene expression (IL-12, IFN $\gamma$  and TNF $\alpha$ ) was more evident to live than to heat killed (HK) promastigotes (PROM). Furthermore, Live-PROM induced significantly more cells to IFN $\gamma$  secretion compared to HK-PROM. Surprisingly, this IFN $\gamma$  secretion was independent of IL-12 (*figure 5*), and addition of exogenous IL-12 did not affect the observed IFN $\gamma$  production.



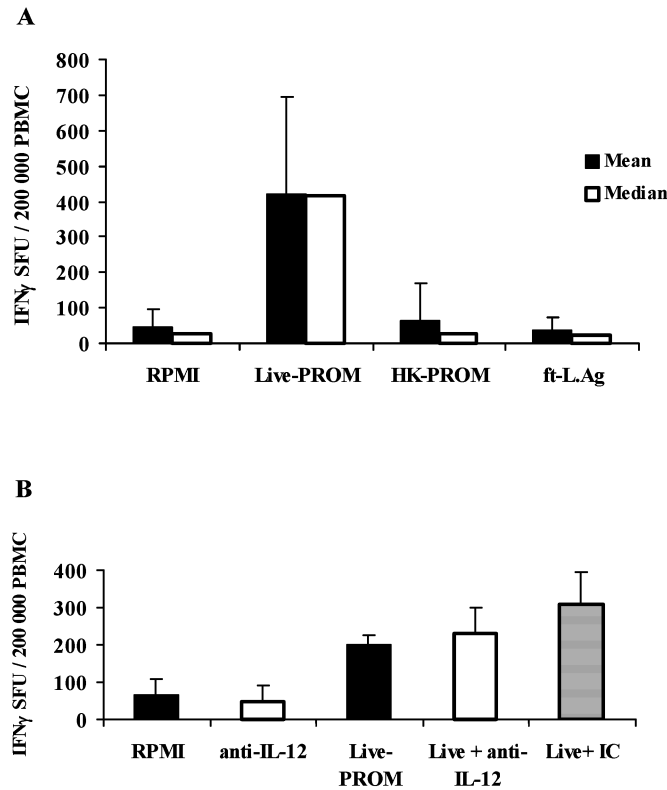


Figure 5: A) Frequency of PBMC secreting IFN $\gamma$  after 48 hours stimulation with live and dead (heat-killed, HK, and freeze-thawed, ft-L.Ag) *Leishmania* promastigotes. B) The effect of 5  $\mu$ g/ml  $\alpha$ -IL-12 on IFN $\gamma$  secretion induced by live-PROM after 48 hours stimulation, IC= isotype control.

Proliferative responses were in adult donors variable; however, in cells from truly naïve donors (cord blood) live promastigotes induced more proliferation than killed. No IFN $\gamma$  secreting cells were detected in cord blood MNC by either stimulation.

The relevance, of this finding, is that live parasites, apart from having the potential ability to persist within the infected host over time, when used as an antigen initially evoke cellular responses that significantly differs from those induced by killed promastigotes. Furthermore, the results show that PBMC of normal healthy donors rapidly respond to viable *Leishmania*, with IL-12 mRNA induction and IFN $\gamma$  secretion, and thus have the potential to inhibit or delay establishment of the infection. Once *Leishmania* parasites have established themselves in the macrophage IL-12 production and MHC expression has been demonstrated to be down-regulated in these cells [97, 100]. We found that live promastigotes induced some IL-12 p40 mRNA 6 /18 hours after stimulation of PBMC, but not later. This could suggest that the monocytes / macrophage become infected and down regulate MHC molecules and their ability to secrete IL-12, which may explain why proliferative response to live promastigotes were scanty in cells of many donors that displayed high proliferative response when stimulated with killed promastigotes. It is also possible that the IL-12 mRNA detected

was never translated and exported. Another explanation could be that monocytes / macrophages were not the source of IL-12 mRNA. The limited attempts we performed to detect the source of the mRNA failed, thus the cellular source of the IL-12 mRNA remains unknown. No IL-12 mRNA was detected 6-48 hours after stimulation with killed promastigotes.

The cytokine responses observed after stimulation with live-PROM would be anticipated to be of benefit to the host. Thus, it was of interest to further dissect the responses involved in these differences.

### **Live *Leishmania* Promastigotes Induced IFN $\gamma$ Secretion by NK Cells while Killed Promastigotes Tend To Induce CD4<sup>+</sup> Cell Proliferation**

Our group has previously demonstrated that both T and NK cells from healthy donors can be induced to proliferation and cytokine secretion when stimulated with freeze-thawed *Leishmania* promastigotes [284]. Healthy donors could be grouped into either responders or non-responders depending on their ability to induce proliferation and cytokine production. To address the question if live and dead promastigotes differentially activated different cell subsets we stimulated PBMC depleted of specific cell subsets with live and HK-PROM (**paper 2**). The data demonstrated that the early IFN $\gamma$  secretion induced by live-PROM was dependent on the presence of CD56<sup>+</sup> cells; CD56 is expressed on both NK and NK-T cells. Removal of CD4 positive and plastic adherent cells (PAC) cells did not significantly affect the observed IFN $\gamma$  production, implying that these cells were not required for the observed IFN $\gamma$  secretion. However, proliferation was almost entirely CD4<sup>+</sup> cells dependent. This was most evident in the cultures stimulated with killed promastigotes, where the most proliferative response was observed. This was demonstrated by depleting PBMC of CD4<sup>+</sup> cells before stimulation and confirmed by assessing the phenotype of blast cells, where results showed that the main cell type induced to proliferate by HK-PROM were the CD4<sup>+</sup> cells. Ft-LAg tended to act similarly to the HK-PROM. Live-PROM induced proliferation was more a feature of CD8<sup>+</sup> and CD56<sup>+</sup> cell.

### **NK Cells (CD56<sup>+</sup>, CD3<sup>-</sup>, CD14<sup>-</sup>) Can Be Activated To IFN $\gamma$ Secretion by Live but Not Dead *Leishmania* Promastigotes in The Absence of Other Cell Subsets and IL-12**

Depletion experiments had shown that CD56<sup>+</sup> cells were required for the induction of IFN $\gamma$  by live promastigotes in healthy donors and that removal of adherent cells did not affect the observed IFN $\gamma$  secretion. Furthermore, we had already shown that IL-12 was not required for the production of IFN $\gamma$  (**paper 1**). While, not proven it had been suggested that carbohydrate structures on microorganisms could have the ability to act directly on NK cell receptors [287]. Taken together, this made us suggest that the activation of NK cells to IFN $\gamma$  secretion by live promastigotes was independent of other cell subsets. To prove this we stimulated FACS sorted NK cells, defined as CD56<sup>+</sup>, CD3<sup>-</sup>, CD14<sup>-</sup>, with live and HK-PROM. Results demonstrated a direct activation of NK cells by live, but not HK-PROM (*figure 6*). Activation of NK cells by live-PROM was observed in all donors tested.

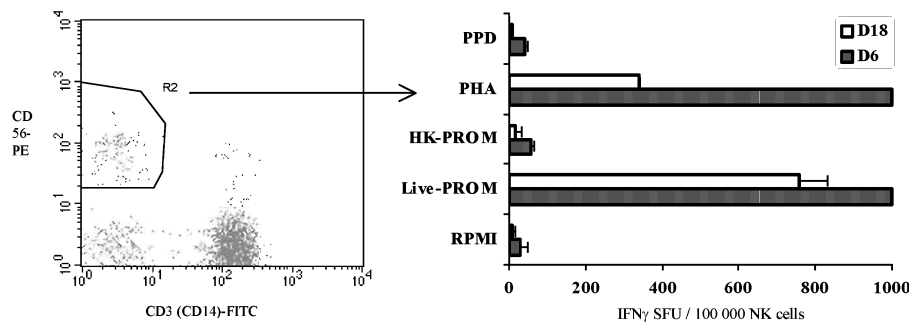


Figure 6: Isolation of NK cells by FACS sorting followed by stimulation with Live- and heat killed (HK) promastigotes (PROM). PPD and PHA were used as control antigen and mitogen respectively. Results from 2 donors (D6 and D18) are shown.

This finding demonstrates a novel mechanism of NK cell activation by an infecting agent. Since, carbohydrates have been suggested as candidates for direct NK cell activation we speculated that the LPG surface coat of *Leishmania* would be important for the direct activation of NK cells. To evaluate this we used *Leishmania* LPG deficient mutants where the LPG gene had been knocked-out. To our surprise the lack of LPG did not significantly affect the frequency of IFNγ secreting cells, induced by the promastigotes, indicating that LPG is not required for this direct activation, although its presence seems to enhance the frequency of IFNγ secreting cells (**paper 2**). Thus, the surface structure/s involved in the direct activation are yet to be described. However, MHC class II molecules do not appear to be involved since, blocking with α-MHC class II did not affect the IFNγ secretion (unpublished data).

The data are consistent with an explanation that live promastigotes primarily activate cells of the innate immune system while responses to killed promastigotes is associated with cells of the adapted immune responses. However, whether or not the activation of CD4<sup>+</sup> cells in healthy donors is due to cross-reactive epitopes remains to be elucidated. Furthermore, it is likely that the killing of the promastigotes degrades the parasite and thus eases the presentation to CD4<sup>+</sup> cells, while the live parasites has to be killed before presentation is efficient.

### Why are The NK Responses Not Described in Cells from Naïve / “Control” Mice?

There are a few reports addressing the role of NK in the very early response against the *Leishmania* parasite in mice. The existing reports consistently demonstrate a role for NK in the early defence against *Leishmania* [84, 112, 288]. Such responses may be sufficient to control a natural infection, but may not be enough to stop the forced laboratory induced infection (with  $\approx 1 \times 10^6$  promastigotes). Direct activation of NK has never been described in mice. If present it would be interesting to test the biological relevance of this activation.

However, direct activation may be a unique feature of humans NK cells. The naïve human NK cells may broadly be divided into CD56<sup>bright</sup> and CD56<sup>dim</sup>, with the bright

being skewed towards cytokine production and the dim being both cytotoxic and cytokine secreting. Direct homologues of CD56<sup>bright</sup> and CD56<sup>dim</sup> have not yet been identified in mice [109]. Although, it remains to be tested, it would appear most likely that it is the CD56<sup>bright</sup> NK cell subset that are involved in the IFN $\gamma$  response we observe.

Using the *L. major* model system may be another one of the reason why NK responses have not been observed in cells from untreated mice. When stimulating human PBMC from healthy donors with *L. major* responses are scanty compared to stimulation with *L. aethiopica*, *L. donovani* or *L. mexicana* (for example see figure 8). Furthermore, analyses of PBMC cells are rarely used in *Leishmania* mouse studies. Most experiments performed use spleen cells. Hence, other cells than lymphocytes, which could have down regulatory effect on NK cells may be present in these cultures. Our inability to detect IFN $\gamma$  secreting cells in NK cells derived from human spleens (unpublished observation) could support this. However if this is due to differences between NK cells in different organs or to the heavy drug treatment the organ donor may have received is very difficult, if not impossible to determine.

Unfortunately focus has been drawn from the role of NK cells once it was demonstrated that NK cells were not required for development of a Th1 type response and resolving the experimental *Leishmania* infection [116]. However there are several things to consider in this model: The dose of promastigotes given was at least 1000-10000 times higher than what would be anticipated by natural transmission. It has clearly been shown that different doses of parasites evoke differential responses in mice [289]. The “CD8<sup>+</sup> T cell story” is an example of how the dose can influence the result. Injection of a high dose of promastigotes indicated that CD8<sup>+</sup> T cells were not required for immunity and resolution of the infection [178]. However, when administered intradermally at low dose, CD8<sup>+</sup> T cells were found to be crucial for immunity [148]. Furthermore, the genetically deficient mice used by Satoskar *et al.* may not be able to reflect functions / cells important in the normal mouse. There was no comparison with antibody depleted normal mice in this study. Thus, the results from Satoskar *et al.* do not exclude an important role for the NK cells in the early response. Moreover, recent evidence of cross-talk between NK cells and DC, suggest that vaccination protocols where DC are used may be influenced by NK cells [290, 291]. Thus it may be of interest to re-visit NK cells in *Leishmania* models.

### **Vaccine Candidate Molecules Can Stimulate Cells from Unexposed Donors To Proliferation and Cytokine Production**

Much *Leishmania* vaccine research has focused on identification of immunogenes and their potential as vaccines in mouse models. We have evaluated the cellular responses to some of these vaccine candidates in healthy donors. In **paper 3** we have tested the effect of LACK a 36-kDa molecule derived from *L. major*. Response to LACK is a very early event in a *Leishmania* infection. Thus, innate elements of immunity could be involved.

In **paper 4** we have tested the stage specific amastigote antigens P-2, P-4 and P-8 derived from *in vitro* cultivated *L. pifanoi* amastigotes. Since, the amastigote is the form present in the mammalian host it is of importance to study molecules that are specific or upregulated in this form and how they could influence the innate responses.

We have additionally evaluated IFN $\gamma$  and proliferative response to the vaccine candidate Leish-111f, that recently entered clinical phase I trials, in PBMC from both healthy donors and patients with active CL.

### **LACK Stimulates Cells from Healthy Donors To Both IFN $\gamma$ and IL-10 Production**

Consistent with previous findings from our group when Ft-LAg was used, LACK induced high proportional increase of proliferation in NK and CD8 $^{+}$  cells from healthy donors (**paper 3**). Although, proliferative responses of CD4 $^{+}$  cells were observed, they did not proportionally increase as much as the NK and CD8 $^{+}$  cells. Proliferation was coupled with IFN $\gamma$  and IL-10 secretion as measured in 72-hour culture supernatants (*table 2*). Our results suggest that LACK induces a mixed Th1 / Th2 response. This is supported by more recent studies of cells from healthy donors that also showed that both IFN $\gamma$  and IL-10 were induced by LACK stimulation [292]. The role of NK cells was not assessed in the latter study. However, Bourreau *et al.* demonstrated that naïve CD45RA $^{+}$ CD8 $^{+}$  T cells produced IFN $\gamma$  and memory CD4 $^{+}$  CD45RA $^{-}$  T cells were responsible for IL-10 production [292]. It was speculated that the responses involved low affinity T cell receptors and T cells cross-reactive to antigens associated with the gut flora or food. As well as in the healthy donors, cells from patients in the early course of infection respond to LACK with both Th1 and Th2 cytokines [255, 292]. It appears as though CD4 $^{+}$  T cells contribute more to the IFN $\gamma$  secretion in these donors compared to healthy subjects. The finding that LACK can induce IL-10 production in patients may indicate that this antigen contributes to down-modulating immune responses during infection. Interestingly, Maasho *et al.* demonstrated that the responses to LACK appear to disappear during the course of leishmaniasis [255]. This could support mouse models that have demonstrated that tolerance to this antigen is associated with protection [252]. The role of the innate responses induced by LACK in resistance vs. susceptibility to *Leishmania* infection is unknown. The cumulative LACK data obtained from studies of LACK response in cells from naïve donors and patients indicate that vaccination with LACK is not straightforward in human and requires to be carefully monitored if to be used.

Table 2: Levels of IFN $\gamma$  and IL-10 over background in 72 hour culture supernatants from PBMC of healthy donors (blood bank and lab workers) after stimulation with LACK and amastigote antigens. As comparison results obtained from stimulation with ft-Lag and PPD are also shown.

Antigen		IFN $\gamma$ pg/ml	IL-10 U/ml	SI (proliferation)
LACK	(n=8) <sup>a</sup>	375 $\pm$ 201 <sup>b</sup>	334 $\pm$ 230	(n=7) <sup>c</sup> 13.2
P-2	(n=11)	194 $\pm$ 191	202 $\pm$ 179	(n=15) 3.7
P-4	(n=11)	6 $\pm$ 18	3 $\pm$ 7	(n=15) 0.6
P-8	(n=11)	39 $\pm$ 81	15 $\pm$ 22	(n=15) 2.5
Ft-LAg	(n=12)	8 $\pm$ 21	4 $\pm$ 9	(n=15) 6.7
PPD	(n=11)	413 $\pm$ 152	56 $\pm$ 35	(n=15) 26

Note: a) data lab exposed donor omitted b) The amount of IFN $\gamma$  secretion measures in cell cultures from healthy Sudanese volunteers (n=12) was 311 $\pm$ 266 pg/ml. c) Analysis of LACK was not always performed at the same time as the other antigens.

### Cells from Healthy Donors Were Mainly Stimulated by The Amastigote Antigen P-2

Since the amastigote is the form found in the mammal host we were interested to see if cells from healthy donors would also respond to molecules derived from this stage (**paper 4**). When tested in mouse models it was demonstrated that P-4 and P-8 induced significant cross protection against *L. amazonensis*, while P-2 provided protection, albeit less and only when a low challenge dose was given. In patients with ACL, cellular responses were mainly observed to P-8 and/or P-4 and not to P-2 [263]. However, when cellular responses were evaluated in patients with CL caused by *L. aethiopica* P-8 and P-2 were the main inducers of cytokine production and proliferation. In cells from unexposed donors we found that P-2 induced the most responses, P-8 induced some responses, while no responses to P-4 were evident (*table 2*). Furthermore, P-2 induced responses in truly naïve cells, obtained from cord blood (*figure 7*).

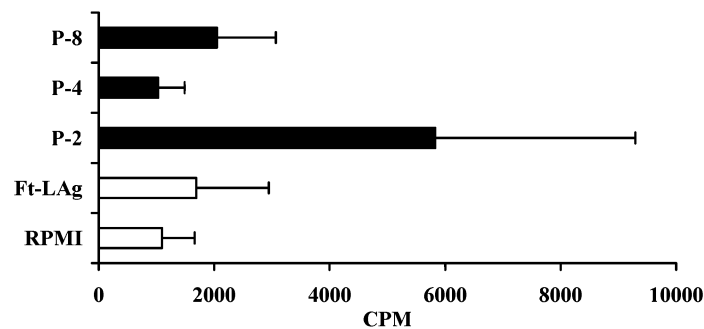


Figure 7: Proliferative responses to amastigote antigens in cord blood derived MNC after six days stimulation (n=14).

Albeit lower in magnitude, the P-2 responses observed in cells from the adult donors were strikingly similar to those observed when stimulating with LACK (*table 2*). In line with the LACK findings P-2 induced a mixed IFN $\gamma$  / IL-10 response in PBMC from healthy adult donors. Furthermore, the cell subsets with the largest proportional increase after stimulation were the CD3 $^+$ , CD16 $^+$ /56 $^+$  and CD4 $^+$ /CD8 $^+$ . It was suggested that the activation by P-2 could be through the enzymatic properties of this cysteine proteinase. However, when another cysteine proteinase, papain, was tested no such activation was found.

### IFN $\gamma$ Secretion and Proliferation To Both LACK and P-2 are Sensitive To MHC-Class II Blocking

Another observation we made was that the innate / cross-reactive *in vitro* responses to both LACK and P-2 showed MHC class II dependency. Our results show that both CD16 $^+$ /56 $^+$  and CD8 $^+$  proliferation were significantly inhibited by  $\alpha$ -MHC class II treatment as was the IFN $\gamma$  secretion, whereas the IL-10 secretion appeared to be enhanced (*table 3*).

Table 3: Effect of blocking of MHC class II on cytokine secretion induced by LACK and P-2. Results from one representative donor out of three tested are shown. As comparison results obtained from stimulation with ft-LAg and PPD are also shown.

IFN $\gamma$ (pg/ml)					
	RPMI	P-2	LACK	Ft-LAg	PPD
Antigen alone	0	85	209	0	446
Antigen + $\alpha$ -MHC class II	0	0	0	0	292

IL-10 (U/ml)					
	RPMI	P-2	LACK	Ft-LAg	PPD
Antigen alone	0	83	67	0	17
Antigen + $\alpha$ -MHC class II	0	132	136	0	0

Others have implied that NK activation in response to *Leishmania* antigen requires T cells [293]. However, our data show that CD4 $^+$  cells are not necessarily required for the IFN $\gamma$  responses induced by P-2 and LACK (*table 4*). However, purified NK cells could not alone be activated by P-2 or LACK and required adherent cells, representing a population of APC, to secrete IFN $\gamma$  (**paper 2, 4** and *table 4*).

Table 4: Levels of IFN $\gamma$  and IL-10 after 24 hours stimulation of PAC alone or PBMC depleted of all lymphocytes subsets, but CD56 $^+$  i.e. NK cells

IFN $\gamma$ (ng/ml)				
	RPMI	P-2	LACK	PHA
PAC alone	1.7	1.4	1.7	1.5
PAC+ CD56 $^+$ cells	0.1	9.5	11.7	0.3

Note: This assay was done using another ELISA kit (new batch) and different IFN $\gamma$  standard.

It should be noted that LACK was selected based on MHC class II restricted presentation to a protective mouse T cell line. In this *in vitro* system the LACK molecule, as well as the P-2, appears to be taken up and be presented by APC to NK cells in an MHC class II dependent manner or induce monocytes / macrophages to secretion of NK cell activating cytokines. Furthermore, NK cells were found to upregulate their MHC class II expression after LACK and P-2 stimulation (data not shown), which could indicate that NK cells may have the ability to present LACK and P-2 to T cells and other NK cells (**paper 3 and 4**).

### **Are NK Cells Involved in Regulation of Monocytes?**

An interesting side observation we made in our studies of P-2, was that NK cell may be involved in the regulation of monocyte / macrophage IL-10 secretion. Unstimulated cultures, depleted of lymphocytes i.e. containing only adherent cells spontaneously secreted IL-10 (490 U/ml in cultures of  $10^6$  PAC /ml, result is mean of two donors tested). However, when  $CD56^+$  cells were left in cultures ( $10^6$  NK + PAC /ml), no IL-10 secretion was detected.  $CD56^{bright}$  NK cells have been demonstrated to spontaneously secrete  $IFN\gamma$  [107] and it is possible the spontaneous  $IFN\gamma$  secretion by these cells are involved in basic control mechanisms of IL-10 secretion by monocytes / macrophages.

### **Activation of Cells from Healthy Donors: Cross-reactivity or A Possibility of *Leishmania* Derived Superantigens?**

Some of the responses to *Leishmania* and *Leishmania*-derived antigens we have observed in presumably naïve PBMC could indicate superantigenic activity. Superantigens (SAg) are molecules that without prior processing can be presented by MHC class II to both  $CD4^+$  and  $CD8^+$  T cells expressing certain  $V\beta$  chains, reviewed in [294, 295]. However, these responses are not exclusively restricted to T cells and  $V\beta$  chains, NK cells have been demonstrated to be directly activated to  $IFN\gamma$  secretion by some SAg [296]. Moreover, NK cells have been reported to present SAg as well as conventional antigens to T cells [297, 298].

The direct activation of NK cells by live promastigotes cannot be ascribed to conventional SAg activity, since MHC class II blocking did not affect this activation.

The proliferative responses induced by ft-LAg, LACK and P-2 were all found to be inhibited by MHC class II blocking. To exclude the possibility that the responses were due to endotoxins (known to have superantigenic properties) in the antigen preparations we pre-treated antigens with polymyxin B and measured endotoxin levels with the LAL test as described in the methodology section. Endotoxin levels in our samples were found to be far below what is accepted by medical companies. Thus, we concluded that the responses we measured were not a feature of contamination by bacterial endotoxins. Additionally it should be noted that another recombinant antigen we have tested, gp63, did not induce proliferative and  $IFN\gamma$  responses in healthy donors [299]. We can however, not exclude the possibility that other non-endotoxin SAg are present in the LACK preparation.



Superantigens would not be desirable in a vaccine as they are thought to corrupt the immune response to the invading pathogen. Thus, a vaccine possessing superantigenic activity could initially evoke a response, but later lead to clonal exhaustion and render specific T cells unresponsive to the antigen, as discussed in [300]. This theory could contribute to the explanation why vaccination with killed *Leishmania* and LACK in the absence of adjuvant even can exacerbate the disease [252, 301].

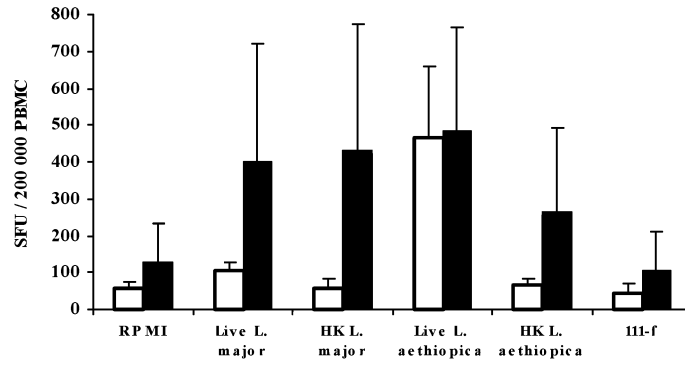
The alternative, or additional, explanation to the responses in cells from healthy donors is cross-reactivity with T cell clones primed by other infectious agents or frequently encountered antigens. This cross-reaction theory would assume that NK cell activation in naïve donors is T cells dependent, as has been suggested by others [293]. What support the cross activation theory are studies showing that mainly CD4<sup>+</sup> T cells of memory phenotype responded when cells from healthy donors were stimulated with *Leishmania* antigens [292, 302, 303]. If the activation is due to cross reactivity and / or SAg remains to be elucidated.

### **No IFN $\gamma$ or Proliferative Responses to Leish-111f in Cells from Healthy Donors or Patients**

Leish-111f is a vaccine candidate that is on its way to be tested in human vaccine trials. This tri-fusion proteins has induced solid protection in mouse models when given together with MLP<sup>®</sup>-SE adjuvant [241, 245]. Furthermore, LmSTII plus TSA using IL-12 and alum as adjuvants was demonstrated to be protective in a non-human primate model [242]. The components of Leish-111f have also been proposed to have therapeutic potential for MCL [248].

We found it interesting to see if this vaccine candidate, like LACK and P-2, induce responses in cells from healthy donors. Such responses could be of both beneficial and harmful nature and would have to be taken into consideration in the context of vaccines. When the vaccine candidate Leish-111f was used to stimulated cells from healthy donors with no history of leishmaniasis, we did not observe IFN $\gamma$  secretion or proliferation. This could be explained by the low inherent immunogenicity of Leish-111f, described in mice [245]. We believe that avoiding interference with the cells of innate immune system and possible cross-reactive T cells may be a desirable feature of a vaccine. These responses are less well characterized in leishmaniasis and may be difficult to monitor. We were furthermore, interested in characterizing the responses that Leish-111f would evoke in patients with active CL caused by *L. major*. To our surprise, but in line with observations made in cells of patients with American CL before vaccination (F. Modabber, personal communication), PBMC from Iranian donors with active CL did not respond when stimulated with Leish-111f (*figure 8*). Apparently, T cells from naturally infected individuals with on-going disease do not respond with proliferation or IFN $\gamma$  secretion to this vaccine candidate molecule.

A



B

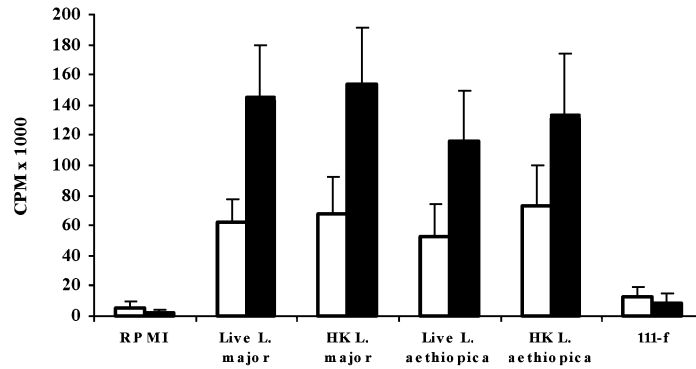


Figure 8: Responses to Leish-111f (111-f) in comparison to live and killed promastigotes of two different species (*L. aethiopica* and *L. major*). Open bars show responses in healthy controls, Iranian and Swedish, (n=5); black bars show responses in patients with active cutaneous leishmaniasis caused by *L. major* (n=6). A) IFN $\gamma$  secretion after 40 hours stimulation B) Proliferation assessed after 6 days stimulation

These findings, or rather lack thereof, made us halt further analyses of Leish-111f.

It may be that T cell clones responding to this antigen are developed late in the course of leishmaniasis and thus cellular responses would only be found in individuals that are on their way to or have cured the disease. It has been reported that cured individuals respond to more antigens than patients with active disease [304]. There is limited data available on responses to Leish-111f in self-cured CL patients. However, those that have responses to either LmSTII or TSA also respond to Leish-111f (personal communication with R. Coler). Thus, an explanation that the coupling of the antigens in the tri-fusion protein affects the ability of recognition by cells from patients with active leishmaniasis has limited support.

Another suggestion that has been brought up is that Leish-111f behaves as a cryptic antigen. Such antigens are mainly discussed in the context of autoimmunity, but may be of interest in other fields [305]. If Leish-111f is a cryptic antigen it would suggest that

the *Leishmania* derived antigens in the construct are poorly presented to the immune system during natural infection. This theory is slightly contradictory to the mouse data, which classified the individual antigens in Leish-111f as immuno-dominant. However, these antigens may be hidden during natural infection, but presented in an immuno-dominant manner when given as a vaccine. Moreover, presentation of antigens may differ between mice and men. However, the mouse studies clearly show that Leish-111f responding T cells clones can recognize the parasite and be protective and the pilot studies where Leish-111f was used as therapeutic vaccine, indicate that Leish-111f induced responses are beneficial also against human leishmaniasis.

### NK Cells from Patients with Active Cutaneous Leishmaniasis Are Less Responsive Compared to Healthy Controls

Previous reports from this and other groups indicate that NK cell activity is impaired in patients with active leishmaniasis [125-127]. We have in this study assessed the ability of NK cells from patients to respond with IFN $\gamma$  secretion when stimulated with live or heat-killed *Leishmania*. We have evaluated IFN $\gamma$  secretion in NK cells from individuals with on-going cutaneous leishmaniasis (CL) after stimulation with *Leishmania* promastigotes. The contribution of NK cells was determined by either assessing PBMC depleted of CD56<sup>+</sup> cells or assessing NK cells purified by depletion of CD3, CD19, CD14 and adherent cells. Although, NK cells from patients responded with IFN $\gamma$  when stimulated our results indicate that purified NK cells from patient with active cutaneous leishmaniasis have a generalized reduced capacity to secrete IFN $\gamma$  compared to healthy controls (*figure 9*). If this reduced ability to secrete IFN $\gamma$  is due to loss of a specific NK subsets or not remains to be elucidated. Too few cells were acquired for satisfactory FACS analysis of the number of CD56<sup>bright</sup> NK cells.

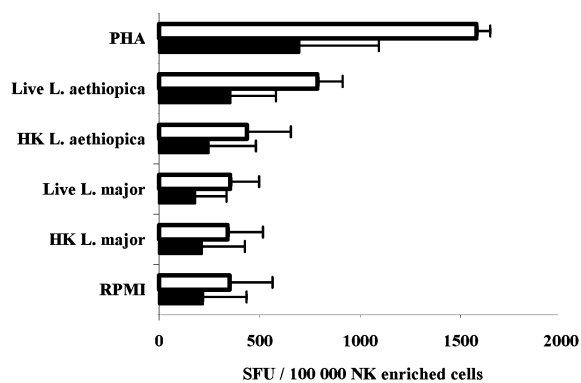


Figure 9: IFN $\gamma$  secretion in negatively selected NK cells from healthy Iranian controls (open bars, n=4) and patients with active *L. major* CL (black bars, n=8) after 40 hours stimulation.

Moreover, although not significant, PBMC from patients tended to respond more to killed than live *L. major* promastigotes, whereas PBMC from healthy controls only responded to stimulation with live *L. aethiopica* (**paper 5**).

## NK cells from Cured Leishmaniasis Patients Are Down-regulated by CD4<sup>+</sup> cells

Preliminary data using cells from individuals that have cured CL suggest that IFN $\gamma$  secretion induced by live-promastigotes was not affected by depletion of CD56<sup>+</sup> cells. Surprisingly depletion of CD4<sup>+</sup> cells did not significantly affect the frequency of the spots induced by live-PROM in cells from cured CL (*figure 10*). However, the spots were significantly smaller. The IFN $\gamma$  spots we observe in healthy donors tend to be small (see paper 5 fig 1b). Using cells from individuals with active or cured CL the spots tend to be larger, with fewer small spots observed (*figure 10c* and paper 5 fig 1b). On depletion of CD4<sup>+</sup> cells, live-PROM stimulation did not significantly affect the frequency of spots in cells from cured CL donors, however, smaller spots were evident (*figure 10c*). This suggest that the large spots are due to antigen specific stimulation of CD4<sup>+</sup> cells and the small spots are responses of CD56<sup>+</sup> cells. In addition the presence of CD4<sup>+</sup> cells appear to inhibit small spots, suggesting regulation of NK cell induced IFN $\gamma$  by CD4<sup>+</sup> cells.

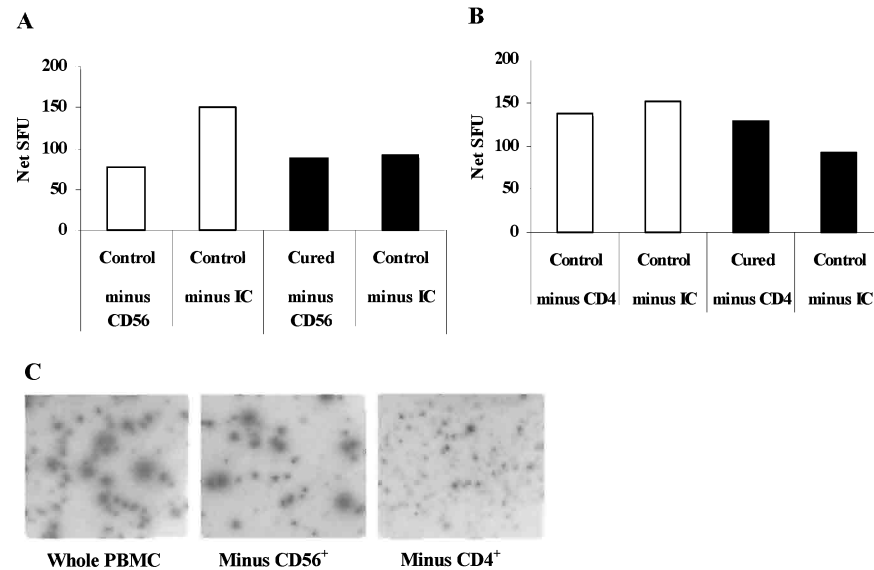


Figure 10: Net number of IFN $\gamma$  secreting cells in individuals cured of CL (black bars) and healthy Iranian controls (open bars) after depletion of A) CD56<sup>+</sup> cells B) CD4<sup>+</sup> cells. Results are given as SFU per 2x10<sup>5</sup> cells counted before depletion of specific cell subset or isotype control (IC). Bars shown mean net value of 5 (control) and 6 (cured) donors tested. C) Appearance of spots of cells from an individual cured of CL stimulated with Live *L. aethiopica* before and after depletion of CD56<sup>+</sup> and CD4<sup>+</sup> cell subsets.

Killed promastigotes did not induce IFN $\gamma$  secretion in cell cultures depleted of CD4<sup>+</sup> cells.

Although, we merely have indications, our hypothesis is that, when cells from cured individuals are stimulated with live promastigotes, NK activity is controlled by *Leishmania* specific CD4<sup>+</sup> T cells. This would be supported by results showing that PBMC from VL patients suppress NK cell activity *in vitro* [127]. Regulatory T cells

(CD4<sup>+</sup>, CD25<sup>+</sup>) have been demonstrated in cancer patients to inhibit NK cell mediated cytotoxicity [306]. It is possible that NK cells are involved in the pathology of leishmaniasis and would need to be controlled. In this respect regulatory T cells are considered to be important in the control of pathogenic responses [307]. Furthermore, endogenous CD4<sup>+</sup> CD25<sup>+</sup> have been shown to accumulate at the site of murine *L. major* infection where, they appear to be involved in the control of persistent parasites [286]. Although, not shown by Belkaid *et al.* [286] regulatory T cells may be important also in the control of the potentially pathogenic inflammatory responses during *Leishmania* infection. Furthermore, once acquired immunity has been established it may be important to limit inflammation caused by innate immune responses. Thus, acquired mechanisms, which can control the innate activation during a secondary infection, are likely to have evolved. Kirby *et al.* recently showed that innate responses differ in primary and secondary *Salmonella* infection [198].

### **Can the Fate of NK Cells Reflect Development of Immunity?**

Surrogate markers of immunity to leishmaniasis in humans are not known. Mouse studies have demonstrated that the presence of CD4<sup>+</sup> T cells secreting IFN $\gamma$  in response to *Leishmania* would be an indicator of protection. Studies of cells from individuals that have cured leishmaniasis would indicate that the same would be true in humans. However, recent data indicate that this may not always be so. Although, CD4<sup>+</sup> T cells of memory phenotype (CD45RA<sup>-</sup>), secreting IFN $\gamma$  in response to *Leishmania* stimulation, were found before infection, lesions still developed. Furthermore, absence of IFN $\gamma$  secreting CD4<sup>+</sup> T cells did not necessarily correlate to susceptibility (unpublished observation).

Base on our results where NK IFN $\gamma$  responses appear to be lost or controlled during the course of CL, we speculated that analysis of IFN $\gamma$  responses by these cells could be of potential use as surrogate markers. Furthermore, we were interested to see if the observation made in leishmaniasis patients on a group basis in cross sectional studies would also be valid on individual basis in longitudinal studies. We got the unique opportunity to test this theory in a control group of volunteers before and after they received leishmanization (i.e. artificial infection). This study was done in connection with a vaccination study supported and approved by TDR/WHO where the efficacy of different doses of ALM + BCG + Alum were evaluated by challenge (leishmanization). The group of donors we tested were not vaccinated (only leishmanized) or belonged to the control group in the vaccine study that only received PBS injection. We evaluated IFN $\gamma$  and IL-13 (as a marker of Th2 response) responses in cells from non-immunized volunteers before and 9 months after artificial injection with viable *L. major* promastigotes. The original protocol included samples taken 7 days after infection, however, the quality of these samples was not adequate for analysis. Limitations in cell number did not allow us to test the effect on the direct activation. Thus, we had to settle for an indirect analysis using PBMC depleted of CD56<sup>+</sup> cells. The drop out rate was rather high and out of 10 donors tested prior to leishmanization only 6 had adequate number of cells before and after leishmanization. Five out of the six donors tested had a lesion or an induration at the site of injection after 9 months when the second blood sample was taken.

CD56<sup>+</sup> cells contributed significantly to both IFN $\gamma$  and IL-13 secretion induced by live *L. aethiopica*, both before and 9 months after leishmanization. To our disappointment there was no significant difference, in frequency or size of the spots, between the two time points. This was not anticipated from the results of the naturally infected donors. The results obtained from the donor who did not develop a lesion or an induration did not stand out from the others. From this we are unable to conclude that NK cell dependent IFN $\gamma$  secretion appears to correlate with protection or development of disease. Moreover, no difference in the over-all frequency of cells secreting IFN $\gamma$  in response to either live *L. aethiopica* or ft-*L. major* was observed before and 9 months after leishmanization (figure 11). When analysed on group basis we found that more cells secreted IL-13 in response to ft-*L. major* 9 months after than before leishmanization. However, we have to consider that the difference, observed in the study group prior to and after leishmanization, may be a consequence of the limited number of donors assessed.

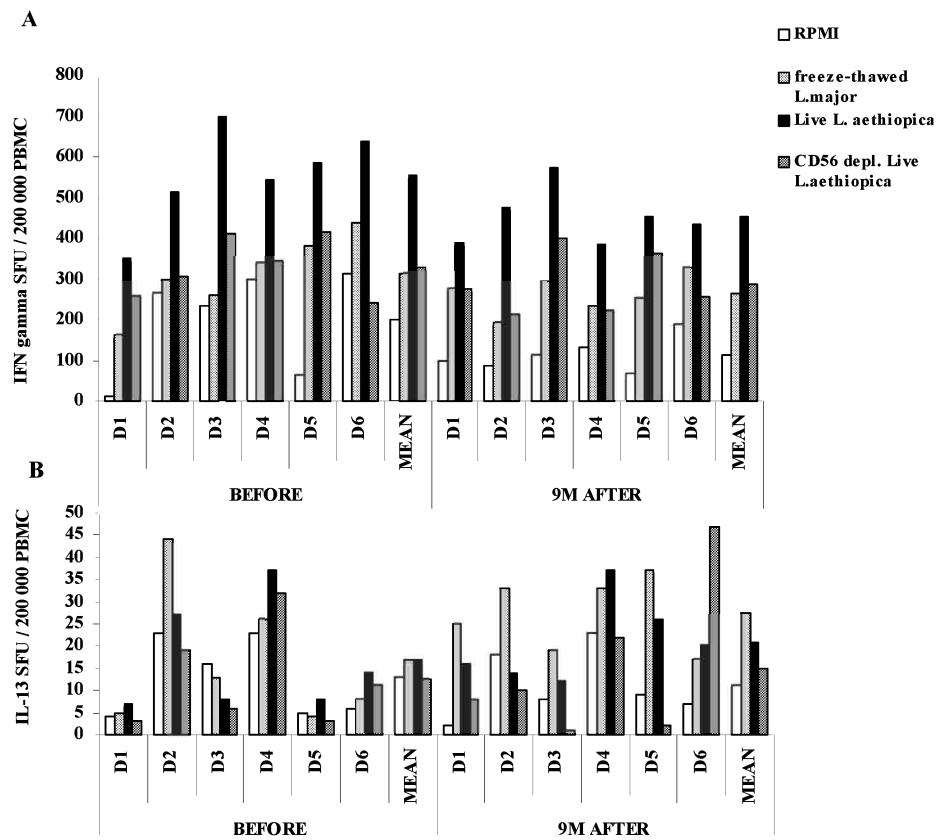


Figure 11: Cytokine responses to *Leishmania* before and after leishmanization. A) IFN $\gamma$  and B) IL-13 spot forming units (SFU). Graphs show mean (of duplicate) number of SFU after 40 hours stimulation with ft-*L. major* (light grey bars) or Live *L. aethiopica* (black bars). Effect of Live *L. aethiopica* induced cytokine secretion after depletion of CD56 positive cells (dark grey bars). Open bars show cytokine secreting cells in control cultures.

What should be noted is that all individuals selected for this study (and the above mentioned vaccine study) bore no sign and had no history of leishmaniasis. Furthermore, they were all Leishmanin skin test (LST) negatives, although living in a *L. major* endemic area of Iran (Ardestan). Subclinical infection has been thought to be reflected by a positive LST reaction with proliferative and cytokine responses being similar to those of overt leishmaniasis [308]. The absence of LST reaction has been interpreted as lack of immunity to *Leishmania*. However, the LST negative donors have most likely been exposed and infected, without developing clinical infection. Thus, it would seem likely that non-T cell mediated immunity is present in these individuals. Hence, innate elements of immunity may be responsible for preventing infection in these donors. Since 5/6 of donors used, in our study, displayed signs of disease these innate response may be overpowered by leishmanization at the dose of promastigotes given ( $\approx 2.5 \times 10^4$ ). Interestingly, overt disease did not seem to correlate with development of characteristic CD4<sup>+</sup> T cell responses (believed to be protective) in a group of LST negative endemic donors following leishmanization (unpublished data), although, characteristic T cell responses with disease were observed in a few of these leishmanized individuals. While, highly speculative, if the above hypothesis of T cell inhibition of Live-PROM induced NK cell IFN $\gamma$  is true the lack of induction of *Leishmania* specific T cell responses at the time of lesions (observed in the vaccine group), could in part explain the significant contribution of CD56<sup>+</sup> cells to the IFN $\gamma$  secretion in the ongoing infection.

A scenario is proposed where NK cells participate in the innate defence against *Leishmania* in individuals lacking T cell mediated immunity i.e. non-immune and possibly also LST negative individuals living in endemic areas (*figure 12*). These innate responses may many times be sufficient to prevent the establishment of disease following natural infection. However, innate defence mechanisms are not always enough to control the infecting parasites, thus disease appears. Control of disease is associated with acquisition of *Leishmania* specific T cells and induction of immunity to re-infection. However, these mechanisms need to be controlled.

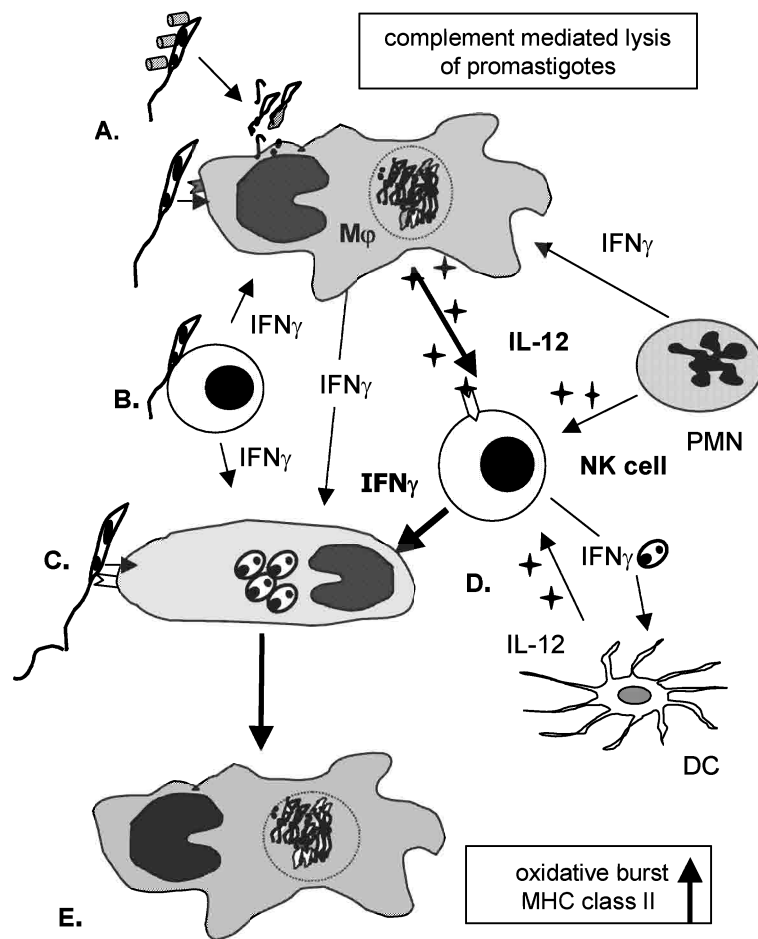


Figure 12: A) Uptake of lysed promastigotes or ineffective infection of macrophage resulting in activation and killing of the promastigote B) Suggested role for direct activation of NK cells. C) Successful infection of macrophages. D) IL-12 induced activation of NK cells. E. Killing of amastigotes, enhanced presentation of parasite antigen to T cells



To avoid tissue damage, once T cell mediated immunity has been acquired, it may be important to control innate inflammatory responses mediated by, amongst others, NK cells. Thus, we suggest a second scenario (*figure 13*) where in individuals, who have establishment T cell mediated immunity after recovery of leishmaniasis, have generated antigen specific  $CD4^+$  T cells that have the potential to control the NK responses. Suppression of NK cells could be mediated by secretion of cytokines like  $TGF\beta$  and IL-10 and / or by cell-to-cell contact. If NK cell responses are controlled by suppressor / regulatory cells secreting down regulatory cytokines, these cytokines would be anticipated to also inhibit protective *Leishmania* specific T cells ( $CD4^+$  and  $CD8^+$ ) and inhibit macrophage activation. However, these cells will (by unknown mechanism) modulate or override the suppressor activity and control a secondary infection. If the control is mediated through cell-to-cell contact it may be the protective  $CD4^+$  cells and / or specific suppressor T cells that exert the down modulating effect on the NK cells by acting on NK cell inhibitory receptors. This scenario is yet to be tested.

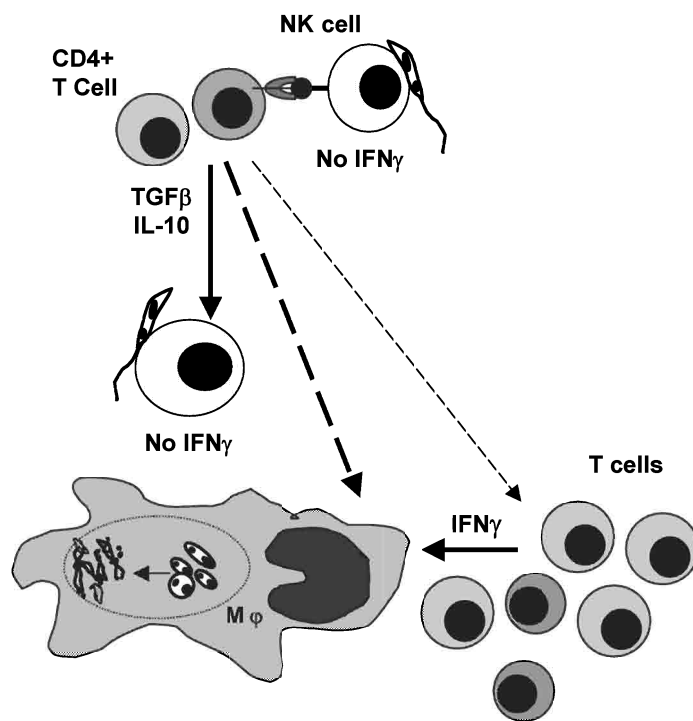


Figure 13: Suggested T cell regulated inhibition of NK cells in individuals cured of leishmaniasis

## CONCLUSIONS

- **Live and dead parasites activate different cells of the immune system**
- **Purified NK cells can be directly activated by Live *Leishmania* promastigotes**
- **The *Leishmania* vaccine candidates LACK and the amastigote antigen P-2 induces cellular responses involving NK cells in PBMC from healthy donors**
- **Not all *Leishmania* derived molecules induce responses; P-4 and Leish-111f did not activate PBMC from unexposed donors**
- **Direct activation of NK cell to IFN $\gamma$  secretion is reduced in individuals with active cutaneous leishmaniasis**
- **Controlled artificial infection through leishmanization shows the complexities of defining surrogate markers of immunity to leishmaniasis in humans**

## CONCLUDING REMARKS

The work of this thesis has mainly addressed how PBMC from donors with no history of leishmaniasis respond when exposed to whole *Leishmania* parasites and vaccine candidates.

We have shown that IFN $\gamma$  NK cell responses to live *Leishmania* are prominent in PBMC from healthy individuals with no history of disease. These responses may be important in preventing establishment of the infection and can contribute to the induction of potentially protective Th1 responses.

Studies of vaccine candidates as well as killed promastigotes in PBMC of healthy donors revealed responses that could be anticipated to both favour and impede the efficacy of the vaccine. Since, it may be difficult to control such responses, vaccine candidates may benefit from avoiding substantial involvement of cross reactive or innate responses. Alternatively vaccines should aim at inducing innate responses that favour only the initiation of acquired T cell mediated immunity.

While, the use of leishmanization should have given a unique opportunity to study the effect of *Leishmania* infection on NK cell responses in humans, perhaps the study population or the dose of promastigotes used may have masked what effect there may have been on the NK cell response. However, we show that NK cell responses can be affected by leishmaniasis and we propose a theory where T cells can control NK cell responses once acquired immunity has been established.

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